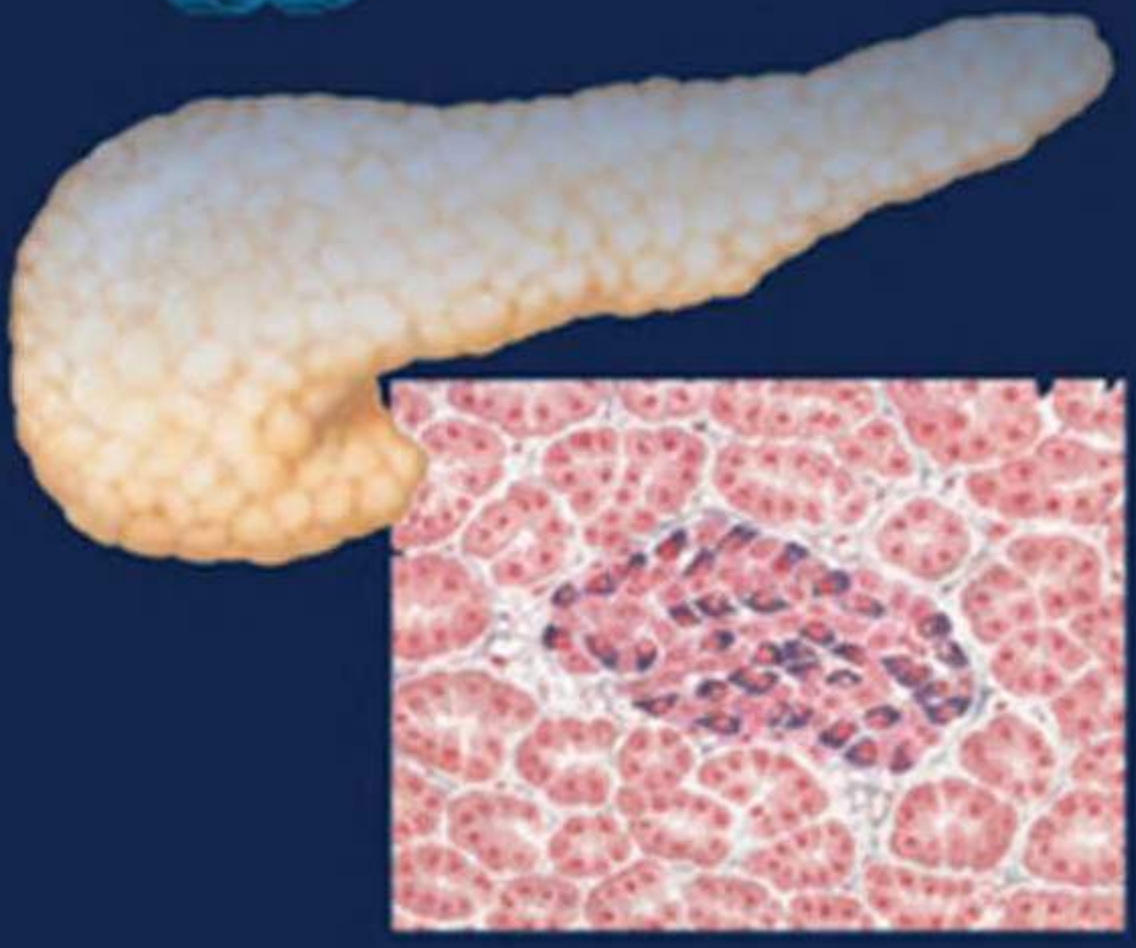
PETER RONNER

NETTER'S ESSENTIAL BIOCHEMISTRY



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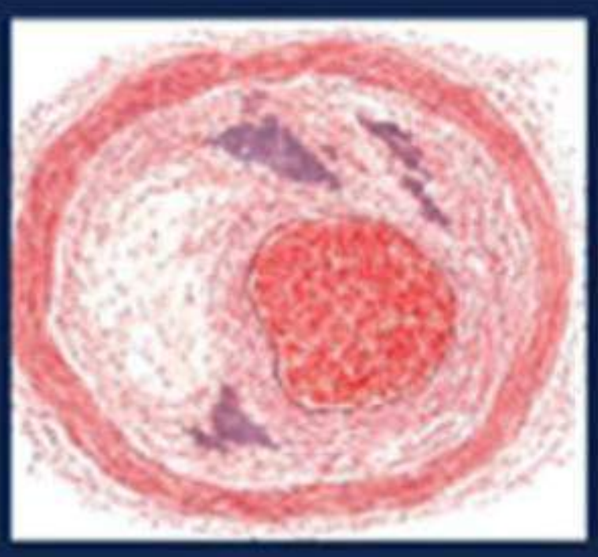

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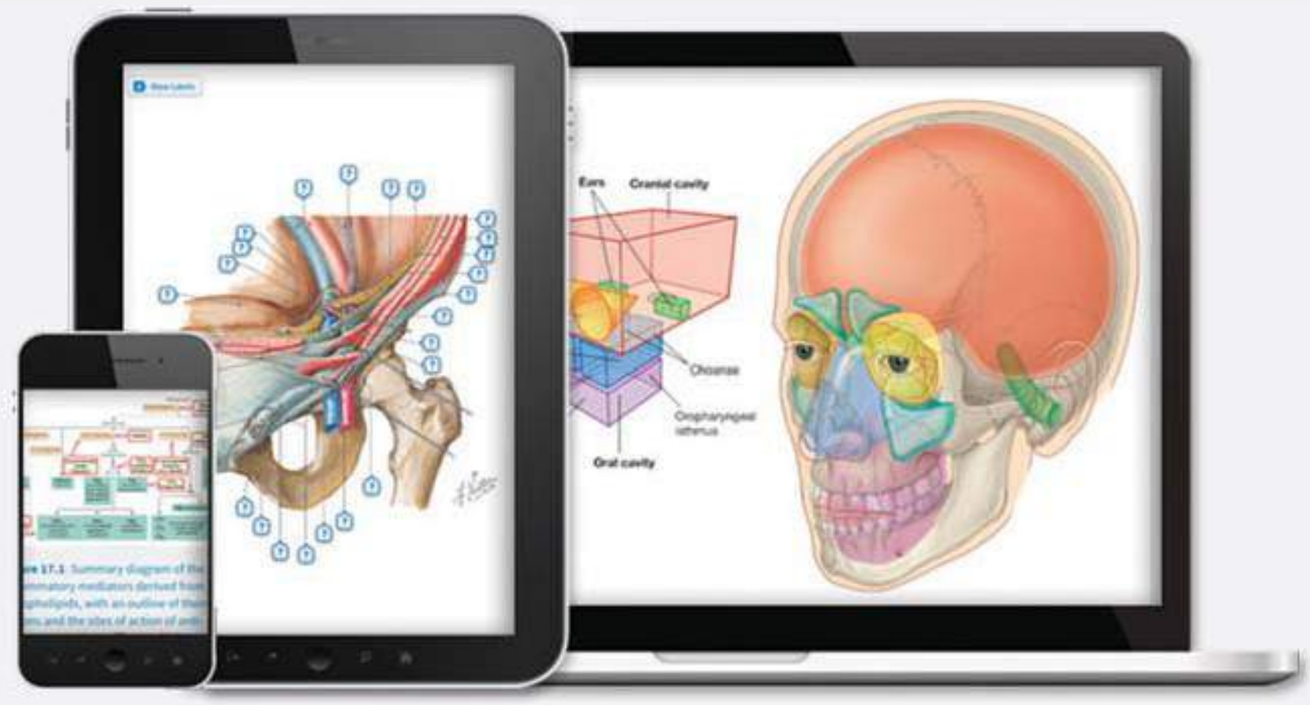

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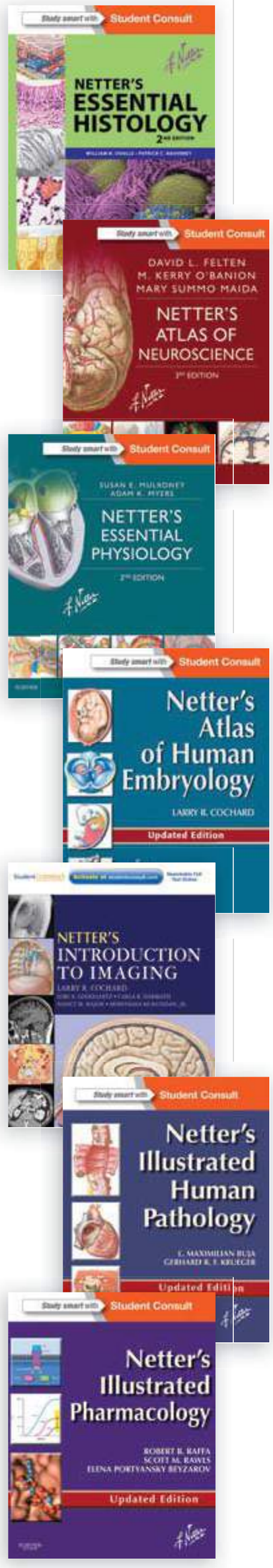

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NETTER'S ESSENTIAL BIOCHEMISTRY

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To Wanda and Lukas

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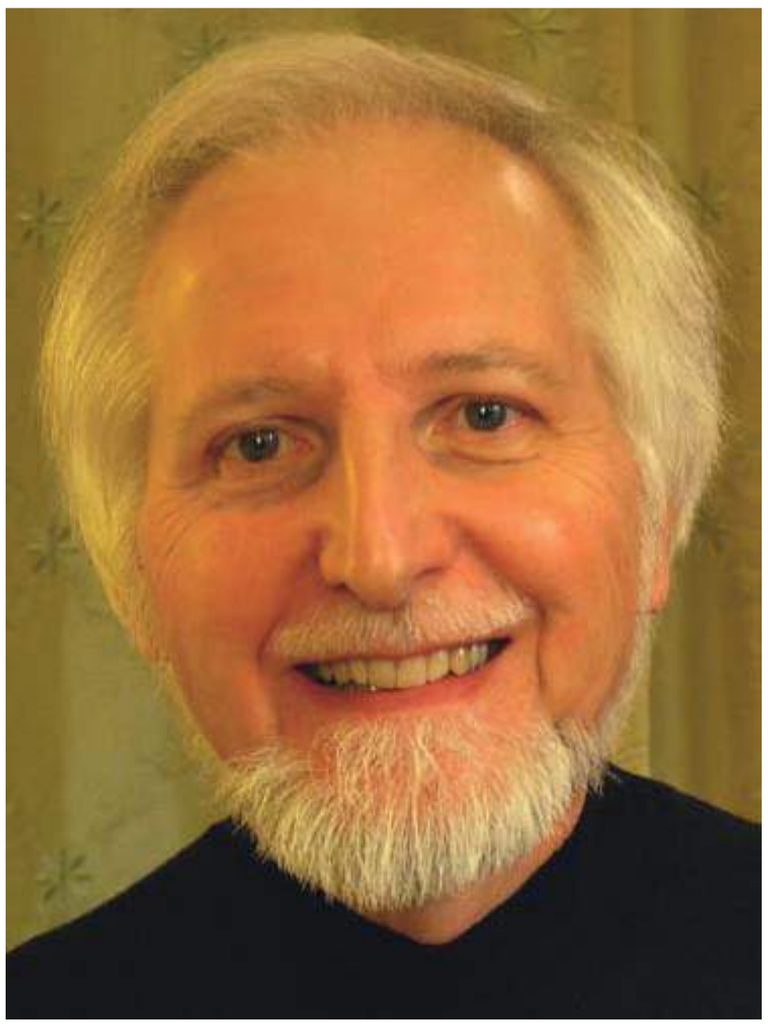

Peter Ronner, PhD, is Professor of Biochemistry and Molecular Biology at the Sidney Kimmel College of Medicine at T omas Jef erson University in Philadelphia. He holds a secondary appointment as Professor of Pharmaceutical Sciences in the College of Pharmacy at T omas Jef erson University. Dr. Ronner received his PhD in Biochemistry from the Swiss Federal Institute of Technology (ETH) in Zurich. His former laboratory research involved studies of pancreatic hormone secretion. Dr. Ronner has taught medical students for nearly 30 years and pharmacy students for almost 10 years. He is also a past chair of the Association of Biochemistry Course Directors (now Association of Biochemistry Educators). At Jef erson, he has received numerous awards for his teaching, including a Lindback Award and a portrait painting.

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Many people have helped me write this book, but Dr. John T omas from New York University School of Medicine deserves a place of honor. He and I worked on this book together until we were forced to pause for a few years.

Years earlier, at the University of Pennsylvania, the late Dr. Annemarie Weber introduced me to teaching biochemistry to medical students. She was a tremendous role model. At T omas Jef erson University, Dr. Darwin Prockop planted in my mind the idea of writing a biochemistry textbook. Many years later, Paul Kelly (then at Icon Learning Systems), approached me with the idea of using Dr. Netter's images for a biochemistry review book. T is appealed to me because biochemistry is taught as a rather abstract science that students have difficulty linking to actual patients. T e Netter images, I hoped, would provide the views of the practicing physician. T anks to the support of my chairman, Dr. Jef rey Benovic, this book project became part of my scholarly pursuits. I am thankful for the invaluable feedback the many students of medicine and pharmacy at Jef erson gave me over the years.

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T is book is dedicated to my wife Wanda and my son Lukas. Wanda has been a key influence on me, because she has continuously given me her perspective as a practicing physician and medical student educator. Lukas, a chemistry major and current medical student, has been my most trusted adviser on questions about young learners, chemistry, and artwork, and he has reviewed much of my writing.

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About the Artist

FRANK H. NETTER, MD

Frank H. Netter was born in 1906 in New York City. He studied art at the Art Student's League and the National Academy of Design before entering medical school at New York University, where he received his MD degree in 1931. During his student years, Dr. Netter's notebook sketches attracted the attention of the medical faculty and other physicians, allowing him to augment his income by illustrating articles and textbooks. He continued illustrating as a sideline after establishing a surgical practice in 1933, but he ultimately opted to give up his practice in favor of a full-time commitment to art. After service in the United States Army during World War II, Dr. Netter began his long collaboration with the CIBA Pharmaceutical Company (now Novartis Pharmaceuticals). T is 45-year partnership resulted in the production of the extraordinary collection of medical art so familiar to physicians and other medical professionals worldwide.

In 2005, Elsevier, Inc. purchased the Netter Collection and all publications from Icon Learning Systems. T ere are now over 50 publications featuring the art of Dr. Netter available through Elsevier, Inc. (in the US: www.us.elsevierhealth.com/Netter and outside the US: www.elsevierhealth.com).

Dr. Netter's works are among the finest examples of the use of illustration in the teaching of medical concepts. T e 13-book Netter Collection *of* Medical Illustrations, which includes the greater part of the more than 20,000 paintings created by Dr. Netter, became and remains one of the most famous medical works ever published. T e Netter Atlas *of* Human Anatomy, first published in 1989, presents the anatomical paintings from the Netter Collection. Now translated into 16 languages, it is the anatomy atlas of choice among medical and health professions students the world over.

T e Netter illustrations are appreciated not only for their aesthetic qualities, but, more important, for their intellectual content. As Dr. Netter wrote in 1949, ". . . clarification of a subject is the aim and goal of illustration. No matter how beautifully painted, how delicately and subtly rendered a subject may be, it is of little value as a medical illustration if it does not serve to make clear some medical point." Dr. Netter's planning, conception, point of view, and approach are what inform his paintings and what make them so intellectually valuable.

Frank H. Netter, MD, physician and artist, died in 1991.

Learn more about the physician-artist whose work has inspired the Netter Reference collection: http://www.netterimages.com/artist/netter.htm.

Preface

This book provides an introduction to and review of biochemistry as it pertains to the competencies required for graduation as a doctor of medicine or pharmacy. Increasingly, the basic sciences are taught alongside clinical science, often organ by organ. This book can help students in such integrated curricula gain a discipline-specific understanding of biochemistry, particularly metabolism. The book is structured so that it is useful for both the novice and the student who needs a quick review in preparation for licensure exams. The chapters are extensively cross-referenced so the material can be used in almost any chapter sequence. Descriptions of disease states are a regular part of the book rather than an addendum in the margin. Students often find it challenging to use their knowledge of basic science to solve clinical problems. Hopefully, Dr. Netter's images ("Medicine's Michelangelo"), as well as the text and other diagrams in this book, will help students build mental bridges between basic science and clinical practice.

The chapters have a structure that makes it easy for the reader to decide what to read and review:

- The Synopsis is an introductory overview of the content of the chapter that requires very little preexisting knowledge.
- The Learning Objectives indicate what the reader should be able to do when mastering the material presented in the chapter.
- Each section starts with a preview.
- Selected terms are printed in bold to make it easier to find relevant text when starting from the index.
- The diagrams contain only the most essential information.
- The Summary provides a brief overview of the chapter material for the expert.
- A Further Reading section provides the reader with a starting point to satisfy deeper interests.
- Review Questions provide the reader with an opportunity to apply newly acquired knowledge. Answers to these questions are at the end of the book.

Writing this text and designing the accompanying graphs has been a wonderful and interesting journey for me. I have also enjoyed many years of teaching biochemistry to future physicians and pharmacists. I hope that you, the reader, will also be amazed by the processes that underlie human existence, both in health and in sickness.

Peter Ronner

P.S.: Please *f*eel *f*ree to email suggestions *f*or improvements to peterronner1@gmail.com.

Contents

Chapter 1 Human Karyotype and the Structure of DNA

SYNOPSIS

- Heritable information is encoded in deoxyribonucleic acid (DNA). DNA is a linear polymer of deoxyribonucleotides, and it is present in the nucleus and mitochondria of cells.
- The DNA of a cell comprises pairs of complementary molecules; each pair assumes a double-helical structure.
- DNA double helices in the nucleus are wound into higher-order structures. The simplest of such structures is the nucleosome; the most complex structures exist in the form of condensed chromosomes during cell division. Light microscopic examination of these chromosomes is part of karyotyping.
- Helicases and topoisomerases change the coiling of DNA for transcription, replication, and repair of DNA.
- Inhibitors of DNA topoisomerases can be used to destroy cancer cells or bacteria.

LEARNING OBJECTIVES

For mastery *of* this topic, you should be able to do the *f*ollowing:

- Describe the components and architecture of a DNA double helix and explain where proteins bind to DNA helices.
- Provide an example of reporting a DNA sequence in the customarily abbreviated style.
- Describe the most basic unit for packaging DNA into the nucleus.
- Describe the normal human karyotype and list the number of DNA double helices that make up a single metaphase chromosome.
- Describe the function of DNA topoisomerases and explain the role of these enzymes in changing the topology of chromosomes.

1. CHEMICAL STRUCTURE OF DNA

Mitochondria and the nucleus o*f* each cell contain DNA that is a polymer o*f f*our basic types o*f* nucleotides. DNA stores heritable in*f*ormation by way o*f* its nucleotide sequence.

DNA is a linear polymer of the **deoxyribonucleotides** deoxyadenosine monophosphate (dAMP), deoxyguanosine monophosphate (dGMP), deoxycytidine monophosphate (dCMP), and thymidine monophosphate (dTMP, TMP; Fig. 1.1). Each deoxyribonucleotide consists of deoxyribosephosphate (derived from a pentose, a 5-carbon sugar) covalently linked to a base that is **adenine**, **guanine**, **cytosine** (or 5-methyl cytosine), or **thymine**. Adenine and guanine structurally resemble purine; hence, they are called **purine** nucleotides (synthesis, turnover, and degradation of these nucleotides are described in Chapter 38). Cytosine and thymine structurally resemble pyrimidine; hence, they are called **pyrimidine** nucleotides (synthesis of these nucleotides is described in Chapter 37).

As part of epigenetic regulation, ~4% of the cytidine nucleotides of DNA in the nucleus are **methylated to 5-methyl deoxycytidine** (see Fig. 1.1). T e term epigenetic regulation refers to changes in the DNA or DNA-associated proteins that do not af ect the sequence of the bases but af ect gene expression. Some of these changes can be heritable and passed from one cell to its descendants (see imprinting in Chapter 5). Quite generally, methylation influences the higher-order packing and transcription of DNA (see Chapter 6). Methylation is required for the inactivation of the second X chromosome in females (see Chapters 5 and 21), the silencing of certain transposons (movable genetic elements), regulation of the expression of genes during development, and determining the expression of particular genes from only the mother or only the father.

Each DNA molecule has a **5′ end** and a **3′ end** (Fig. 1.2). To distinguish the atoms of the deoxyribose from those of the base, the deoxyribose carbon atoms are given a **prime** as a postfix (e.g., 3′). T e dinucleotide shown in Fig. 1.2 has a phosphate group at the 5′ position of nucleotide 1 and a hydroxyl group at the 3′ position of nucleotide 2, which is typical of DNA. T e nucleotides are linked by **phosphodiester bonds**. DNA is normally elongated at the 3′ end (see Section 1 in Chapter 3).

By convention, the **sequence** of a DNA is written as the sequence of the bases in the **5′→3′** direction, using **A** for adenine, **C** for cytosine, **G** for guanine, and **T** for thymine. If the sequence is instead written 3′→5′, this must be indicated. T e sequence of bases in DNA contains heritable information. DNA is found in the nucleus (see Section 4) and in mitochondria (see Section 3 in Chapter 23).

2. HYDROGEN BONDING BETWEEN COMPLEMENTARY BASES

In the *f*ashion o*f* a zipper, complementary DNA molecules associate by hydrogen bonding. A and *T* can be linked by two hydrogen bonds, C and G by three hydrogen bonds.

In Watson-Crick base pairing, A and T are hydrogen bonded to each other, and so are C and G. Each base of a nucleotide contains one or more hydrogen donors (–OH and $–NH_2$) and one or more hydrogen acceptors (=O and =N–). A hydrogen acceptor can form a partial bond to a donor's hydrogen atom; such a bond is called a **hydrogen bond**. A and T each contain one hydrogen donor and one hydrogen acceptor in suitable positions, such that A and T can be linked by a total of two hydrogen bonds (Fig. 1.3). C has one hydrogen donor and two hydrogen acceptors, while G has two hydrogen donors

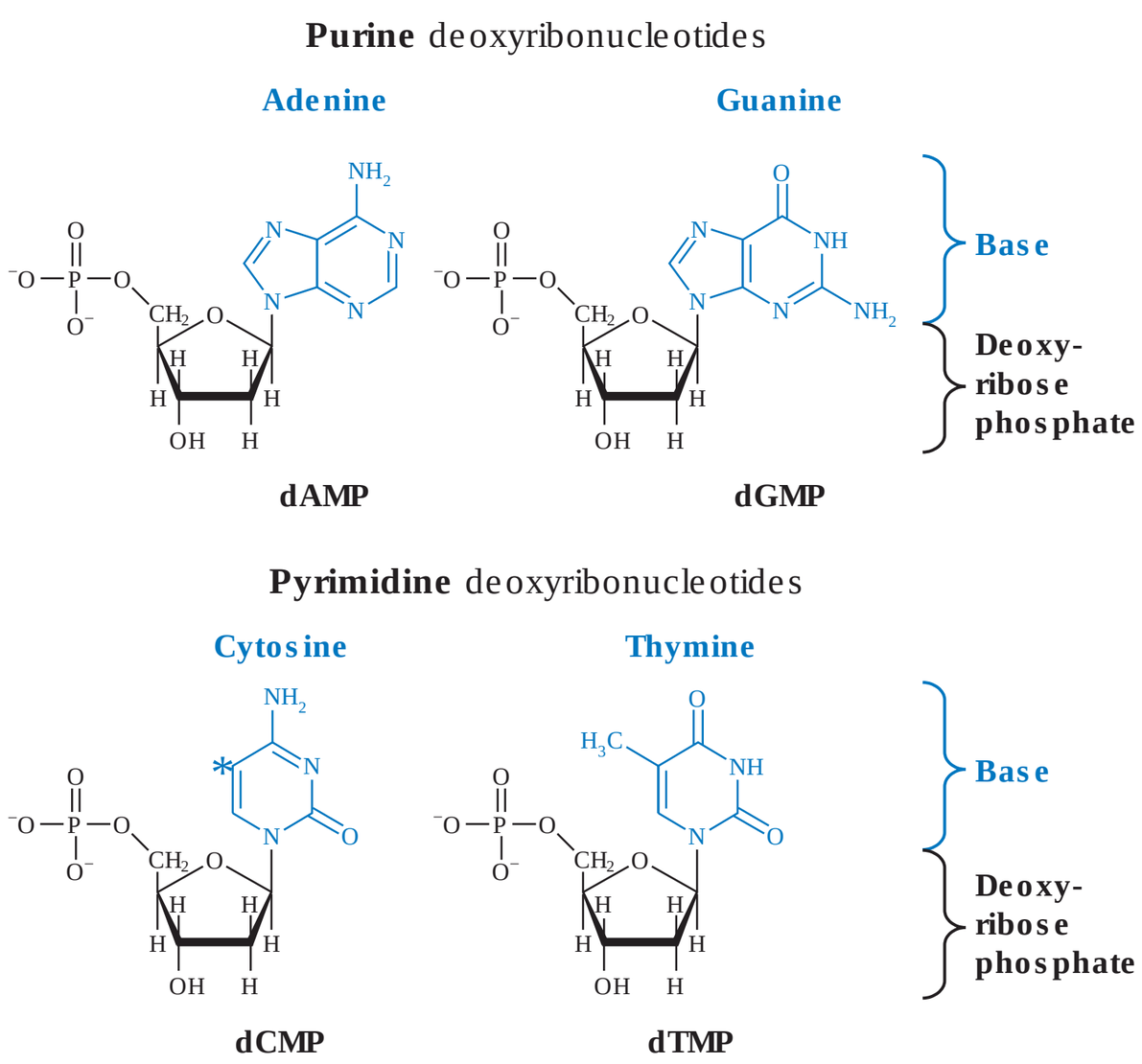

Fig. 1.1 Structures of deoxyribonucleotides found in DNA. The asterisk indicates the site of potential cytosine methylation.

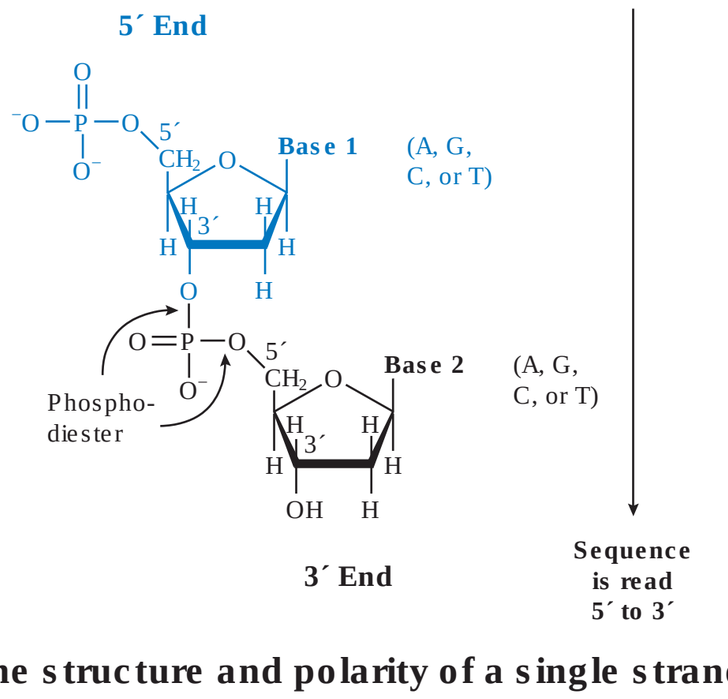

Fig. 1.2 The structure and polarity of a single strand of DNA.

and one hydrogen acceptor in suitable positions so that C and G can be linked by a total of three hydrogen bonds. Since they form hydrogen bonds with each other, A and T are called **complementary bases**; likewise, C and G are complementary bases. CG base pairs are harder to separate than AT base pairs because they have more hydrogen bonds. (Non–Watson-Crick base pairing is observed predominantly in RNA, where it is common.)

In two complementary DNA molecules, all bases form hydrogen-bonded AT and GC base pairs, and the molecules are paired in an antiparallel fashion. For instance, the molecules 5′-AACGT-3′ and 3′-TTGCA-5′ are complementary (Fig. 1.4). The nucleotide at the 5′ end of one DNA strand is thus hydrogen bonded to the nucleotide at the 3′ end of its complementary DNA strand. All heritable human DNA exists in complementary strands that, in vivo, usually assume a double-helical structure (Fig. 1.5). In mitochondria, each DNA strand consists of about 16,000 nucleotides; in the nucleus, each DNA strand consists of more than 45 million nucleotides.

When a DNA sequence is reported, the sequence of the complementary strand is usually omitted because it can easily be inferred.

According to the **Chargaf rule**, DNA contains equimolar amounts of A and T, as well as equimolar amounts of C and G. AT and CG base pairing are the basis of Chargaf 's finding.

3. DNA DOUBLE HELIX

Most human DNA assumes a double-helical structure. The double helix consists of two complementary strands that run in opposite directions.

Complementary hydrogen-bonded DNA molecules normally assume the structure of a **DNA double helix** (see Fig. 1.5). In this structure, the hydrogen-bonded bases are close to the central long axis of the DNA helix. The covalently linked deoxyribose phosphates of the two DNA strands wind around the periphery of the helix cylinder, akin to the threads of an unusual screw (a typical screw has only one thread). As is evident from Fig. 1.3, the bonds between the bases and the deoxyribose moieties (i.e., the N-glycosidic bonds) do not point in exactly opposite directions. Hence, the two strands of linked deoxyribose phosphates are closer together on one side of the base pair than on the other side. Thus, the DNA double

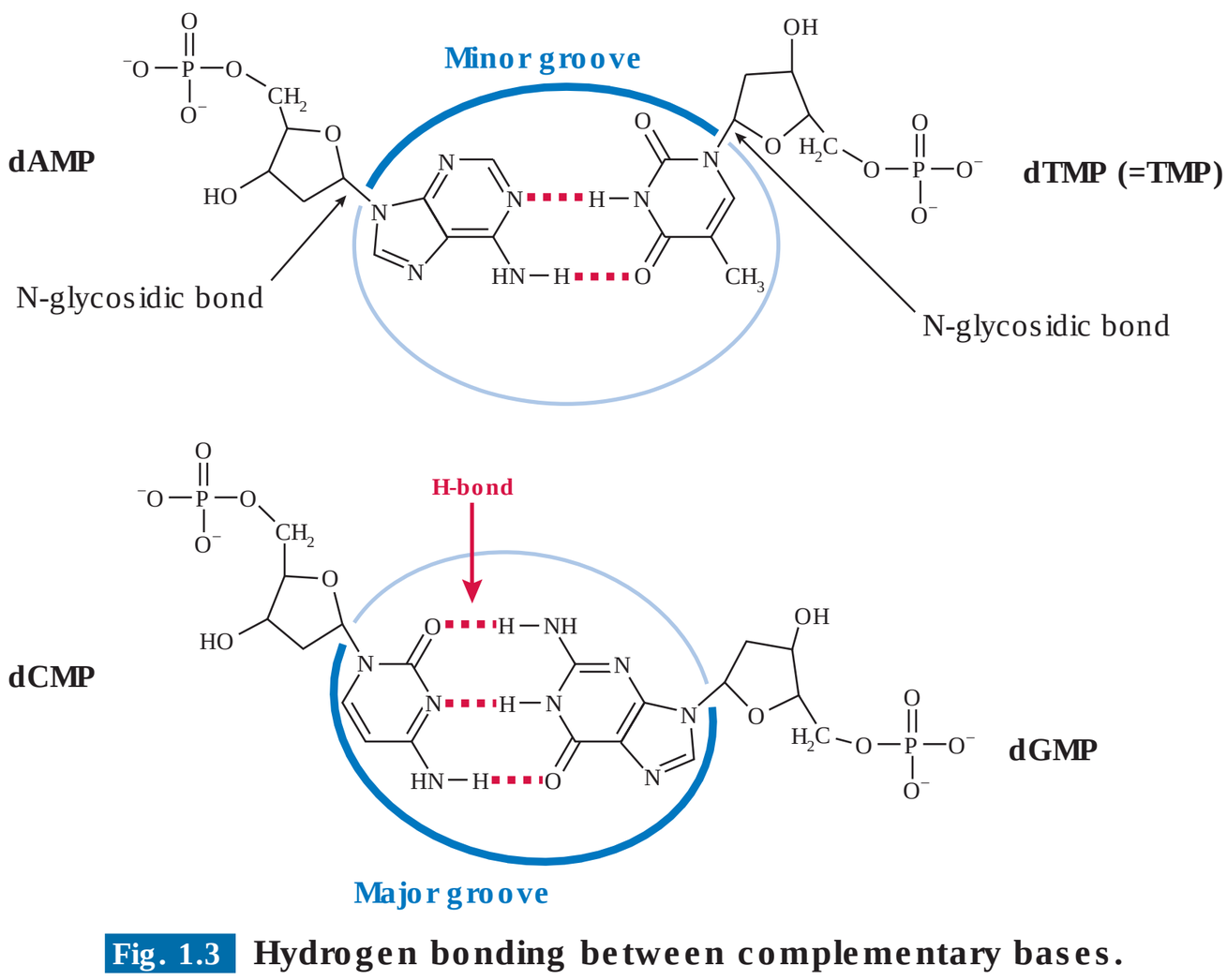

Fig. 1.3 Hydrogen bonding between complementary bases.

helix has unequal grooves: a **minor groove** and a **major groove**.

There are several double-helical DNA structures that differ in handedness, diameter, and rise per turn. The most prominent of these structures are referred to as **A-DNA**, **B-DNA**, and **Z-DNA**. In cells, most DNA is in a double-helical form that resembles B-DNA.

Transcription factors that bind to DNA (see Chapter 6) bind to atoms at the surface of the major or minor groove and can thereby recognize a particular nucleotide sequence. Some transcription factors increase the contact with DNA further by bending or partially opening the double helix.

Certain positively charged side chains of **DNA-binding proteins**, as well as certain positively charged **stains** used in histochemistry (e.g., the basic dyes **hematoxylin**, **methylene blue**, and **toluidine blue**), bind to DNA by interacting with the negatively charged phosphate groups. These phosphate groups line the backbone of DNA and are exposed on the outside of the double helix (see Fig. 1.5). Among DNA-binding proteins, positive charges are found on some amino acid side chains of histones (see below) and of certain transcription factors (see Chapter 6). Complexes of DNA and the DNA-binding histone proteins are referred to as **chromatin**. The negative charges of the phosphate groups of DNA alone give rise to an overall negative charge of DNA that is taken advantage of in the **electrophoresis** of DNA (see Chapter 4).

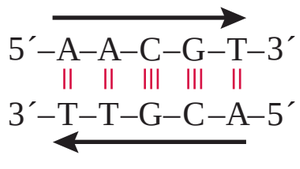

Fig. 1.4 Basic structure of double-stranded DNA. Double-stranded DNA can form a double helix (see Fig. 1.5).

In vivo, hydrogen bonds between bases of complementary DNA strands are broken and reformed during replication, repair, or transcription of DNA (see Chapters 2, 3, and 6). In vitro, the separation and "rejoining" (hybridization) of complementary DNA strands are an important part of many diagnostic DNA-based procedures (see Chapter 4).

4. PACKING OF DNA DOUBLE HELICES INTO CHROMATIDS

The length of human DNA molecules far exceeds the diameter of the cell nucleus. DNA is compacted into orderly structures ranging from nucleosomes to metaphase chromatids.

In the nucleus, DNA is folded into nucleosomes, which in turn are part of increasingly higher orders of folding. The greatest degree of DNA compaction is needed for cell division. The longest human chromosome (chromosome 1) contains about 246 million base pairs and has a length ~15,000 times the diameter of a typical nucleus. The organization of DNA also affects the transcription of genes. The basic unit of folding is the **nucleosome**, of which several types exist. Nucleosomes contain a **core particle** that consists of eight histone proteins, a DNA helix of ~147 base pairs that encircles the **histones** ~1.7 times (Fig. 1.6), and **linker DNA** of ~40 base pairs to which histone H1 is often bound. N- and C-terminal **tails** of the histones protrude from nucleosome core particles. Certain amino acids in these histone tails can be modified (Table 1.1). The resulting structure of the histone tails affects the packing, **replication** (see Chapter 3), and **transcription** of DNA (see Chapter 6).

Nucleosomes can be organized into **30-nm** diameter **chromatin fibers**. Chromatin fibers, in turn, can be condensed into yet higher-order structures, and finally into

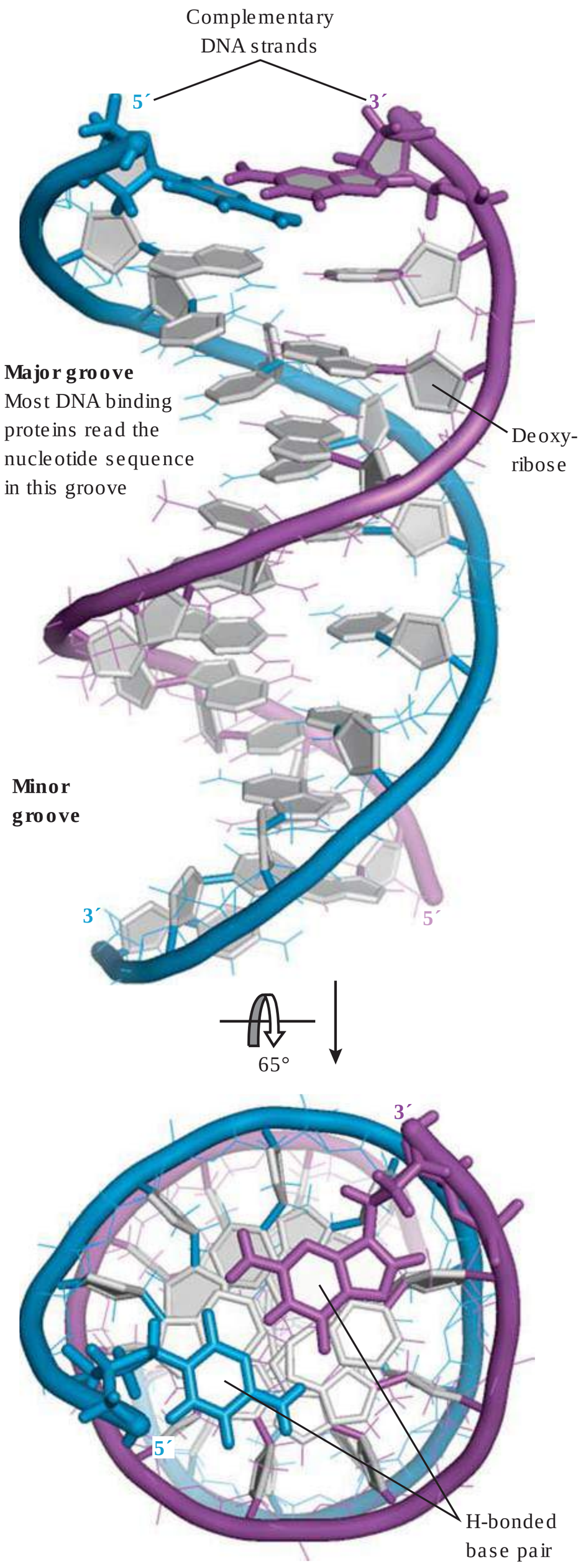

Fig. 1.5 The double-helical structure of DNA. The structure of an 11-base-pair segment of the human N-ras gene is shown (the sequence of the purple strand is 5′-GGCAGGTGGTG; this sequence frequently undergoes mutation and then promotes the development of a tumor). The bases are in the center, and the riboses are located in the periphery of the helix. The blue and purple snaking cylinders are imaginary forms that connect the phosphorus atoms and show the progress of the helix. The bonds that connect phosphates and riboses and that form the true backbone of a DNA strand are generally situated just outside the calculated cylinders. The planes of the rings of deoxyriboses and bases are shown in light gray. The two DNA strands are antiparallel; the blue strand is winding downward (5′ to 3′), while the purple strand is winding its way up (5′ to 3′). The structure of this oligomer mostly resembles the structure of B-DNA. (Based on Protein Data Bank [www.rcsb.org] file 1AFZ from Zegar IS, Stone MP. Solution structure of an oligodeoxynucleotide containing the human N-ras codon 12 sequence refined from 1H NMR using molecular dynamics restrained by nuclear Overhauser effects. *Chem Res Toxicol.* 1996;9:114–125.)

chromatids. Chromatids are found only in dividing cells during mitosis.

5. CHANGES IN DNA TOPOLOGY

T e cellular processes of DNA repair, replication, and transcription (discussed in Chapters 2, 3, and 6) require, at times, the unwinding of DNA from its structures (e.g., the 30-nm chromatin f ber, the nucleosomes, and the double helix) followed by rewinding. Changes in the winding of DNA are catalyzed by helicases and topoisomerases.

Topology is a field of mathematics that describes the deformation, twisting, and stretching of objects such as DNA. As outlined above, DNA of human chromosomes is organized into nucleosomes and higher-order structures. T e chemical structure of DNA can accommodate only a limited amount of torsional strain, and the chromatin structure prevents the dispersion of strain over a large distance. (As an analogy, consider how winding af ects the three-dimensional shape of a phone cord or garden hose.) T us, the winding of DNA (the topological state of DNA) matters. Torsional strain can result from the partial opening of a DNA helix (e.g., during repair, replication, or transcription) or from a nonrotatable complex of enzymes that moves in between the two strands of a DNA double helix (Fig. 1.7). Replication and transcription, for example, cause overwinding, or positive supercoiling, within the chromosomes.

Helicases can use energy from ATP hydrolysis to separate the two strands of the double helix. T e energy input from ATP is needed to pay the penalty for breaking hydrogen bonds between bases in DNA. Humans produce several dif erent helicases. T e physiological roles of these helicases are largely unknown. Mutations in a few helicases are known to cause disease: a deficiency in WRN causes **Werner syndrome** (predominantly characterized by premature aging); a deficiency in BLM causes **Bloom syndrome** (accompanied by an increased rate of tumorigenesis); and a deficiency in RECQ4 causes **Rothmund-T omson syndrome** (associated with skin

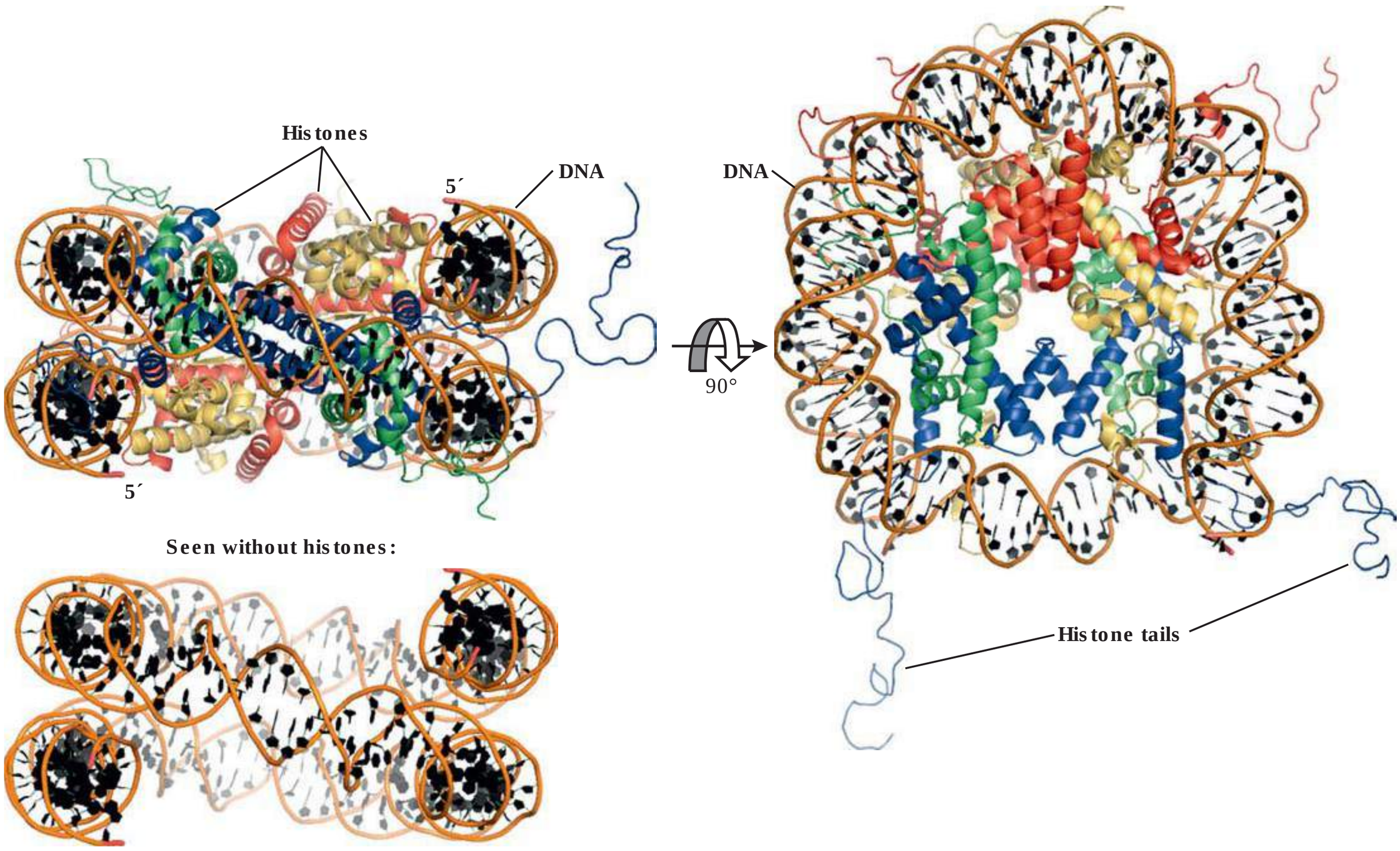

Fig. 1.6 Packing of DNA into a nucleosome core particle in the nucleus. Nucleotides are shown in black. An idealized cylinder through all phosphorus atoms is shown in light brown. DNA winds almost twice around a core of eight histone proteins. There are two copies each of histone H2A (gold), H2B (red), H3 (blue), and H4 (green). (Based on Protein Data Bank [www.rcsb.org] file 1KX5 from Davey CA, Sargent DF, Luger K, Maeder AW, Richmond TJ. Solvent mediated interactions in the structure of the nucleosome core particle at 1.9 Å resolution. *J Mol Biol.* 2002;319:1097–1113.)

Table 1.1 Modifications of Histones

Amino Acid	Side Chain Modification
Lysine	Methylation (mono-, di- or tri-; CH_3– is a methyl group) Acetylation (CH_3–CO– is an acetyl group) Ubiquitylation (ubiquitin is a 76-residue protein) Sumoylation (SUMO = small ubiquitin-like modifier, a small group of ~100-residue proteins) ADP-ribosylation (conjugation with a ribose that in turn forms a phosphodiester with ADP)
Arginine	Methylation (mono- or di-; there are two possibilities for dimethylation) Deimination (exchange of =NH for =O, turning arginine into citrulline)
Glutamate	ADP-ribosylation
Serine	Phosphorylation
Threonine	Phosphorylation
Tyrosine	Phosphorylation

ADP, adenosine diphosphate.

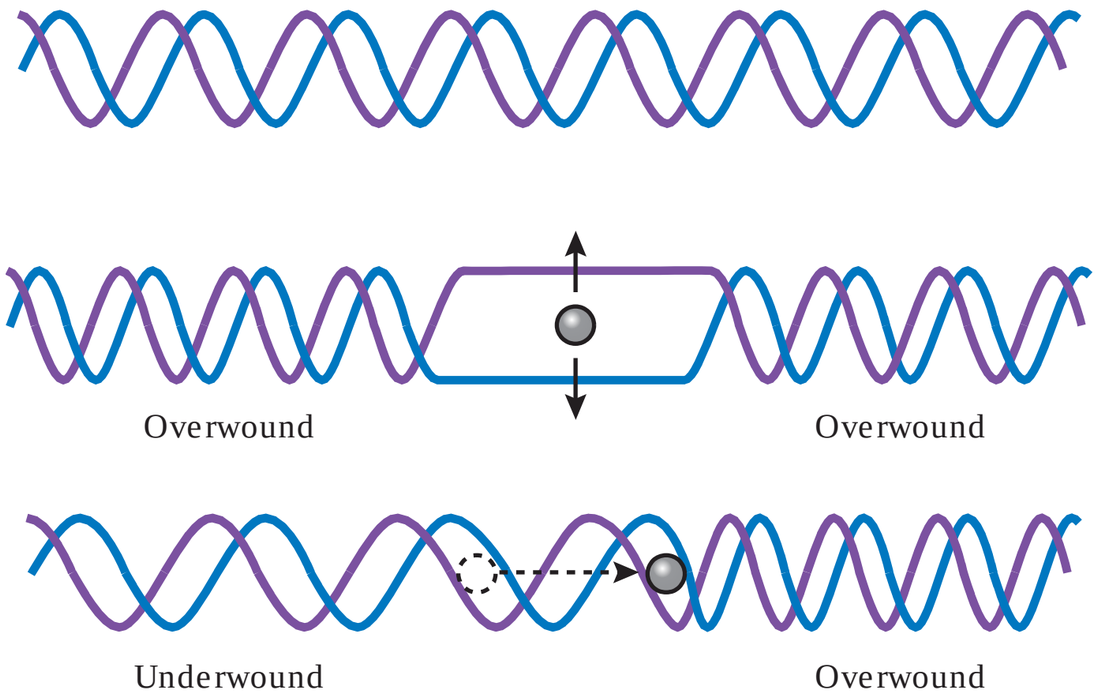

Fig. 1.7 **Strain imposed on double-helical DNA when the helix is opened up partially, or when a nonrotatable object moves in between the two complementary strands.**

abnormalities). All of these disorders are rare and show autosomal recessive inheritance.

Once a part of two complementary DNA strands has been separated, **single-strand binding proteins** (e.g., replication protein A [RPA]) can prevent the pairing of bases.

Topoisomerases can relieve strain in DNA and thus alter the topology of DNA. **Supercoiled DNA** is DNA that has folded back on itself to accommodate under- or overwinding (negative or positive supercoiling, respectively) of the double helix. Topoisomerase I and topoisomerase II both relax supercoiled DNA during replication and transcription. Topoisomerase II also untangles (decatenates) DNA for chromosome segregation during mitosis. Type I topoisomerases cut one strand, whereas type II topoisomerases cut both strands of a double helix. In both cases, the enzyme forms a transient covalent link with either the 5′ or 3′ end of the broken DNA.

Type I topoisomerases (including topoisomerase I) relieve the torsional strain of DNA by cutting one strand of the double helix, swiveling around the intact strand or passing the intact strand through the break, and then ligating the cut strand again (Fig. 1.8).

Inhibitors of topoisomerase I of er a means of preferentially damaging tumor cells that divide more frequently than normal cells. Analogs of **camptothecin** prolong the lifetime of a covalent DNA–topoisomerase I complex that is formed as a normal reaction intermediate. As the genome is copied during replication (see Chapter 3), the obstructing DNA–topoisomerase I–camptothecin complex can result in permanent strand breaks, which the cell may attempt to repair. When the number of double-strand breaks exceeds a cell's capacity for repair (see homologous recombination repair in Chapter 2), the cell undergoes apoptosis (i.e., programmed cell death; see Chapters 2 and 8). Camptothecin analogs (e.g., **topotecan** and **irinotecan)** are used predominantly in the treatment of advanced malignancies (e.g., relapsing small-cell lung cancer or metastatic ovarian cancer).

Type II topoisomerases (topoisomerase II in humans, and DNA gyrase and topoisomerase IV in bacteria) cleave both

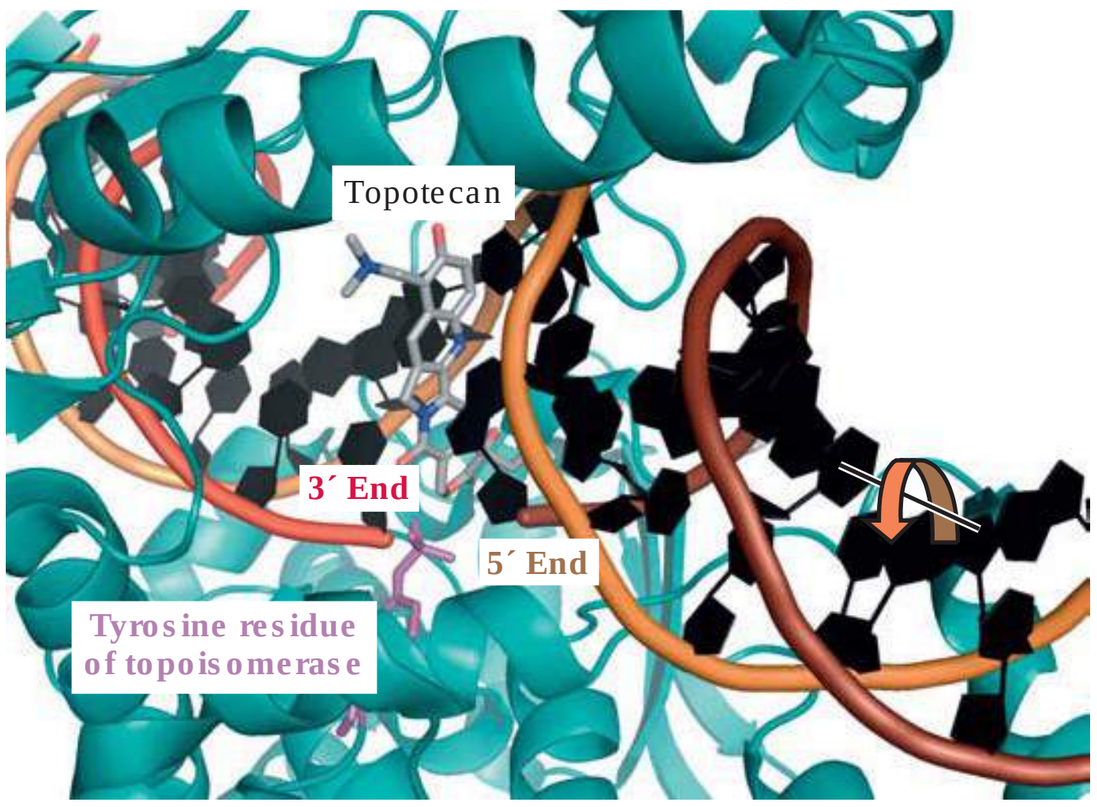

Fig. 1.8 **Topoisomerase I cuts one strand of DNA and swivels around the other strand.** The riboses and bases of DNA are shown in black. An artificial, smoothed backbone is drawn through the phosphorus atoms in red, brown, or orange (there are three segments of DNA). The enzyme is shown in greenish blue. A tyrosine residue (magenta) is covalently linked to the 3′ end of the "red" DNA chain. The "brown" DNA chain, together with a portion of the "orange" DNA chain, can rotate and thereby relieve torsion stress. Normally, the 5′ end of the "brown" chain then reconnects with the 3′ end of the "red" chain. Here, the chemotherapeutic drug topotecan (shown as a stick model with C in grey, N in blue, and O in red) binds in between the 3′ base of the "red" chain and the 5′ base of the "brown" chain; topotecan thereby prevents religation of these chains, which leads to cell death. (Based on Protein Data Bank [www.rcsb.org] file 1K4T from Staker BL, Hjerrild K, Feese MD, Behnke CA, Burgin Jr. AB, Stewart L. The mechanism of topoisomerase I poisoning by a camptothecin analog. *Proc Natl Acad Sci.* 2002; 99:15387–15392.)

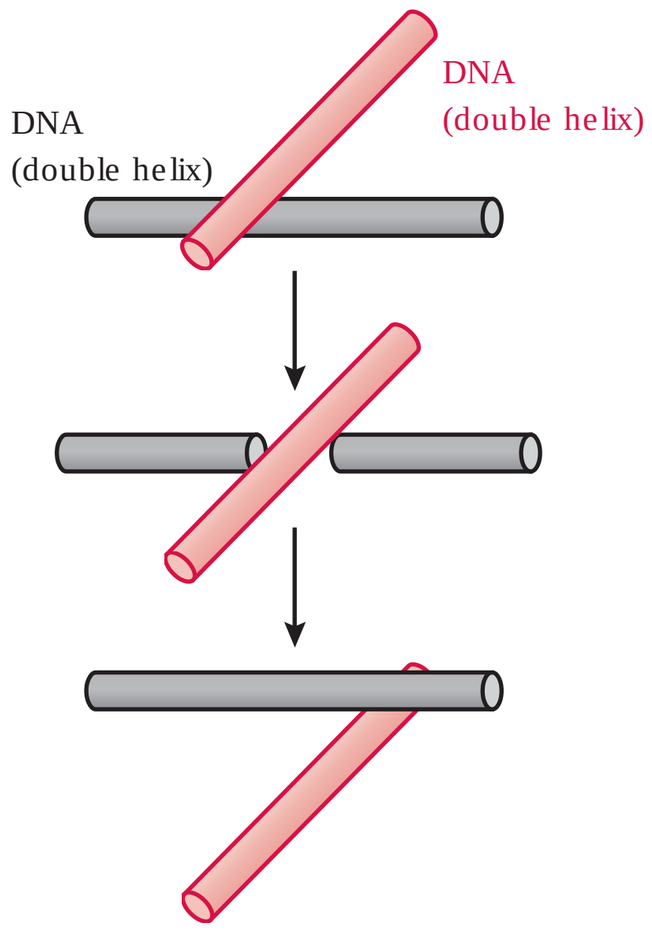

Fig. 1.9 **Reaction catalyzed by topoisomerase II.**

strands of one double helix, use conformational changes in the enzyme subunits to pass a separate DNA segment between the break, and then ligate the cut strands (Figs. 1.9 and 1.10). T is process requires ATP. Type II topoisomerases are involved in relaxing the supercoils that result from DNA replication or

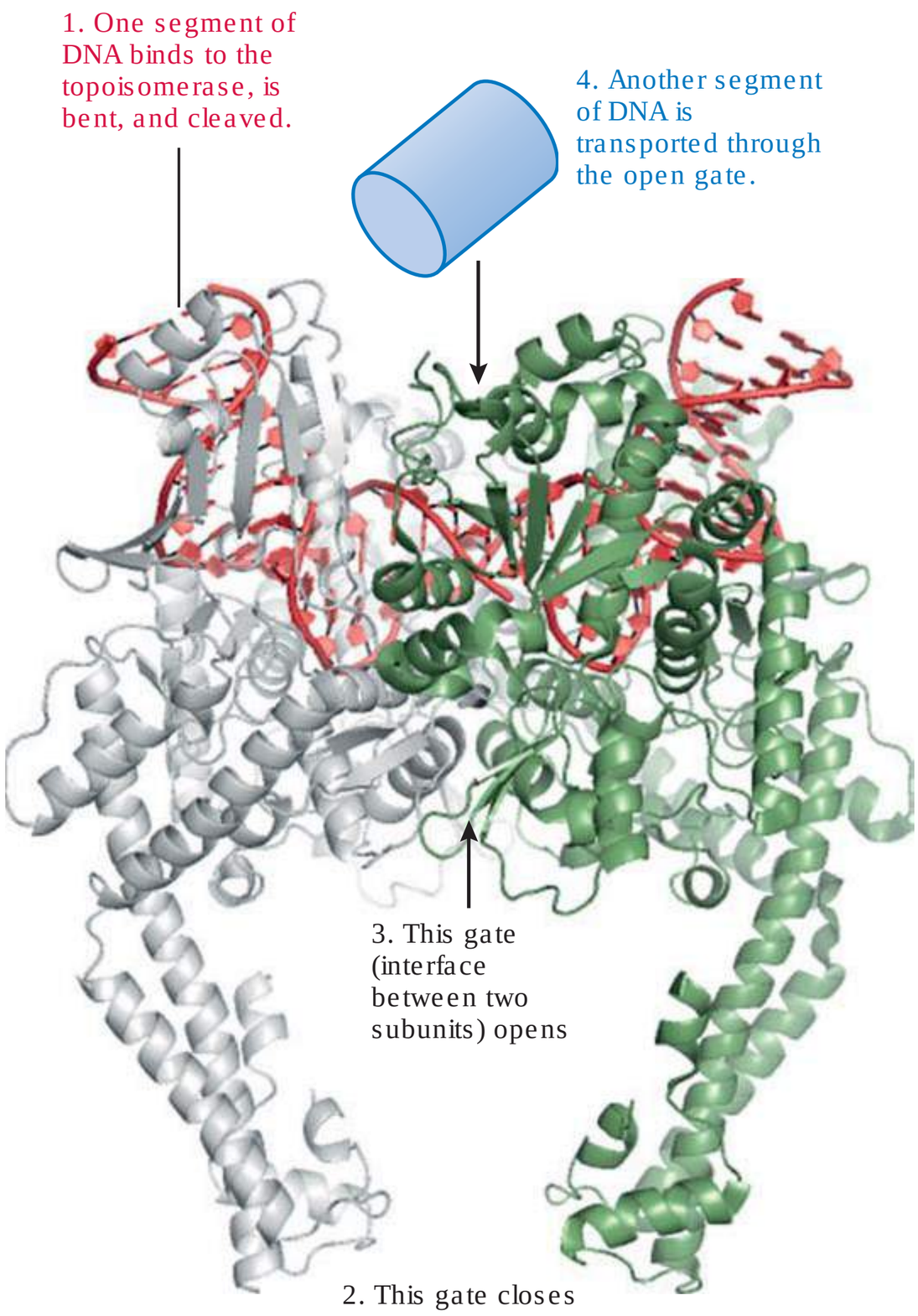

Fig. 1.10 Human topoisomerase IIα catalyzes the passage of one DNA strand through another DNA strand. The enzyme functions as a dimer. The image shows the catalytic core domain, including the central gate and the lower, C-terminal gate; not shown is the ATPase domain, which is at the top of the structure. (Based on Protein Data Bank [www.rcsb.org] file 4FM9 from Wendorff TJ, Schmidt BH, Heslop P, Austin CA, Berger JM. The structure of DNA-bound human topoisomerase II alpha: conformational mechanisms for coordinating inter-subunit interactions with DNA cleavage. *J Mol Biol.* 2012;424: 109–124.)

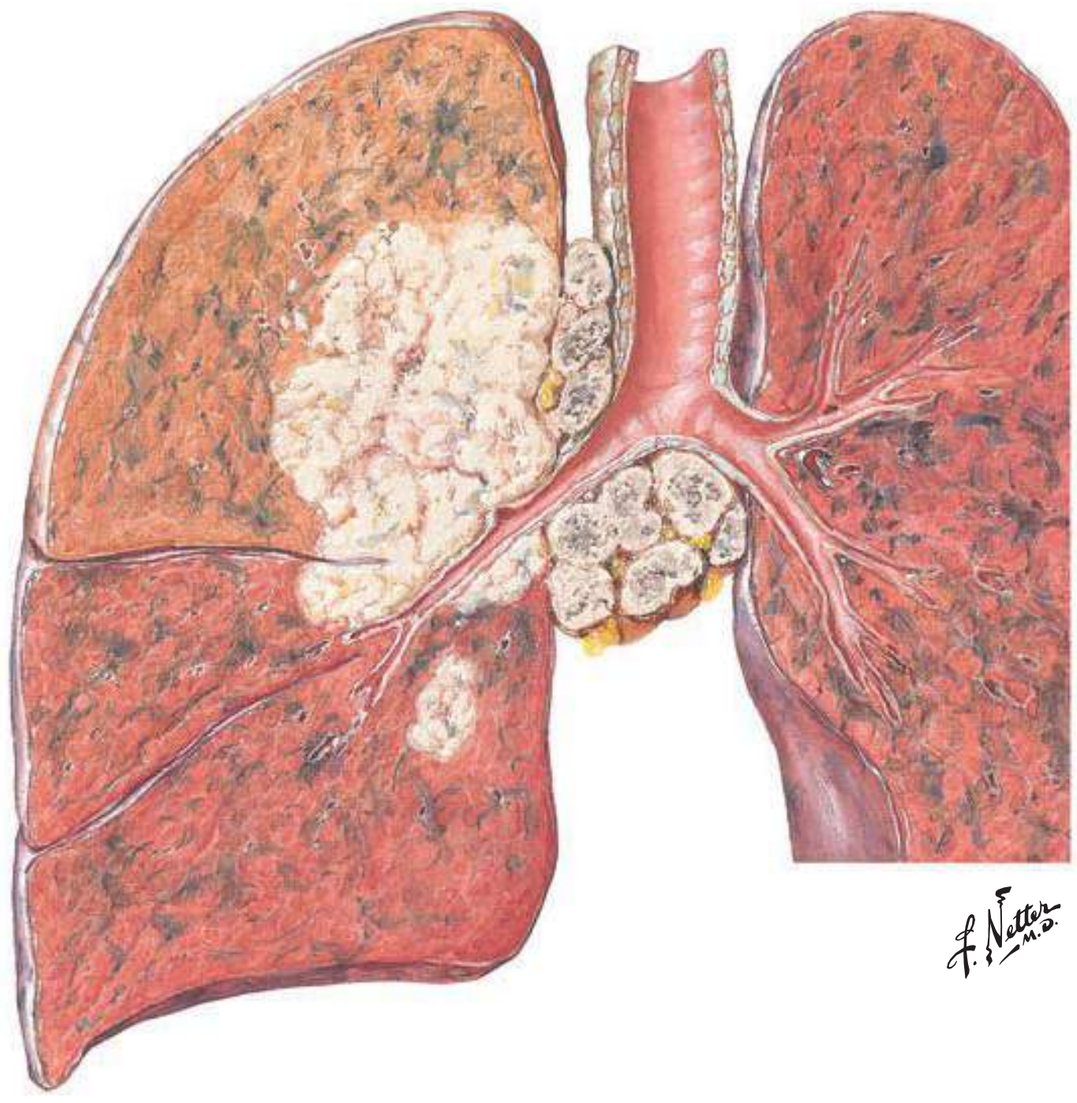

Fig. 1.11 Etoposide is an inhibitor of topoisomerase II and is often used in the treatment of extensive small-cell lung cancer. These patients typically have disseminated disease. Etoposide is often combined with a platinum drug.

transcription. Sister chromatids become intertwined during DNA replication; this linking is called catenation. The essential function of type II topoisomerases, which cannot be performed by type I enzymes, is the separation (decatenation) of replicated chromosomes before compaction and cell division.

Inhibitors of topoisomerase II are useful as anticancer agents. Most of these inhibitors are part of a class called topoisomerase II poisons. Two drugs of this class that are widely used in chemotherapy are **doxorubicin** (an anthracycline) and **etoposide** (an epipodophyllotoxin; Fig. 1.11). In the presence of these drugs, topoisomerase II can cleave DNA but cannot ligate it. Therefore, DNA replication and transcription are both inhibited. As a consequence, DNA strand breaks accumulate and lead to apoptosis (programmed cell death). However, these drugs are also mildly mutagenic and thus increase a patient's risk of developing therapy-related leukemia. Aside from poisons, catalytic inhibitors of topoisomerase II inhibit other portions of the catalytic mechanism of the enzyme (e.g., ATP hydrolysis) and cause cell death without inducing DNA strand breaks.

Some polyphenols in our diet also poison topoisomerase II. Soybeans contain **genistein**, which binds to estrogen receptors and can help ameliorate symptoms of menopause. Genistein also poisons topoisomerase II. Genistein appears to have anticancer activity, but in pregnant mothers it also confers a higher risk of childhood leukemia in the of spring. Green tea contains the polyphenol **epigallocatechin gallate** (EGCG), which also poisons topoisomerase II. The biological impact of these poisons has not been fully established, and there is some evidence that these agents may be chemopreventive.

Fluoroquinolone antibacterials inhibit bacterial DNA gyrase (the name given to the positively supercoiling topoisomerase II in bacteria) and topoisomerase IV. Commonly used quinolones are the broad-spectrum antibiotics **ciprofloxacin**, levofloxacin, ofloxacin, and moxifloxacin.

6. HUMAN KARYOTYPE

The human karyotype consists of 46 chromosomes. Stained metaphase chromosomes are used for karyotyping.

Each normal human cell nucleus in the G_0 phase of the cell cycle (see Chapter 8) contains 46 chromosomes (i.e., 46 DNA double helices). In preparation for cell division, the 46 double helices are replicated to form 92 double helices (see

Chapter 3). T en, each one of these helices is greatly condensed into **chromatids** (see Section 4). Proteins join pairs of identical chromatids at their centromeres to form **metaphase chromosomes**.

With basophilic stains (e.g., **Giemsa stain**), metaphase chromosomes can be visualized under a light microscope (Fig. 1.12). Images of stained chromosomes are used to characterize the chromosomes of an individual (i.e., to describe an individual's **karyotype)**. Stains used in karyotyping produce various diagnostically useful **banding patterns**, which depend on the staining procedure used, the degree of DNA compaction, and the presence of DNA-bound proteins.

Two of the 46 chromosomes are called **sex chromosomes**; the remaining 44 chromosomes are called **autosomes**. Humans typically inherit one sex chromosome and 22 autosomes from each parent. T ere are two types of sex chromosomes, X and Y. Each female with a normal karyotype has two X chromosomes (one of which gets inactivated by methylation; see Chapter 5). Each male with a normal karyotype has one X and one Y chromosome. T e 22 autosomes are numbered from 1 to 22 in approximate order of decreasing size (see Fig. 1.12).

Segregation of chromosomes occurs during both cell division (mitosis) and gamete formation (meiosis). During **mitosis**, pairs of chromatids are pulled apart so that each daughter cell gets 46 chromatids (i.e., 46 DNA double helices). In nondividing cells, the term **chromosome** is used to designate a single chromatid (i.e., a single DNA double helix). Every cell in the G_0 phase of the cell cycle has 46 chromosomes (in this case, 46 DNA double helices). During **meiosis I**, homologous chromosomes form pairs that are then pulled to separate poles (yielding only 23 chromosomes per cell, whereby each chromosome contains two chromatids). During **meiosis II**, paired chromatids are pulled apart to yield cells that contain only 23 chromatids (i.e., 23 DNA double helices).

Cells contain more than 100 times more DNA in their nucleus than in their mitochondria. Although a cell's network of mitochondria contains thousands of copies of **mitochondrial DNA**, even the shortest of the 46 chromosomes contain more than 3000 times the number of base pairs in the mitochondrial genome.

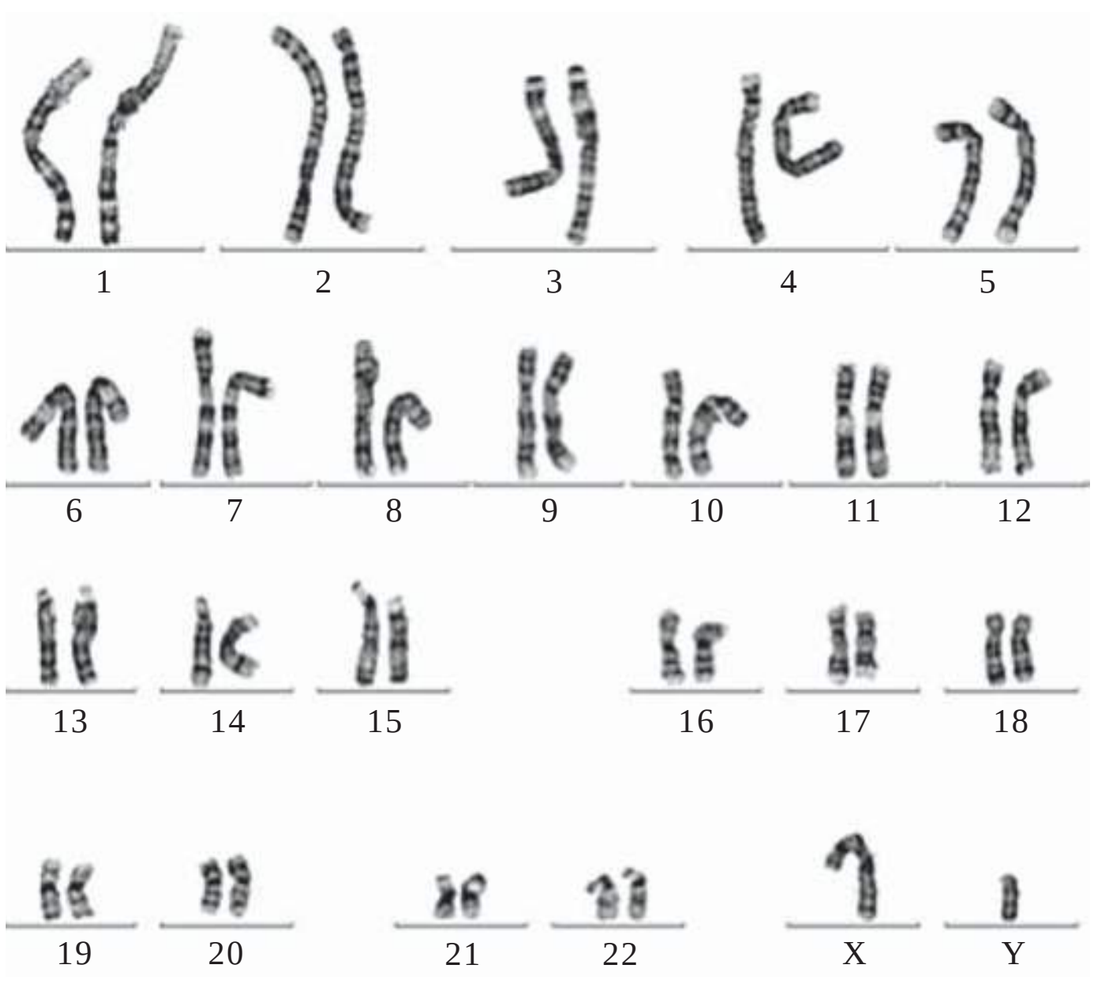

Fig. 1.12 Normal male karyogram. For karyotyping, cultured cells are arrested in metaphase. This karyogram shows the light-microscopic images of stained chromosomes from a single cell. The chromosomes are sorted and analyzed according to their size and banding pattern. (Courtesy Dr. Barry L. Barnoski, Oncocytogenetics Laboratory, Cooper University Hospital, Camden, NJ.)

SUMMARY

- DNA is a polymer of dAMP, dCMP, dGMP, and dTMP. T e bases of these nucleotides can hydrogen bond to form AT or GC base pairs. A and T are thus complementary bases, as are G and C.
- DNA is mostly present as double helices that consist of two complementary DNA strands. Complementary strands pair in a head-to-tail fashion (i.e., the 5′ end of one strand is paired with the 3′ end of its complementary strand). Unless indicated otherwise, DNA sequences are written in a 5′→3′ direction.
- DNA binding proteins can bind selectively to a specific DNA sequence by interacting with the atoms of bases that are at the surface of the DNA helix grooves.
- T e length of nuclear DNA molecules far exceeds the diameter of the nucleus. Inside the nucleus, most of the DNA is condensed into nucleosomes; this, in turn, is condensed into higher-order structures. T ese structures play critical roles in the regulation of transcription and make the orderly separation of DNA molecules possible during cell division.
- Helicases separate complementary strands of DNA. Single-stranded DNA binding proteins prevent the pairing of separated strands. Topoisomerases cut one or both strands of double-helical DNA, relieve torsional strain (topoisomerases I and II) or untangle chromosomes in preparation for mitosis (topoisomerase II), and then religate the strands. Inhibitors of topoisomerases are used in chemotherapy for cancer.
- Human cells with a normal karyotype contain 46 chromosomes: 23 from the mother and 23 from the father. Of the chromosomes, 44 are autosomes and two are sex chromosomes.

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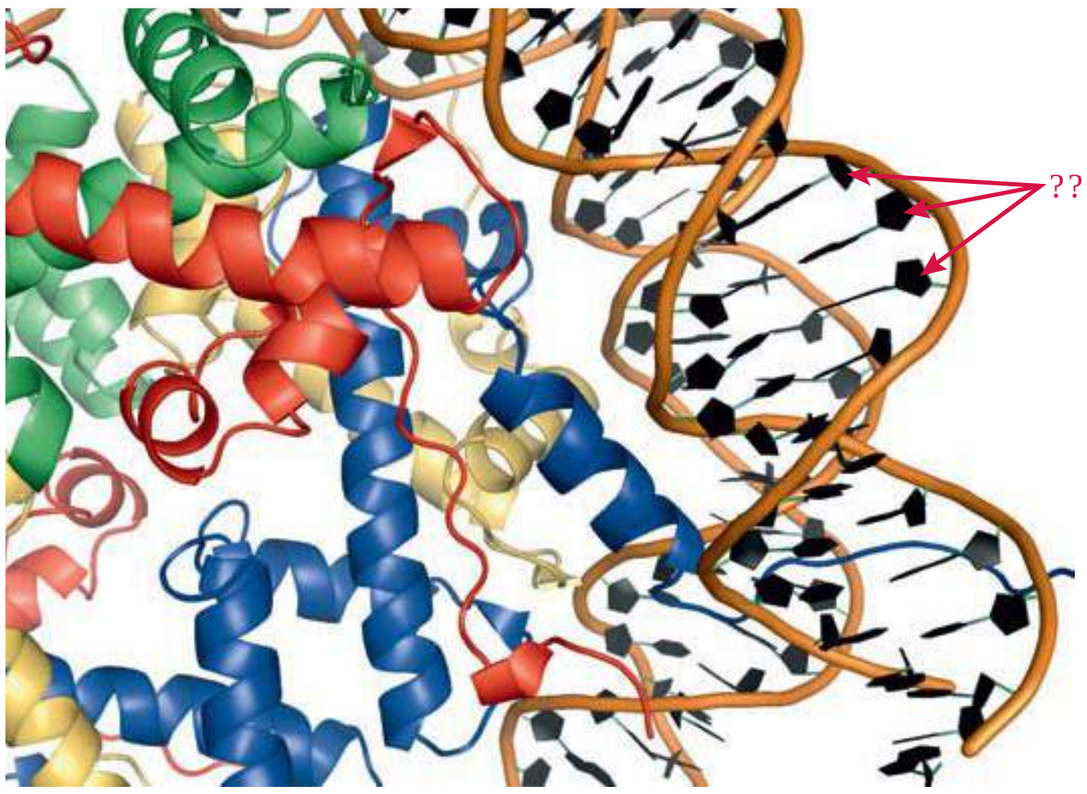
Review Questions



1. T e figure above shows part of a nucleosome. T e three pentagons identified by arrows represent which of the following?

   A. Deoxyriboses
   B. Phosphate groups
   C. Proline residues
   D. Pyrimidine bases

2. In the image shown in Question 1, the DNA binds to positively charged amino acid residues of histones via which of the following?

   A. Covalent bonds
   B. Electrostatic interactions
   C. Hydrogen bonds

3. A 69-year-old male patient with metastatic colon cancer receives treatment with a cocktail of chemotherapeutic drugs that contains irinotecan. T is drug inhibits which one of the following processes?

   A. Modification of histone tails
   B. Pairing of complementary bases
   C. Reading of bases in the major groove
   D. Relaxation of supercoiled DNA

4. Many DNA-based diagnostic tests use a DNA polymerase from T ermus aquaticus, a bacterium that can survive high temperatures. Compared with the DNA of bacteria that grow at 25°C, the DNA of *T.* aquaticus is expected to have a higher fraction of which of the following nucleotides?

   A. A and C
   B. A and G
   C. A and T
   D. C and G
   E. C and T
   F. G and T

Chapter 2 DNA Repair and Therapy of Cancer

SYNOPSIS

- DNA damage may be due to the inherent properties of DNA or the damaging effects of ultraviolet light, radiation, drugs, or noxious agents in the environment. Damage may manifest as lesions to nucleotides, DNA adducts, crosslinks within or between DNA strands, or single- or double-strand breaks in the DNA. Diverse DNA repair mechanisms exist, ensuring near constancy of the genome.
- Knowledge of DNA repair is important for understanding how inadequate DNA repair leads to tumorigenesis and how chemotherapy and radiotherapy of cancer can lead to overwhelming damage and death of tumor cells, as well as neoplasms among previously normal cells.
- DNA repair has been studied extensively in bacteria and yeast. Although humans have more complex DNA repair pathways than these single-cell organisms, DNA repair proteins are highly evolutionarily conserved. Appropriately, many human DNA repair proteins are named after their counterparts in bacteria and yeast.
- The base-excision repair pathway becomes active when a single nucleotide is altered. The faulty nucleotide is excised and replaced with a new one that fits the complementary DNA strand.
- The mismatch repair pathway detects mismatches of base pairs and bulges due to missing or excess nucleotides that arise from faulty DNA replication. The most recently synthesized portion of DNA is removed, and new DNA is synthesized based on the complementary DNA strand.
- The nucleotide-excision repair pathway repairs damage that grossly distorts DNA. Such damage may stem from exposure to the sun, cigarette smoke, or platinum chemotherapeutic drugs. A section of the damaged strand is cut out, and the DNA is then resynthesized.
- Nonhomologous end-joining repairs double-strand breaks by joining the broken ends. The product is often different from the original DNA. Double-strand breaks can arise from DNA damage by x-rays or chemotherapeutic drugs.
- The homologous recombination pathway repairs double-strand breaks by producing long, single-strand overhangs that invade a homologous DNA strand. The invaded strand then serves as a template for resynthesis of the lost DNA.
- Hereditary and acquired defects in DNA repair favor tumorigenesis (e.g., in the colon, breast, ovaries, pancreas, and skin).

LEARNING OBJECTIVES

For mastery *of* this topic, you should be able to do the *f*ollowing:

- Summarize the major DNA repair pathways.
- Describe how ultraviolet light and high-energy x-rays damage DNA, and how this damage is repaired.
- Describe how polycyclic aromatic hydrocarbons in cigarette smoke damage DNA and how this damage is repaired.
- Explain how platinum drugs and nitrogen mustards (e.g., cyclophosphamide) damage DNA and how this damage is repaired.
- Describe and explain commonly used lab tests for DNA mismatch repair in biopsy tissues.
- Explain the term microsatellite instability, describe a lab test for microsatellite instability, and link microsatellite instability to a deficiency in a DNA repair pathway.
- List hereditary cancer syndromes and specify the associated defects in DNA repair, as well as the pattern of inheritance. List any modifications in chemotherapy or radiotherapy of tumors that must be made for affected patients.
- Describe how chemotherapy and radiotherapy kill tumor cells and how these treatments can be tumorigenic in normal cells.

1. BASE-EXCISION REPAIR

The base-excision repair (BER) pathway deals with common *f*orms o*f* damage to a single nucleotide. It removes an altered nucleotide, adds the proper deoxyribonucleotide, and seals the cut in the DNA. About 1% o*f* all patients who have colon cancer have two de*f*ective copies o*f* the **MUTYH** gene that encodes an enzyme needed *f*or BER.

Every day, in every cell, thousands of bases in DNA are altered (Fig. 2.1). Bases (mostly adenine or guanine) are spontaneously **lost** from the DNA deoxyribose backbone. Bases can be **deaminated**, especially 5-methylcytosine and cytosine, which thus give rise to thymine and uracil, respectively. **Hydroxyl radicals** can react with bases; this happens especially with guanine, forming **8-oxo-guanine** (also called **8-hydroxyguanine)**. The physiological methyl-group donor S-adenosylmethionine can react with adenine to form **3-methyladenine**. **Ionizing radiation** (e.g., **x-rays**, **γ-rays**) can ionize water (thereby giving rise to a hydroxyl radical), oxidize a base, cleave a base from the deoxyribosephosphate backbone, or fragment a deoxyribose and thereby cut one of the complementary DNA strands.

In **the short-patch BER** pathway, enzymes recognize a damaged nucleotide and then replace it (Fig. 2.2). Humans produce many **DNA glycosylases** that slide along DNA, recognize deaminated, hydroxylated, or methylated bases, and remove them. This generates a substrate that is recognized by AP endonuclease 1, which cuts the DNA where the base is missing. Polynucleotide kinase/phosphatase (PNKP) then phosphorylates the free 5′ end and dephosphorylates the adjacent 3′ end. **Poly-ADP-ribose polymerase** (**PARP**) binds to the strand break and recruits the protein **XRCC1**, which serves as a platform for recruiting other repair proteins. Then, **DNA polymerase β** excises the abasic deoxyribose and replaces it with a proper new nucleotide. Finally, **DNA ligase**

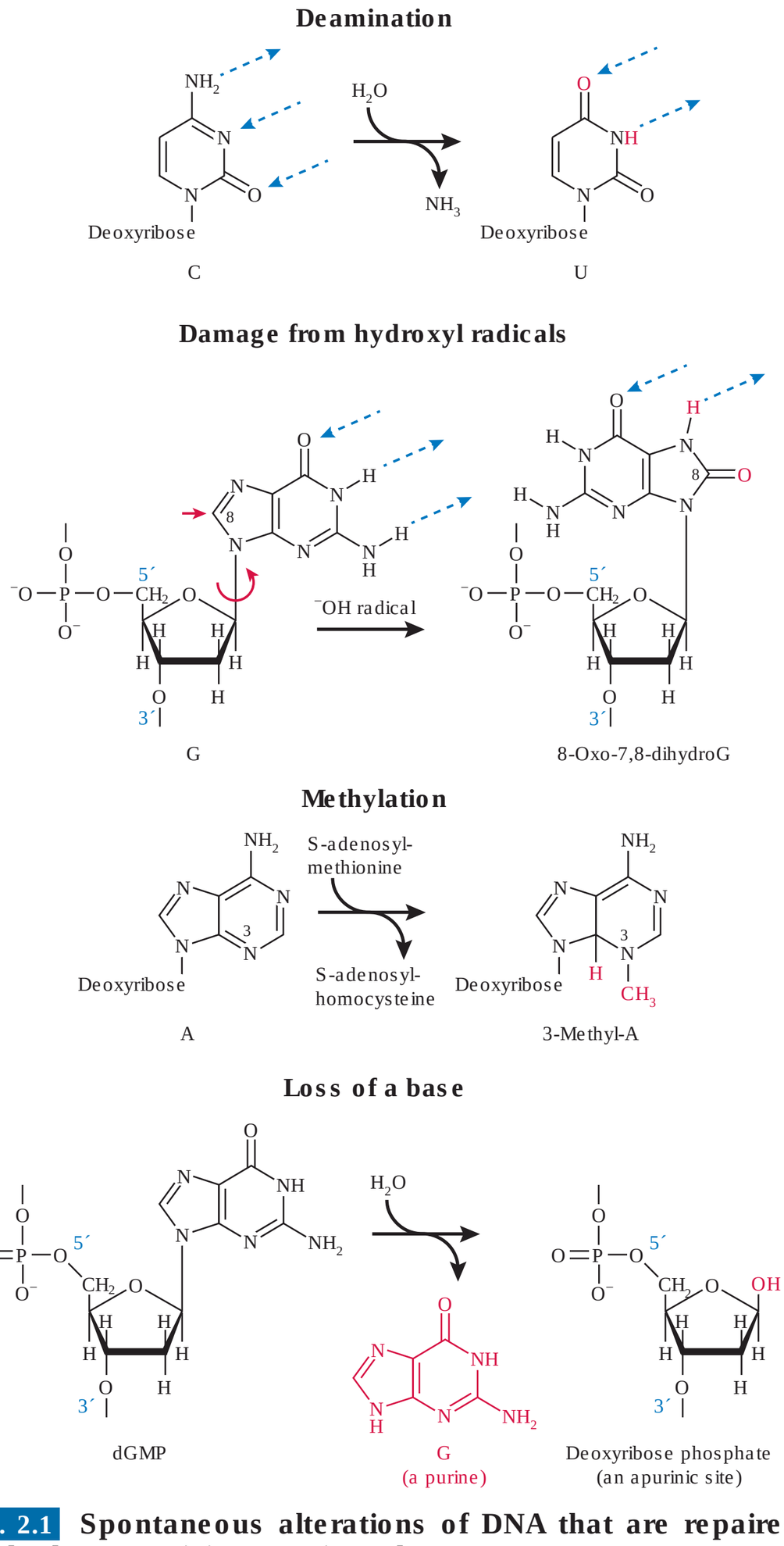


Fig. 2.1 Spontaneous alterations of DNA that are repaired by the base-excision repair pathway.

IIIα seals the nick in the DNA strand to reestablish a contiguous DNA molecule.

A single defect also triggers the **long-patch BER pathway**, which replaces 2 to 10 consecutive nucleotides. Certain oxidation products of a **deoxyribose** must be removed via the long-patch pathway. In addition, this pathway completes some of the repairs that cannot be completed by single-patch BER.

If damage to a base is not repaired, DNA **replication** (see Chapter 3) may insert an incorrect nucleotide, or it may come to a halt until the damaged base is repaired. Replication stops when methyladenine (see Fig. 2.1) is present so that BER can replace methyladenine with adenine. When a base is missing, **translesion DNA synthesis** inserts a nucleotide into the newly synthesized DNA strand, but this nucleotide may be the wrong one. If replication inserts an inappropriate base opposite a damaged one, the **mismatch repair (MMR)** pathway (see Section 2) often detects the error. For instance, it recognizes an A opposite a U (from deamination of C) or opposite an oxo-G (from the hydroxylation of guanine).

The enzyme **DNA MYH glycosylase**, encoded by the MUTYH gene, partners with the MMR pathway (see Section 2) to excise A opposite 8-oxo-guanine (see Fig. 2.1). The BER pathway then replaces the 8-oxo-G with G. MUTYH stands for MutY homolog (from bacteria).

About 1% of all patients who have **colorectal cancer** have **MUTYH-associated polyposis** (**MAP**), a disease that is caused by deficient **DNA MYH glycosylase** activity. The disease shows autosomal recessive inheritance. In patients with MAP, G→T mutations accumulate (persistent A opposite 8-oxo-G yields a T in place of 8-oxo-G in the next round of replication). Interestingly, such G→T mutations are found in the same genes that are mutated or no longer transcribed in some patients who have sporadic colorectal cancer (i.e., in patients who do not have MAP). In patients with MAP, colon cancer typically occurs in the late forties. At this time, the colon often contains tens to hundreds of polyps.

PARP inhibitors are in clinical trials for patients who have tumors with defective homologous recombination (HR) repair (see Section 4) and therefore rely unusually heavily on PARP-dependent BER and nonhomologous end joining (NHEJ; see Section 4). PARP inhibitors inhibit BER and NHEJ because poly-ADP-ribose recruits DNA repair proteins (e.g., XRCC1) to damaged sites in the DNA. While PARP inhibitors are relatively innocuous to normal cells, they are especially toxic to tumor cells that are deficient in BRCA1 or BRCA2, proteins that play a role in HR repair.

2. MISMATCH REPAIR

The mismatch repair (MMR) system handles improper base matches, as well as single-strand loops that stem from insertions or deletions during replication. Repair is directed to the most recently synthesized DNA strand. MMR is defective in ~10% to 20% of sporadic cancers of the colon, rectum, stomach, or endometrium. Hereditary mutations that affect MMR are the cause of Lynch syndrome, which most often causes colorectal or endometrial cancer.

The MMR pathway detects noncomplementary base pairs and repairs them. Mismatches may stem from the following (Fig. 2.3): (1) spontaneous tautomerization of bases during DNA polymerization; (2) deamination of cytosine to uracil or of 5-methylcytosine to thymine; (3) DNA polymerase error of inserting a base that is not complementary to the DNA strand that is being copied; and (4) DNA polymerase slippage in nucleotide sequence repeats.

In MMR, **MSH** proteins recognize the damage and **MLH** proteins help initiate the excision of a stretch of the most recently synthesized DNA (Fig. 2.4). Heterodimeric MSH proteins (homologs of bacterial MutS proteins; Table 2.1) detect mispaired and unpaired bases. MLH proteins (homologs of bacterial MutL proteins) then attach to the MSH proteins. In

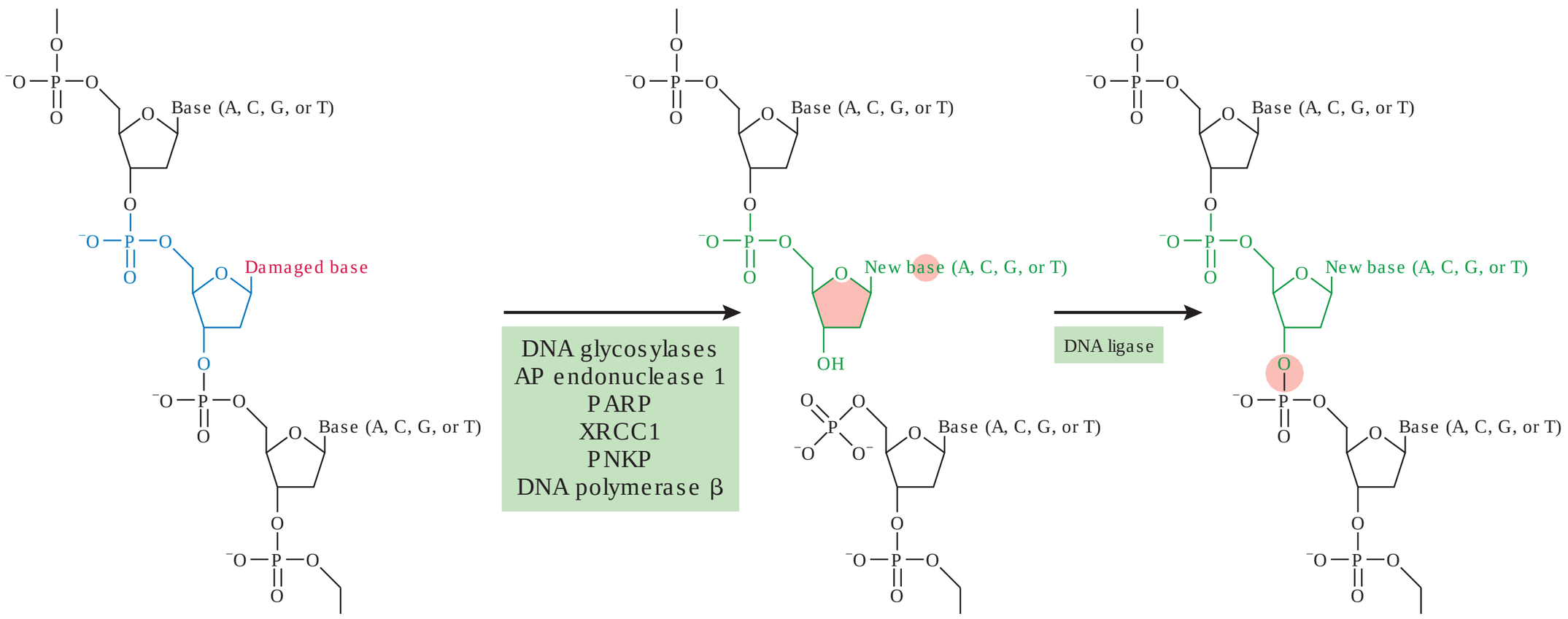


Fig. 2.2 **The short-patch base-excision repair pathway.**

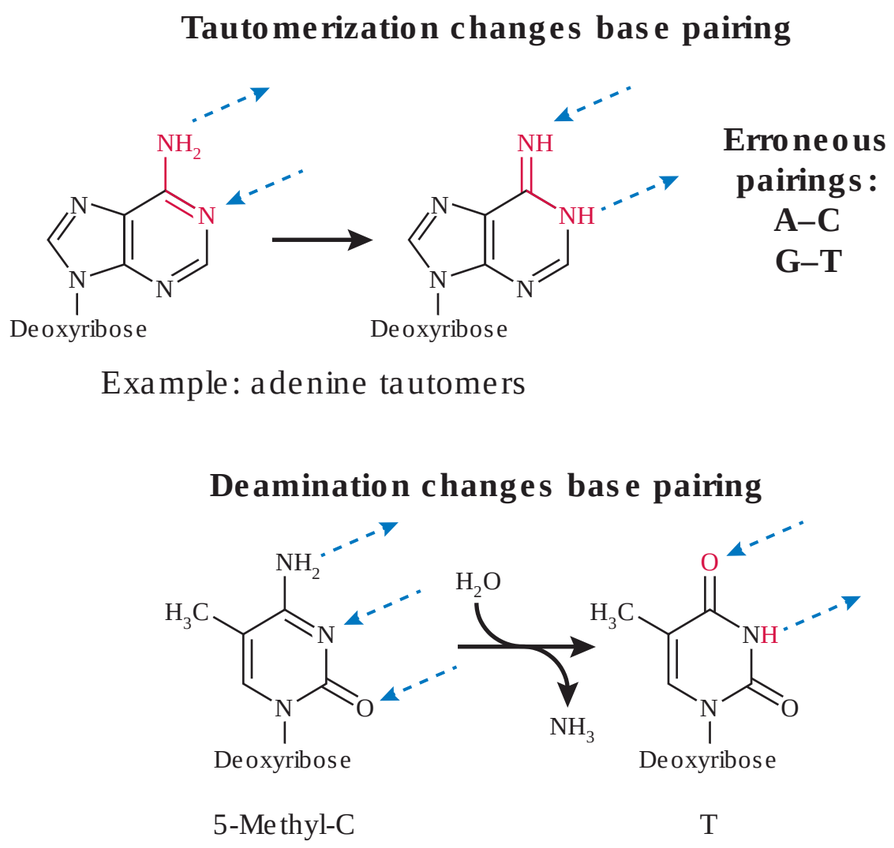


Fig. 2.3 **Causes of base mismatches that are repaired by the mismatch repair pathway.** *Blue arrows* indicate hydrogen bonding for base pairing.

addition, MLH proteins bind to an exonuclease. Tethered to MSH and MLH proteins, the **exonuclease 1** (Exo1) begins the degradation of the most recently synthesized DNA strand at a nearby cut in the DNA (it is unclear how this cut arises). **DNA polymerase** δ then resynthesizes the missing portion of

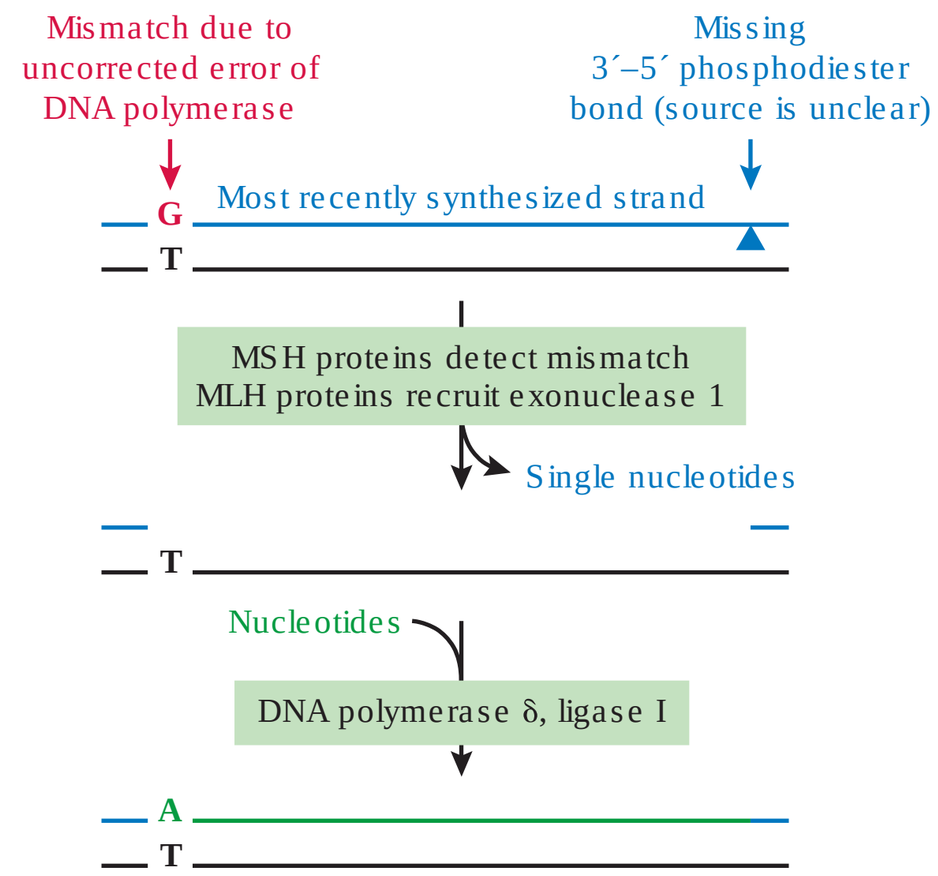


Fig. 2.4 **DNA mismatch repair.** Shown is the repair of a G–T mismatch.

the DNA strand, using the complementary strand as a template. Finally, **DNA ligase I** ligates the pieces of DNA.

Inactivation of the DNA MMR system leads to an increased susceptibility to cancer, especially **cancer** of the colon, stomach, or endometrium. Cells with impaired MMR accumulate mutations at a vastly increased pace. T is leads to frameshift mutations that impair the production and function of tumor suppressors, prevent programmed cell death, or alter signaling, transcription, or immune surveillance.

T e tumors from ~15% of patients who have **colon cancer** and ~20% each of patients who have **endometrial** or **gastric cancer** have defective MMR systems. In patients with a **sporadic** form of this cancer, the MMR deficiency is usually due to the **methylation** of the promoter of both copies of the **MLH1** gene. T e methylation essentially abolishes the expression of the MLH1 protein.

Table 2.1 Proteins That Are Involved in DNA Mismatch Repair in the Nucleus

Absolutely Required Protein	Possible Partners to Form a Heterodimer*	Heterodimer
RECOGNITION OF MISMATCH		
MSH2†	MSH3 (for up to 15 extra or missing nucleotides in microsatellites)	MutSβ
	MSH6† (for mismatches and ≤2 extra or missing nucleotides in microsatellites)	MutSα
INITIATION OF NUCLEOTIDE EXCISION		
MLH1†	PMS1	MutLβ
	PMS2† (used in most mismatch repair processes)	MutLα
	MLH3	MutLγ

*One partner can partially make up for another.
†Most patients with Lynch syndrome inherited an inactivating mutation in MSH2, MSH6, MLH1, or PMS2.

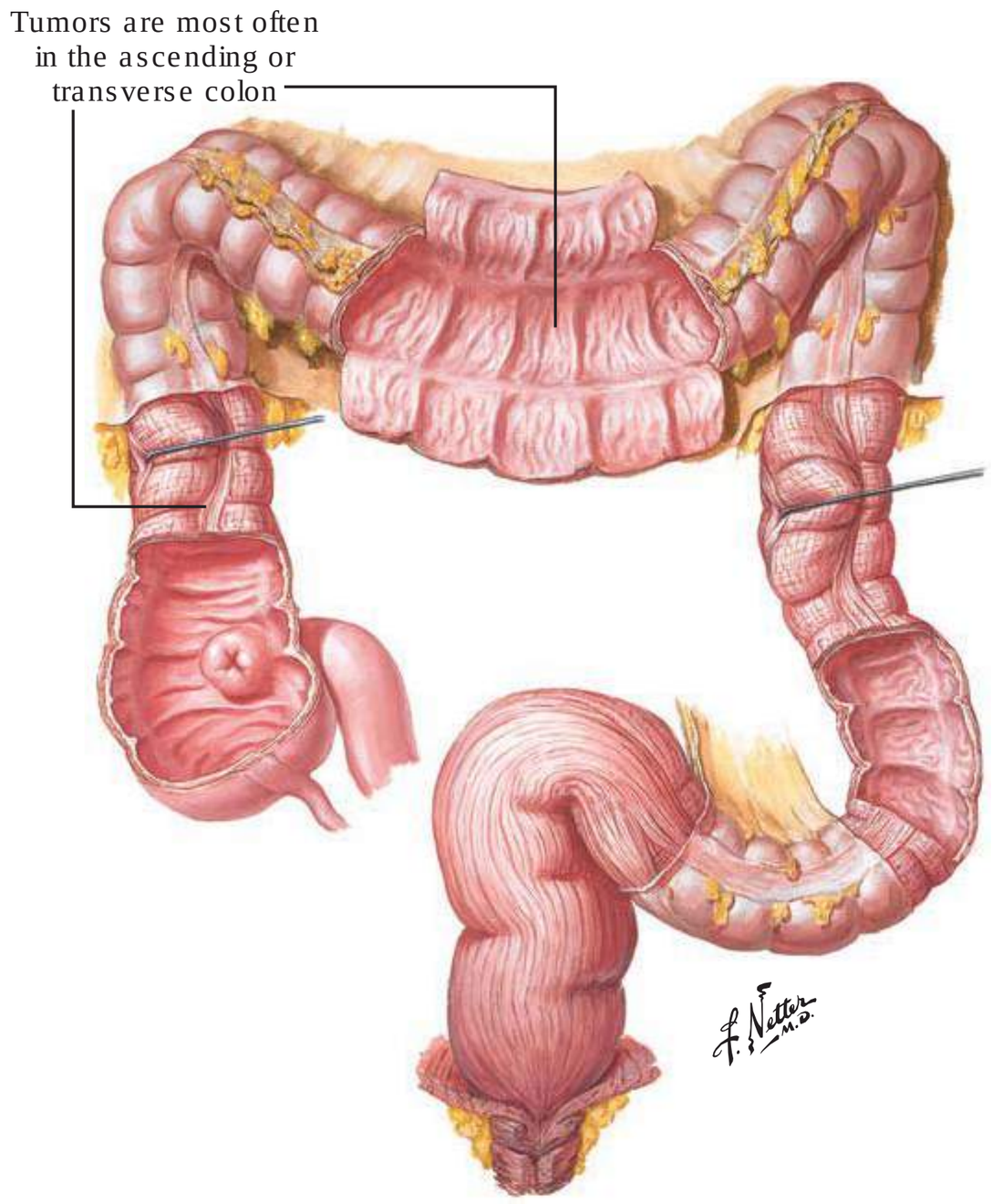


Fig. 2.5 Patients with hereditary defects in mismatch repair have Lynch syndrome and are at a high risk of colon cancer.

Lynch syndrome is a **hereditary** predisposition to cancer that is due to a germline mutation in a DNA MMR gene (Fig. 2.5). At least 1 in 1,000 individuals has Lynch syndrome, and about 3% of all patients who have colon cancer have Lynch syndrome. Most patients with Lynch syndrome inherited a mutation that inactivates MSH2, MSH6, MLH1, or PMS2 (see Table 2.1). The second copy of the gene or its associated promoter then undergoes mutation or epigenetic inactivation (via DNA methylation) in certain cells of the body, such that no functional protein is produced in these cells (e.g., in the colon). Patients who have Lynch syndrome have a ~70% lifetime risk for cancer of the colon, ~40% risk for cancer of the endometrium, and ~15% risk for cancer of the stomach or an ovary. These cancers occur at an unusually early age (e.g., colon cancer typically in the mid-40s). In contrast to patients who have a sporadic tumor with defective MMR mechanisms, patients who have Lynch syndrome also have a mutant MMR gene in blood lymphocytes. Since only one defective allele needs to be inherited, the disease shows autosomal dominant inheritance (see Chapter 5). This means that if only one parent is affected, each offspring has a 50% chance of inheriting the disease.

Immunohistochemical detection of MMR proteins in a tumor of the colon or endometrium is often part of the diagnosis of an MMR deficiency. The tissue is commonly stained for MLH1, MSH2, PMS2, and MSH6. Patients who have sporadic colon cancer or endometrial cancer due to hypermethylation of the promoter for the MLH1 gene do not show immunoreactivity for MLH1. Similarly, patients who have Lynch syndrome, and therefore only a mutant version of a particular MMR protein, exhibit an absence or greatly reduced immunoreactivity for that protein. To complicate matters (see Table 2.1), a lack of MLH1 often leads to the degradation of the PMS2 protein, and a lack of MSH2 leads to the loss of the MSH6 protein (but not vice versa). Results of immunohistochemical assays can be used for guidance in DNA-based testing for mutations, hypermethylation, and microsatellite instability (see below).

Defective MMR can be detected as the **microsatellite instability (MSI)** of DNA. Microsatellites are 5- to 100-fold repeats of sequences that contain one to five nucleotides [e.g., $(A)_{16}$ or $(GT)_9$]. Microsatellites are also called **short tandem repeats**. When a patient is tested for MSI, DNA is obtained from the excised tumor and occasionally also from peripheral blood lymphocytes (the DNA in lymphocytes is assumed to be representative of the DNA in the germline). By using PCR (see Chapter 4), DNA that contains certain microsatellites (e.g., the mononucleotide repeats BAT25 and BAT26 and the dinucleotide repeats D2S123, D5S346, and D17S250) is amplified and analyzed for size. BAT26 is within the MSH2 gene, but the remaining microsatellites are outside the genes that encode MMR proteins. In MMR-deficient tumor cells, these repeats usually become shortened, giving rise to a shorter piece of PCR-amplified DNA. Most tumors that have MSI show abnormal lengths of four or five of the five microsatellite sequences mentioned. A tumor is usually said to have **high MSI** instability if two or more of the five tested microsatellites

show a change in length. If only one of the five microsatellites is unstable, there is **low MSI**. If all five microsatellites are stable, the tumor is said to be **microsatellite stable**.

In patients who have tumors that show MSI, pathogenic changes can occur in the lengths of the A_{26} microsatellite BAT26 in the MSH2 gene, in the C_8 microsatellite of the MSH6 gene, in the A_{10} microsatellite of the **tumor growth factor β receptor 2** gene, or in the G_8 tract of the gene for the cell-death-inducing protein **BAX**. Changes in the repeat lengths of the other four diagnostically measured microsatellites (BAT25, D2S123, D5S346, D17S250) are not known to be pathogenic.

Patients who have a colon tumor that demonstrates microsatellite instability do not derive any benefit from adjuvant chemotherapy with **5-fluorouracil** (the mechanism of action of fluorouracil is described in Chapter 37). In contrast, 5-fluorouracil is often part of adjuvant chemotherapy for microsatellite-stable colon tumors.

Persons who are **homozygous** or compound heterozygous for mutations in DNA MMR proteins not only develop gastrointestinal cancer but also have brain tumors and hematologic malignancies in childhood. T is disorder is called **constitutional MMR deficiency syndrome** (**CMMR-D**).

3. NUCLEOTIDE-EXCISION REPAIR

T e nucleotide-excision repair (NER) pathway recognizes distortions o*f* the DNA double helix that arise *f*rom environmental insults (e.g., sunlight or smoking) or chemotherapeutic agents (e.g., platinum drugs). In addition, it repairs lesions that lead to the stalling o*f* transcription. A section o*f* ~30 nucleotides o*f* one DNA strand is removed in one piece and then resynthesized.

NER can be divided into two subpathways: **global genome** (GG) repair and **transcription-coupled** (TC) repair. GG-NER occurs throughout the genome when helix-distorting lesions (e.g., interstrand crosslinks) are recognized. By contrast, TC-NER occurs when RNA polymerases, which negotiate helix-destabilizing lesions inefficiently, become stalled on the DNA. In TC-NER, the transcribed strand of the DNA is repaired more efficiently than the nontranscribed strand. A network of histone-modifying processes appears to assist access to histone-bound DNA. GG-NER and TC-NER activate the same set of NER enzymes to repair the DNA.

Distortions of DNA helices, recognized by GG-NER, are a hallmark of many types of DNA damage. GG-NER deals mostly with **intrastrand DNA adducts** and **crosslinks**. Common intrastrand DNA adducts include the following lesions: (1) TT-, TC-, CT-, and CC-**cyclobutane dimers** that are caused by **ultraviolet** (**UV) light** (either from the sun or tanning lights; Fig. 2.6); (2) adducts between adenine or guanine and **polycyclic aromatic hydrocarbons** (found in **cigarette smoke** and environmental contaminants; Fig. 2.7); and (3) adducts between GG or AG sequences and **platinum drugs** (e.g., **cisplatin**, **carboplatin**, or **oxaliplatin**, which are used in chemotherapy for solid tumors; Figs. 2.8 and 2.9).

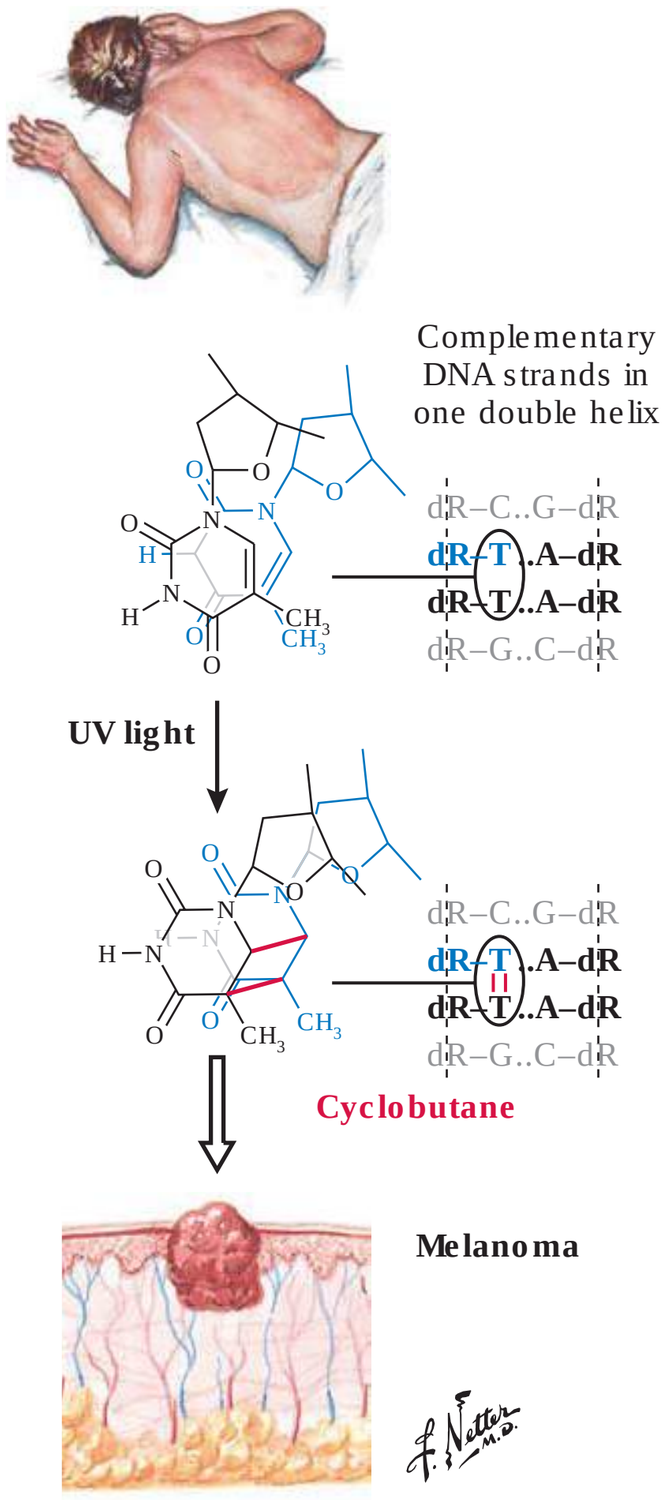


Fig. 2.6 UV light induces intrastrand crosslinking of pyrimidine bases into cyclobutane dimers.

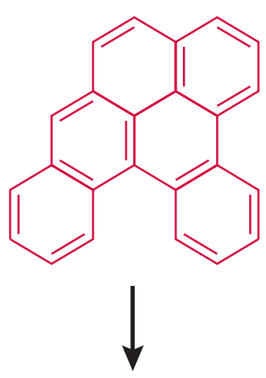
Once the damage is detected, helicases in **TFIIH** (a protein complex with roles in both GG-NER and TC-NER) unwind nearby DNA and verify the presence of damage (Fig. 2.10). RPA binds to single-stranded DNA and prevents the reformation of hydrogen bonds between base pairs. T e **ERCC1-**XPF endonuclease complex cuts unwound DNA 5′ of the lesion. A section of ~30 nucleotides is removed and a **DNA polymerase** (e.g., δ, ε, or κ) resynthesizes the missing region. Finally, a **DNA ligase** (I or III) links the 3′ end of the newly synthesized region to the rest of the DNA strand.

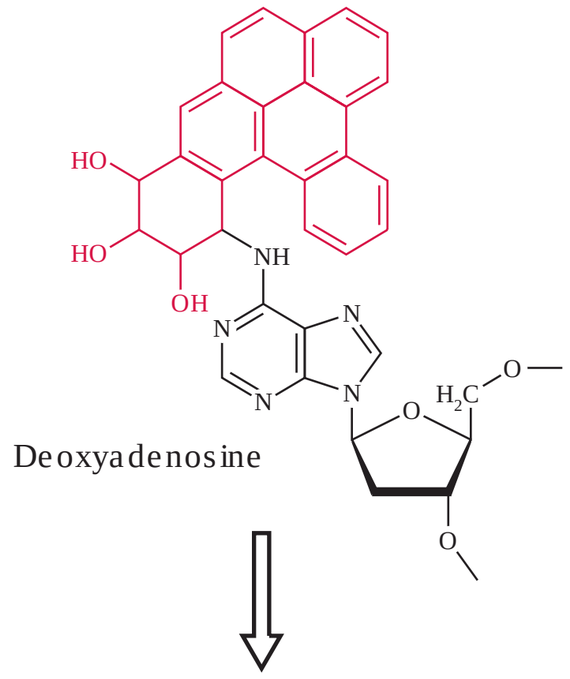
Patients with deficient TC-NER mostly show impaired development, premature aging, and neurodegeneration; some also have increased sensitivity to UV light. If many transcription sites are halted and stopped for long periods, the cell undergoes programmed cell death (**apoptosis**; see Chapter 8).

Patients who have abnormalities specifically in GG-NER tend to have very early-onset cancer (in part induced by UV light) because error-prone translesion DNA polymerases (see Chapter 3) bypass the many unrepaired lesions during DNA replication.

T e inadequate repair of intrastrand crosslinks promotes the formation of **tumors**. Inadequate repair of UV-induced DNA damage plays a role in the development of **basal cell**

Dibenzo[a,l]pyrene:





Bronchogenic carcinoma, squamous cell type

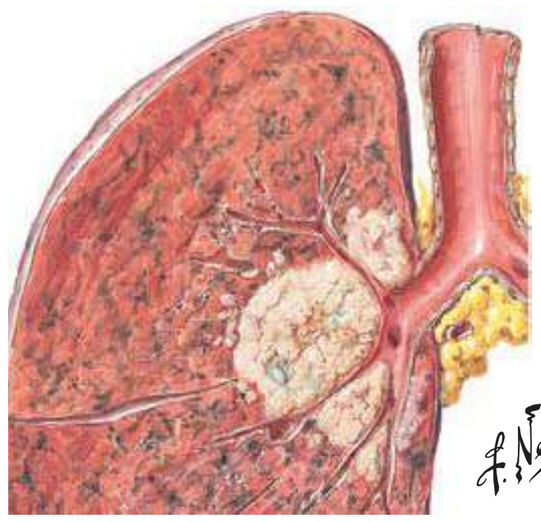


Fig. 2.7 Adduct of the principal metabolite of the carcinogen dibenzo[*a*,*l*]pyrene with deoxyadenosine in DNA.

carcinomas, **squamous cell carcinomas**, and **melanomas** of the skin (see Fig. 2.6). Inadequate repair of smoking-induced damage plays a role in the development of **lung cancer** (see Fig. 2.7).

Debilitating heritable deficiencies in NER are seen in the rare autosomal recessively inherited diseases **xeroderma pigmentosum**, **Cockayne syndrome**, and a form of light-sensitive **trichothiodystrophy** (all occur in less than 1 in 100,000 people). All these diseases can, in turn, be subdivided into several types, depending on the protein that is mutated. Patients with xeroderma pigmentosum readily develop tumors when exposed to UV light, and they also have an increased susceptibility to cancer that results from smoking or carcinogens in the diet. Cockayne syndrome is characterized by emaciation and short stature as well as neurological impairment, often also by photosensitivity. Trichothiodystrophy is characterized by brittle hair and sometimes also by photosensitivity. These disorders dramatically reveal the importance of components of the NER system.

Inadequate repair of drug-induced damage is taken advantage of in the treatment of **cancer.** Testicular cancer cells, for instance, have a low capacity for NER and thus readily undergo programmed cell death (see Section 5 and Chapter 8) when exposed to platinum drugs. This is drug sensitivity is a major reason for the high cure rate of testicular cancer that is achieved with therapy that includes **cisplatin** (see Figs. 2.8 and 2.9).

The NER pathway works together with HR (see Section 4.2) to repair interstrand crosslinks, such as those generated by platinum compounds, nitrogen mustards, or psoralen.

4. REPAIR OF DOUBLE-STRAND BREAKS AND INTERSTRAND CROSSLINKS

Nondividing cells repair double-strand breaks chiefly via NHEJ. Dividing cells repair double-strand breaks and interstrand crosslinks via a combination of NHEJ and HR repair. HR repair involves the copying of information from a nearby sister chromatid or homologous chromosome. Patients who have hereditary deficiencies of HR repair have a variety of cancer syndromes.

4.1. Nonhomologous End Joining

Ionizing radiation (e.g., in the form of high-energy **x-rays**) can give rise to single- and **double-strand breaks**. Ionizing radiation damages DNA directly or indirectly by forming DNA-damaging free radicals (mostly hydroxyl radicals, $OH^{\bullet}$, from water). When ionizing radiation cuts both DNA strands within 10 to 20 base pairs of each other, the cuts generate a double-strand break. The ends of the breaks often contain an inappropriate phosphate group or a fragment of deoxyribose.

In nondividing cells, double-strand breaks are largely repaired by **NHEJ** (see Fig. 2.11). The proteins **Ku70** (also called **XRCC6**) and **Ku80** (also called **XRCC5**) bind to the broken ends of the DNA. The Ku proteins recruit the DNA-dependent protein kinase catalytic subunit and the **nuclease** Artemis, which processes the ends of the strands if needed. End processing may be accompanied by a loss of nucleotides. As needed, the **DNA polymerases** λ and μ then insert nucleotides with or without a template. A **DNA ligase** complex (consisting of XLF, XRCC4, and DNA ligase IV) then ligates the ends of the DNA; this ligase complex tolerates some gaps and mismatches. (In contrast to double-strand breaks, single-strand breaks are repaired by BER; see Section 1.)

NHEJ can introduce mutations and is therefore a potentially tumorigenic process. Nonetheless, these mutations are

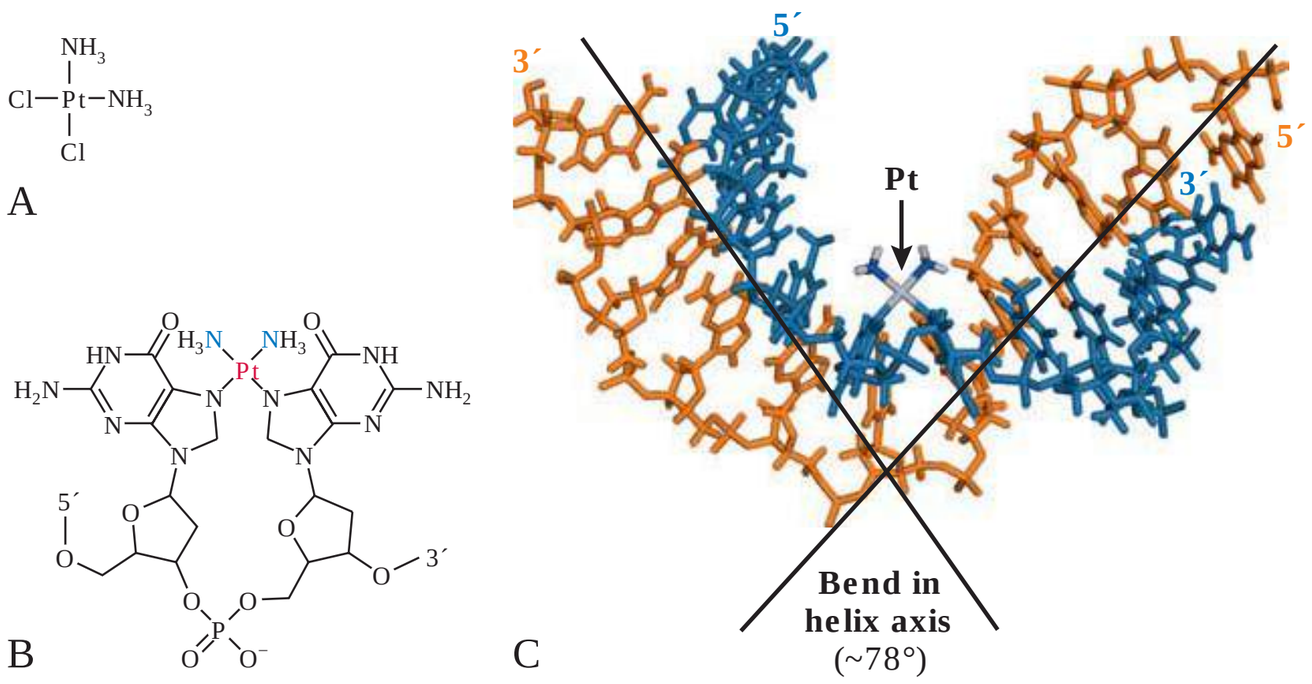


Fig. 2.8 Cisplatin-induced intrastrand crosslinking between two adjacent guanine bases. **A**, Cisplatin. **B,** Cisplatin adduct with guanine bases in DNA. **C,** Solution structure of a cisplatin-DNA intrastrand crosslink. Platination causes partial unwinding of the double helix, an unusual angle of the planes of the guanine bases, and an overall bend in the long axis of the helix. Platinum drugs also generate *inter*strand crosslinks, which have to be repaired via homologous recombination repair (see Section 4.2). (Based on Protein Data Bank [www.rcsb.org] 1A84 from Gelasco A, Lippard SJ. NMR solution structure of a DNA dodecamer duplex containing a cis-diammineplatinum [II] d[GpG] intrastrand cross-link, the major adduct of the anticancer drug cisplatin. *Biochemistry*. 1998;37:9230–9239.)

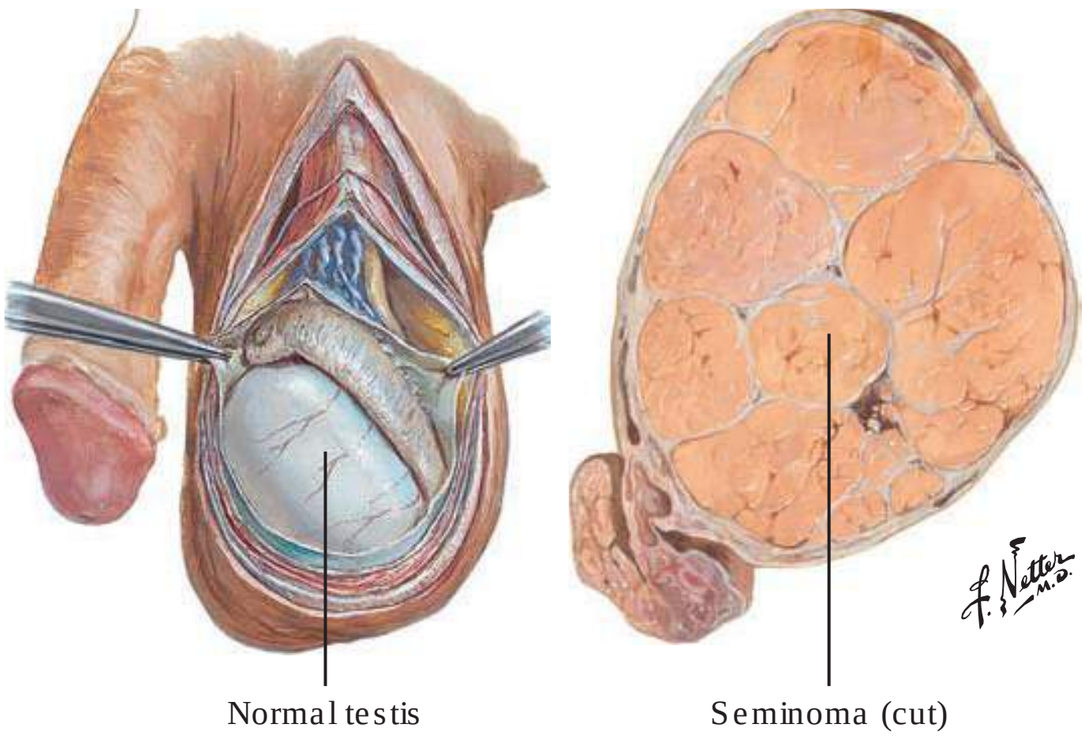


Fig. 2.9 Use of cisplatin in the treatment of testicular cancer. Patients usually undergo orchidectomy and then often receive adjuvant chemotherapy with cisplatin. Treatment is successful in part largely because the tumor cells have a low capacity for nucleotide-excision repair and then undergo apoptosis.

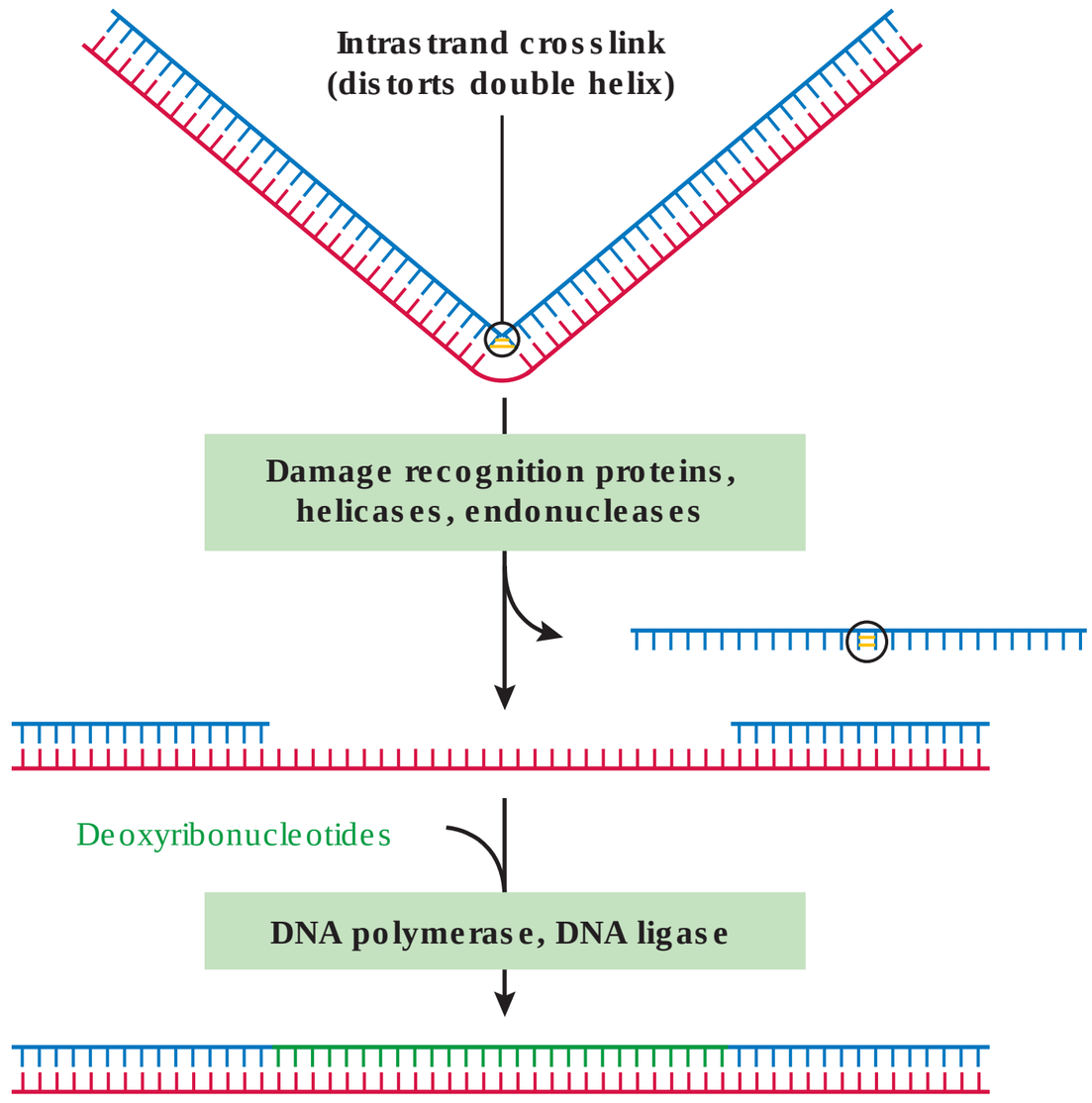


Fig. 2.10 Nucleotide-excision repair of intrastrand crosslinks. The crosslinks can be TT-, CT-, or CC-cyclobutane dimers (UV induced; see Fig. 2.6), single nucleotides linked to polycyclic aromatic compounds (as found in cigarette smoke; see Fig. 2.7), or platinum-linked purine nucleotides (e.g., cisplatin induced; see Fig. 2.8).

thought to be less damaging to cells than unrepaired DNA double-strand breaks because unprotected ends at the breakpoint would be degraded. Furthermore, some of the unrepaired DNA segments would lack centromeres or telomeres, which would be catastrophic for the genome of a cell. NHEJ can take various paths even with the same starting damage. Mutations caused by NHEJ often consist of one to 10 nucleotide **deletions** or three or fewer nucleotide **insertions**. When a nucleus contains numerous double-stranded DNA fragments, NHEJ even carries a risk of joining the wrong DNA fragments, which results in **translocation** (material from one chromosome is joined to another chromosome). T is can cause a protein-coding segment of a gene to be controlled by a promoter that induces aberrant transcription and thus pathogenic protein production. As described in Chapter 8, an increased rate of mutations paves the way for the development of a tumor.

Intense irradiation of cells with **ionizing radiation** produces such extensive and persistent DNA damage that af ected, heavily damaged cells undergo programmed cell death; this is the basis of **radiation therapy** of tumors. Radiation therapy

aims not only to introduce double-strand breaks but also additional DNA lesions within the region of the break. Cells that have DNA with such **clustered lesions** are especially likely to die.

In the **adaptive immune system**, NHEJ is involved in recombining V, D, and J segments of antibodies and T-cell receptors. T e inaccuracies of NHEJ help increase the diversity of antibodies and T-cell receptors. Patients who have a deficiency in NHEJ can also have a deficiency in their adaptive immune system.

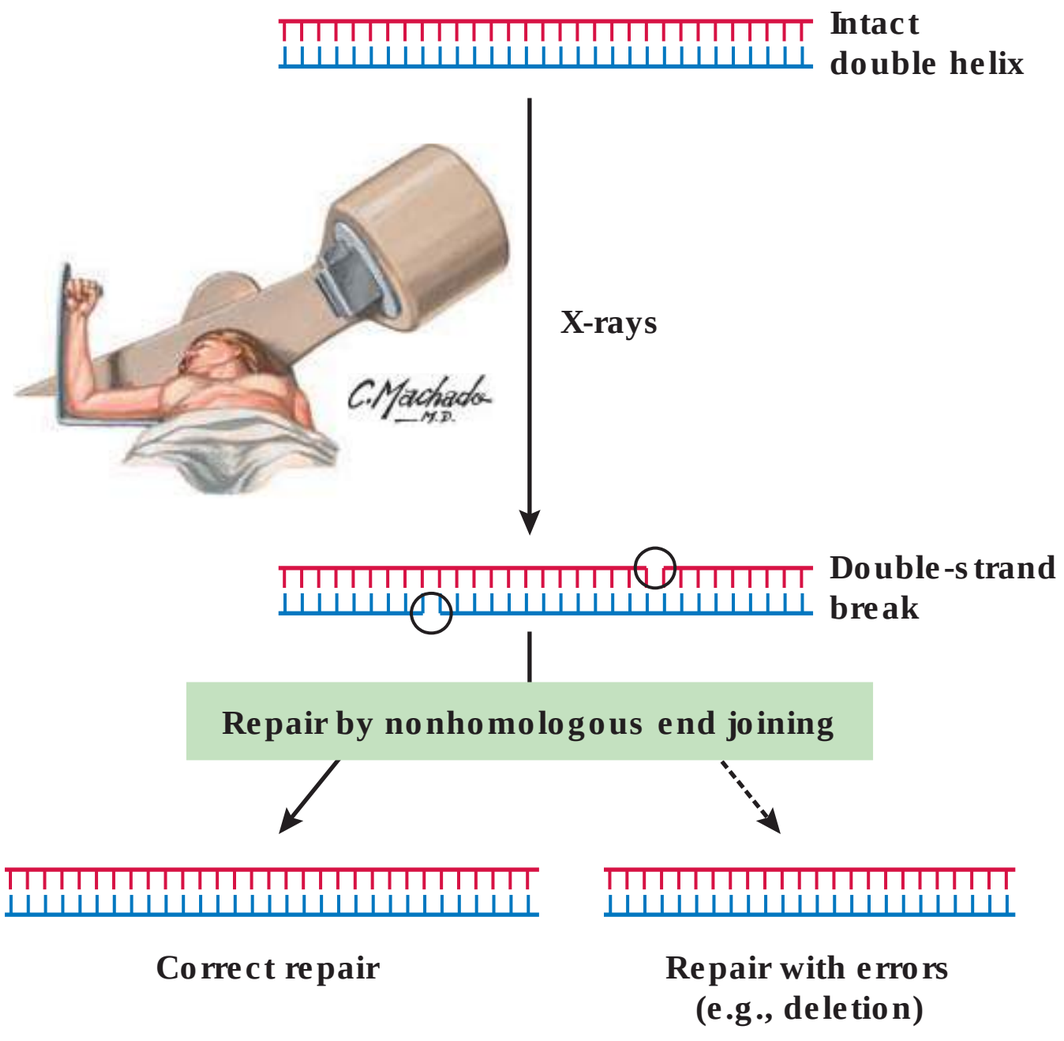


Fig. 2.11 Repair of radiation-induced double-strand breaks by nonhomologous end joining. Some radiation is natural. Radiation is also a mainstay of cancer treatment.

4.2. Homologous Recombination Repair (Homology-Directed Repair)

HR repair, like NHEJ described above, repairs DNA double-strand breaks. HR most often uses a **sister chromatid** as a template for repair. In contrast to NHEJ, HR is generally accurate. In the S and G2 phases of the cell cycle, double-strand breaks can arise mostly from problems with DNA replication (see Chapter 3), such as unrepaired **single-strand breaks** or complexes of DNA with **poisoned topoisomerase**. It is estimated that a normal dividing cell under physiological circumstances needs to repair ~50 double-strand breaks per cell cycle, and most of these breaks are handled by HR.

Interstrand crosslinks are repaired by NER alone (GG-Ner and/or TC-NER; see Section 3) or by an obligatory combination of HR repair and NER. (Intrastrand crosslinks are repaired by NER.) Interstrand crosslinks result from platinum drugs, nitrogen mustards, or psoralens. **Platinum drugs** (e.g., **cisplatin**, **carboplatin**) and **nitrogen mustards** (e.g., **cyclophosphamide**) are used in chemotherapy to kill tumor cells. **Psoralens** (e.g., **methoxypsoralen**) are used for the treatment of **psoriasis** and **vitiligo**. UV light induces psoralens to form cyclobutanes with staggered pyrimidine bases on the two strands of a DNA double helix. Psoriasis (Fig. 2.12) is a common skin disorder that is marked by the hyperproliferation of keratinocytes; treatment with a psoralen plus light reduces this hyperproliferation. Vitiligo (see Fig. 35.18) is a condition involving the patchy loss of skin pigmentation that af ects 1% to 2% of the population. T e loss of pigmentation is due to an absence of melanin pigment-producing cells, which in turn may be due to inflammation (see Chapter 35). Treatment with a psoralen plus light is ef ective and might work by diminishing inflammation. Although the psoralens kill some cells as intended, they also increase the rate of mutation in other cells, which explains the side ef ect of an increased rate of skin cancer.

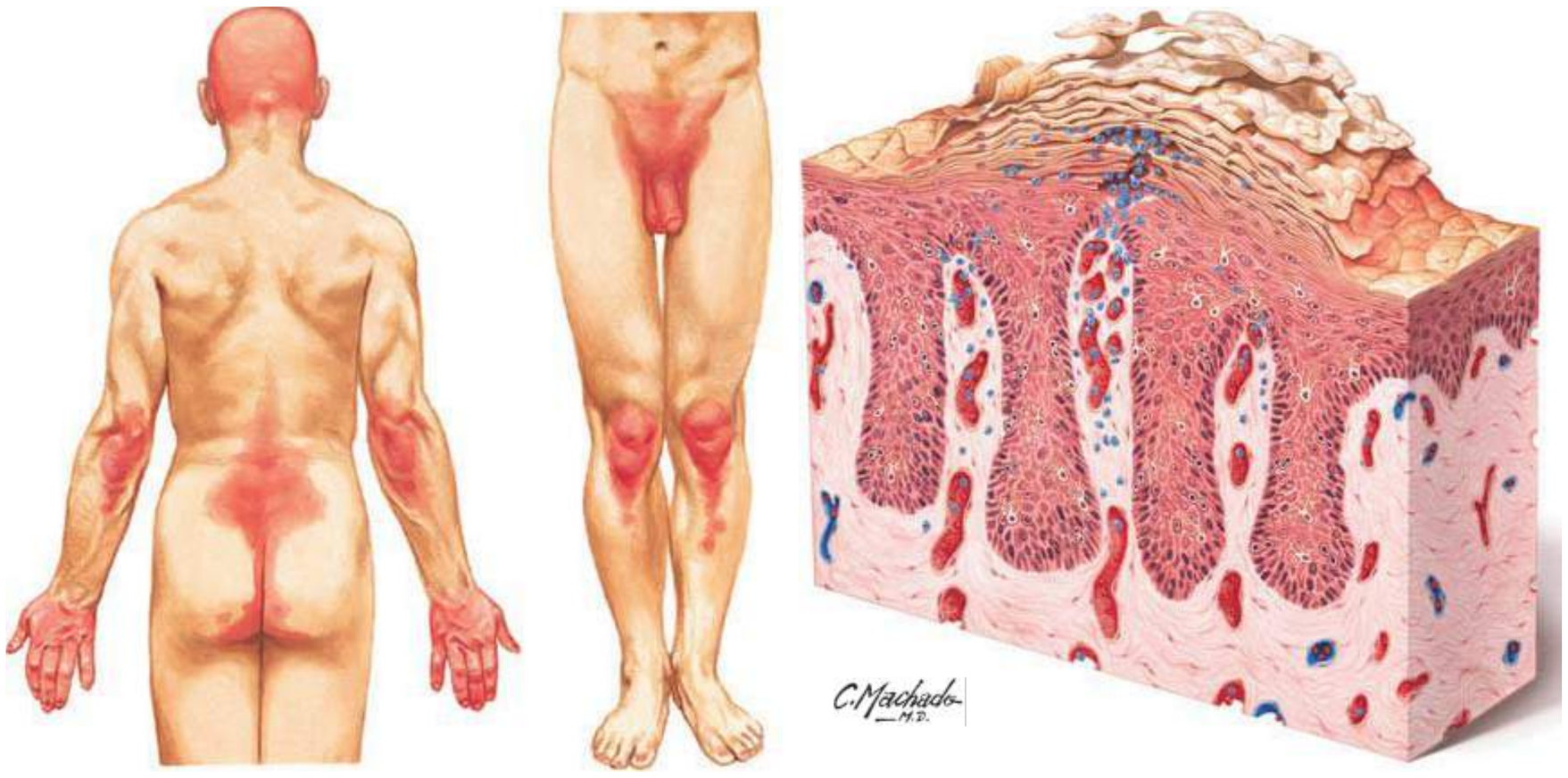


Fig. 2.12 Psoriasis is sometimes treated with photoreactive psoralens, which cause DNA intrastrand and interstrand crosslinks.

After a double-strand break occurs, the **MRN complex** (consisting of Mre11, Rad50, and Nbs1) binds to the ends of the DNA and activates the signaling kinase **ATM** (ataxia telangiectasia mutated). ATM, in turn, phosphorylates numerous proteins, some of which halt the cell cycle, while others increase the DNA repair activity. The MRN complex cuts one DNA strand ~100 to 200 base pairs from the break and then resects this strand toward the break. Another protein complex resects the same strand in the opposite direction so that a single-strand overhang is generated that can be greater than 1000 bp long. One such overhang is generated in each piece of broken DNA. Meanwhile, MRN also tethers together the ends of the two pieces of DNA. The **BRCA1** protein (breast cancer 1) forms a dimer with the **PALB2** protein and recruits the **BRCA2** protein, which in turn helps load the recombinase RAD51. RAD51 then fosters the invasion of a sister chromatid or a homologous chromosome by a 3′ overhang. The invaded DNA strand serves as a template for an elongation of the 3′ overhang. Subsequently, helicases and nucleases resolve the entangled DNA strands.

The use of a homolog for HR repair leads to **gene conversion**, which can be the cause of a **loss of heterozygosity** (**LOH**). The homologous chromosomes, derived from the mother and father, contain similar but not identical sequences. Gene conversion refers to the finding that the sequence of one parental allele converts to the sequence of the other parental allele. Hence, a somatic cell with one functional and one nonfunctional copy of a gene may give rise to a cell with two nonfunctional copies. LOH can also be used to describe the deletion of the examined sequence. The term **uniparental disomy** is applied when there is a loss of a chromosome or chromosome segment (usually containing multiple genes) from one parent and a gain of the lost sequence from the other parental chromosome. In patients who are heterozygous for a deficiency of a tumor suppressor, HR repair may lead to the complete loss of tumor suppressor activity, which may be tumorigenic (see Chapter 8).

Impairment of the activity of the **MRN** complex or the **ATM** kinase leads to an increased rate of mutation, and to an increased sensitivity toward therapeutic ionizing radiation. **Ataxia-telangiectasia**, seen in about one per 300,000 births, is due to homozygosity or compound heterozygosity for inactivating mutations in the ATM gene. **Nijmegen breakage syndrome** and **ataxia-telangiectasia–like disease** are much rarer diseases that are due to homozygosity or compound heterozygosity for inactivating mutations that affect the MRN complex. Reduced amounts of the MRN complex are also observed in about 20% of **breast tumors.**

About 5% of women who have breast cancer (Fig. 2.13), or ~0.1% of all men and women, have inherited a mutation in the BRCA1 or BRCA2 genes. Over time, the remaining, normal BRCA allele becomes lost or inactivated. Without functional BRCA proteins, cells accumulate DNA alterations at an increased rate and are thus prone to tumorigenesis. Patients with a heritable BRCA mutation are at an increased risk for **breast cancer**, **ovarian cancer**, and other tumors. The propensity for tumor formation is inherited in **autosomal dominant** fashion (as in Lynch syndrome; see Section 2). Heterozygosity for a defective BRCA1 or BRCA2 allele is a frequent cause of the **hereditary breast and ovarian cancer syndrome** (**HBOCS**).

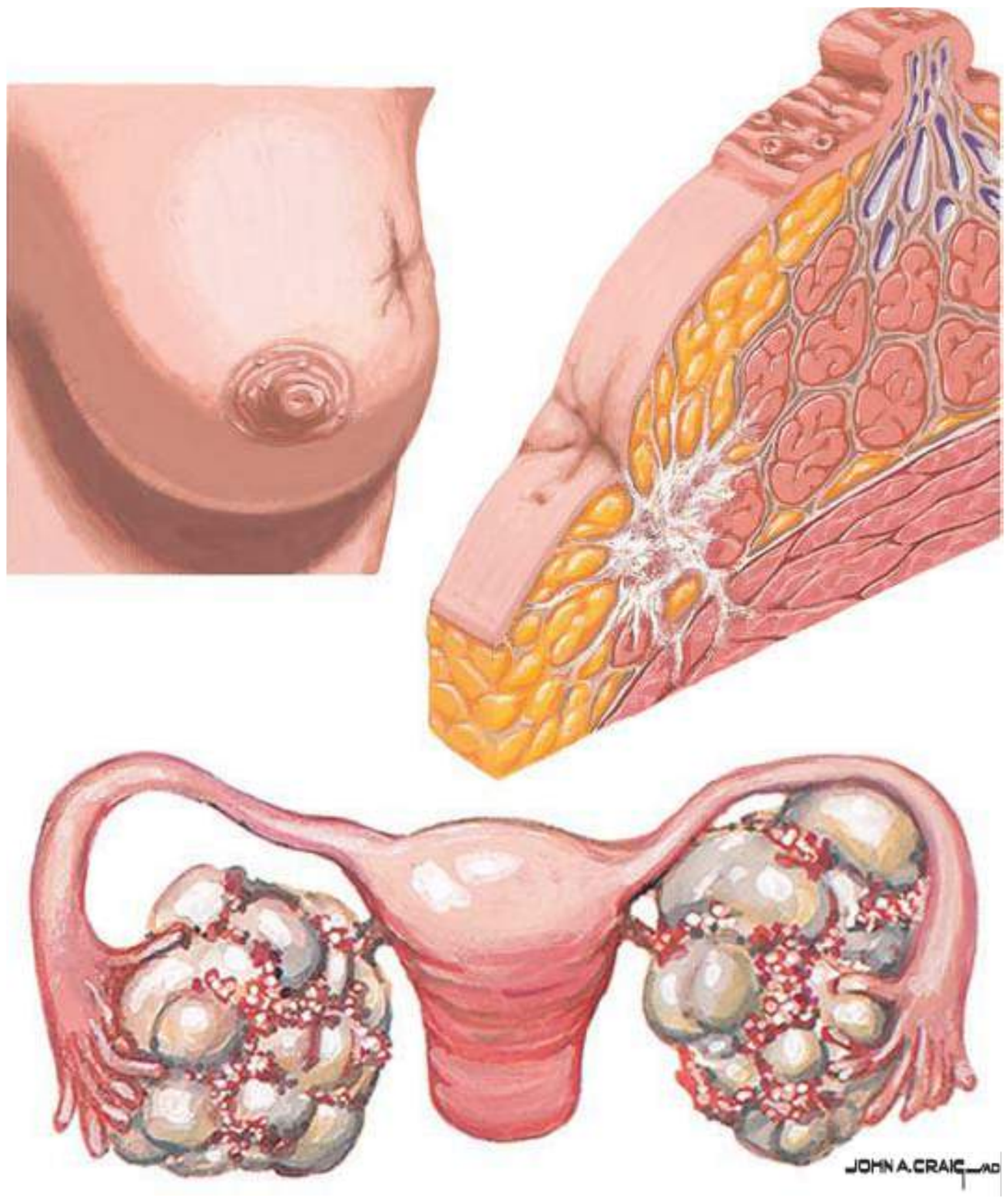


Fig. 2.13 Approximately 10% of women have breast cancer during their lifetime, and about 5% of these patients inherited one mutant BRCA allele that encodes a protein with impaired function. With time, the other, normal allele becomes nonfunctional, thereby impairing homologous recombination repair of DNA. Cells without functioning homologous recombination repair accumulate mutations at an increased rate and are more likely to give rise to a tumor. Patients can be tested for *BRCA* gene mutations.

Germline heterozygosity for a mutation in the PALB2 gene is associated with an increased risk of **pancreatic cancer** and **breast cancer**. Together, mutations in PALB2 and BRCA2 are responsible for much of hereditary pancreatic cancer (other contributors are mutations in a gene for a MMR protein and mutations in the CDKN2A gene). PALB2 mutations are responsible for only a few percent of patients who have hereditary breast cancer.

Fanconi anemia is a heritable, rare syndrome that is characterized by bone marrow failure, resulting in anemia, leukopenia, and thrombopenia, as well as malformations. Affected persons are at high risk of developing hematological malignancies and solid tumors. Patients who have Fanconi anemia are homozygous, compound heterozygous, or hemizygous for a mutant protein of the **Fanconi anemia network** (also called **Fanconi anemia pathway**). The complete network consists of at least 16 proteins that play a role in DNA repair. PALB2 and BRCA2 are members of the Fanconi anemia network. Patients

who have two mutant BRCA2 alleles have the D1-type of Fanconi anemia, and patients who have two mutant PALB2 alleles have the N-type. Protein complexes of the Fanconi anemia network coordinate some of the DNA excision, strand invasion, and resolution of HR. In the general population, several types of sporadic tumors are deficient in one of the proteins of the Fanconi anemia network, which bears on the susceptibility of these tumors to DNA crosslinking agents. Patients who have Fanconi anemia are hypersensitive to ionizing radiation and DNA crosslinking agents (e.g., cisplatin or cyclophosphamide).

HR not only plays a role in DNA repair but also in forming crossovers in **meiosis** for the purpose of identifying and pairing homologous chromosomes, thereby increasing genetic diversity among of spring.

5. DNA DAMAGE RESPONSE HALTS THE CELL CYCLE AND REGULATES APOPTOSIS

As is outlined in Chapter 8, cancer is the result of damage to the genome such that cell growth and survival are no longer properly regulated. Inadequate DNA repair increases the rate of mutation and thus favors the formation of a tumor. Cells have means of assessing DNA damage and determining whether to survive or self-destruct.

A cell's **DNA damage response** senses DNA damage, slows progression through the cell cycle, and coordinates this with DNA repair; when DNA damage is persistent, the response can also initiate apoptosis. Most DNA repair pathways are discussed in Sections 1 to 4. Translesion DNA synthesis is discussed in Section 2 of Chapter 3. Fig. 2.14 provides an overview of these repair pathways.

The DNA damage response is best studied in cells that contain double-strand breaks. Such breaks eventually lead to the activation of the kinases ATR and ATM, which in turn activate the checkpoint kinases Chk1 and Chk2. Through various means, the checkpoint kinases lead to a halt in the cell cycle by blocking the G1/S transition, S phase, the G2/M transition, or M phase (see Section 1 in Chapter 8). This allows DNA repair pathways, including translesion DNA synthesis, to repair DNA damage.

DNA repair pathways are often redundant: although a particular type of damage is typically repaired mostly by one pathway, it can often be repaired by an alternative pathway. If DNA damage is repaired, the signal blocking the cell cycle is eliminated, and progression through the cycle resumes. If the damage is not repaired, the DNA damage signal persists and can trigger apoptosis.

Some **chemotherapeutic drugs** kill cells by inducing DNA damage that is so overwhelming that the cells undergo apoptosis. The sensitivity of normal and abnormal cells to chemotherapy-induced DNA alterations depends on many variables, including drug uptake and efflux, the capacity for DNA repair, the ability of cells to sense and transduce the DNA damage response, and the likelihood that DNA damage leads to apoptosis. Many tumor cells have altered sensitivity to DNA damage-induced apoptosis. Cells that survive damage from chemotherapeutic drugs (e.g., because entry into apoptosis is misregulated or DNA damage-sensing mechanisms fail to detect the damage) may give rise to a new tumor or aggravate the behavior of the existing tumor.

Intense **ionizing radiation**, such as that used for radiation therapy of cancer, causes cell death not just by the sheer volume of damage to DNA, but also by clustering damage within one to two turns of the DNA helix. It is unclear why clustered damage is particularly lethal.

SUMMARY

- The base-excision repair (BER) pathway mends the damage from deamination, hydroxylation, methylation, the loss of a base, from the alteration of a deoxyribose, or from a single-strand break. Such damage is a result of the chemical

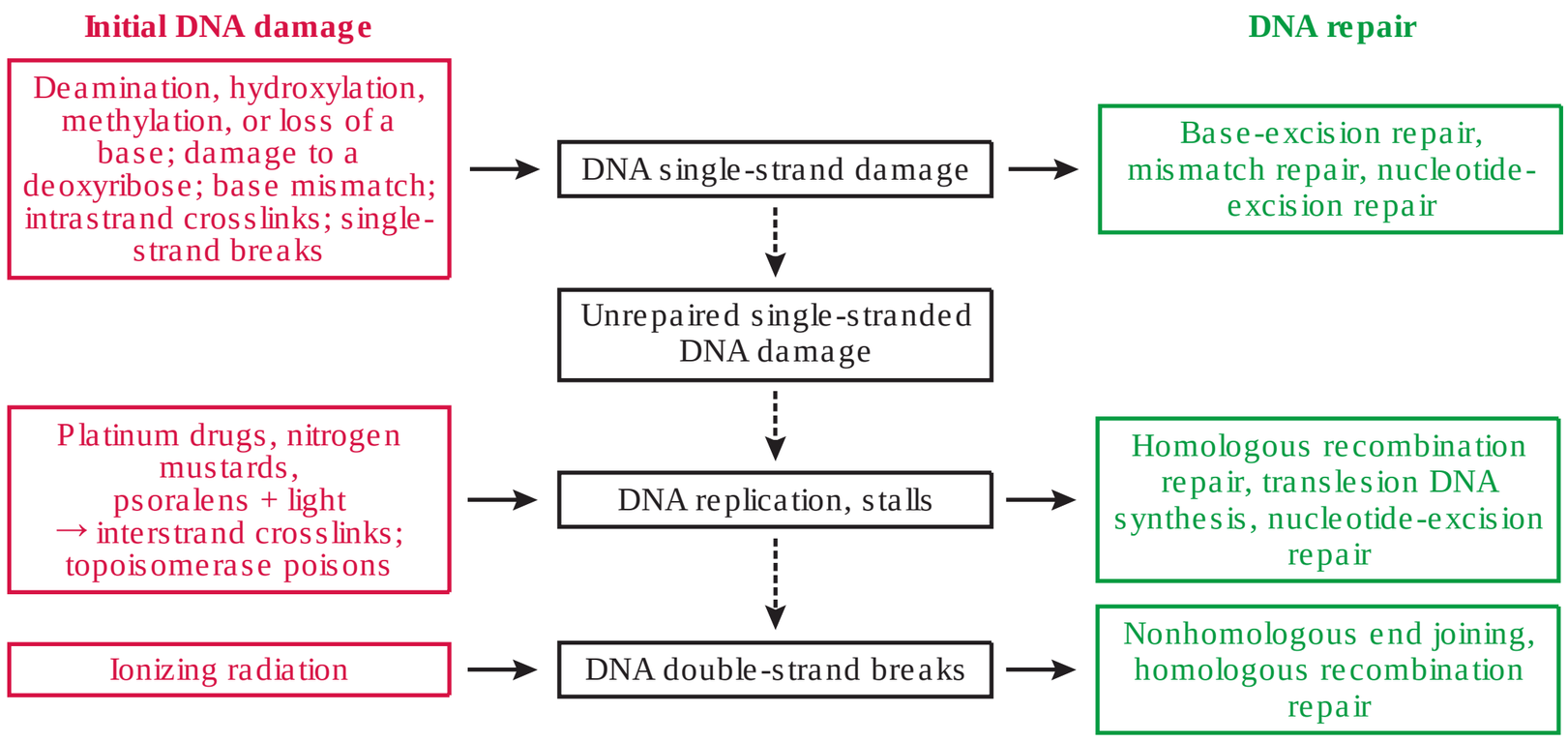


Fig. 2.14 Overview of DNA repair pathways. DNA replication and translesion DNA synthesis are explained in Chapter 3.

properties of DNA, the destructive effects of other cellular constituents, or ionizing radiation. The short patch repair pathway replaces a single damaged nucleotide, while the long-patch repair pathway replaces a stretch of 2 to 10 consecutive nucleotides. The complementary DNA strand serves as a template for the insertion of nucleotides.

- MUTYH-associated polyposis (MAP) is caused by a homozygous or compound heterozygous deficiency of DNA MYH glycosylase, which plays a role in repairing 8-oxoguanine (produced from guanine by a radical). The disease is associated with the formation of numerous polyps in the colon, as well as early colorectal cancer.
- DNA mismatch repair (MMR) processes tackle single-base DNA mismatches and DNA loops, which arise from errors in the synthesis of DNA, from the spontaneous deamination of C to U or methyl-C to T, or from homologous recombination (HR) repair. Enzymes of the DNA MMR pathway degrade and resynthesize a portion of a DNA strand, which is often the most recently synthesized strand.
- Lynch syndrome is due to an inherited MMR deficiency. Both inherited and acquired deficiencies in MMR can cause cancer, especially of the colon, uterus, and ovaries. An MMR deficiency in tumor cells can often be detected as deficient immunohistochemical staining of MMR proteins and by DNA microsatellite instability (MSI) (i.e., a shortening of the lengths of certain nucleotide repeats).
- Nucleotide-excision repair (NER) involves the removal and appropriate replacement of a contiguous stretch of ~24 to 32 nucleotides around a helix-distorting lesion on one strand of a DNA double helix. Such lesions commonly stem from the crosslinking of the pyrimidine bases (T or C) by exposure to UV light, from the reaction of metabolites of polycyclic aromatic hydrocarbons (found in smoke) with purine bases (A or G), or from the crosslinking of adjacent purine bases by platinum drugs (used in chemotherapy of tumors). A high rate of production of helix-distorting DNA lesions is associated with an increased risk of cancer (e.g., melanoma and lung cancer).
- After a double-strand break, HR involves resection of one strand to produce a long single-strand overhang, invasion of the homologous chromatid, copying of the information, and separation of the two chromatids.
- Ionizing radiation (e.g., high-energy x-rays) causes double-strand breaks that can be repaired by NHEJ or by HR.
- The therapeutically used platinum drugs, nitrogen mustards, and photoreactive psoralens produce interstrand crosslinks that can be repaired by HR repair. The BRCA1 and BRCA2 proteins participate in HR repair. Mutations in the BRCA genes can convey increased susceptibility to breast and ovarian cancer.
- DNA damage-sensing pathways can halt the cell cycle to allow time for DNA repair. Cells with excessive unrepaired DNA damage often undergo apoptosis. Some of the drugs used in the chemotherapy of cancer kill cells by causing DNA damage in excess of the cells' capacity for repair. These drugs are inherently mutagenic to all cells.

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Review Questions

1. A 48-year-old woman has endometrial cancer and undergoes a hysterectomy. Immunohistochemistry of tumor tissue reveals the presence of MLH1 and PMS2, but an absence of MSH2 and MSH6. Based on this finding, the most likely diagnosis is which of the following?

   A. Cockayne syndrome
   B. Hereditary breast and ovarian cancer syndrome
   C. Lynch syndrome
   D. MUTYH-associated polyposis

2. Some nitrofurans form adducts with bases in DNA that cannot be repaired via the base-excision repair pathway. The adduct leads to distortion of the DNA double helix. The lesion is most likely repaired by which one of the following DNA repair pathways?

   A. Homologous recombination repair
   B. Mismatch repair
   C. Nonhomologous end joining
   D. Nucleotide-excision repair

3. On a colonoscopy, a 45-year-old patient with a family history of colon cancer is found to have about 90 polyps. A genetic workup revealed homozygosity for a common mutation in the MU*T*YH gene. This patient most likely developed adenomas in the colon due to a deficiency in which one of the following DNA repair pathways?

   A. Base-excision repair
   B. Homologous recombination repair
   C. Mismatch repair
   D. Nonhomologous end joining
   E. Nucleotide-excision repair

4. Aflatoxin is a polycyclic aromatic hydrocarbon that is produced by Aspergillus species, which often grow on cereals, peanuts, and nuts. T e liver converts ingested aflatoxin to a compound that reacts with guanine in DNA. A stable adduct of guanine and the aflatoxin derivative is predominantly repaired by which one of the following DNA repair pathways?

   A. Base-excision repair
   B. Homologous recombination repair
   C. Mismatch repair
   D. Nonhomologous end joining
   E. Nucleotide-excision repair

5. Loss of heterozygosity (LOH) is frequently observed in tumor tissue. T ereby, the allele from one parent is lost. Which one of the following DNA damage (1) and repair (2) pathways most readily gives rise to LOH?

   A. (1) Deamination of C to U and (2) mismatch repair
   B. (1) Double-strand break and (2) homologous recombination repair
   C. (1) Formation of TT cyclobutane dimer and (2) nucleotide-excision repair
   D. (1) Opening of deoxyribose ring and (2) base-excision repair

Chapter 3 DNA Replication

SYNOPSIS

- During DNA replication, each DNA strand serves as a template for the synthesis of a new, complementary DNA strand. Replication starts at many sites on each chromosome. As double-stranded DNA is opened up for replication, each strand can be copied continuously in one direction, but it must be copied in many small segments in the opposite direction.
- Translesion DNA polymerases help DNA replication continue through unrepaired DNA lesions.
- Telomeres, the ends of chromosomes, shorten with each round of replication. This shortening plays a role in senescence. Cells of the germline and stem cells use telomerase to keep the length of their telomeres constant.

LEARNING OBJECTIVES

For mastery of this topic, you should be able to do the following:

- Outline the replication of DNA.
- Describe the factors that contribute to the fidelity of DNA replication.
- Describe the structure of telomeres, explain how replication leads to shortening of telomeres, and describe how select cells maintain an adequate length of their telomeres.

1. DNA REPLICATION

During replication, each strand of a DNA double helix serves as a template for the synthesis of a new complementary DNA strand. Topoisomerase I releases the superhelical strain and helicases catalyze the separation of complementary DNA strands. Replication of a section of DNA starts with the synthesis of an RNA primer. Then, DNA polymerase catalyzes the addition of deoxyribonucleotides. Finally, the RNA primer is replaced by deoxyribonucleotides.

The packing of nuclear DNA into nucleosomes, 30-nm chromatin fibers, and higher-order structures is described in Chapter 1. In preparation for **DNA replication** (i.e., the copying of the genome), higher-order DNA structures are dismantled by **chromatin remodeling factors**. Such factors include enzymes that modify proteins in chromatin (e.g., acetylases and deacetylases, methylases and demethylases, kinases, and phosphatases) and proteins that replace existing proteins in chromatin.

Nuclear DNA is replicated during **S phase** of the cell cycle (see Chapter 8), which usually takes a few hours. Mitochondrial DNA is replicated on demand, which can occur independently of the replication of nuclear DNA. The following is an account of DNA replication in the nucleus.

Replication of a DNA double helix is **semiconservative**: each strand of an existing DNA double helix serves as a template for the synthesis of a new complementary strand. At the end of this process, each of the two double helices contains one of the old DNA strands and one of the newly synthesized DNA strands (Fig. 3.1).

During replication, DNA synthesis proceeds in a 5′ to 3′ direction (Fig. 3.2). The 3′ hydroxyl group at the end of a growing DNA strand performs a nucleophilic attack on the phosphorus atom of the incoming deoxyribonucleoside triphosphate that is closest to the sugar (i.e., the α-phosphate of the incoming dNTP). If the nucleotide at the 3′ end of a DNA strand lacks the 3′ hydroxyl group, the DNA strand cannot be elongated.

Replication can start at thousands of predetermined regions, the **origins of replication** (**ORI**s). ORIs are spaced ~50,000 to 300,000 base pairs apart. The use of these origins is regulated. There are early- and late-replicating origins. Furthermore, most origins are never used under normal circumstances (i.e., they are dormant). Dormant origins can become active when replication stalls.

The following processes ensure that the DNA around each ORI is replicated only once per cell cycle: During the G_1 phase of the cell cycle, just before S phase (see Chapter 8), multiprotein **origin-recognition complexes** assemble on the ORIs in a process termed licensing. This happens only in the presence of loading factors, which in turn are present only during this G_1 phase. In the G_1-to-S-phase transition and during S phase (when loading factors are no longer present), an ATP-driven helicase in the assembled complex is activated, and the complex departs from the ORI. The activity of cyclin-dependent kinases (CDKs; see Chapter 8) is high as cells exit from G_1 and enter S phase, and it remains high until chromosome segregation takes place during M phase. This high CDK activity inhibits licensing during the S and M phases, thus preventing rereplication.

Starting at an ORI, separation of the complementary DNA strands gives rise to two **replication forks** (see Fig. 3.3). As ATP-driven helicases separate double-helical DNA into single strands, **single-strand–binding proteins** such as **RPA** (**replication protein A**) partially wrap around the single-stranded DNA and prevent the hybridization of bases within the same strand or with the complementary DNA strand. **Topoisomerase I** removes the superhelical stress from the DNA (see Chapter 1). Each strand of the DNA double helix is read in a 3′→5′ direction, giving rise to new, complementary DNA strands that are called **leading strands** (see Figs. 3.3 and 3.5).

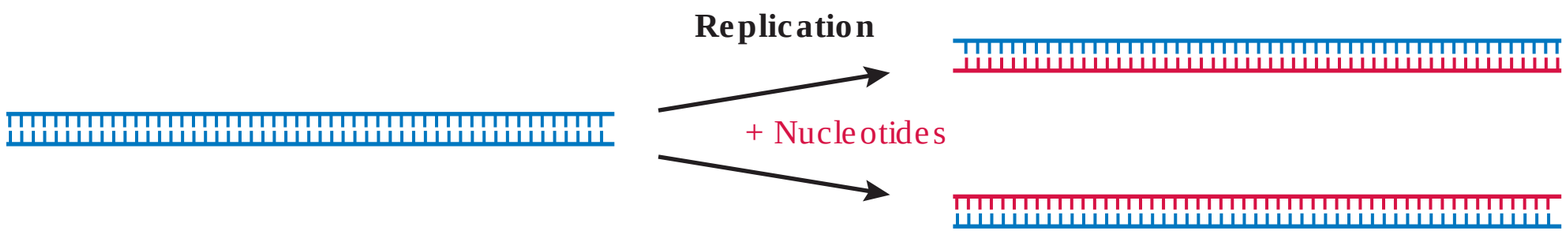
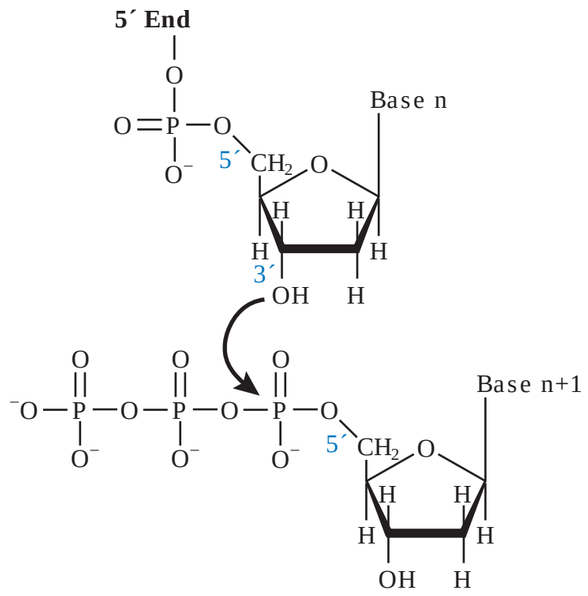


Fig. 3.1 **DNA replication is semiconservative.**



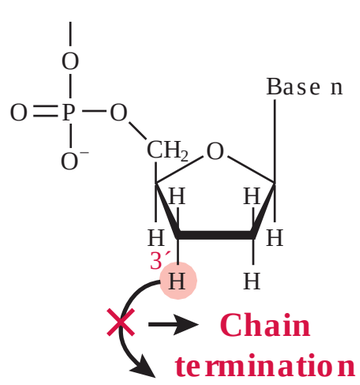


Fig. 3.2 **DNA replication occurs 5′ to 3′ and is stopped by incorporated synthetic dideoxyribonucleotides that lack the 3′ hydroxyl group.** Dideoxyribonucleotides are used clinically to treat cancer and in the laboratory for DNA sequencing and various DNA-based tests.

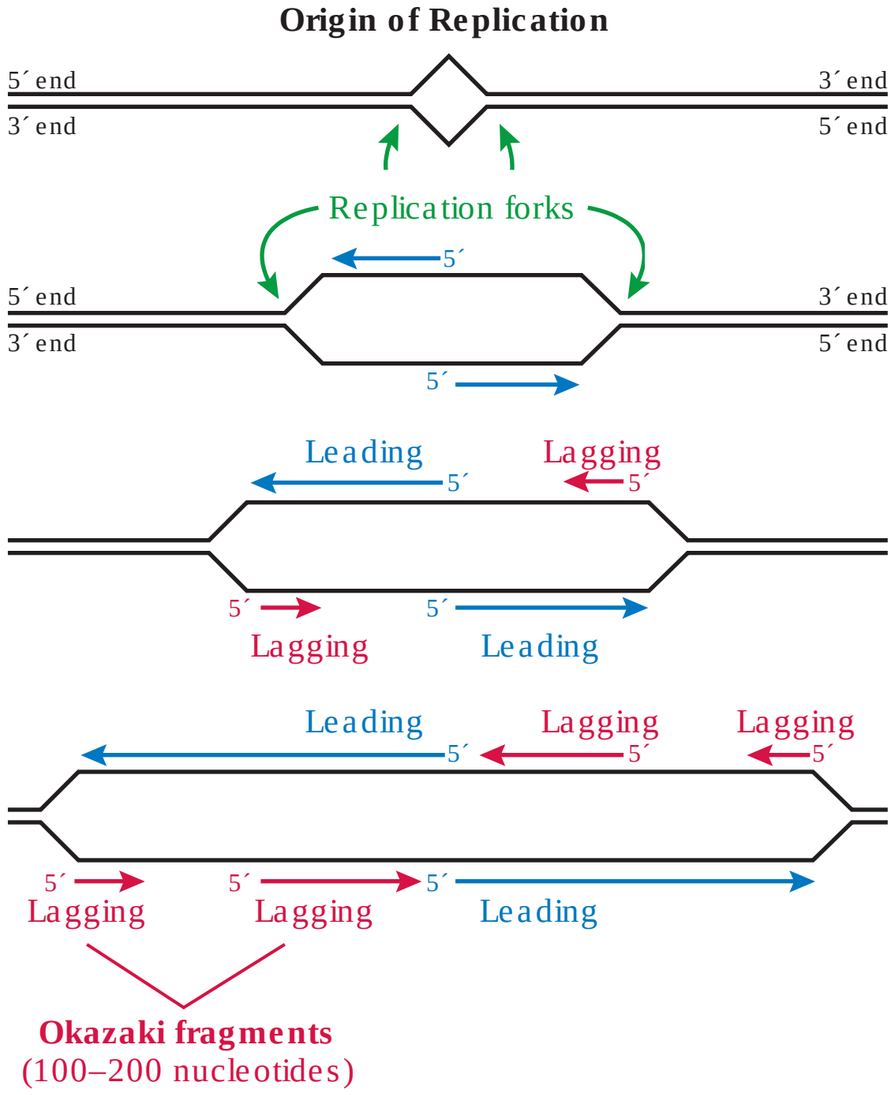


Fig. 3.3 **Replication forks during DNA replication.**

Fig. 3.4 **Primer synthesis during DNA replication.**

As replication proceeds, an increasing length of DNA on the 3′ side of the ORI remains uncopied, because the template can be read only in the 3′→5′ direction. Once approximately 100 to 200 uncopied bases are exposed, a DNA polymerase works on these strands as well, producing 100 to 200 nucleotide-long pieces of DNA that are called **Okazaki fragments** (see Figs. 3.3 and 3.5). T e Okazaki fragments are eventually ligated, and this strand is called the **lagging strand**. T us, synthesis of the leading strand is continuous, while synthesis of the lagging strand is discontinuous.

DNA polymerase α is a multisubunit enzyme complex that contains a DNA polymerase and an RNA polymerase. It uses ribonucleoside triphosphates (i.e., ATP, CTP, GTP, UTP) to synthesize a complementary **RNA primer** that is ~7 to 12 nucleotides long (Figs. 3.4, 3.5). T e DNA polymerase then extends the RNA primer by ~20 nucleotides. All RNA and DNA is synthesized by the addition of a nucleoside 5′-triphosphate to the 3′-hydroxyl group of the preceding nucleotide (i.e., the newly synthesized strand grows in a 5′→3′ direction). Neither the RNA polymerase or the DNA polymerase in the DNA polymerase α complex can carry out proofreading; the complex therefore incorporates noncomplementary nucleotides at a higher frequency than DNA polymerase δ (see below).

DNA polymerase δ (the DNA polymerase responsible for the bulk of nucleotide incorporation) and **DNA polymerase ε** (which plays a minor role not shown in Fig. 3.5) elongate the strand synthesized by DNA polymerase α. Each polymerase has a proofreading function; if the bases of the growing DNA strand do not match the template, the enzyme stalls, excises the mismatched nucleotide, and then continues polymerization.

When DNA polymerase δ reaches an RNA primer on the lagging strand, the primer and up to ~30 deoxyribonucleotides that follow it are displaced and excised. DNA polymerase δ inserts the missing complementary nucleotides, and the **DNA ligase** joins the 3′ end of the most recently synthesized piece of DNA with the appropriate 5′-end of the previously synthesized piece of DNA.

DNA replication has a very high **fidelity**. On average, each replication of the human genome (involving ~3 billion base pairs) introduces only about three base changes. T e high accuracy is in large part due to the substrate specificity

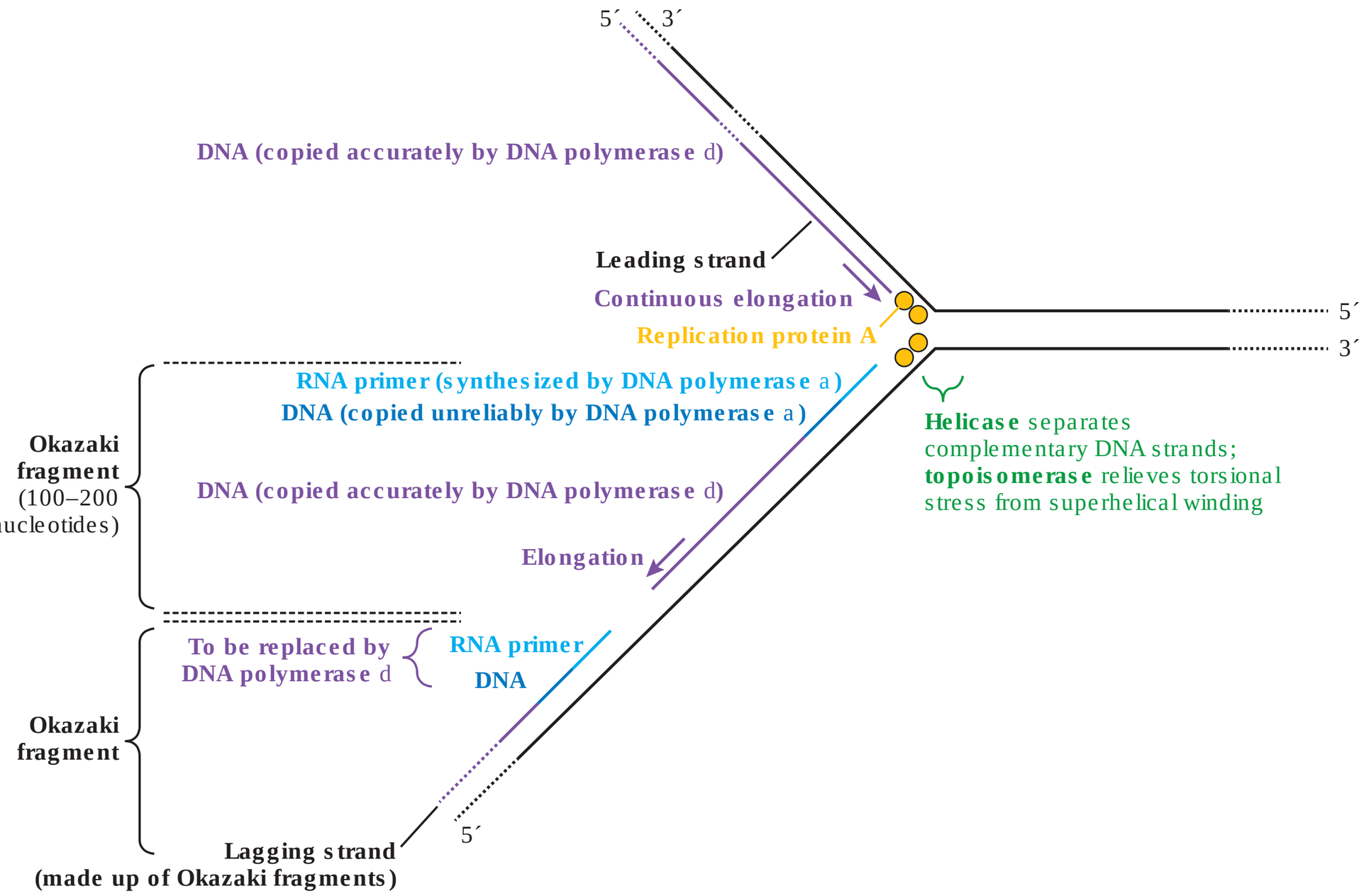


Fig. 3.5 **DNA replication, showing the leading and lagging strand at a replication fork.**

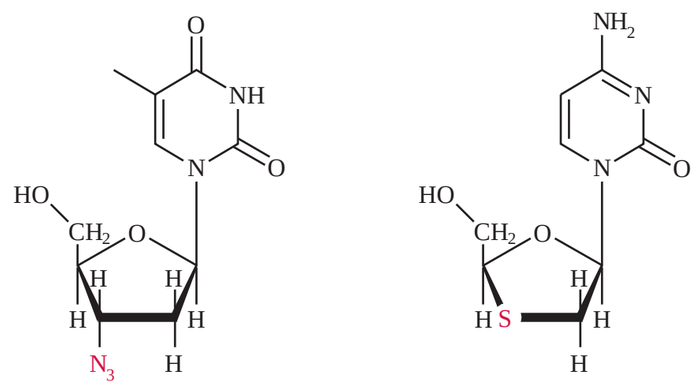
and proofreading function of DNA polymerase δ, as well as the efficiency of postreplication DNA mismatch repair (see Section 2 in Chapter 2).

Following replication, DNA is assembled into nucleosomes and higher-order chromatin structures using chromatin assembly factors, including existing and newly synthesized histones.

DNA polymerases and **DNA ligases** from a variety of organisms are used for in vitro DNA diagnostic methods (see Chapter 4).

Dideoxyribonucleotides that interfere with DNA replication are used in cancer chemotherapy, as antiviral drugs, in DNA diagnostics, and in Sanger-type DNA sequencing (see Chapter 4). Nucleotides without a 3′-hydroxyl group (e.g., **ddATP**, **ddGTP**, **ddCTP**, and **ddTTP)**, can be incorporated into the DNA, but because they lack a 3′-hydroxyl group, they are chain terminators (see Fig. 3.2).

Zidovudine and **lamivudine** (Fig. 3.6) both inhibit viral reverse transcriptases (enzymes that copy viral RNA into DNA) but have only a minor ef ect on human DNA polymerases. Both drugs are used against infections with **retroviruses** (i.e., viruses that contain an RNA genome). Zidovudine is an analog of thymidine. Enzymes in the cell convert zidovudine to the triphosphate form, and reverse transcriptase subsequently incorporates it into growing DNA strands. T ese DNA strands cannot be elongated because zidovudine lacks a 3′-hydroxyl group. Lamivudine likewise is phosphorylated inside cells and then markedly inhibits viral reverse transcriptases but not human DNA polymerases. Like zidovudine,

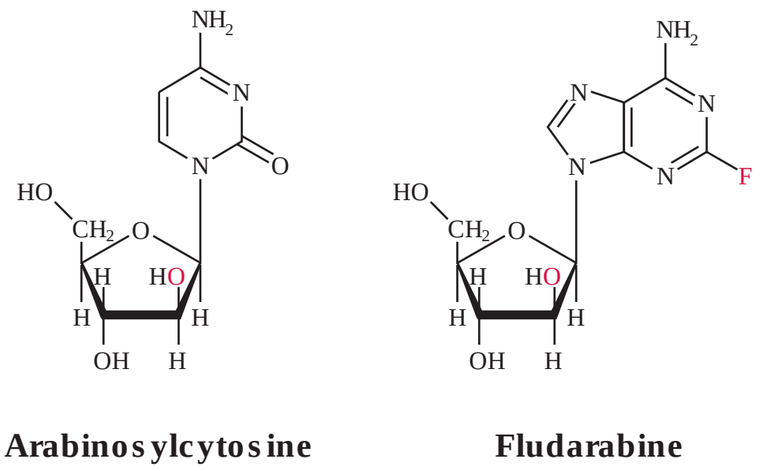


Zidovudine **Lamivudine**

Fig. 3.6 **Drugs that preferentially inhibit DNA synthesis in retroviruses.** Zidovudine is an analog of thymidine, and lamivudine is an analog of deoxycytidine. Residues that differ from the physiological nucleoside are shown in red.

lamivudine also acts as a chain terminator. Lamivudine is used in the treatment of hepatitis B and human immunodeficiency virus-1.

Arabinosylcytosine and **fludarabine** (Fig. 3.7) interfere with DNA replication, and both drugs are used for the treatment of acute **leukemias**. Inside cells, these drugs are phosphorylated. DNA polymerase incorporates arabinosylcytosine triphosphate and fludarabine triphosphate into DNA. However, these synthetic nucleotides are poor substrates for excision and replacement, as well as for continued replication. T e decrease in replication leads to double-strand breaks. In addition, fludarabine at the 3′ terminus of a piece of DNA prevents ligation by DNA ligase, leading to persistent single-strand breaks (i.e., nicked DNA). Once a cell has incorporated



Arabinosylcytosine **Fludarabine**

Fig. 3.7 Drugs that inhibit DNA replication. Arabinosylcytosine is an analog of cytidine, and fludarabine is an analog of adenosine. Residues that differ from the physiological nucleoside are shown in red.

about 100,000 molecules of fludarabine into its DNA, it undergoes apoptosis.

2. TRANSLESION DNA SYNTHESIS

*T*ranslesion DNA synthesis is carried out during DNA replication by polymerases that recognize damaged nucleotides that escape DNA repair mechanisms. T ese DNA polymerases usually incorporate the correct nucleotide opposite a damaged one, but the damaged DNA template strand is not repaired.

During DNA replication, **bypass DNA polymerases** (**translesion DNA polymerases**) are recruited to DNA lesions that escape repair by the pathways of base excision, mismatch repair, and nucleotide excision (see Chapter 2). T e major replicative DNA polymerases α and δ generally cannot copy damaged nucleotides. In contrast, bypass DNA polymerases copy a wide spectrum of DNA damage, but they also have lower copy fidelity than DNA polymerase δ. Bypass polymerases are much more active with damaged DNA as a template than with intact DNA; this characteristic prevents translesion DNA polymerases from inaccurate copying of appreciable stretches of undamaged DNA. Bypass DNA polymerases do not repair the damage to nucleotides in the template DNA.

Humans have several types of bypass DNA polymerases: η (eta), ι (iota), κ (kappa), and others. Collectively, these polymerases can insert A, T, C, or G opposite an abasic site, insert G opposite U (U derives from the deamination of C), or insert C opposite 8-oxoguanine, for lesions that should have been repaired by the base excision repair pathway (see Chapter 2). Bypass DNA polymerases can also insert an AA opposite the cyclobutane TT dimers, T opposite adducts of polycyclic aromatic hydrocarbons with A, C opposite adducts of polycyclic aromatic hydrocarbons with G, or CC opposite cisplatin GG intrastrand crosslinks, for lesions that should have been repaired by the nucleotide excision repair pathway (see Chapter 2).

T e physiological importance of translesion DNA synthesis is apparent from a variant form of **xeroderma pigmentosum** that is due to a deficiency in DNA polymerase η (eta). T is DNA polymerase inserts an AA opposite cyclobutane TT dimers (it catalyzes DNA synthesis opposite other damaged nucleotides as well). Af ected patients are extremely sensitive to sunlight and have an elevated risk of cancer.

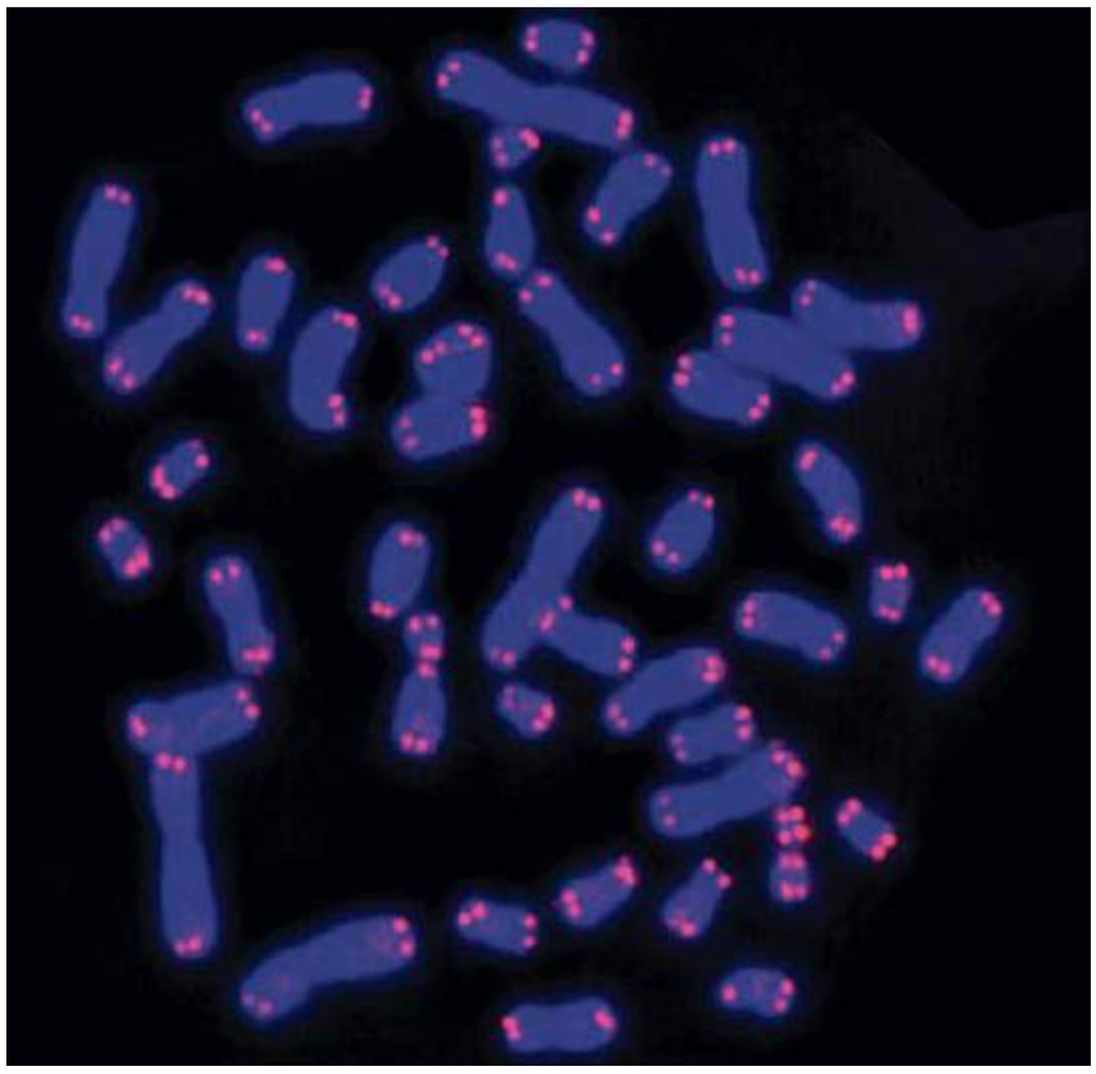


Fig. 3.8 Metaphase chromosomes with telomeres. A metaphase chromosome consists of two sister chromatids (see Section 5 in Chapter 1). Each chromatid has two ends and thus two telomeres. The chromosomes were stained with a telomere-specific, pink fluorescent peptide nucleic acid probe and the chromosomes with the blue fluorescent DNA-binding molecule DAPI (4′,6-diamidino-2-phenylindole). (From Olaussen KA, Dubrana K, Domont J, Spano JP, Sabatier L, Soria JC. Telomeres and telomerase as targets for anticancer drug development. *Crit Rev Oncol Hematol.* 2006;57:191–214.)

3. REPLICATION OF THE ENDS OF CHROMOSOMES (TELOMERES)

T e ends o*f* the chromosomes are called telomeres; they consist largely o*f* repeats o*f* the sequence *TT*AGGG. So that DNA repair complexes do not mistake telomeres *f*or the ends o*f* damaged DNA, the ends o*f* the DNA strands are *f*olded over, and the telomeric repeats are covered by proteins. In most somatic cells, upon replication, each DNA strand loses telomeric DNA until, eventually, short telomeres induce cell senescence. By contrast, telomerase maintains the lengths o*f* the telomeres in germ cells, some stem cells, and most tumor cells.

T e ends of the chromosomes are called **telomeres** (Fig. 3.8), and they play an important role in aging and cancer. Telomeres prevent DNA repair mechanisms from attacking the ends of the chromosomes, provide a means to limit the number of times a cell can divide, and can prevent the irreversible loss of genetic information from the ends of the chromosomes during DNA replication. Immortal cancer cells circumvent the telomere-limitation of cell division.

A telomeric nucleoprotein structure shields the telomeres from recognition by DNA repair complexes (Fig. 3.9).

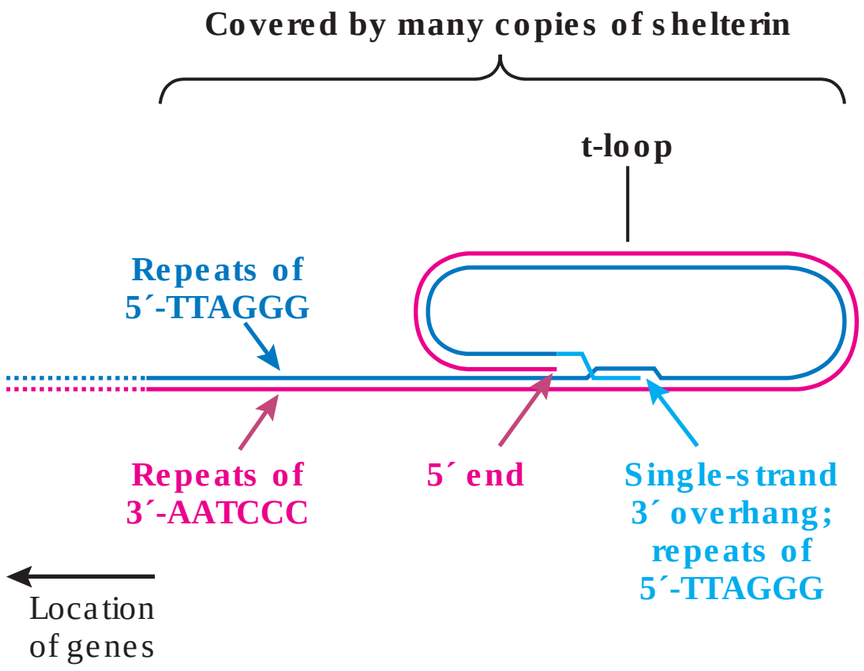


Fig. 3.9 **Structure of a telomere.**

Telomeres are about 4,000 to 15,000 bases long and consist mostly of repeats of the sequence **TTAGGG** (5′-CCCTAA on the complementary strand). The last 20 to 35 TTAGGG repeats at the 3′ end of each DNA strand have no partners in the complementary strand (i.e., they form a single-strand 3′ overhang). A double-strand portion of the telomere loops back, and the single-strand 3′ overhang invades a double-strand portion of the telomere, forming a **t-loop**. Telomeric DNA is covered by **shelterin** protein complexes that specifically bind to telomeric DNA repeats and protects telomeres from being recognized by DNA repair proteins, thereby preventing fusion of chromosomes.

In most somatic cells, the **telomeres shorten** with each cell division (Fig. 3.10). Therefore, telomere length provides the cells with a potential mechanism to count and limit the number of cell divisions. Since the DNA polymerases that are involved in DNA replication require template DNA 3′ to their catalytic site, the normal replication machinery described in

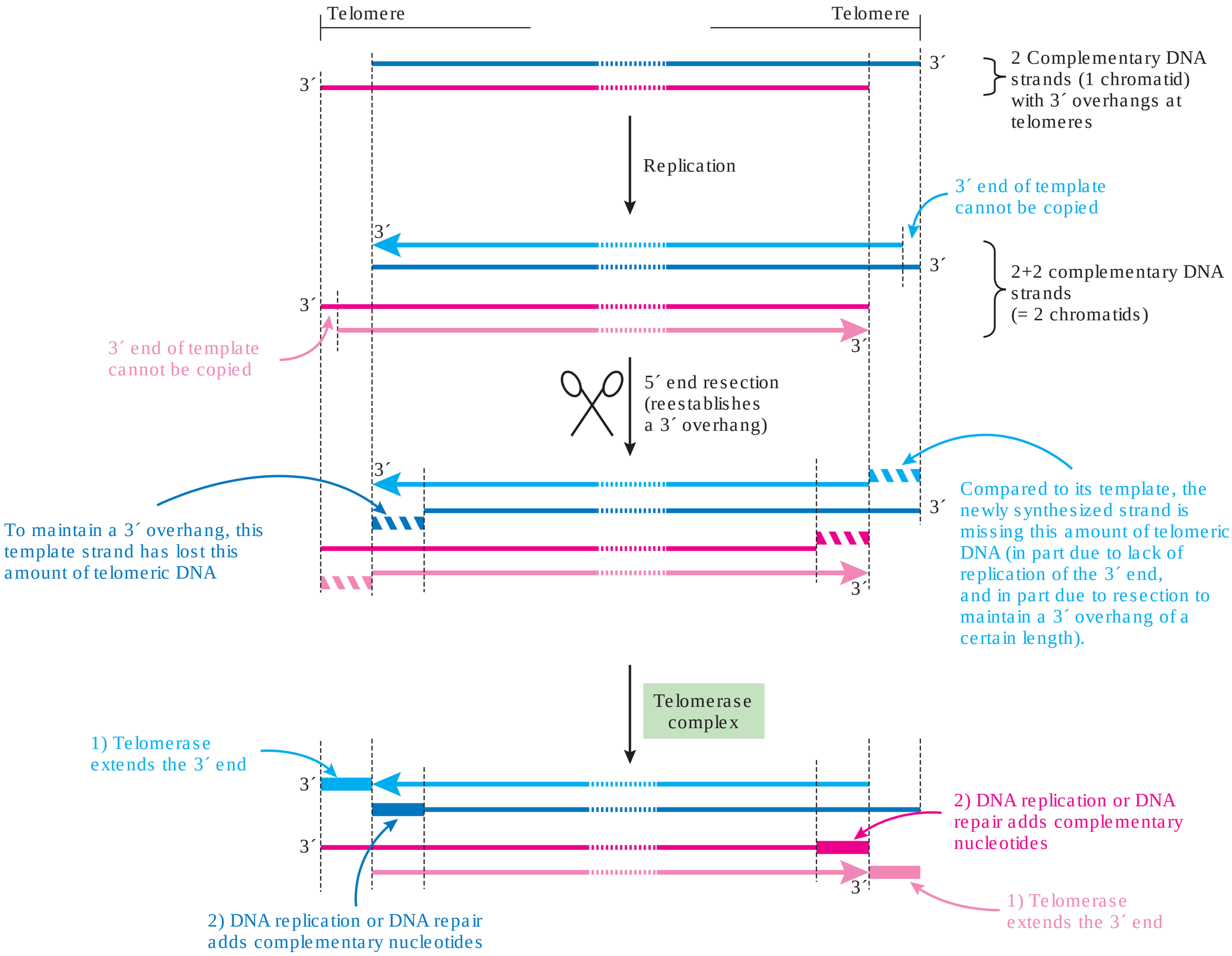


Fig. 3.10 **Telomere shortening and lengthening.** Telomere shortening occurs during replication of the genome. Cells that express telomerase can rebuild the telomere length.

Section 1 cannot replicate the 3′ end of the DNA strand. In addition, an exonuclease degrades a portion of each 5′ end to maintain the length of the 3′ overhang. In vitro measurements show that **fibroblasts** on average lose 40 to 120 bp per telomere with each cycle of replication. Fibroblasts that have a relatively small number of TTAGGG repeats no longer divide (i.e., they enter replicative **senescence)** and stay in the G_0 phase of the cell cycle (see Chapter 8).

T e **telomerase** protein complex can maintain telomere length by adding telomeric DNA repeats, nucleotide by nucleotide, using an RNA template (**telomerase RNA component**, **TERC**) that is stably bound to the protein complex. T us, telomerase has reverse transcriptase activity that can repeatedly copy the sequence 3′-CAAUCCCAAUC-5′ contained within the long TERC RNA to produce the 5′-TTAGGG-3′ telomeric DNA repeats. Numerous proteins regulate the placement of telomerase on single-strand telomeric DNA. Once telomerase is displaced from the telomere, DNA replication or repair processes add complementary nucleotides (deoxy-C, T, and deoxy-A) to the 5′ end of the complementary DNA strand.

Cells with a high or unlimited potential for replication, such as embryonic stem cells, germ cells, stem cells in the bone marrow or the villi of the intestine, activated lymphocytes, and tumor cells express telomerase. Otherwise, normal somatic cells typically have little or no telomerase activity.

For unknown reasons, despite the presence of telomerase in precursor cells, the telomeres of many cells (e.g., circulating white blood cells, muscle, skin, and adipose tissue) become shorter by ~25 bp per year of age. Factors such as smoking, psychological stress, asthma, and chronic obstructive pulmonary disease (COPD) hasten the shortening.

Short telomeres are associated with high mortality, while long repeats are associated with an increased risk for a select number of neoplasms.

T e **heritable telomeropathies** (**inherited telomere syndromes**) known to date are caused by mutations in a subunit of telomerase or in a protein that binds to telomeres. Most pathogenic mutations are accompanied by haploinsufficiency, and telomeropathies therefore usually show autosomal dominant inheritance. Impaired telomere maintenance causes a spectrum of symptoms that may include leukoplakia (white patches on the mucosa of the mouth), skin hyperpigmentation, nail dystrophy, fibrosis of the lungs, bone marrow failure, and liver cirrhosis.

Tumor cells have the means to avoid replicative senescence and apoptosis due to telomere shortening. Tumor precursor cells may avoid senescence by inactivating a cell cycle checkpoint (see Chapter 8). As telomeres continue to shorten as a result of DNA replication, the cells reach a crisis point, at which the telomeres no longer protect the genetic information and apoptosis normally ensues; only cells that can establish protective telomeres survive. Indeed, most immortal tumor cells have active **telomerase** that helps them maintain telomeres, although these telomeres are relatively short. Telomerase is thus a potential target for antitumor drugs. In a minority of tumors, telomeres are maintained by the recombination of chromosome ends or short pieces of circular DNA that contain telomeric repeats; this is called the **alternative lengthening of telomeres** (**ALT**) pathway. Telomeres that are maintained by the ALT pathway are unusually long and dif er greatly in length. T e ALT pathway is found most frequently in sarcomas.

SUMMARY

- Most nuclear DNA is replicated during the S-phase of the cell cycle. Replication starts at the origins of replication (ORIs). Helicases separate complementary DNA strands, while topoisomerases relieve torsional superhelical strain. DNA polymerase α, which shows low fidelity, synthesizes an RNA primer and then extends it with deoxyribonucleotides. DNA polymerase δ and ε extend the growing DNA strand with high fidelity and also replace the RNA primer and the inaccurately copied DNA region with an accurate copy of DNA. DNA ligase joins contiguous pieces of the newly synthesized DNA.
- As a DNA double helix is opened and replicated at an ORI, each parental strand is read continuously on one strand and discontinuously on the other strand. Discontinuous replication of DNA gives rise to Okazaki fragments that are subsequently ligated.
- Nucleotides without a 3′-hydroxyl group can inhibit DNA replication.
- During replication, the translesion DNA polymerases ε (eta), ι (iota), and κ (kappa) read damaged nucleotides on the template DNA that escaped the repair processes. T ese damaged nucleotides include 8-oxoguanine, cyclobutane thymine dimers, cisplatin intrastrand crosslinks, and adducts of polycyclic hydrocarbons with a base. T ese polymerases insert a nucleotide in the growing DNA strand, but they do not repair the damaged nucleotides in the template strand.
- T e ends of the chromosomes are capped by telomeres. Telomeres consist of 700 to 2,500 TTAGGG repeats, the end portion of which forms a t-loop. Shelterin covers the repeats. In most somatic cells, the telomeres shorten with each cycle of replication, and short telomeres induce replicative senescence. Telomerase lengthens telomeres by adding TTAGGG repeats based on its own internal RNA template. Germline cells and some stem cells use telomerase to maintain their telomeres. Tumor cells use telomerase or a recombination-based pathway (termed alternative lengthening of telomeres) to maintain telomeres. Abnormal function of the telomerase complex can lead to premature aging of a number of tissues (e.g., bone marrow, lungs, and liver).

FURTHER READING

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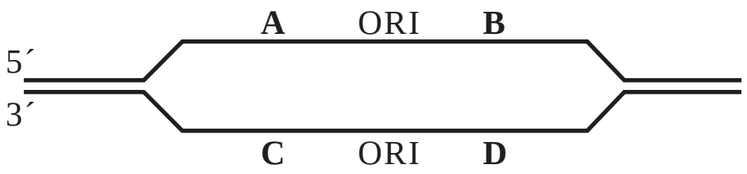
Review Questions



1. The figure above represents DNA undergoing replication. Lagging strand synthesis occurs in which of the following areas?

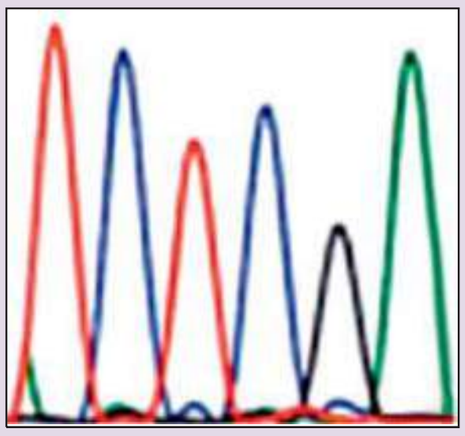
A. A and B
B. A and C
C. A and D
D. B and C
E. B and D

2. Translesion DNA synthesis accomplishes which of the following?

A. Extends DNA strands that end in a dideoxyribonucleotide
B. Inserts nucleotides opposite the damaged nucleotides
C. Joins the lagging strands
D. Refills deletions of DNA nucleotides
E. Replicates the DNA of adjoining Okazaki fragments

3. Which of the following is true about telomeres?

A. A metaphase chromosome contains two telomeres and consists of two chromatids.
B. Telomere length positively correlates with mortality rate.
C. Telomeres shorten during DNA replication.
D. The shelterin complex elongates telomeres.



Chapter 4 Clinical Tests Based on DNA or RNA

SYNOPSIS

- DNA- and RNA-based tests are widely used in the clinic, for instance prenatal diagnosis, diagnosis of hereditary diseases, screening for infectious agents, and optimization of cancer treatment.
- For traditional cytogenetic determination of the karyotype (the set of chromosomes), cells from biopsy material are grown in vitro and arrested in mitosis. The chromosomes are then stained and analyzed under a light microscope.
- In fluorescence in situ hybridization (FISH), fluorescently labeled DNA probes are hybridized with chromosomes. The main application of FISH is detecting chromosome alterations.
- In the polymerase chain reaction (PCR), synthetic DNA primers are used to define a segment of DNA that is then amplified, sometimes by a factor of more than 10^9.
- DNA microarrays consist of an inert surface onto which numerous known DNA sequences are deposited in known locations. Hybridization of test DNA or of test DNA plus competing control DNA, followed by detection, allows analysis of the test DNA for deletions, duplications, and, in special cases, even sequence. There is great variation in the resolving power of DNA microarray–based tests.
- Sanger sequencing is the traditional method for sequencing nucleic acids. More recently, massive parallel sequencing has revolutionized molecular diagnostics; it is used, for example, to detect inherited mutations and analyze mutations in a tumor.
- DNA from the placenta circulates in the pregnant mother's blood and can be analyzed by massive parallel sequencing to estimate the chromosome complement of the fetus.

LEARNING OBJECTIVES

For mastery of this topic, you should be able to do the following:

- Describe the sample requirements for a traditional cytogenetic analysis and the level of detail one can expect to find in the final karyotype report.
- Describe FISH and explain the results one can expect to obtain.
- Explain how PCR can amplify one or more specific DNA fragments. Describe quantitative (real-time) PCR. Explain how the threshold cycle is used for quantification of test DNA.
- Explain how restriction enzymes and capillary electrophoresis can be used to determine the presence or absence of a mutation.
- Explain how DNA melting curve analysis can be used to determine the presence or absence of a mutation.
- Describe the type of result one can expect from aCGH (array comparative genomic hybridization) and from DNA SNP (single nucleotide polymorphism) microarrays.
- Compare and contrast Sanger sequencing and massive parallel sequencing.
- Explain how DNA from a pregnant woman's blood can be used to estimate the karyotype of her fetus.

1. CONVENTIONAL CYTOGENETICS AND FLUORESCENCE IN SITU HYBRIDIZATION

In conventional cytogenetics, viable cells from biopsied tissue, blood, or bone marrow are grown in vitro and arrested in metaphase, when the chromosomes are maximally condensed and clearly separated from each other. The results of analyzing stained chromosomes are summarized in a karyotype report. For FISH, one or more fluorescently labeled nucleic acid probes for genetic loci of interest are allowed to hybridize with nucleic acids that have been made single stranded. Then, fluorescent spots are then assessed for rearrangements.

For a traditional cytogenetic analysis and determination of karyotype, viable cells from a fetus or a patient are grown in vitro, arrested at metaphase (when chromosomes are condensed), dropped onto a glass slide, stained, and analyzed for abnormalities based on the unique banding pattern of each type of chromosome (Fig. 4.1; also see Fig. 1.12). Cells used for cytogenetic analyses must therefore be able to divide and be free of infection. The most commonly used chromosome stain is Giemsa in a technique called **G-banding,** or Giemsa banding. The two most common indications for determining a karyotype are cancer and prenatal diagnosis.

Aneuploidy is the presence of a number of chromosomes that is not an exact multiple of 23, such as 45 chromosomes instead of the 46 present in a normal diploid karyotype (see Fig. 4.1).

To be visible in a cytogenetic karyotype analysis, a deletion or insertion must be at least approximately 5 million base pairs (bp) long.

For **FISH**, metaphase or interphase chromosomes of cells from a patient are hybridized with a DNA segment that is specific for the genetic locus of interest and conjugated with a fluorescent molecule (Fig. 4.2). FISH on metaphase chromosome spreads allows determination of the chromosome location of the signal. FISH on interphase chromosomes is performed on nondividing cells and can therefore be used on histology tissue sections, so that the FISH signal can be correlated with histologic features. In cells that have a normal karyotype, two signals (fluorescent spots) per nucleus are seen, one for each gene in the two autosomes. If there is partial or complete duplication of a chromosome, or if the genetic locus under study is amplified, more than two signals are seen. Conversely, the deletion of the gene under study leads to a reduction in the number of signals. FISH detects deletions larger than approximately 150,000 bp. Accordingly, FISH can detect **microdeletions** and **microduplications**, which have a size of approximately 1 million to 3 million bases and cannot

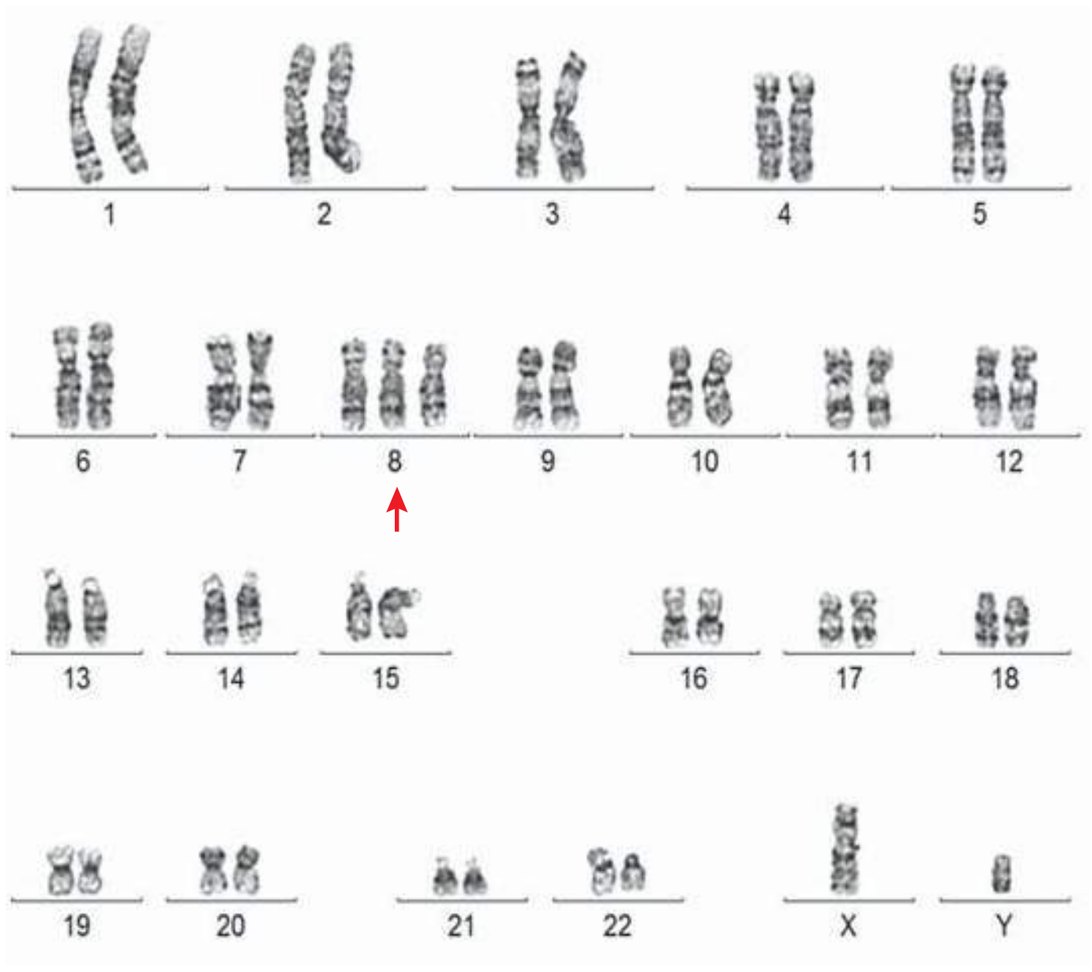


Fig. 4.1 Abnormal karyogram of a bone marrow cell from a patient with acute myeloid leukemia. Cells in the bone marrow acquired a third copy of chromosome 8 *(arrow)*. (Courtesy Dr. Barry L. Barnoski, Oncocytogenetics Laboratory, Cooper University Hospital, Camden, NJ.)

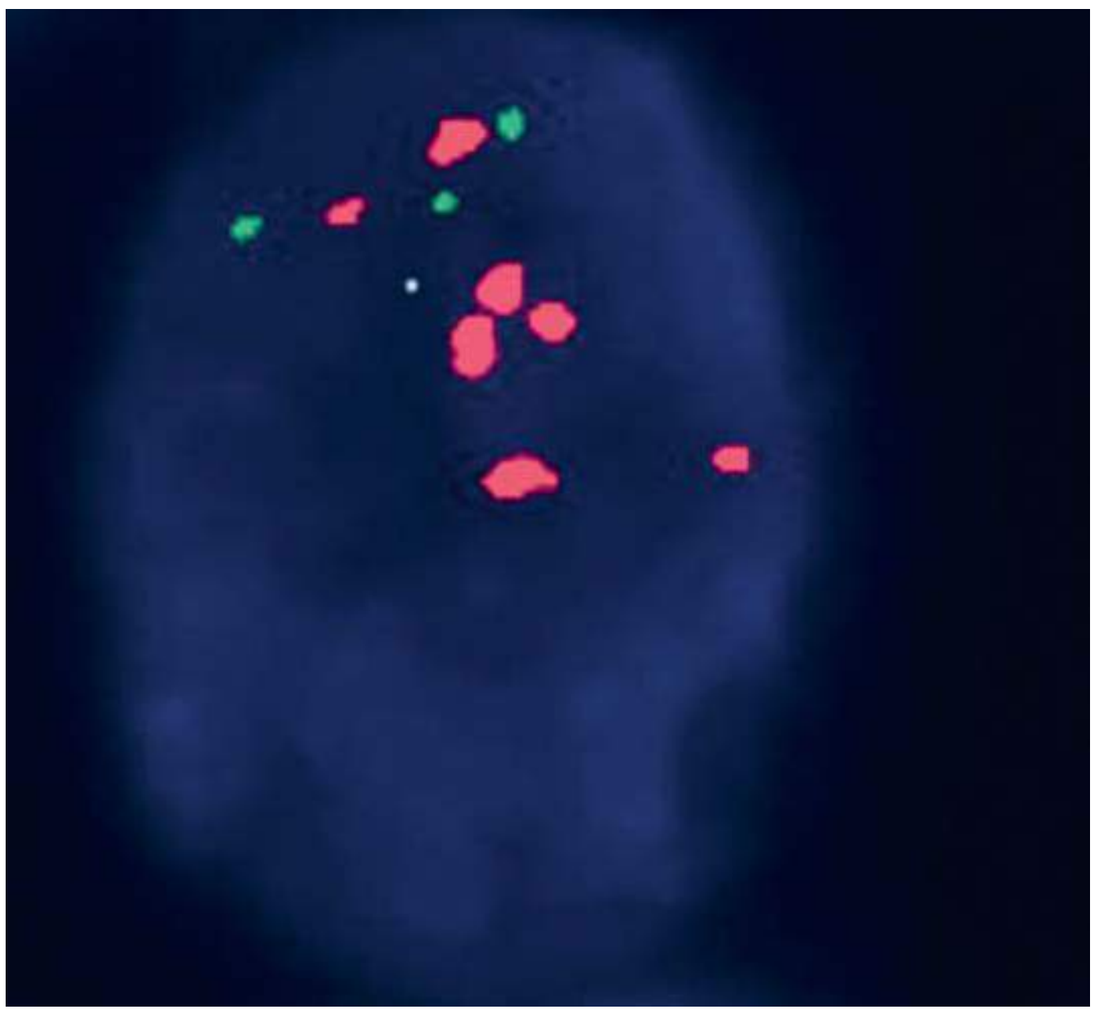


Fig. 4.2 Example of fluorescence in situ hybridization to interphase chromosomes. Breast cancer cells were tested for epidermal growth factor 2 (*HER2*) status. The red fluorescent probe binds to *HER2*, and the green fluorescent probe binds to the centromere of chromosome 17. In a normal cell, two red and two green signals are present. In tumors that meet criteria for *HER2* amplification, the number of red signals is ≥6/cell, or the number of red signals is ≥4/cell and the ratio of red to green signals is ≥2. The image shows a single-cell nucleus *(blue)*. *HER2* is amplified. (Data from Pu X, Shi J, Li Z, Feng A, Ye Q. Comparison of the 2007 and 2013 ASCO/CAP evaluation systems for HER2 amplification in breast cancer. *Pathol Res Pract*. 2015;211:421-425.)

be detected with G-banding of metaphase chromosomes. FISH is not sensitive enough to detect point mutations or small deletions. It provides data on single cells.

FISH can also be used to detect **rearrangements** within and between chromosomes (e.g., translocations). The rearrangement may lead to **separation** or **fusion** of two differently colored FISH probes for two different genetic loci.

2. DNA AMPLIFICATION BY POLYMERASE CHAIN REACTION

PCR is used to amplify select DNA segments in a DNA sample. Primers determine the start and end of a sequence to be amplified. In clinical lab tests, PCR is often used to determine the amount of a particular DNA in a sample, but the technique also has other applications (see Sections 3 and 4).

PCR is a frequently used template amplification technique in RNA- or DNA-based clinical tests. PCR produces multiple copies of a segment of DNA that is bounded by two specifically chosen oligonucleotide sequences called **primers** (Fig. 4.3). The oligonucleotide primers (in vitro synthesized single-stranded DNA approximately 15 to 20 nucleotides long) are needed for **DNA polymerase** to be active. As shown in Chapter 1, complementary DNA strands are by convention presented with the 5′-end of the coding strand in the top left. In such a representation, the two primers are sometimes referred to as the **forward primer** and the **reverse primer**.

A **PCR cycle** involves heat denaturation to separate complementary DNA strands, cooling to allow hybridization (annealing) of primers to the complementary sequences of the template, and moderate heating to increase the activity of DNA polymerase, which extends the primers, using the hybridized DNA strand as a template (Fig. 4.4). To simplify DNA amplification, a heat-tolerant DNA polymerase is chosen (typically from an organism that lives in a hot spring). A full PCR cycle typically takes approximately 1 to 2 minutes.

After 20 and 30 PCR cycles, the template DNA molecule has been copied approximately 1 million (2^{20}) to 1 billion (2^{30}) times, respectively. At this point, the nonamplified DNA (the

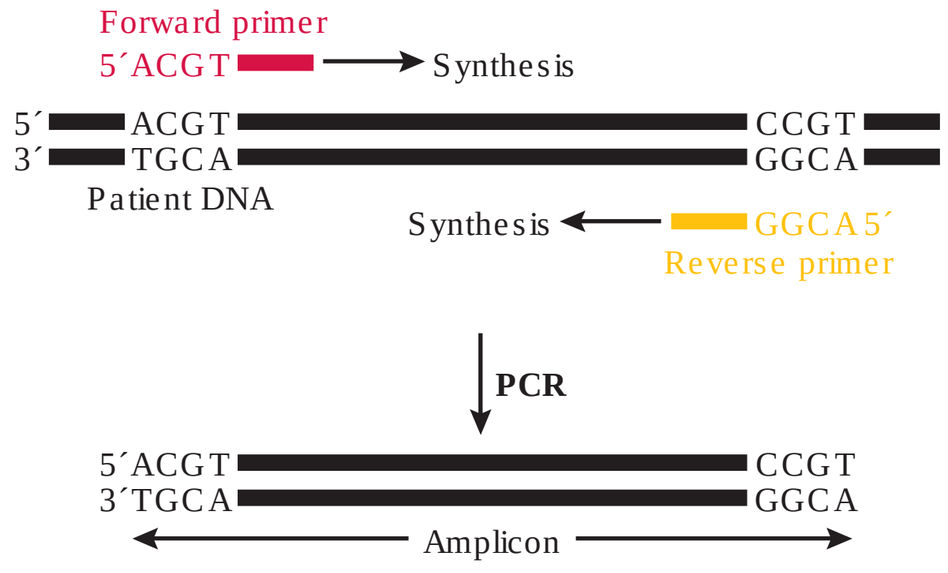


Fig. 4.3 Primer-directed in vitro DNA synthesis. The forward primer uses the bottom strand as a template and thereby synthesizes a new top strand. The reverse primer uses the top strand as a template and thereby synthesizes a new bottom strand.

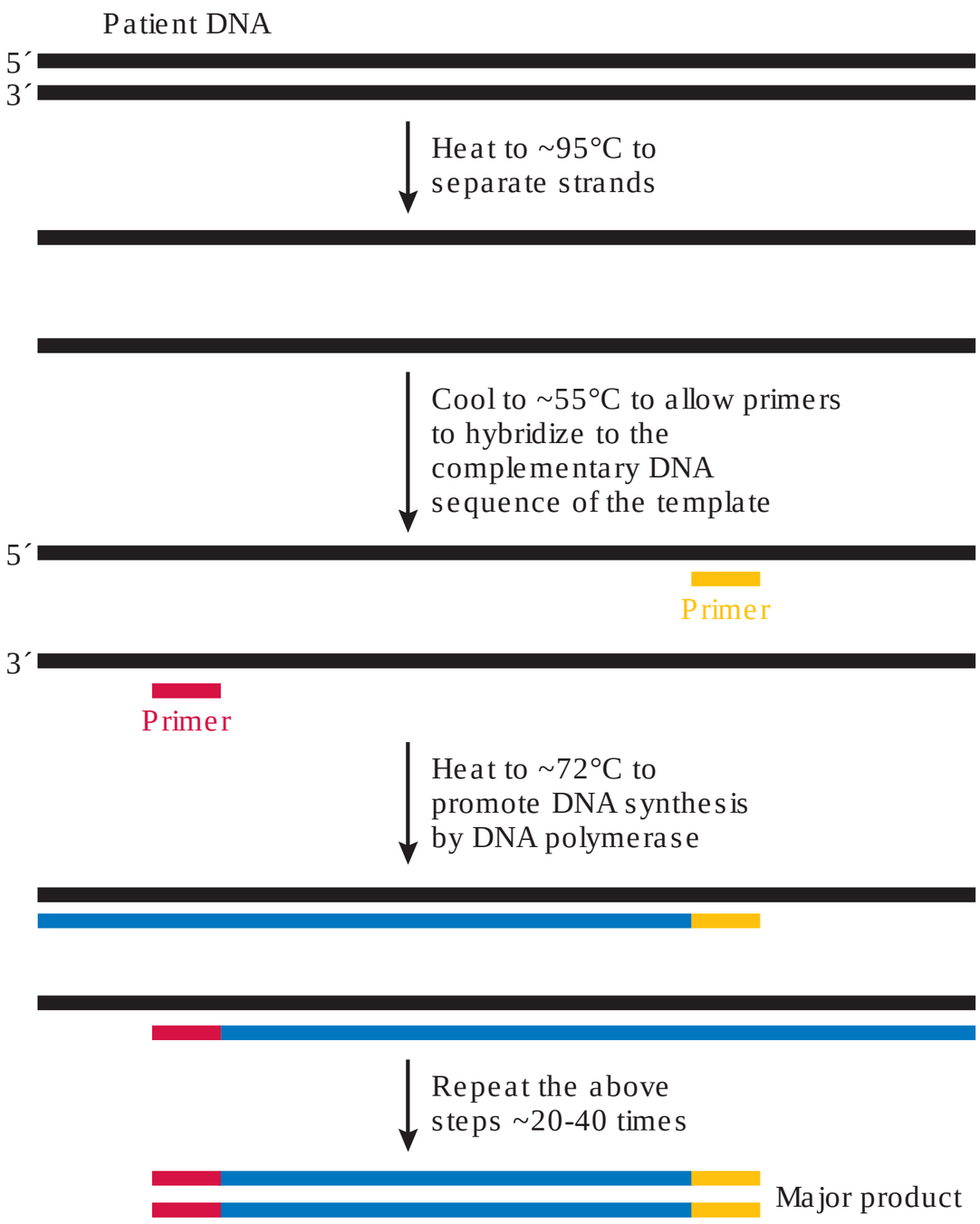


Fig. 4.4 Principle of the PCR amplification of a DNA sequence. PCR requires two different primers so that both strands can be amplified with a defined start and end sequence. The DNA polymerase stems from an organism that is adapted to high temperature.

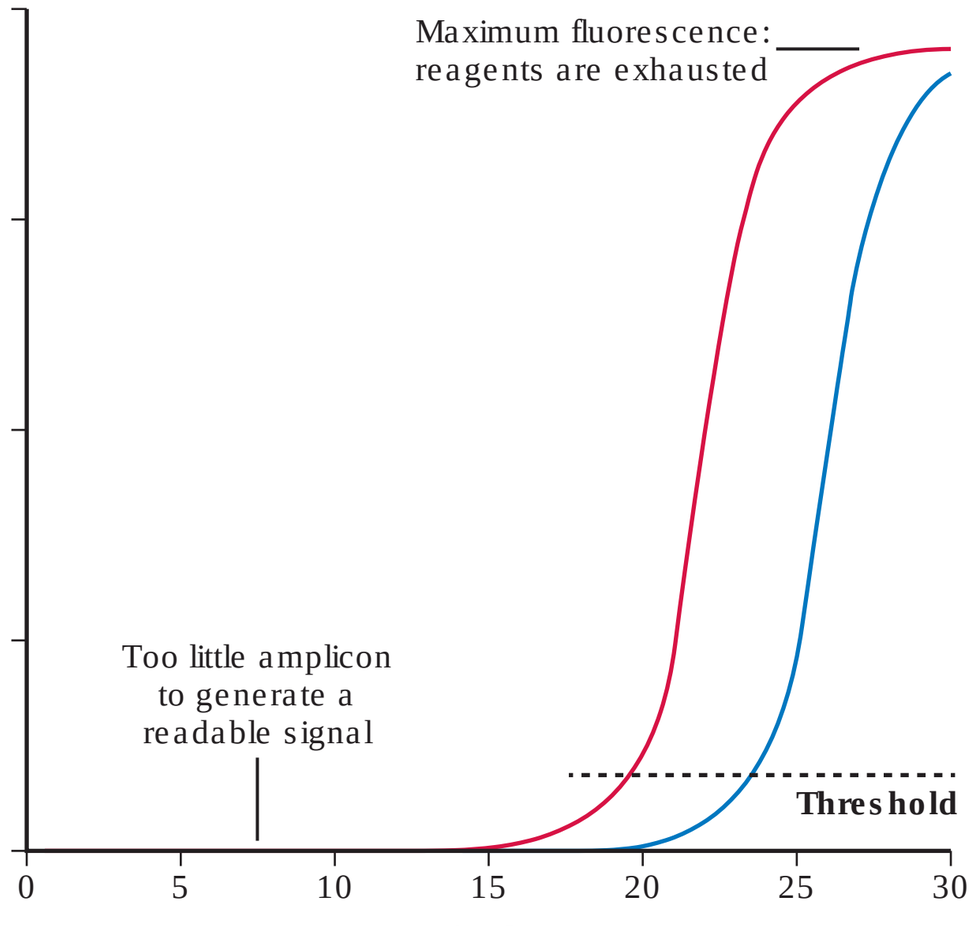


Fig. 4.5 Quantitative PCR amplification. The amount of DNA in the sample increases exponentially and is followed with a fluorescent dye during each cycle. The cycle at which the amount of DNA crosses the predetermined threshold is a measure of the amount of DNA in the initial sample.

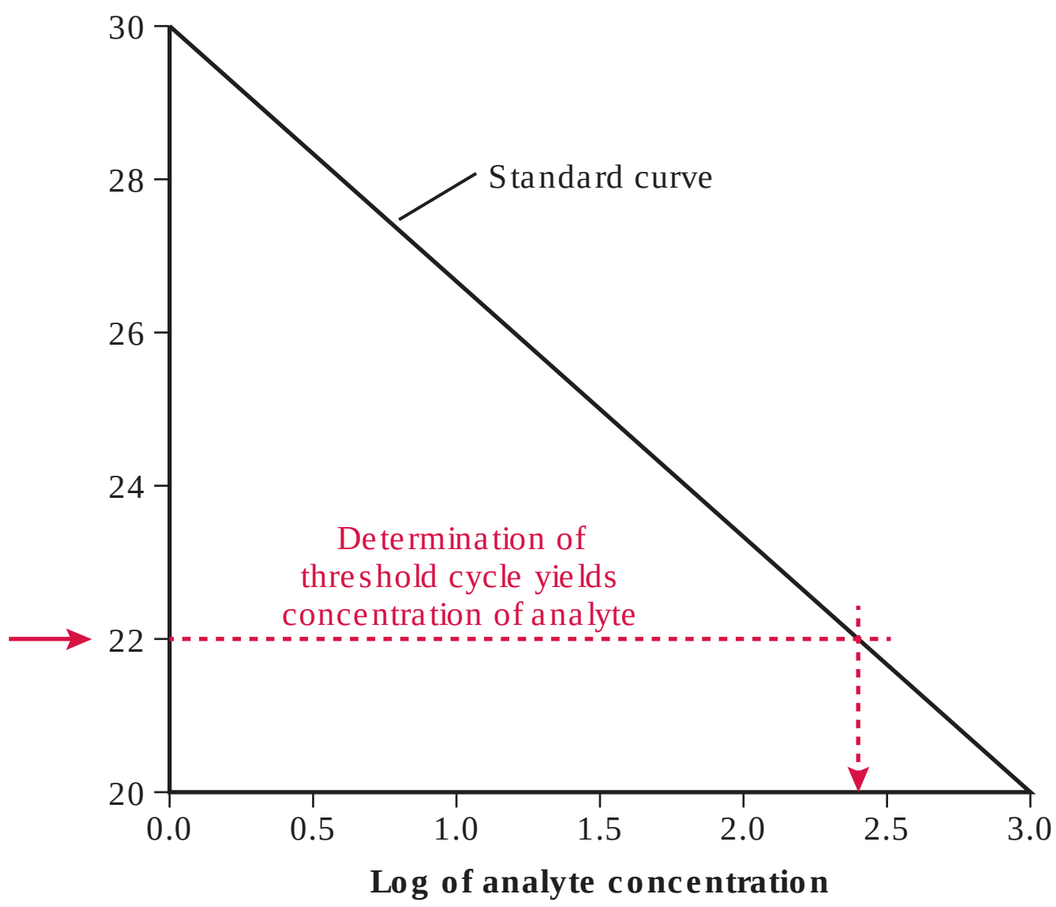


Fig. 4.6 Standard curve for real-time PCR that relates analyte concentration to threshold cycle. Fig. 4.5 explains the threshold cycle. The higher the threshold cycle number for a sample, the lower the concentration of the analyte.

DNA that is not of interest) has faded into nonsignificant background abundance.

In **reverse transcription PCR** (**RT-PCR**), an **RNA** template is reverse transcribed into complementary DNA (**cDNA**), which is then amplified as in regular PCR.

Multiplex PCR refers to a procedure during which more than two primers are present so that more than one DNA (or cDNA) sequence can be amplified simultaneously. Multiplex PCR can be used to analyze several regions of interest at the same time (one of these regions may be used for quality control).

After PCR amplification, the amplified DNA segment (**amplicon**) is analyzed further, often by one of the methods described in Sections 3 and 4.

T e handling of amplicons for further analysis poses a risk of **contamination** because of the very high concentration of amplicon compared with the DNA segment of interest in patient samples. Many labs perform preamplification and postamplification steps in separate areas.

For **real-time PCR**, fluorescently labeled probes that are specific for the sequence of interest are added to the reaction, and fluorescence is measured at each PCR cycle (Fig. 4.5). T is technology is often used for quantification and is sometimes called **quantitative PCR** (**qPCR**). A threshold fluorescence is set, and a calibration curve (Fig. 4.6) is established that relates the threshold cycle (i.e., the cycle at which the fluorescence crosses the threshold) to the amount of template DNA. T is permits quantification of template DNA in patient samples. Since real-time PCR provides the results directly during the run, no post-PCR handling of the amplicons is necessary, which significantly reduces the risk of contamination.

3. ELECTROPHORESIS AND MELTING CURVE ANALYSIS OF DNA

Electrophoresis allows determination of the size of a nucleic acid fragment. Restriction enzyme digestion permits probing of a small part of the sequence of a DNA segment. Melting curves allow the detection of a difference between DNA sequences that match a probe perfectly and ones that match imperfectly. All of these tests are commonly performed on PCR-amplified DNA.

Electrophoresis is commonly used for size separation of nucleic acid fragments. An electrical field pulls the negatively charged molecules through a matrix, such as an agarose or polyacrylamide gel or a polymer-filled capillary. Small DNA molecules migrate the fastest because they experience the least amount of drag in the matrix; conversely, large DNA molecules migrate the slowest.

Capillary electrophoresis is used in modern Sanger sequencing (see Fig. 4.10) and to determine the fragment length after PCR or digestion with a restriction enzyme (see below).

Digestion of PCR-amplified DNA with **restriction enzymes** (**restriction endonucleases**) allows one to gain limited sequence information (Fig. 4.7). Hundreds of restriction enzymes are available that cut only at specific sequences (these are mostly palindromic; i.e., they read the same on both DNA strands) and in a specific manner. It is likely that a restriction enzyme can be found that cuts either only the normal or the known mutant sequence. After the reaction has gone to completion, the size of the products is determined by electrophoresis.

Instead of digestion with a restriction enzyme, **melting curve analysis** by real-time PCR (Fig. 4.8) can be used to identify small known sequence variations. The real-time PCR is performed with the usual primers and also with two labeled, sequence-specific nucleic acid probes. At low temperature, one of the two probes anneals with the segment where the known mutation is located, regardless of the presence or absence of the mutation. The probe that anneals closer to the 5′ end of the amplified DNA segment has a **donor chromophore** at its 3′ end, and the second probe (which hybridizes directly to the first probe) has an **acceptor chromophore** at its 5′ end. When the two probes bind next to each other on an amplified DNA, and when they are illuminated with the light that the donor chromophore absorbs efficiently, **Förster resonance energy transfer** to the acceptor chromophore takes place. The acceptor chromophore then fluoresces at its specific wavelength, which is measured by the real-time PCR instrument. At the end of the amplification cycles, the probes are annealed and the fluorescence intensity is high. The sample is then heated gradually, causing the probes to melt of the amplified DNA. If the probe does not perfectly match the patient's DNA, it dissociates at a relatively low temperature, thereby causing a loss in fluorescence. By contrast, the probe that perfectly hybridizes to the patient's DNA dissociates at a relatively high temperature. For interpretation, the melting curve for the test DNA is compared to a standard.

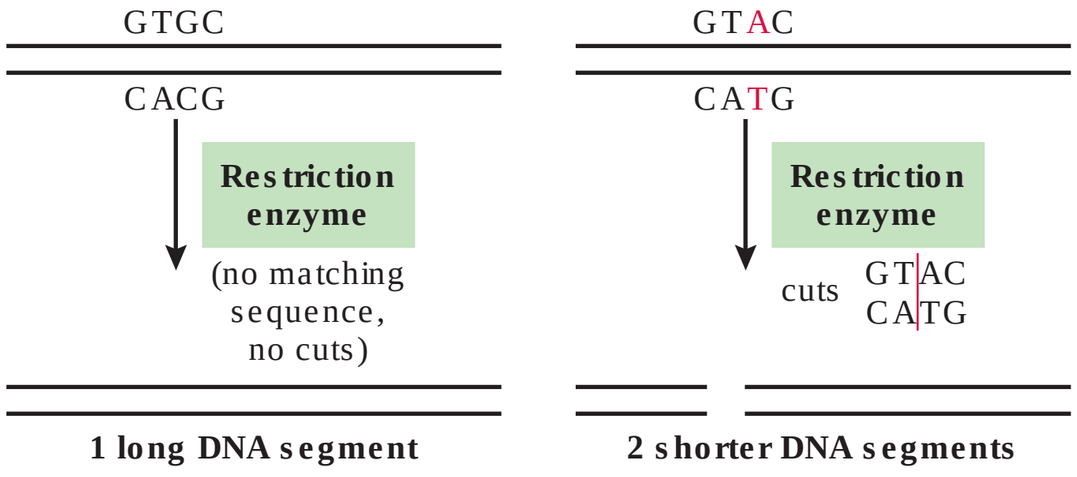


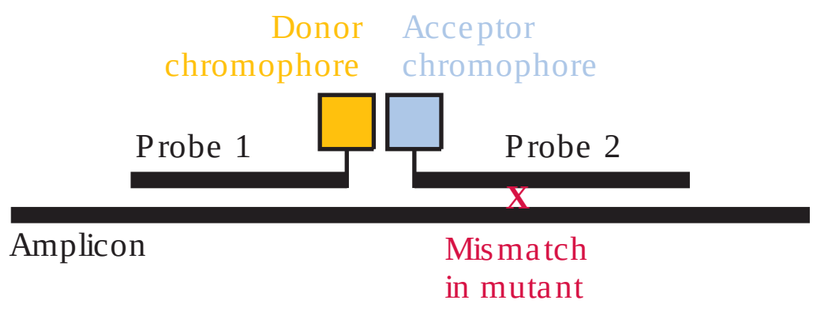
Fig. 4.7 Digestion of PCR–amplified DNA with a restriction enzyme. After digestion with the restriction enzyme, DNA fragment length is determined by electrophoresis. The procedure can be used, for example, to test for the C282Y mutation in the *HFE* gene of patients suspected of having *HFE* hemochromatosis (see Section 9.2 in Chapter 15).

4. DNA MICROARRAY–BASED TECHNOLOGIES

DNA microarrays contain known DNA sequences at known locations and can be used to probe test DNA for deletions, duplications, and—at a limited number of chosen locations—sequence.

A **DNA microarray** (sometimes called a **chip**) consists of a treated flat surface (often glass) approximately 25 × 25 mm^2 in size that has been densely printed with several copies each of thousands to more than 1 million different segments of DNA (often called **probes**). The sequence of each probe is known, as is its location in the array. Sample DNA (and sometimes control DNA) is allowed to hybridize with the probes. The extent of hybridization is then measured.

Depending on the length, diversity, and design of the probes in a DNA microarray, different resolutions and goals are achieved. The longer probes bind DNA fragments regardless of a minor sequence variation. This is used to scan the entire genome for **copy number variations** (CNVs; i.e.,



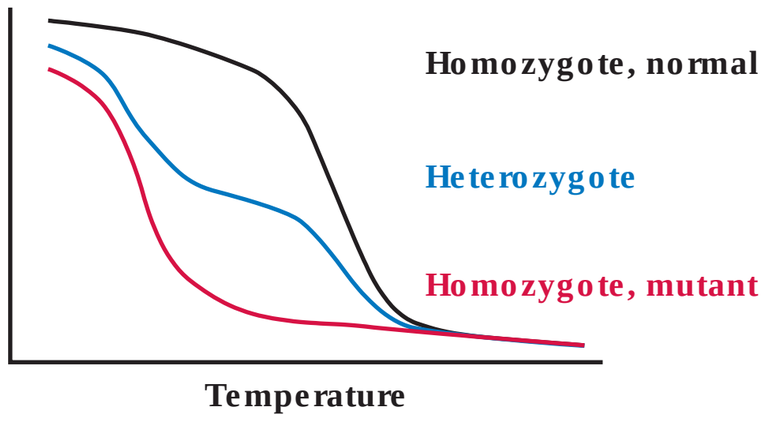


Fig. 4.8 Melting curve analysis.

deletions or amplifications; see array comparative genomic hybridization below). Balanced translocations are not detectable because DNA is fragmented before analysis. The shorter probes can be designed to differentiate between short sequences that differ by a single nucleotide (see single nucleotide polymorphism microarrays below).

In one of the most common clinical applications, **array comparative genomic hybridization** (**aCGH**), microarrays are exposed simultaneously to fragments of control DNA labeled with one particular fluorophore and fragments of test DNA labeled with a different fluorophore (Fig. 4.9). The test and the control DNA fragments compete for hybridization with complementary probes on the microarray. The fluorescence of each fluorophore is determined for every printed array location. At each location, the ratio of the fluorescence of the two fluorophores is indicative of the ratio of test DNA to control DNA.

DNA **single nucleotide polymorphism (SNP) microarrays** are designed to determine the identity of single nucleotides at thousands of specific loci in the genome. A SNP (pronounced "snip") is a locus in the genome at which a different single base (A, C, G, or T) is commonly found in the population. SNPs are so polymorphic (diverse) in the population that people likely inherit two different versions of the SNP from their parents. The array includes probes for both DNA strands and for all relevant SNPs. Fluorescently labeled DNA of a patient is hybridized to the SNP array and binds mostly to perfectly complementary probes on the array.

SNP arrays can be used to identify sequence variations at SNPs, to compare the SNP distribution of related individuals and perform linkage analysis, and to determine sites of **loss of heterozygosity.** DNA segments that are involved in loss of heterozygosity contain numerous SNPs. If there is loss of heterozygosity (see Section 4.2 in Chapter 2), stretches of neighboring SNPs lose their natural diversity (because of uncompensated loss of a DNA segment or homologous recombination repair of DNA).

In clinical practice aCGH and SNP arrays are often combined.

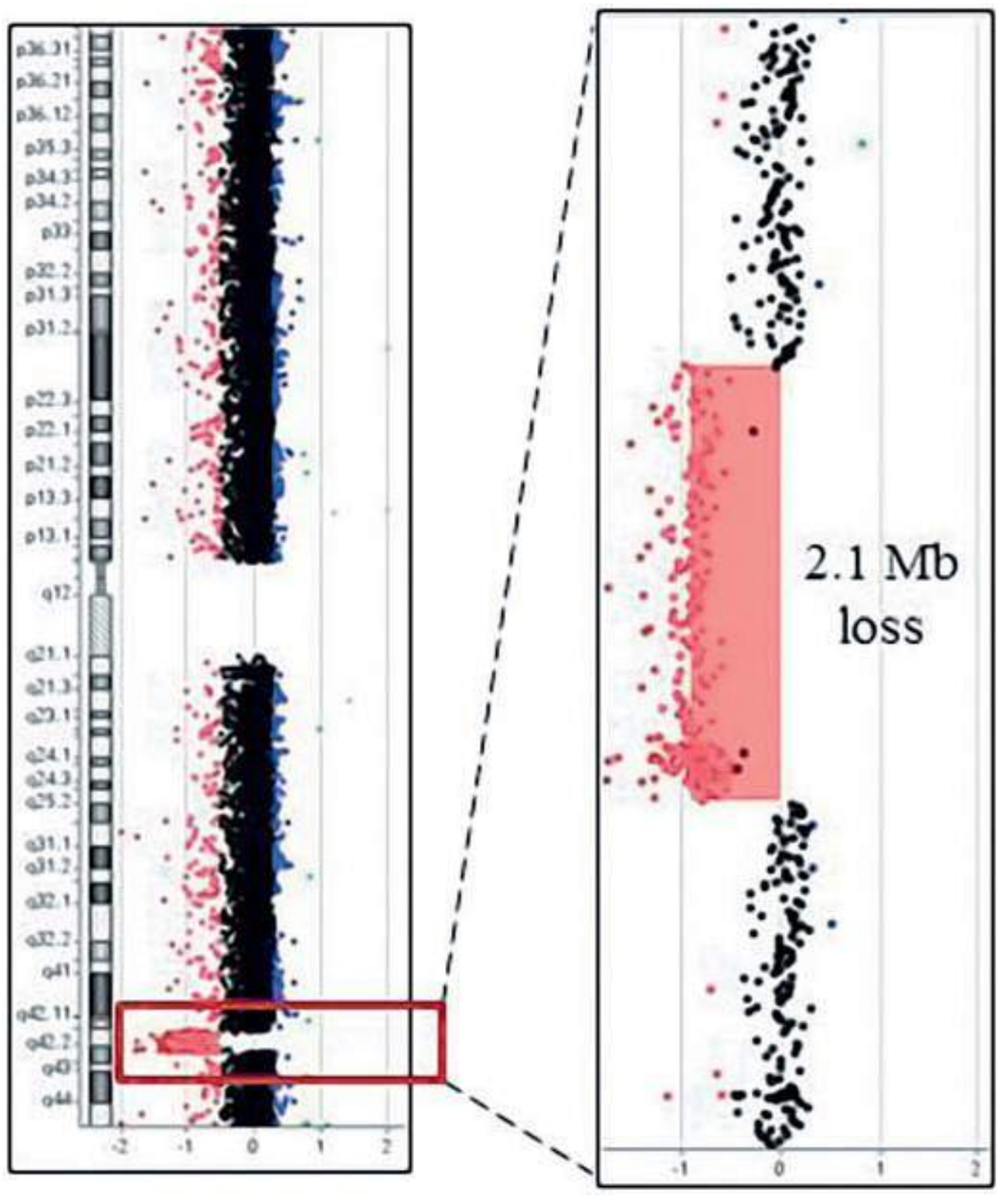


Fig. 4.9 Microarray analysis of chromosome 1. The leftmost part of the figure shows a schematic of chromosome 1. Each dot in the graph represents the reading of one oligonucleotide probe. In the area that is free of dots (approximately the centromere and q12 to q21.1), no oligonucleotide probes were present. The numbers on the horizontal axis refer to the numbers of DNA segments lost or gained relative to control DNA. The data relate to a newborn with brain malformation. (From Peters DG, Yatsenko SA, Surti U, Rajkovic A. Recent advances of genomic testing in perinatal medicine. *Semin Perinatol.* 2015;39:44-54.)

5. DNA SEQUENCING

Sanger sequencing is a highly accurate but slow and expensive way of sequencing DNA with chain-terminating nucleotides. Massive parallel sequencing is used extensively in molecular diagnostics. It involves attaching DNA fragments to a surface, multiplying them locally, and then sequencing them at their known location; with algorithms, the sequence data are assembled into a genome (akin to a puzzle).

5.1. Sanger Sequencing

Sanger sequencing is built on the principle that DNA-synthesizing DNA polymerase can incorporate a **dideoxynucleotide**, which causes **chain termination** (dideoxynucleotides lack a 3′-hydroxyl group that could form a phosphodiester with an incoming nucleotide; see Fig. 3.2). If a mixture contains approximately 99% normal deoxynucleotides and approximately 1% chain-terminating dideoxynucleotides, a chain ends whenever a dideoxynucleotide is incorporated. Typically, the four different chain-terminating dideoxynucleotides (A, T, C, G) are labeled with four different fluorescent dyes. This generates DNA fragments that fluoresce with the color that befits the nucleotide at the 3′-end of the chain. Separating the resulting chains with one base resolution (now generally done with capillary electrophoresis; see Section 3) and detecting the four different fluorescence colors allow determination of the nucleotide sequence (Fig. 4.10).

5.2. Massive Parallel Sequencing

Massive parallel sequencing (**second-generation sequencing**, **next-generation sequencing**, or **deep sequencing**) refers to the simultaneous sequencing of multiple DNA segments. This is now often achieved by fragmenting DNA, enriching the DNA for regions of interest, creating a DNA library attached to a solid support, and then sequencing the attached DNA fragments at their specific location step by step while

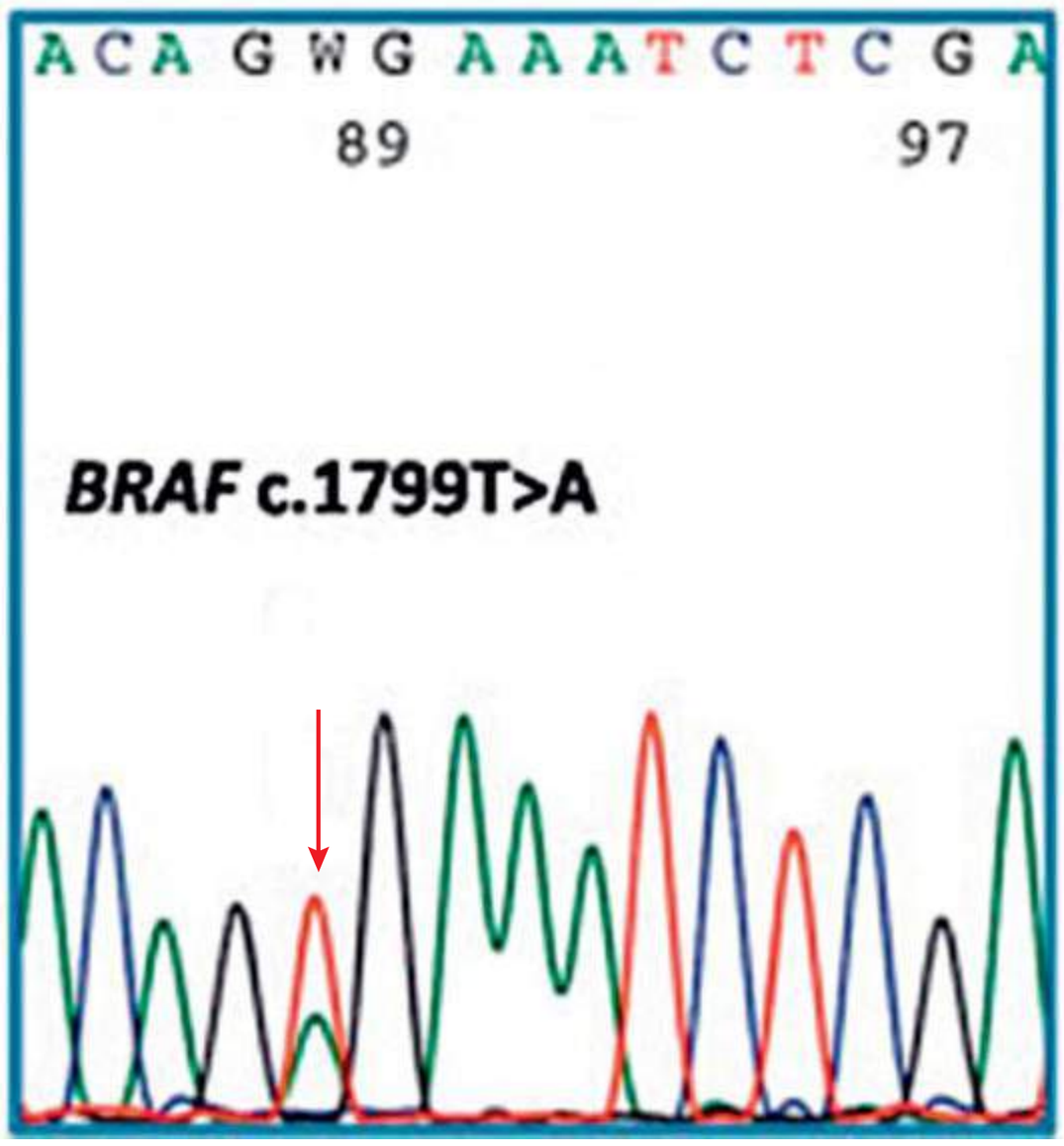


Fig. 4.10 Sample result of Sanger sequencing of a lung nodule. The nodule contained some cells that had a normal *BRAF* sequence with the expected red peak, signifying a T (fifth base shown, *red arrow*), and cells that contained a T→A mutation, seen as a smaller green peak at the same location. (From Yousem SA, Dacic S, Nikiforov YE, Nikiforova M. Pulmonary Langerhans cell histiocytosis: profiling of multifocal tumors using next-generation sequencing identifies concordant occurrence of BRAF V600E mutations. *Chest*. 2013;143: 1679-1684.)

following the reaction with imaging. Multiple competing technologies are available.

After massive parallel sequencing, the obtained sequences (**read**s) are assembled into whole chromosomes using computer-based algorithms (Fig. 4.11). The algorithms take advantage of overlapping sequences and knowledge of the human genome. For genetic testing (when all tested cells are expected to be genetically identical), there should be 30 or more reads. When searching for somatic mutations in tumors, the sequencing depth can be as high as 1000 reads; this permits the detection of a mutation in as little as approximately 5% of all cells.

In search of a genetic alteration, next-generation sequencing can cover the entire genome (**whole-genome sequencing**) or be limited to all exons (**exome sequencing**). The exome makes up only ~2% of the genome, yet it contains ~85% of disease-causing mutations. The cost of sequencing ~95% of the exome is currently ~10% of the cost of sequencing ~98% of the whole genome.

For sequencing, hotspot mutation panels are available that cover up to several hundred DNA segments in which mutations are known to occur. These panels can be used to determine the spectrum of significant mutations in a

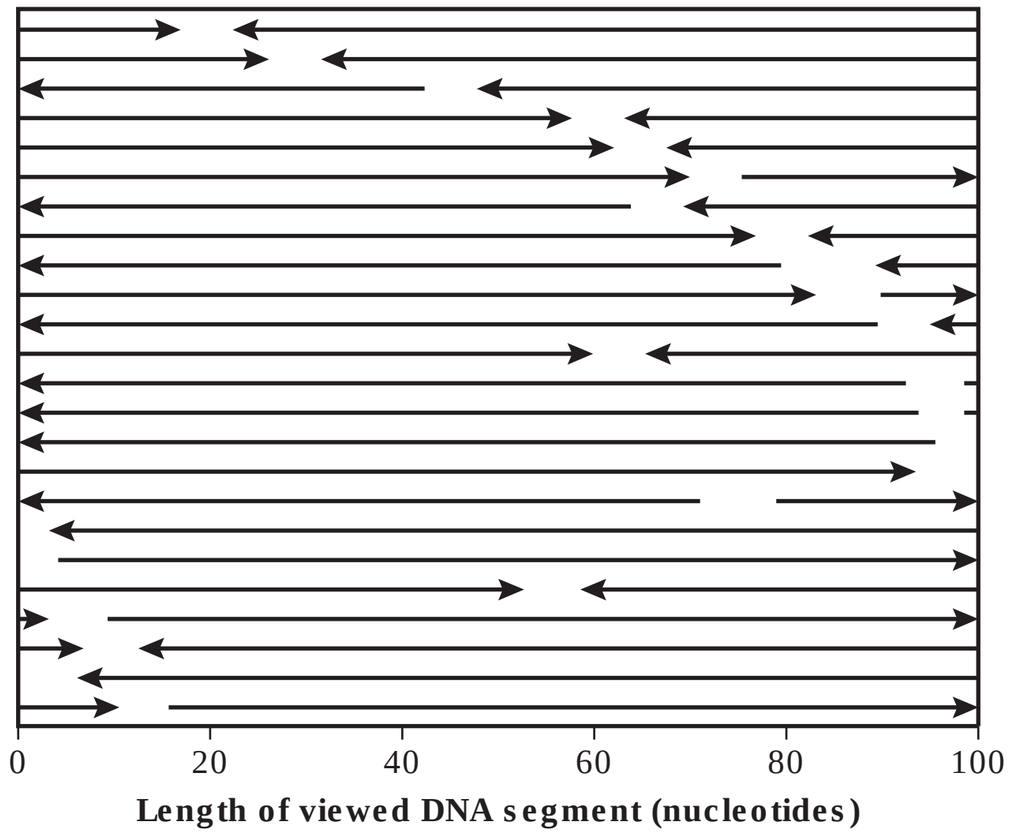


Fig. 4.11 Processed results of massive parallel sequencing. Arrows indicate the direction of the sequenced strands (DNA has two antiparallel, complementary strands). Fragments with arrows that point to the very left or right end of the graph extend beyond the viewed frame. The maximum read depth for the depicted 100-base segment is 24.

tumor or elucidate the genetic basis of a patient's heritable disorder.

At present, the challenge is not so much the generation of sequencing data at an affordable price and with reasonable speed, but the curation and interpretation of the highly complex data. For instance, exome sequencing of a single patient's DNA yields approximately 25,000 variants, and genome sequencing of the same DNA yields approximately 3 million variants. It is challenging to separate variants of clinical significance from **variants of unknown significance (VUS)**. Furthermore, it is difficult to determine which data should be disclosed and how the complex information should be explained to individual patients.

6. SELECTED CLINICAL APPLICATIONS OF DNA-BASED TESTING

DNA-based testing is extensively used in prenatal diagnosis, in determining the cause of infections, in diagnosing certain hereditary disorders, and in assessing tumors for genetic alterations relevant to diagnosis, prognosis, or treatment.

6.1. Prenatal Diagnosis

Several approaches are used for prenatal testing: proteins in the mother's serum (triple screen, quad screen, penta screen; see Section 2.3 in Chapter 31), ultrasound examination of the fetus, and analysis of DNA from the fetus.

About 1% to 2% of fetuses are **aneuploid**, and about another 3% to 4% have a **microdeletion** or **microduplication**. The major aneuploidies include trisomy 13 (**Patau** syndrome), trisomy 18 (**Edwards** syndrome), trisomy 21 (**Down**

syndrome), **Turner** syndrome (only 1 X chromosome), and **Klinefelter** syndrome (XXY). Fetuses with most other aneuploidies are not viable. The major microdeletion syndromes are a microdeletion in the long arm of chromosome 7 causing **Williams** syndrome, a microdeletion or imprinting error of a region in the long arm of chromosome 15 near the centrosome causing either **Prader-Willi** or **Angelman** syndrome, and a microdeletion in the long arm of chromosome 22, near the centrosome (causing **DiGeorge** syndrome in its most severe manifestation).

DNA made by the fetus can be obtained by withdrawing **amniotic fluid** (**amniocentesis**), taking a biopsy of **chorionic villi** (**chorionic villus sampling**), or using a sample of the mother's **blood**. The invasive procedures of amniotic fluid sampling and chorionic villus sampling carry up to ~1% risk of pregnancy loss. Taking a blood sample of a pregnant woman for analyzing cell-free DNA carries no such risk, but the analysis of this DNA is far less sensitive and more complicated.

Fetal cells obtained from amniotic fluid or chorionic villus sampling can be cultured, arrested in metaphase, stained, and analyzed by **G-banding** (see Section 1). G-banding readily identifies aneuploidies but not microdeletions. **FISH** probes can detect the most common aneuploidies, and they are also used to find or confirm microdeletions and microduplications. A combination of **aCGH** and **SNP DNA microarray** of fetal DNA uncovers the same aneuploidies and unbalanced chromosome alterations as G-banding, but it cannot detect balanced translocations or triploidy (see also Section 4). However, this combined microarray technology reveals uniparental disomy (see Section 4.2 in Chapter 2), and it uncovers small chromosome changes that are below the level of resolution of G-banding. A sizable fraction of these small alterations are currently of unknown clinical significance.

Fragments of **fetal DNA** less than 200 bp in length circulate briefly in the pregnant mother's blood. These fetal DNA fragments are also referred to as **fetal cell-free DNA**. The DNA originates from the placenta. The peak size of fetal cell-free DNA is approximately 140 bp and that of maternal cell-free DNA is approximately 160 bp. The maternal DNA pieces correspond to the DNA in a nucleosome plus a linker, while the fetal DNA lacks the linker sequence (it must have been clipped). At 10 to 20 weeks of gestation, the serum of the mother contains approximately 10 times more DNA from the mother than from her fetus. After delivery, the concentration of fetal DNA in the mother's plasma drops rapidly because the half-life of fetal DNA is only about 15 minutes.

By using serum from the pregnant mother, there are various ways of determining abnormalities of fetal DNA in the sea of maternal DNA. In one approach, all DNA from the mother's blood is sequenced by **massive parallel sequencing**. The DNA fragments are then assembled into whole chromosomes with computer-based algorithms. If the fetus has a trisomy (e.g., trisomy 21, which gives rise to Down syndrome and occurs in approximately 1 in 750 pregnancies), a greater number of chromosomes 21 is apparent relative to the other chromosomes. Massive parallel sequencing is often limited to the clinically most relevant chromosomes. Other approaches include sequencing of the father's genome.

Sequencing of the cell-free DNA in the plasma of the mother is currently best established for detecting **aneuploidies**.

One or more of the aforementioned techniques are often used to determine the complement of **sex chromosomes** of the fetus. This is of special importance in families who are affected by X-linked disorders (e.g., Lesch-Nyhan syndrome; see Section 2.4 in Chapter 38) or by congenital adrenal hyperplasia (see Section 4.2 in Chapter 31).

Cell-free DNA can also readily be used to test for **rhesus D** positivity in the fetus in a rhesus D–negative mother. The condition requires treating the mother with anti-rhesus D immunoglobulin.

6.2. Other Common Nucleic Acid–Based Tests

To guide **cancer** treatment, some companies offer a combination of bioinformatics and **massive parallel sequencing** of known genetic alterations in tumor cells. Examples of such tests are FoundationOne, FoundationOne Heme, and Caris Molecular Intelligence.

Massive parallel sequencing of select DNA segments is also available for **hereditary diseases** that can be caused by a mutation in one of multiple genes, such as hereditary breast cancer (Section 4.2 in Chapter 2), Lynch syndrome (a form of hereditary colon cancer; see Section 2 in Chapter 2), or neonatal diabetes (see Sections 1 and 3 in Chapter 39).

The diagnosis of many **infectious agents** relies on **real-time PCR**. DNA- or RNA-based identification of infectious agents is much faster than tests that involve culture. Furthermore, not all infectious agents can be cultured. A short turnaround time is especially important in patients who are in critical care and have life-threatening infections. Rapid molecular testing for pathogens can be performed in 1 to 2 hours. Viral load testing is important in monitoring the effectiveness of human immunodeficiency virus, hepatitis C virus, and other viral infections. In the practice of gynecology, there are commonly used molecular tests for RNA from Chlamydia trachomatis, Neisseria gonorrhoeae, and *T*richomonas vaginalis, as well as DNA from herpes simplex virus. Detection of DNA or mRNA from human papillomavirus plays an important role in determining which patients are at an increased risk of cancer of the cervix.

Leukemias and lymphomas are increasingly classified by their genetic alterations to guide diagnosis and treatment. For instance, chronic myelogenous leukemia and some forms of acute lymphoid leukemia are caused by a reciprocal translocation between chromosome 9 and chromosome 22, leading to the synthesis of a pathogenic BCR-ABL1 fusion protein. Therapy is available that blocks the kinase activity of the fusion protein. After initiating treatment, the amount of BCR-ABL1 mRNA is determined with **RT-PCR** (see Section 2)

every few months to monitor the effectiveness of the treatment.

SUMMARY

- Traditional cytogenetic testing requires sterile cells that grow in vitro. Cytogenetic testing can detect all alterations in chromosome number, as well as deletions, duplications, inversions, and translocations that are larger than approximately 5 million bp.
- Fluorescence in situ hybridization (FISH) is ideal for detecting deletions and amplifications of DNA segments of more than approximately 150,000 bp, such as HER2 gene amplification in breast cancer. FISH is also used for rapid identification of translocations and inversions.
- In the polymerase chain reaction (PCR), the forward and reverse primers determine the 5′ end and the 3′ end of the amplicon. With every temperature cycle, DNA melts, primers anneal, and the heat-stable DNA polymerase extends the primers. In quantitative PCR (qPCR), quantitative information is derived from the threshold cycle number relative to a calibration curve. In multiplex PCR, multiple forward and reverse primers exist. In reverse transcription (RT)-PCR, RNA is first reverse transcribed into DNA and then amplified by PCR.
- Capillary electrophoresis is used extensively in clinical tests to determine the size of DNA fragments.
- Melting curve analysis provides information on sequence mismatches compared with a chosen probe. Restriction enzyme digestion is another way to test for the presence of a pathogenic point mutation in an amplicon.
- Array comparative genomic hybridization (aCGH) and single nucleotide polymorphism (SNP) DNA microarrays printed with oligonucleotides can provide detailed information on aneuploidies, microdeletions, microduplications, and uniparental disomy but not about balanced translocations.
- Sanger sequencing is based on dideoxynucleotide-induced chain termination, followed by electrophoresis (usually capillary electrophoresis).
- Massive parallel sequencing depends on attaching DNA fragments of the genome to a solid support and then following the sequencing reaction on the surface of the support. The DNA sequence fragments are assembled by computer-based algorithms.
- Cells obtained from amniocentesis of chorionic villus sampling can be analyzed by G-banding, FISH, aCGH, DNA SNP microarray, or massive parallel sequencing.
- By massive parallel sequencing, short fetal DNA segments in a pregnant woman's blood can be discerned from the cell-free DNA of the mother and used to uncover an aneuploidy of the fetus, as well as determine the sex chromosome complement of the fetus.
- Massive parallel sequencing of selected DNA segments is available to characterize genetic alterations in tumors.
- Infectious agents are often quantified by molecular methods, sometimes after reverse transcribing RNA into cDNA.

FURTHER READING

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- Wong AI, Lo YM. Noninvasive fetal genomic, methylomic, and transcriptomic analyses using maternal plasma and clinical implications. *T*rends Mol Med. 2015;21: 98-108.

Review Questions

1. DNA-based diagnosis of a known point mutation that causes iron overload is best accomplished with which one of the following techniques?

   A. aCGH
   B. FISH
   C. Karyotype by G-banding
   D. PCR, then melting curve analysis

2. A part of the sequence of a section of a double-stranded DNA segment is as follows (only one strand is shown): 5′-..**GCTAT**…**ACTAC**..-3′. Which one of the following sets of primers can be used to amplify the DNA sequence that is bounded by the nucleotides that are in bold? (Only the 5′ ends of the primers are shown.)

   A. 5′-AAGCT.., 5′-TCAGC..
   B. 5′-CGATA.., 5′-CATCA..
   C. 5′-GCGAA.., 5′-AGTTC..
   D. 5′-GCTAT.., 5′-GTAGT..
   E. 5′-GCTAT.., 5′-AGTCG..

3. A 20,000,000-bp balanced translocation from one chromosome to another is most readily detected with which one of the following techniques?

   A. aCGH
   B. Karyotype by G-banding
   C. Massive parallel sequencing
   D. PCR, then melting curve analysis

4. A 500,000-bp inversion in chromosome 7 is most easily detected with which one of the following techniques?

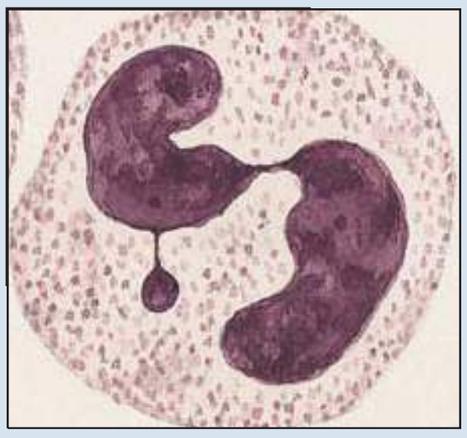
   A. aCGH
   B. FISH
   C. G-banding
   D. Massive parallel sequencing

5. Patients who are homozygous for a mutant aldolase B have hereditary fructose intolerance. Asymptomatic carrier parents requested genetic testing of their newborn daughter for this condition. A defined portion of the aldolase B gene in the DNA of the newborn and her parents was amplified with PCR. T e product was exposed to the restriction enzyme AhaII, which cuts the amplified DNA of a common pathogenic allele into 185 bp and 125 bp fragments; AhaII does not cut the amplified DNA of the normal allele. T e digest was subjected to electrophoresis, and DNA fragments of the following number of base pairs were found:

   Father: 310, 185, and 125
   Mother: 310, 185, and 125
   Daughter: 185 and 125

   T ese results show which of the following?

   A. T e amplicons should have been exposed to more AhaII and for a longer time.
   B. T e daughter has hereditary fructose intolerance.
   C. Each parent has three copies of the aldolase B gene.
   D. T e PCR tubes with the parents' DNA were contaminated.



Chapter 5 Basic Genetics for Biochemistry

SYNOPSIS

- This chapter explains some basic terms in genetics that are used throughout this book. It covers relevant and recurring essentials only and is not intended to provide an introduction to genetics.
- Normal nucleated human cells have 46 chromosomes, half of which are inherited from the mother and half from the father. In each parent, during meiosis, these chromosomes are newly assembled from existing chromosomes through a process of multiple crossing-over events.
- A person who is homozygous for a particular DNA sequence (e.g., a gene) has two exact copies of the sequence, while a person who is heterozygous has two different copies.
- X-linked diseases typically occur with greater frequency in males than in females. Early in female development, each cell randomly chooses one X chromosome for inactivation. Each cell then gives rise to daughter cells that maintain the inactivation of the same X chromosome. As a result, females are mosaics in terms of the X chromosomes they express.
- A small set of genes is imprinted in each oocyte and another small set in each sperm, such that these maternal and paternal copies of genes are not expressed in the offspring.
- An analysis of the association between disease and small sequence differences in known locations of the genome is often performed in the hope of elucidating the genetic basis of a disease.

LEARNING OBJECTIVES

For mastery *of* this topic, you should be able to do the *following*:

- Describe the normal human karyotype and define the meaning of aneuploidy.
- Explain the terms allele, homozygosity, heterozygosity, and compound heterozygosity.
- Compare and contrast penetrance and variable expressivity.
- Compare and contrast dominant and recessive inheritance. Explain the meaning of the terms haploinsufficiency, gain-of-function mutation, loss-of-function mutation, and dominant negative effect, thereby relating these terms to dominant and recessive inheritance. Give an example for each of these terms.
- Compare and contrast the expected phenotypes of X-linked disorders in males and females.
- Compare and contrast Mendelian inheritance and non-Mendelian inheritance due to imprinting.
- Describe the pattern of inheritance of DNA in the mitochondria.
- Explain the terms germline and somatic cells.
- List common types of mutations.
- Describe the use of polymorphic markers in linkage analysis.

1. CHROMOSOMES AND ALLELES

Except *for* the sex chromosomes, healthy humans have two copies o*f* each chromosome. T e mitochondria o*f* a typical cell contain 1,000 or more copies o*f* their own genome. A person who is homozygous *for* a piece o*f* nuclear DNA has two identical copies o*f* the piece, and a person who is heterozygous or compound heterozygous has two di*ff*erent copies.

A normal nucleus in a human cell contains **46 chromosomes** (i.e., two copies each of **22 autosomes** and **two sex chromosomes)** (XX or XY; see Chapter 1). Half of these chromosomes stem from the mother and half from the father.

Since a typical, normal human cell has two copies of every chromosome (except possibly the sex chromosomes) it is said to be **diploid** (or 2n). By contrast, a normal egg and sperm are **haploid** (or 1n) (i.e., they have only 23 chromosomes [22 autosomes and 1 sex chromosome]). A cell that is **aneuploid** has more or less than 46 chromosomes.

T e **karyotype** of cells is a description of the chromosome composition. T e normal human karyotypes are 46,XX and 46,XY (number of chromosomes, followed by a description of the sex chromosomes). Fig. 1.12 shows an image of a normal karyotype.

Aneuploidy is typically pathogenic. Examples include Down syndrome (three copies of chromosome 21; karyotype: 47,XX,+21 or 47,XY,+21), Turner syndrome (45,X), and Klinefelter syndrome (47,XXY). Many tumor cells are aneuploid.

Homologous chromosomes have a similar architecture yet stem from dif erent parents. For instance, chromosome 1 from the mother is homologous to chromosome 1 from the father.

Most human cells contain mitochondria, which have their own genome in the form of **mitochondrial DNA** (**mtDNA**; see Chapter 23). A typical cell contains 1,000 or more copies of mtDNA within its network of mitochondria (i.e., a "mitochondrion" has multiple copies of mtDNA). mtDNA is inherited exclusively from the mother. T e inheritance of mtDNA and associated diseases is complex because mitochondria can contain mixtures of dif erent mtDNAs that are passed on in a chance distribution (see Section 3 in Chapter 23).

Each chromosome contains many **genes**. A gene is often defined as a region of DNA that is transcribed into RNA. To the extent that we have two copies of every chromosome, we also have two copies of every gene; these two copies are referred to as **alleles**. We typically have one allele from the mother and one allele from the father.

A person who is **homozygous** has two similar copies of a gene; these can be two normal copies or two pathogenic copies. A person who is **heterozygous** has two dif erent copies

of the same gene, for example, one normal and one pathogenic copy. A person who is **compound heterozygous** has two different abnormal copies of the same gene. For example, a person who makes only a normal phenylalanine hydroxylase is homozygous for normal phenylalanine hydroxylase. A person who is a carrier for phenylketonuria has one normal and one abnormal allele for phenylalanine hydroxylase. A person who has two identical pathogenic mutant copies of the enzyme is homozygous for phenylalanine hydroxylase deficiency. A person who has two different mutant alleles for phenylalanine hydroxylase (of which there are many different mutants in the population) is compound heterozygous for a mutant phenylalanine hydroxylase.

Females have two X chromosomes, and males have one X and one Y chromosome. An XY male is **hemizygous** for all alleles on the X chromosome.

2. IMPRINTING AND PATTERNS OF INHERITANCE

*T*raits encoded by chromosomes in the nucleus show dominant or recessive patterns o*f* inheritance, whereby the type o*f* inheritance depends on both the definition o*f* the phenotype and the behavior o*f* the relevant molecules. A small *f*raction o*f* genes is expressed only when inherited *f*rom the mother or only *f*rom the *f*ather because o*f* imprinting (DNA methylation). *T*raits encoded by mtDNA are inherited only via the mother.

The **phenotype** describes the attributes of a person; this may include, for instance, physical characteristics, behavior, laboratory measurements, or the risk of neoplasms.

Penetrance refers to the frequency with which a particular disease-causing genotype leads to disease. Penetrance generally increases with age. Hemochromatosis (see Chapter 15), for example, has incomplete penetrance in that only a subset of persons who have an HFE gene with the C282Y mutation develops an iron overload (e.g., older men who abuse alcohol and are infected with hepatitis C virus show very high penetrance).

Variable expressivity refers to symptoms seen with a particular mutation. For instance, Marfan syndrome (see Chapter 13) is due to a mutation in fibrillin-1. Among family members who all have the same mutation, penetrance may be 100% (i.e., all clearly having Marfan syndrome), but there can be a large variation in phenotype. Some members, for instance, may have a normal chest while others have a sunken chest that needs to be corrected surgically.

In **autosomal dominant** inheritance, the phenotype is determined by the dominant allele. In osteogenesis imperfecta, one allele encodes a mutant type I collagen that impairs the assembly of type I collagens into fibrils (see Section 2.2 in Chapter 13). This allele is dominant (it causes disease on its own) and on an autosome. Hence, osteogenesis due to this particular allele is inherited in autosomal dominant fashion.

Autosomal dominant inheritance can be due to the following (examples are provided in Table 5.1):

- **Haploinsufficiency**: A single functional copy of a gene is not sufficient for normal function.
- **Gain-of-function mutation**: A protein acquires a new and pathogenic function (e.g., uncontrolled activity, aggregation, or the catalysis of a new reaction); it is also possible that extra copies of a gene are made and inserted into a chromosome (a process called gene duplication or gene amplification), or that a gene is transcribed at a greater rate.
- **Dominant negative effect**: An abnormal protein destroys the function of the normal protein. This is often seen in proteins that are active as dimers, trimers, tetramers, and so forth.

In **autosomal recessive** inheritance, the phenotype is present when there are two recessive alleles. This is commonly seen in deficiencies of enzymes that function as monomers.

Table 5.1 Examples of Mutation Types That Exhibit Autosomal Dominant Inheritance

Type of Mutation	Example 1	Example 2	Example 3
Haploinsufficiency	Mutant glucokinase in maturity-onset diabetes of the young (MODY) type 2 (Chapter 39)	16/heme, mutant porphobilinogen deaminase in acute intermittent porphyria (Chapter 14)	Mutant LDL receptor in heterozygous familial hypercholesterolemia (Chapter 29)
Gain of function	Mutant subunit of succinate dehydrogenase in hereditary pheochromocytoma and paraganglioma (Chapter 22)	Mutant fumarase in hereditary leiomyomatosis and renal cell cancer (Chapter 22)	Mutant PCSK9 in a type of congenital hypercholesterolemia (Chapter 29)
Dominant negative	Variant low-activity aldehyde dehydrogenase in East Asians that leads to flushing after the consumption of ethanol (Chapter 30)	Mutant type I collagen (which assembles into fibrils) in osteogenesis imperfecta (Chapter 12)	Mutant fibrillin-1 (which assembles into fibrils) in Marfan syndrome (Chapter 13)

LDL, low-density lipoprotein.

Half the normal activity is often sufficient, but an activity of less than 10% of normal may be pathogenic. **Consanguinity** of parents frequently gives rise to an otherwise rare autosomal recessive disorder among of spring. In genetics, consanguinity refers to a close genetic relationship between two individuals. Two first-degree relatives (e.g., a parent and a child, or two siblings) have a coefficient of relationship of 0.5. Two second-degree relatives (e.g., a child and an aunt or grandmother) have a coefficient of relationship of 0.25. Two third-degree relatives (e.g., two first cousins) have a coefficient of relationship of 0.125.

Keep in mind that the type of inheritance (dominant or recessive) depends on both the definition of the phenotype or trait and the characteristics of the molecules that play a role in generating the phenotype. While a particular disease is inherited in a recessive fashion 99% of the time, 1% of all af ected patients may carry a less common mutation in the same gene that shows dominant inheritance. Hence, there are always exceptions to statements such as "T e disease shows autosomal recessive inheritance."

Loss-of-function mutations (**inactivating mutations**) are the norm in traits that show autosomal recessive inheritance.

In disorders that show **X-linked** inheritance, **males** are always af ected. Female heterozygotes are clearly af ected in **X-linked dominant** inheritance but may be unaf ected in **X-linked recessive** inheritance.

As suggested in 1961 by Mary Lyon (the Lyon hypothesis), females **inactivate** all but one **X chromosome** per cell in the embryo in a patchwise fashion, a process referred to as **dosage compensation**. One cell inactivates the X chromosome from the father. A neighboring cell may inactivate the same X chromosome or the X chromosome from the mother in a process that is determined entirely by chance. T ese early cells give rise to progeny that maintain the same patterns of X inactivation. Hence, women are mosaics for X chromosome expression.

Statistically, it is most likely that a female expresses the maternal X chromosome in 50% of her cells and the paternal in the other 50%. It is least likely that a female expresses the X chromosomes from only one parent in all of her cells. Everything in between these two extremes is possible. If the inactivation is unequal, it is called **skewed X inactivation**. For this reason, an X-linked recessive disorder can produce very different phenotypes in heterozygous women.

Examples of X-linked disorders are glucose 6-phosphate dehydrogenase deficiency (see Chapter 21), ornithine carbamoyltransferase deficiency (see Chapter 35), and Lesch-Nyhan disease (see Chapter 38).

X chromosome inactivation in females occurs through DNA methylation. T e inactivated X chromosome is condensed throughout the cell cycle and may be visible in the nucleus as the **Barr body** (Fig. 5.1).

A person is a genetic **mosaic** if some cells have a recognizably dif erent genome from other cells. Mosaicism is typically a result of mutations that take place during development. Mosaicism can af ect the germline or the somatic cells.

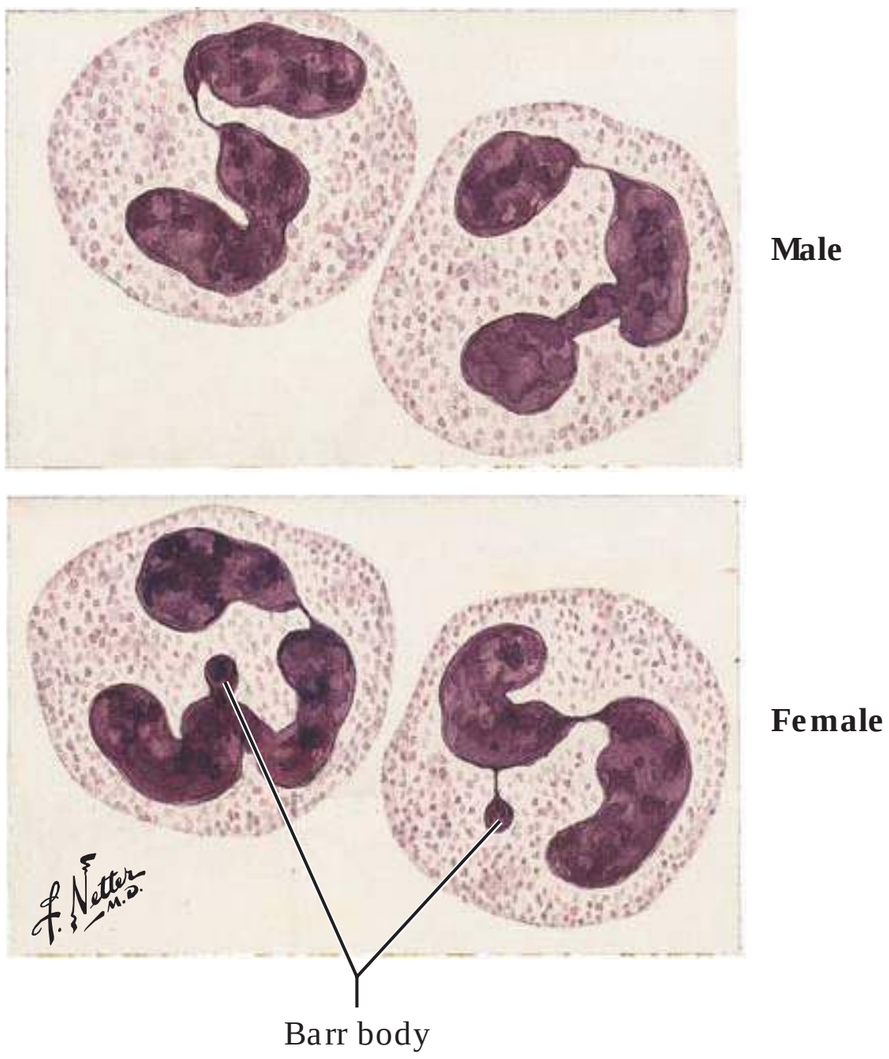


Fig. 5.1 Barr body in neutrophils of a female.

T e term **Mendelian inheritance** refers to inheritance patterns that are dominant or recessive, X linked or autosome linked, whereby it does not matter whether an allele is derived from the mother or father.

About 100 genes encoded on chromosomes in the nucleus show **non-Mendelian inheritance** due to **imprinting**. Imprinting disables the expression of small regions of chromosomes via the **methylation** of certain CpG dinucleotides. Maternally imprinted genes cannot be expressed in the mother's of spring, but that of spring can pass on these genes to the next generation. T e same applies to paternally imprinted genes.

Existing imprinting is erased after fertilization in the cells that give rise to primordial germ cells, and it is reestablished later such that all oocytes have one customary set of genes imprinted, and all sperm have another customary set imprinted. For instance, the daughter of two parents cannot express the maternally imprinted genes; she will therefore express only the homologous paternal alleles. T e reverse is true for paternally imprinted genes. T is daughter's oocytes will imprint a predetermined set of genes; half of the alleles for these genes stem from her mother and half from her father.

3. MUTATIONS AND MARKERS

Mutations are o*ft*en classif ed by their e*ff*ect on transcription and translation. Markers are known sequences in known locations o*f* the genome. T ey are use*f*ul in linking a disease to a DNA location.

T e term **germline** refers to the cells in the gonads that give rise to the eggs and sperm. All other cells are called **somatic cells**. Germline mutations are heritable but somatic mutations are not. Most neoplasms occur in somatic cells.

A mutation is called a **de novo mutation** if it is seen in of spring but not in the peripheral blood or normal tissues of the parents. T e mutation most likely occurred in the germline of a parent and is therefore heritable. It is also possible that only a part of a parent's germline carries the de novo mutation. Mutations in somatic cells occur frequently and may give rise to neoplasms; these mutations are simply called "mutations," not "de novo mutations."

Mutations are often subclassified into the following types:

- A **coding region** mutation occurs in a region of a gene that is transcribed and becomes part of mRNA that is translated into protein.
- A **frame-shift mutation** shifts the reading frame for codons.
- A **missense mutation** converts an amino acid codon to a dif erent amino acid codon.
- A **nonsense mutation** converts an amino acid codon to a stop codon.
- A **promoter mutation may** alter the binding of transcription factors and thereby alter the amount of mRNA that is synthesized.
- A **silent substitution** (**synonymous substitution**) does not change the amino acid sequence of the encoded protein because the genetic code is degenerate (see Chapter 7).
- A **splice site mutation** may af ect splicing efficiency and hence, alter the amount of normally spliced mRNA that is produced. In addition, abnormally spliced mRNA is usually degraded by nonsense-mediated decay.
- A **3′ end-processing mutation** may af ect the efficiency of mRNA 3′ end processing and thereby alter the amount of mRNA that is produced.

Trinucleotide repeats are repeats of the same sequence of three nucleotides (e.g., CAG). Some trinucleotide repeats are unstable, expand, and give rise to disease once they are too long.

A **polymorphism** usually refers to a sequence variation that is less common but still occurs with some frequency (e.g., in more than ~1% of all persons).

A **single-nucleotide polymorphism** (**SNP**) is a polymorphism that af ects only a single nucleotide.

Polymorphic markers are DNA sequences at known locations that show some sequence variation in the population. Notably, they allow a geneticist to determine whether a person inherited a particular sequence from the mother or the father and whether the sequence is associated with a disorder.

Linkage analysis refers to linking a DNA region to a phenotype. Linkage analysis is based on knowledge of marker sequences throughout the human genome and also on the fact that two DNA sequences that are close to each other on a chromosome are more likely to be inherited together than two DNA sequences that are far apart on a chromosome or even on dif erent chromosomes. Crossing over during meiosis is the reason that two DNA sequences that are far apart on a chromosome are not necessarily inherited together.

Many diseases show **genetic heterogeneity** because a certain phenotype can be the result of a mutation in one of several dif erent genes. Often, these genes play a role in the same pathway of metabolism or signaling pathway.

SUMMARY

- 46,XX and 46,XY are the normal human karyotypes. Aneuploidy is an abnormal complement of chromosomes. It occurs, for instance, in Turner syndrome (45,X), Klinefelter syndrome (47,XXY), and Down syndrome (47,XY,+21).
- A compound heterozygous individual typically has two different disease-causing alleles.
- Penetrance refers to the frequency with which a pathogenic genotype gives rise to a defined phenotype. Variable expressivity refers to interindividual variability in the phenotype of the same pathogenic genotype.
- Haploinsufficiency indicates that a single normal copy of a gene is insufficient to maintain a normal phenotype. A gain-of-function mutation gives rise to a new or increased activity of a protein. A dominant-negative ef ect is often caused by a mutant molecule forming dimers and multimers with normal molecules that greatly impair the function of the resulting complex.
- Maternal and paternal imprinting of a small set of genes, which takes place in developing oocytes and sperm, leads to non-Mendelian inheritance.
- Linkage analysis uses polymorphic markers to elucidate a link between DNA and disease.

Review Question

1. Within a family, individuals who have the same telomeropathy-causing mutation present with dif erent abnormal phenotypes. T e diversity of phenotypes is best referred to as which of the following?

   A. Dominant-negative ef ect
   B. Gain-of-function mutation
   C. Haploinsufficiency
   D. Incomplete penetrance
   E. Variable expressivity

Chapter 6 Transcription and RNA Processing

SYNOPSIS

- Transcription is the process of synthesizing an RNA based on a DNA template.
- DNA can be packaged into nucleosomes and compacted further into heterochromatin so that it is inaccessible to the transcription machinery.
- Upstream of a gene is a promoter region that contains binding sites for DNA-binding regulatory proteins called transcription factors. The availability of these transcription factors depends on development, cell type, and various environmental cues.
- Transcription also requires general transcription factors and RNA polymerase.
- During transcription, a protein complex recognizes the 5′-end of the RNA and adds a methyl guanosine cap, while another complex recognizes a polyadenylation site further downstream on the growing RNA chain, cleaves the RNA, and adds a poly(A) tail.
- Spliceosomes remove large segments (introns) from pre-mRNA and connect the remaining sequences (exons).
- Alternate transcription start sites, alternative polyadenylation sites, alternative splicing, alternative translation start sites, and alternate posttranslational protein modifications each contribute to protein diversity that far exceeds the diversity of genes.

LEARNING OBJECTIVES

For mastery of this topic, you should be able to do the following:

- Describe the effect of chromatin structure on transcription, taking into account DNA methylation, histone methylation, and histone acetylation.
- Explain the meaning of the terms CpG dinucleotide and CpG island.
- Describe the basis of epigenetic inheritance.
- Describe the relationships of coding strands, noncoding strands, and template strands to each other and to an entire chromosome.
- Compare and contrast promoter elements, enhancers, activators, repressors, and silencers.
- Outline the assembly of a transcription initiation complex, paying special attention to steps that can be regulated by metabolites or hormones (particularly steroids).
- Interpret a graph of the structure of a promoter and a gene.
- Describe the modifications of precursor mRNA (pre-mRNA) at the 5′- and at the 3′-end and explain the purpose of these modifications.
- Describe the splicing of pre-mRNA and provide an example of alternative splicing.
- Compare and contrast exons and introns and relate these to a gene, as well as the final product of translation.
- Explain how a point mutation can alter splicing and hence the amino acid sequence of a protein.
- Explain the term cryptic splice site and provide an example.

1. DNA METHYLATION AND PACKING IMPEDE TRANSCRIPTION

Methylation of DNA and histones generally leads to packing of methylated DNA into heterochromatin, which is not transcribed. The pattern of DNA methylation can be passed from cell to cell in a process called epigenetic inheritance. This is a normal part of development and cell differentiation. Methylation is abnormal in many neoplasms and also in most cases of Rett syndrome, which is associated with impaired development of the nervous system. Acetylation generally has the opposite effect of methylation in that it makes chromatin available for transcription.

The term transcription refers to the synthesis of an RNA based on a DNA template.

Transcription is controlled at several levels: the compaction of DNA into nontranscribable heterochromatin, the formation of nucleosomes, the methylation of promoter regions, and the availability and activity of factors that are part of the transcription machinery.

In cells, DNA together with **histones** and other proteins forms **chromatin**. Light microscopy and electron microscopy reveal two forms of chromatin: **euchromatin** and **heterochromatin** (Fig. 6.1). Cells typically have more euchromatin than heterochromatin. Heterochromatin is somewhat concentrated near the periphery of the nucleus, and it is more densely packed than euchromatin. The physical structure of heterochromatin is not known, but it likely involves packing nucleosomes into a 30-nm chromatin fiber that is then folded into an even more compact form (see Fig. 1.6 and Section 4 in Chapter 1).

The nucleus is surrounded by an **inner** and an **outer membrane** that contains nuclear **pores**. The two membranes represent a layer of endoplasmic reticulum that envelops the nucleus. The membranes are stabilized by the **nuclear lamina**, which contains lamins, a type of protein that forms so-called intermediate filaments. Other proteins bind heterochromatin to the nuclear lamina.

Genes in **euchromatin** can be transcribed but those in **heterochromatin** cannot. In heterochromatin, nucleosomes are compacted into higher-order structures that prevent the access of proteins that are needed for transcription. DNA in euchromatin is also packaged in nucleosomes, but various mechanisms exist for modifying and moving nucleosomes to enable transcription.

Heterochromatin forms in a tissue-specific manner. During development and differentiation, perhaps one-third of the genome moves from euchromatin to heterochromatin or vice versa. Other parts of the genome, such as telomeres,

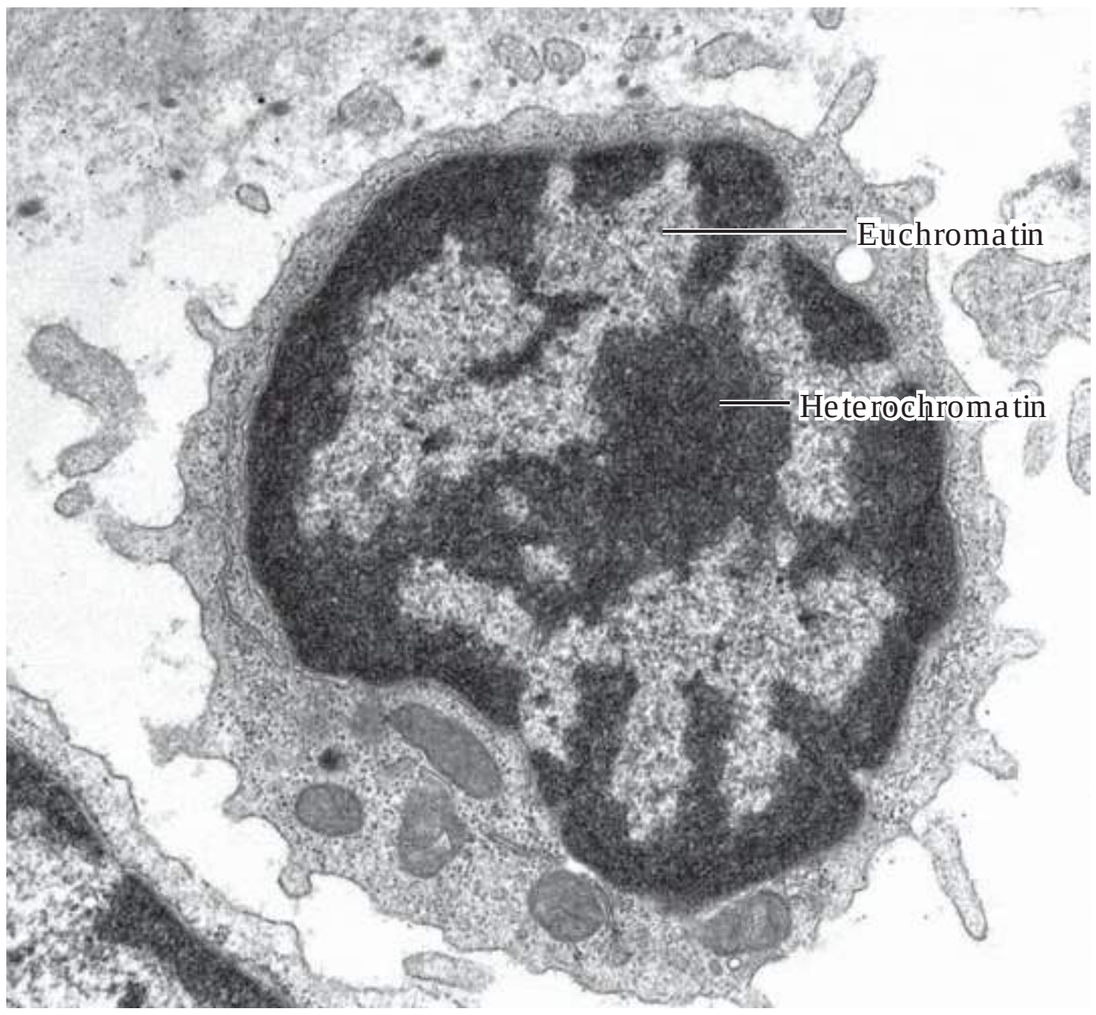


Fig. 6.1 Euchromatin and heterochromatin in the nucleus of a lymphocyte as seen by transmission electron microscopy. The nucleus fills almost the entire lymphocyte.

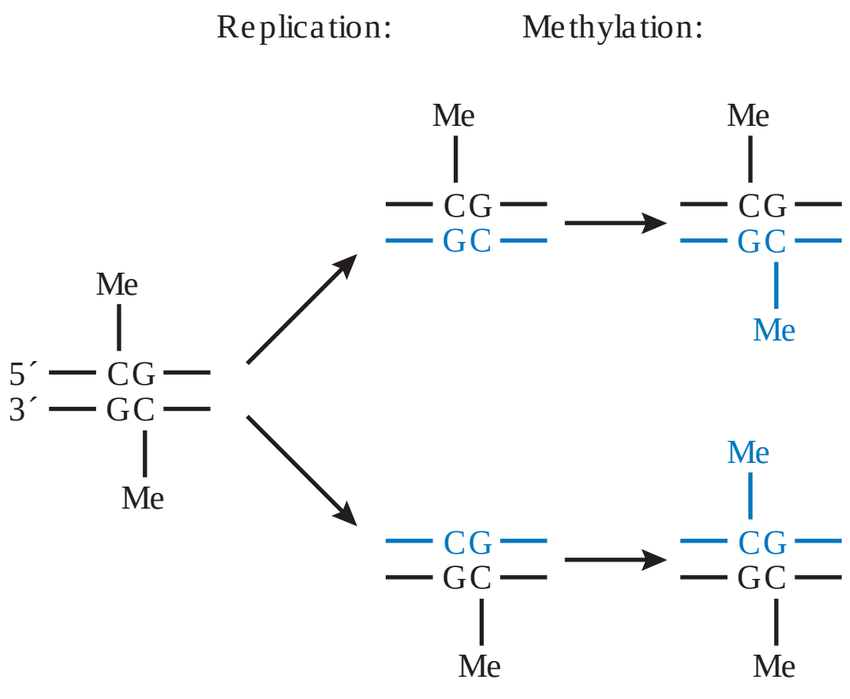


Fig. 6.2 Maintenance of CpG methylation, an epigenetic modification. Methylation occurs only where a CpG on the complementary strand is already methylated. Me, methyl group.

centromeres, the long arm of the Y chromosome, and large segments of chromosomes 1, 9, and 16, are always condensed into heterochromatin. Since the DNA in these regions is not transcribed, even large DNA insertions and deletions have no known effect on a person's phenotype. Indeed, cytogenetics reports often mention an unusual size of one of these heterochromatic regions as being a normal polymorphism.

Methylation of cytosine bases by a **DNA methyltransferase** favors the incorporation of DNA into heterochromatin. DNA methylation occurs principally on the C of a 5′-CG-3′ sequence (often called a CpG dinucleotide, where p refers to the phosphate that forms a phosphodiester; see Fig. 1.2). For every 5′-CG on one DNA strand, a 5′-CG exists on the complementary DNA strand (Fig. 6.2). The Cs on both strands are typically methylated.

CpG islands are segments of DNA that contain a relatively high fraction of CG dinucleotides. Methylcytosine is mutagenic because occasional spontaneous deamination gives rise to thymine. Fittingly, the genome contains fewer CG dinucleotides than would be expected on the basis of statistics. By contrast, CpG islands contain almost the expected fraction of CG dinucleotides. Although ~70% of CG dinucleotides are methylated in the entire genome, CGs in CpG islands are mostly unmethylated. A typical CpG island is ~1000 bp long.

The pattern of CG methylation differs between cell types, and it can also change over time.

Methylation of C on DNA is passed on during cell division and thus gives rise to **epigenetic inheritance** (see Fig. 6.2). A cell therefore inherits not only the DNA of the parent cell (genetic inheritance) but also the pattern of chromatin packaging of the parent cell (epigenetic inheritance). Epigenetic events play a role, for instance in imprinting, development, cell differentiation, and X-inactivation in females (see Chapter 5). During DNA replication, a **DNA methyltransferase** methylates C of a CpG dinucleotide, but only if the C on the complementary strand is already methylated. In this way, methylated sequences remain methylated, and unmethylated sequences remain unmethylated. DNA methyltransferases use **S-adenosyl methionine** (see Fig. 36.6 and Section 4 in Chapter 36) as a methyl group donor.

The formation of blood cells (see Fig. 16.3 and Section 1.2 in Chapter 16) provides an example of epigenetic inheritance. Hematopoietic stem cells in the bone marrow give rise to red blood cells and various types of white blood cells. Each of these developing cell types possesses a different pattern of chromatin condensation. Erythroblasts (precursors to red blood cells) package the genes for immunoglobulins into heterochromatin, whereas white blood cells package the hemoglobin genes into heterochromatin.

A deficiency of the **methyl CpG-binding protein 2** (**MECP2**) causes **Rett syndrome**, a progressive neurologic disorder that is the most common cause of a low IQ in females. MECP2 is widely expressed but especially abundant in mature neurons. The function of MECP2 is poorly understood. It binds to methylated Cs and nucleosomes, competes with a histone, bends DNA, and represses or activates transcription. Rett syndrome is inherited in X-linked dominant fashion and has a prevalence of ~1 in 12,000 births. In males, the disease is often lethal in utero. Females who have Rett syndrome start to regress at age 1 to 4 years.

In many **neoplasms**, CpG islands are methylated, which leads to suppression of transcription of the associated genes, typically **tumor suppressors** (see Chapter 8).

Compaction of DNA into heterochromatin is largely driven by the state of some of the **histone modifications** listed in Table 1.1. The packing of DNA and histones into nucleosomes is described in Fig. 1.6 and Section 4 in Chapter 1. Histone modifications (e.g., methylation and acetylation) occur on the tails of histones, which protrude from the nucleosomes. Compaction of nucleosomes into 30 nm fibers and then further into heterochromatin starts largely at the sites of repetitive or

highly methylated DNA sequences, spreads, and is stopped by certain DNA elements and RNAs.

Methylated DNA can attract **histone methyltransferases** that methylate histones, and methylated histones can attract proteins that contain a **chromodomain** (chromatin organization modifier domain) and favor the formation of heterochromatin. Histone methyltransferases (like DNA methyltransferases) use S-adenosyl methionine (see Fig. 36.6 and Section 4 in Chapter 36) as a methyl group donor. Histone methyltransferases can methylate histone H3 at lysine residue 9 (shorthand H3K9). Proteins that contain a chromo domain can bind to the methylated histone H3 (H3K9me). By contrast, increased methylation of lysine-4 of the same histone H3 (H3K4me) favors the formation of euchromatin.

Lysine acetyltransferases in the nucleus and cytosol can use acetyl-coenzyme A (acetyl-CoA) to add an acetyl group to the amino group of the side chain of a lysine residue. The **acetyl-CoA** for this reaction stems from citrate that has been exported from mitochondria into the cytosol as described in Fig. 27.3 and Section 2 in Chapter 27.

Acetylation of histone lysine side chains leads to a more relaxed structure of chromatin. Lysine acetylation is read by proteins that contain an acetyl-lysine reader domain, such as a **bromodomain**.

Histone deacetylases (**HDAC**s) can remove an acetyl group from a lysine side chain of a histone. Deacetylation of histones favors formation of heterochromatin. Humans produce 18 different HDACs.

Drugs in current use that are HDAC inhibitors include the following:

- **Vorinostat** and **romidepsin** are used in the treatment of cutaneous T-cell lymphoma, a disorder in which malignant T cells form tumors in the skin.
- **Belinostat** and romidepsin are used in the treatment of peripheral T-cell lymphoma, a disease in which malignant T cells are found in a variety of tissues, such as lymph nodes, liver, and bone marrow.
- **Panobinostat** is used together with bortezomib (a proteasome inhibitor) and dexamethasone (a glucocorticoid) in the treatment of multiple myeloma, a form of lymphoma caused by abnormal B cells.
- **Valproic acid** is used in patients who have seizures; some of its effects may be due to inhibition of HDACs.

Conjugation of lysine residue 120 of histone H2B with a single 76–amino acid protein **ubiquitin** by a ubiquitin ligase complex favors the transition from heterochromatin to euchromatin. Ubiquitin is a small protein and therefore much larger than a methyl or an acetyl group. Deubiquitinases remove ubiquitin from histones and thereby facilitate the incorporation of nucleosomes into heterochromatin.

2. THE PROCESS OF TRANSCRIPTION

During transcription, one strand of the DNA is used as a template to synthesize an RNA. The RNA sequence is complementary to the DNA template strand and identical to the coding strand, except that the RNA contains uracil in place of thymine. Upstream of a gene is a promoter region that contains multiple elements to which transcription factors bind. Formation of these protein-DNA complexes is essential to specific and regulated transcription of individual genes. The activity of some transcription factors depends on the binding of ligands, such as steroid hormones. Through the use of different promoter elements and transcription start sites, a single gene can give rise to several different proteins. A number of drugs in clinical use affect the activity of transcription factors.

During transcription, RNA is synthesized in a 5′ → 3′ direction based on the sequence of the DNA **template strand** (the DNA template strand is read in a 3′ → 5′ direction). The 5′ → 3′ synthesis of RNA resembles the 5′ → 3′ synthesis of DNA during replication (see Fig. 3.4), except that RNA contains **ribonucleotides** (not deoxyribonucleotides) and **uracil** in place of thymine.

The **coding strand** (**sense strand**) has the same directionality and sequence (except T in place of U) as the RNA that is made from the template strand. The sequence of both the coding strand and the RNA is complementary to the sequence of the template strand. Sometimes, the template strand is called the **noncoding strand** or the **antisense strand**.

A **gene** is often defined as a segment of DNA that is transcribed into RNA.

By convention, the direction of a chromosome is from the end of the short arm toward the end of the long arm, and the direction of a gene is the direction of the coding strand (5′→3′; Fig. 6.3). A gene that has the same direction as the chromosome is said to be in **forward orientation**; if the directions are opposite, the gene is in **reverse orientation**. Each chromosome contains many genes that are in forward orientation and many that are in reverse orientation.

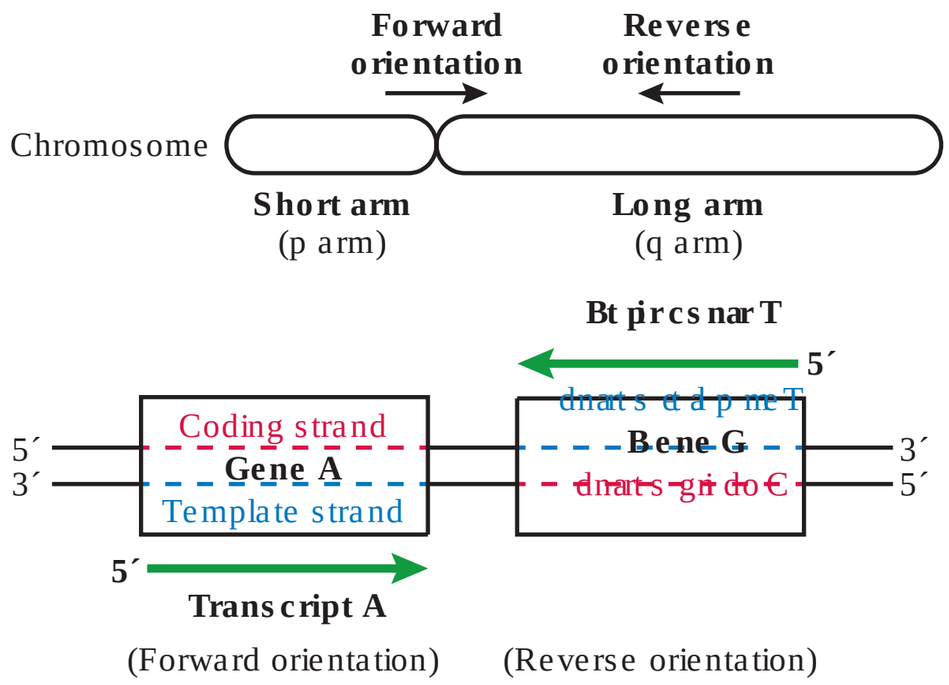


Fig. 6.3 Genes in a chromosome can be oriented in two ways. By convention, the orientation refers to the coding strand. The forward orientation is from the short-arm end of the chromosome toward the long-arm end of the chromosome. RNA is elongated at its 3′-hydroxyl group. The direction of transcription is the same as the direction of the green transcript arrow.

The genes on **mitochondrial DNA** (see Fig. 23.7) are likewise distributed between the two DNA strands (called heavy and light strand).

Transcription is often divided into initiation, elongation, and termination. Initiation refers to the highly regulated assembly of a complex of transcription factors and coactivators to which RNA polymerase then binds. Elongation refers to the synthesis of an RNA by RNA polymerase. Termination refers to the process that halts RNA polymerase and removes it from the DNA.

Upstream (5′) of a gene is a **promoter** region that consists of many **promoter elements** (**cis-acting regulatory elements**, Fig. 6.4), which affect the rate of transcription as detailed further in the chapter.

All **transcription factors** have a DNA-binding domain and bind to promoter elements, enhancers, or silencers (see below) on DNA. The promoter elements are often ~5 to 15 nucleotides long. Note that the term transcription *f*actor excludes the general transcription factors (GTFs) introduced below.

Transcription factors are either transcription **activators** or transcription **repressors**; the binding of transcription factors to regulatory elements on DNA determines how often a gene is transcribed. Figs. 6.5 and 6.6 show examples of a part of a transcription factor bound to a segment of DNA.

Transcription factors bind to the periphery of a DNA double helix; that is, to the grooves in the helix (see Figs. 6.5 and 6.6); this most often involves both the coding strand and the template strand. The binding site of a transcription factor is usually only reported as the 5′ → 3′ sequence because the complementary sequence can easily be inferred.

Many transcription factors bind to DNA as homodimers or heterodimers and therefore contain a **dimerization domain**. A **leucine zipper** (see Fig. 6.6) is a common motif among some of these transcription factors. Apposition of leucine side chains generates a hydrophobic effect that holds the monomers together in a reversible fashion.

Some transcription factors contain a **ligand binding domain** (see Fig. 6.5). Among these transcription factors is the family of **nuclear hormone receptors**, which contains 48 members, including receptors for steroids, vitamin D, retinoic acid, and thyroid hormone, among many others.

The **steroid hormone receptors** encompass the receptors for glucocorticoids, mineralocorticoids, estrogen, progesterone, and dihydrotestosterone. The steroids are membrane permeable, and the steroid receptors that affect transcription are either in the cytosol or in the nucleus. A description of glucocorticoid receptors serves as an example of the complexity of steroid hormone receptor function.

In the absence of a glucocorticoid, **glucocorticoid receptors** reside mostly in the cytosol, where they are bound to a chaperone complex that includes heat shock protein 90 (HSP90; see also Section 4.1 in Chapter 7). When cortisol (the major glucocorticoid) binds to the glucocorticoid receptor, the receptor dissociates from HSP90 and moves to the nucleus thanks to its nuclear localization sequence. In the nucleus, the activated receptor can bind to a glucocorticoid response element (GRE). A typical GRE sequence is nGnACAnnnnGTnC (n = variable nucleotide). The GRE can be close to the promoter or far away from it (up to >100,000 nucleotides). The glucocorticoid receptor can directly bind to DNA and recruit coactivators and corepressors; alternatively, it can be tethered to DNA by binding to other transcription factors. There are also negative GREs (nGREs); when a GR binds to an nGRE, transcription is repressed. The nGREs have a different consensus sequence than the GREs.

Glucocorticoid action is complex and can vary from tissue to tissue as a result of receptor isoforms and epigenetic effects. Only one gene exists for the glucocorticoid receptor. **Alternative splicing** of RNA (see Section 3.4 and Fig. 6.14) yields

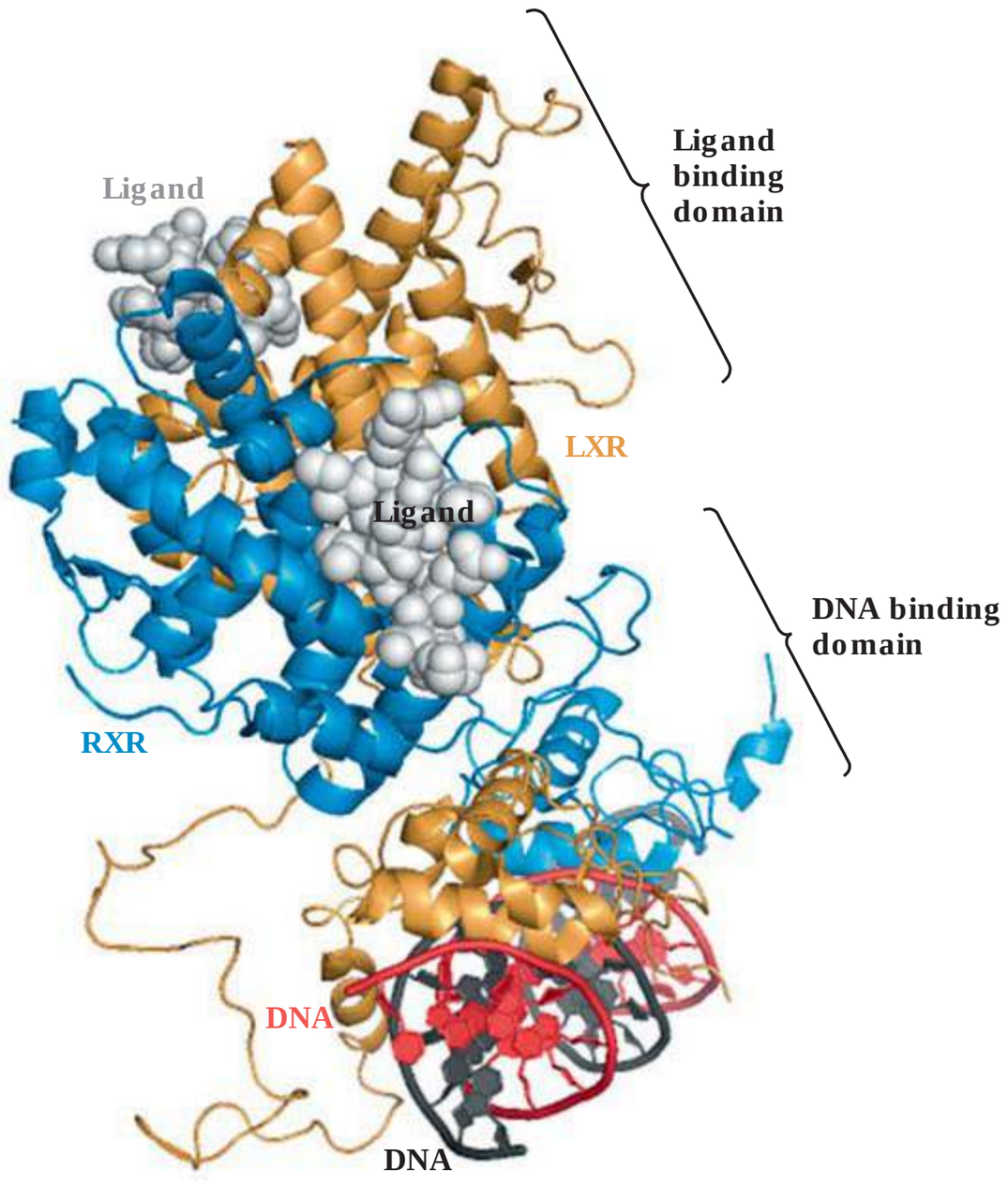


Fig. 6.5 Structure of the liver X receptor–retinoid X receptor transcription factor bound to DNA. The transcription factor binds to two AGGTCA sequences that are four nucleotides apart. The ligand binding domains bind oxysterols (e.g., 27-hydroxycholesterol) and retinoic acid, respectively (see Section 4.1 in Chapter 29). (Based on Protein Data Bank file 4NQA from Lou X, Toresson G, Benod C, et al. Structure of the retinoid X receptor α-liver X receptor β [RXRα-LXRβ] heterodimer on DNA. *Nat Struct Mol Biol.* 2014;21:277-281.)

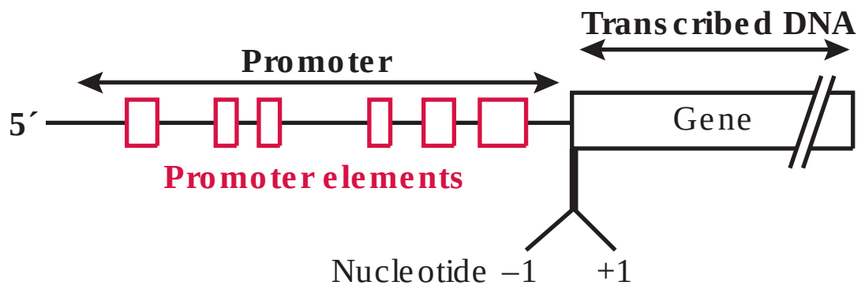


Fig. 6.4 Promoter upstream of a gene. By convention, the first nucleotide that is transcribed is +1, and the nucleotide 5′ to it is −1. All promoter elements are made up of nucleotides that carry negative numbers.

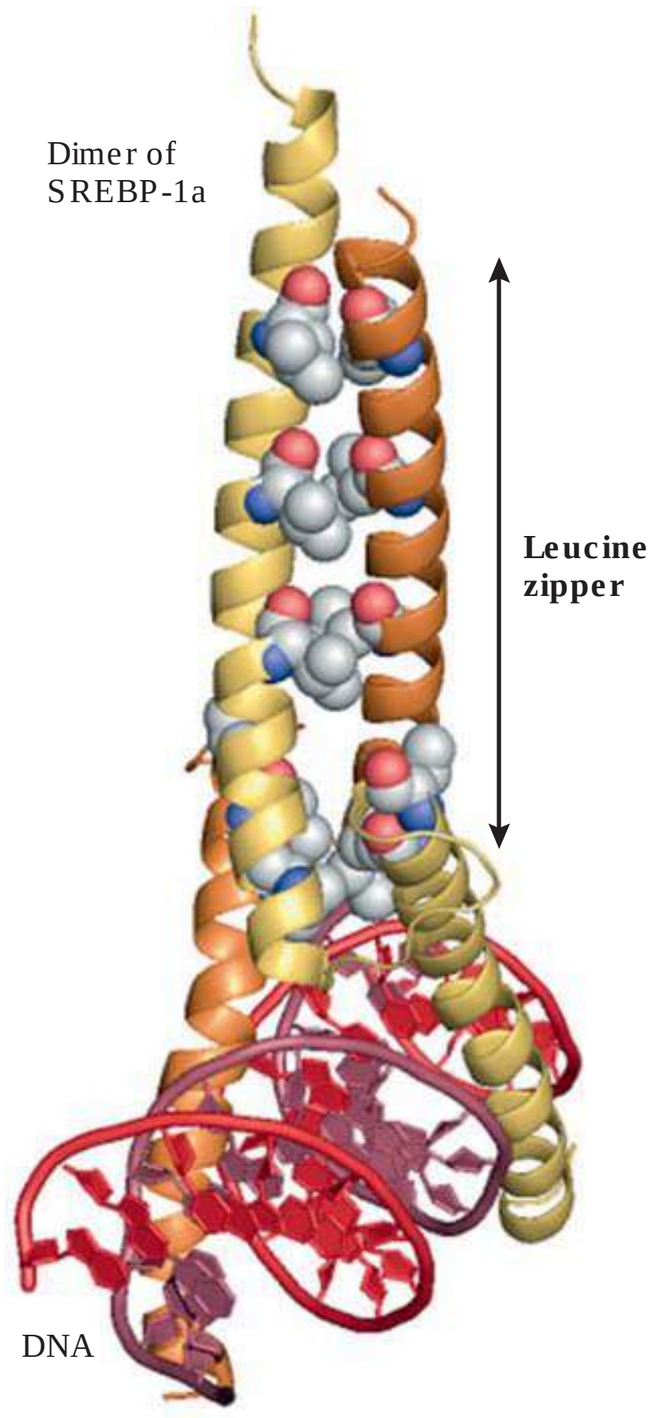


Fig. 6.6 SREBP-1a as an example of a transcription factor that uses a leucine zipper to dimerize. Leucine atoms are shown as spheres (O, red; N, blue; C, gray). (Based on Protein Data Bank file 1AM9 from Párraga A, Bellsolell L, Ferré-D'Amaré AR, Burley SK. Co-crystal structure of sterol regulatory element binding protein 1a at 2.3 A resolution. *Structure*. 1998;6:661-672.)

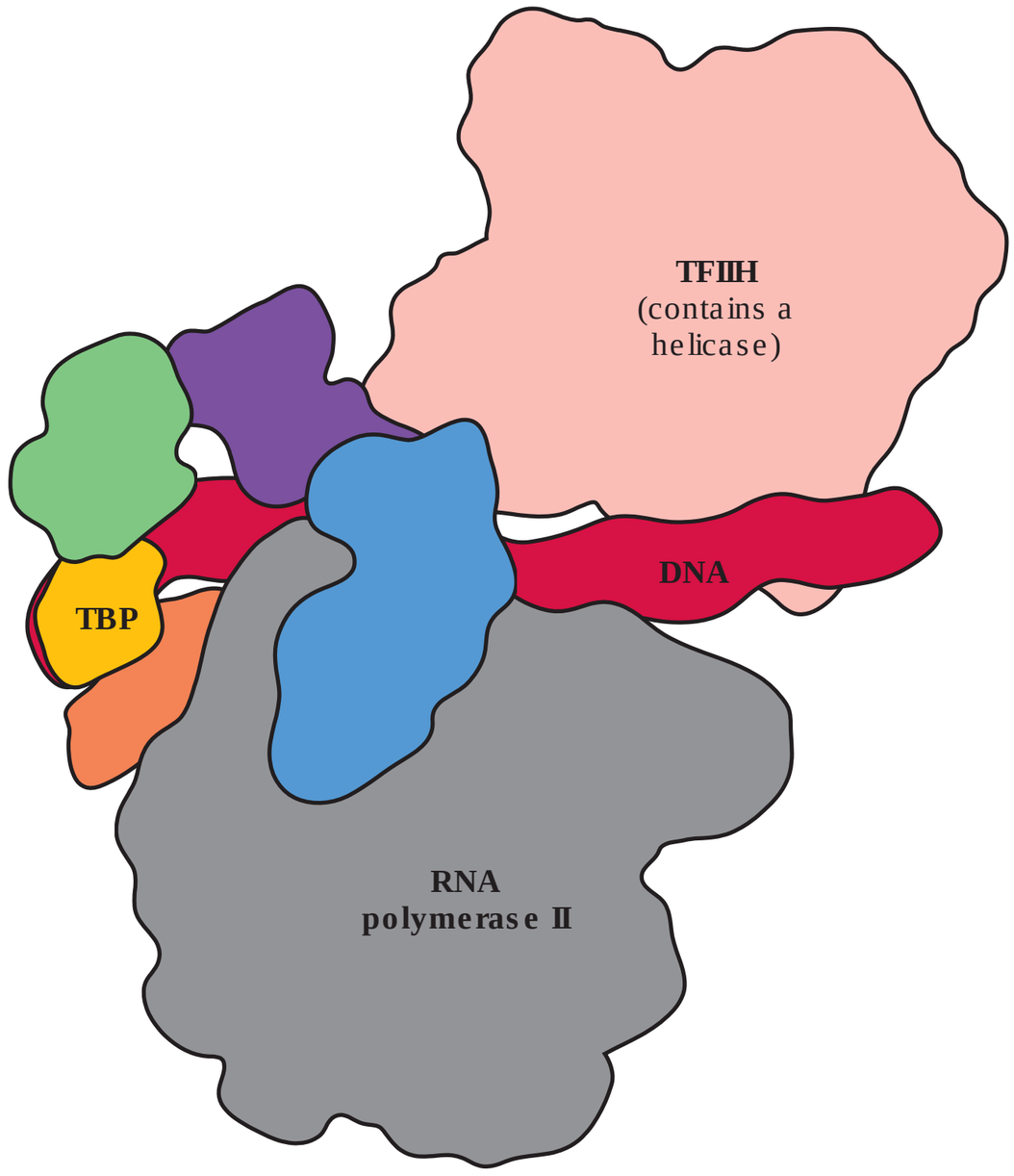


Fig. 6.7 Model of general transcription factors (GTFs) that are part of a preinitiation complex. The complex contains GTFs and RNA polymerase II. Transcription factors and coactivators are not shown. (Data from Murakami K, Tsai KL, Kalisman N, Bushnell DA, Asturias FJ, Kornberg RD. Structure of an RNA polymerase II preinitiation complex. *Proc Natl Acad Sci U S A*. 2015;112:13543-13548.)

several isoforms. GRα is usually the predominant glucocorticoid receptor that responds to glucocorticoids. GRβ is an isoform that does not activate transcription and can therefore antagonize GRα. Indeed, the increased expression of GRβ observed in **asthma** and in **rheumatoid arthritis**, for example, is accompanied by a decreased response to glucocorticoids. Other GR isoforms can also antagonize GRα. Variations in the use of the **translation start site** and in **posttranslational modification** (e.g., phosphorylation and acetylation) can yield further isoforms (see also Chapter 7). Although a GRE is a prerequisite for the binding of GR, great variation is seen from cell to cell in the GREs that are available to bind GR due to a cell-specific **chromatin structure**.

Transcription depends on the formation of a **preinitiation complex** by transcription activators, transcription coactivators, GTFs (general transcription factors), and RNA polymerase in the promoter region of a gene (Fig. 6.7).

For transcription, transcription factors bind to **promoter elements** (**DNA response elements**). Typically, a promoter contains many and diverse promoter elements (see Fig. 6.4).

Some transcription factors contain a **transactivation domain** through which they can bind **transcription coregulators**. Transcription coregulators can either be **transcription coactivators** or **transcription corepressors**. Transcription coregulators bind to transcription factors but not to DNA. Some transcription coactivators facilitate formation of the preinitiation complex, others favor the binding of enzymes that modify histones and thus make DNA available for transcription, and yet other coactivators remove proteins from the DNA that would impede transcription.

The transcription of some genes is markedly enhanced when transcription factors bind to **enhancer sequences** in DNA. Enhancers can be part of the promoter, they can be thousands of nucleotides upstream or downstream of the promoter, and they can even be found inside genes. Enhancers increase transcription only when transcription activators are bound to promoter elements. Enhancers are equally active in both orientations relative to the transcription start site. For a transcription factor bound to an enhancer to increase the formation of a preinitiation complex, the DNA containing the enhancer may loop back. Chromatin is organized such that certain enhancers are physically close to their target promoter regions; this increases the specificity of transcription enhancement.

Repressors (proteins) bind to **silencer elements** on DNA and thus reduce the rate of transcription. Like enhancers, silencers may be in the promoter region, gene, or thousands of nucleotides from the promoter region, and they are equally

active in both orientations relative to the transcription start site.

Transcription factors bound to promoter elements recruit **GTF**s (**TFII** proteins) that bind to the **core promoter**. In contrast to other transcription factors, the GTFs are used for the transcription of most genes. The core promoter is part of the promoter region and is close to the start site of the transcription. Some core promoters contain a **TATA box** (consensus sequence TATAAA) at about nucleotide −30; others contain an **initiator motif** at the transcription start site and a **downstream promoter element** (DPE) at about nucleotide +30 (i.e., downstream of the transcription start site). Transcription factors bound to DNA favor the binding of the GTF **TFIID** to the core promoter. The TFIID complex has a variable composition and contains **TATA-binding protein** (TBP), which binds to the TATA box. Subsequently, the GTFs TFIIA, TFIIB, TFIIC, TFIIE, TFIIF, TFIIG, and TFIIH bind to TFIID, thereby forming the **preinitiation complex**. TFIIB binds to the B recognition element (BRE) in the core promoter at approximately nucleotide –35 (immediately upstream of the TATA box, if there is one).

Once the initiation complex is assembled, the **helicase** of a GTF (see Fig. 6.7) unwinds the DNA helix so that a single strand can enter the active site of the **RNA polymerase**. RNA polymerase can then start transcribing the gene.

The **transcription start site** is determined by the location of promoter elements, including the TATA box (if present), by the local DNA conformation (a result of nucleotide composition), by the position of nucleosomes near the start site, and by the histone composition of nucleosomes (Table 6.1). The transcription start sites of some genes are limited to a single nucleotide, whereas those of others can extend over 30 to 100 nucleotides. Some genes contain a transcription start site that is in or near a particularly labile nucleosome (because of a special histone composition).

When alternate transcription start sites are used, multiple different RNA transcripts and proteins can be made from a single gene.

Eukaryotes contain three RNA polymerases. Under most circumstances, the activities of RNA polymerases I and III account for most of the transcription activity in a cell. We know most about transcription performed by RNA polymerase II.

RNA polymerase II transcripts encompass **messenger RNA** (**mRNA**), some of the **small nuclear RNAs** (**snRNAs**) that are used for splicing (see Section 3.3), and the **micro RNAs** (**miRNAs**) that can alter mRNA stability and translation (see Section 3 in Chapter 7). RNA polymerase uses the template DNA strand to synthesize a complementary RNA, inserting U in places where DNA polymerase would place T. The RNA itself is synthesized in a 5′ → 3′ direction (like DNA synthesis). Consequently, the DNA template strand is read in a 3′ → 5′ direction (like the DNA template strand during DNA replication). RNA polymerase II transcribes ~60 nucleotides per second.

The **3′ end of mRNAs** is not formed by termination of transcription but by **cleavage** of the RNA downstream of a polyadenylation signal. RNA polymerase II then terminates anywhere from a few to a few thousand base pairs downstream from the cleavage site via mechanisms that are still under investigation. As long as a preinitiation complex is present, RNA polymerase can start a new round of transcription.

Together, complexes of GTFs and **RNA polymerases I** and **III** accomplish transcription of genes that encode **ribosomal RNAs** (**rRNA**s; see Section 3 in Chapter 7), **transfer RNAs** (**tRNA**s; see Section 2 in Chapter 7), and some of the **snRNAs** that are part of the spliceosome (see Section 3.3). The abundance of these RNAs influences growth and cell replication.

Mitochondria contain a simpler transcription system compared with the nucleus. In its simplest form, the mitochondrial system requires **mitochondrial transcription factor A**, **mitochondrial transcription factor B2**, and **mitochondrial RNA polymerase**. The mitochondrial DNA encodes only two rRNAs, 22 tRNAs, and 13 proteins that play a role in oxidative phosphorylation (see Fig. 23.3 and Section 3 in Chapter 23). Hence, the entire transcription machinery is encoded in the nucleus, synthesized in the cytosol, and then imported into mitochondria. Mitochondrial DNA contains three promoters and generates various transcripts that give rise to multiple RNAs.

Some clinically used **drugs** and over-the-counter **supplements** influence transcription. **Glucocorticoids** are used widely for immunosuppression (see Section 3 in Chapter 31). In women, various **estrogens** and **progestins** are used for contraception, to treat infertility, and to reduce symptoms of menopause (see Section 2.4 in Chapter 31). **Fibrates** are used to reduce hypertriglyceridemia by activating peroxisome proliferator–activated receptor (PPAR)-α transcription factors (see Section 4 in Chapter 27 and Section 8.1 in Chapter 28). **Thiazolidinediones** are sometimes used to lower blood glucose by activating PPAR-γ transcription factors (see Section 5.3 in Chapter 39). **All-trans retinoic acid** is used in treating acne and acts at least in part by inducing activation of retinoic acid receptors and retinoid X receptors (RXR; see Section 7 in

Table 6.1 Characteristics of Transcription Start Sites

Transcription Start Site	Type of Transcription	Promoter Region	Nucleosomes
One specific nucleotide	Tissue-specific or highly regulated	Has TATA box	Positioning is flexible
~30 to 100 nucleotides	Always transcribed (housekeeping)	Has CpG island (no TATA box)	Nucleosome-free DNA segment near the promoter and start site

Chapter 28, Section 4.1 in Chapter 29, and Section 5 in Chapter 31). **Vitamin D**, which acts via heterodimeric transcription factors consisting of a vitamin D receptor and an RXR, regulates calcium and phosphate homeostasis (see Fig. 31.22 and Section 5 in Chapter 31).

3. PROCESSING OF RNA DURING AND AFTER TRANSCRIPTION

During transcription, the 5′ end of the growing RNA chain is capped with methylguanosine triphosphate. In response to a polyadenylation signal, the 3′ end of the growing RNA chain is cut and a poly(A) tail is added. Alternate polyadenylation sites can influence mRNA stability and even protein sequence. The RNA contains long segments (introns) that are removed and shorter segments (exons) that are retained and spliced together during and after transcription. Most RNAs can be spliced in multiple alternate ways by including or excluding whole exons or parts of exons, thus giving rise to diverse proteins. Mature mRNA is exported from the nucleus into the cytosol.

3.1. Capping of Pre-mRNA

During transcription, the 5′ end of the growing RNA chain is capped with 7-methyl guanosine triphosphate (Fig. 6.8). The methyl guanosine is added in "reverse" orientation, as it is joined to the RNA in a 5′ to 5′ fashion (not the usual 5′ to 3′). Furthermore, a triphosphate group is present between the 7-methyl guanosine and the 5′-end of the RNA. The cap structure is often abbreviated as m^7GpppN (by analogy, the remainder of the RNA would be described as pNpNpN…).

For capping, a phosphate group is first removed from the triphosphate group at the 5′ end of the newly synthesized RNA, then guanosine monophosphate is attached, and then the guanine base is methylated at N^7. The enzymes that catalyze capping bind to RNA polymerase transiently. Capping is complete by the time the RNA polymerase has produced a ~30-nucleotide transcript.

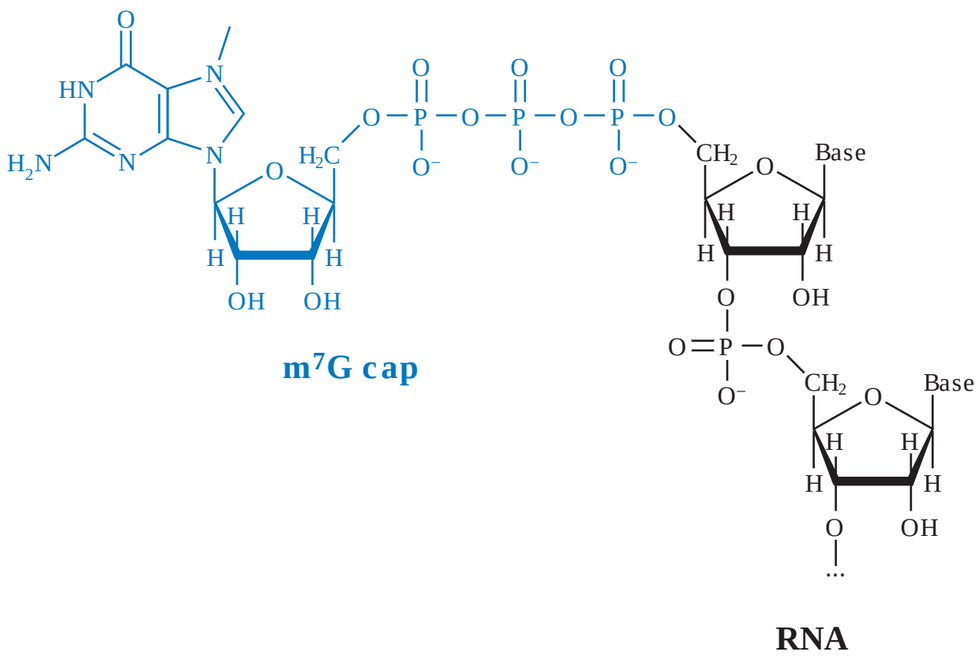


Fig. 6.8 Capping of RNA with methylated guanosine triphosphate.

The cap is essential for the export of mRNA from the nucleus (see Section 3.5) and for translation (see Section 3 in Chapter 7).

3.2. Polyadenylation of Pre-mRNA

Most precursor mRNAs (pre-mRNAs) are polyadenylated; that is, their 3′-end is extended with a ~50- to 100-nucleotide **poly(A) tail**. During transcription, a large protein complex recognizes the sequence AAUAAA (or a similar sequence) on pre-mRNA as a **polyadenylation signal** and then cuts the pre-mRNA between the polyadenylation signal and a U- or GU-rich **downstream sequence element** (DSE). A poly(A) polymerase then generates a poly(A) tail without using a DNA template (Fig. 6.9). The actual site of polyadenylation is ~10 to 35 nucleotides downstream of the polyadenylation signal.

Polyadenylation stabilizes an mRNA against degradation, and it also favors export from the nucleus into the cytosol.

The use of alternate polyadenylation sites can alter mRNA stability (Fig. 6.10) and protein amino acid sequence (Fig. 6.11). Through the use of alternate polyadenylation signals, transcription of most genes gives rise to multiple pre-mRNAs. The choice of signal site used depends on the exact nucleotide sequence of the signal, other elements of the pre-mRNA, and proteins that bind to pre-mRNA.

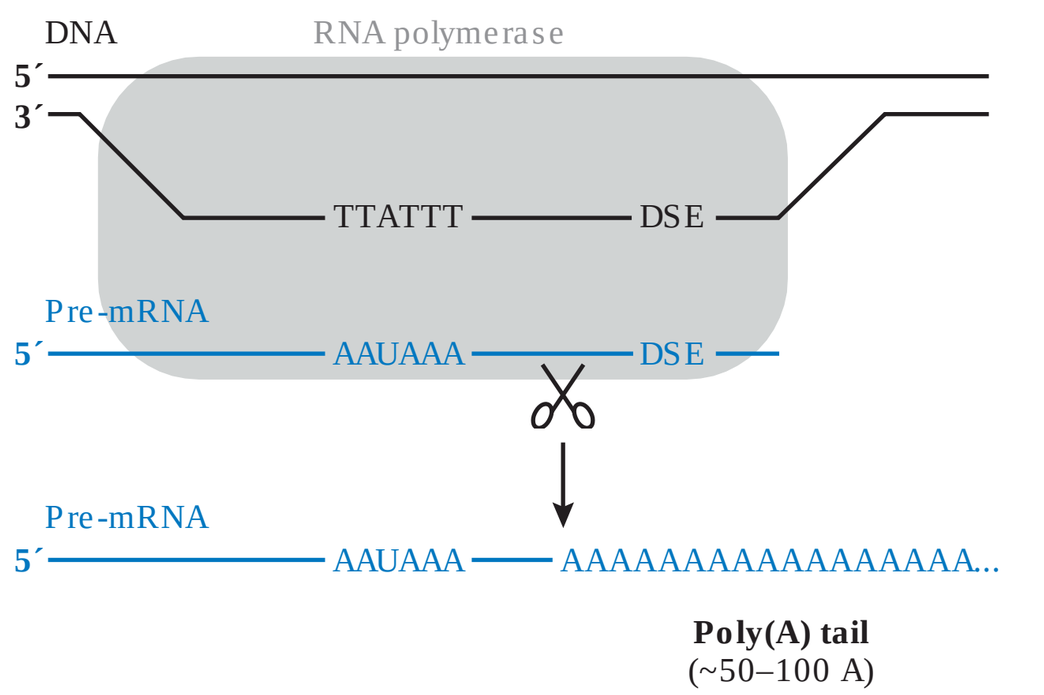


Fig. 6.9 Polyadenylation of RNA. DSE, dowstream sequence element.

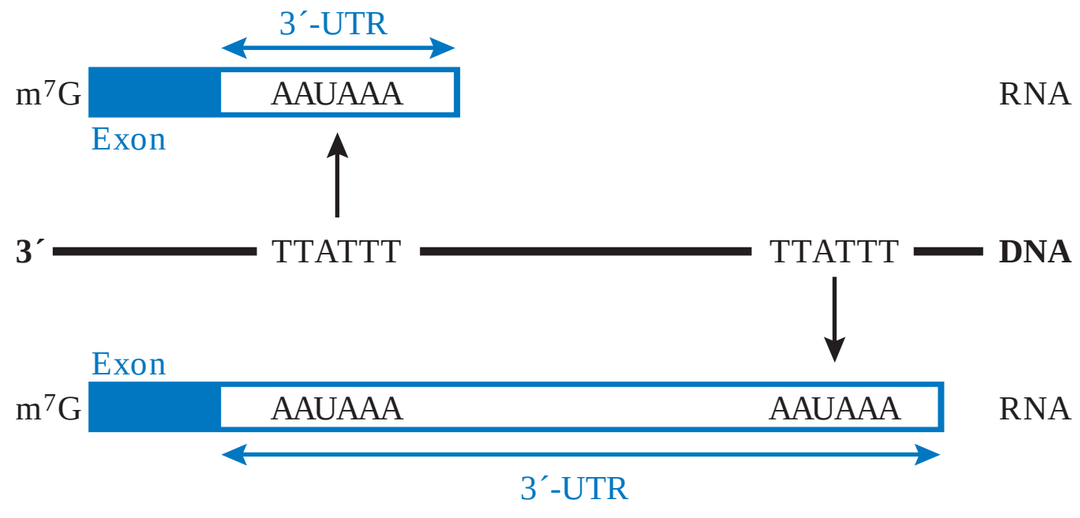


Fig. 6.10 Use of an upstream poly(A) site leads to termination of transcription and a shortened 3′-untranslated region (UTR). The longer 3′-UTR may contain sequences that regulate mRNA half-life. Exons are explained in Section 1.3.

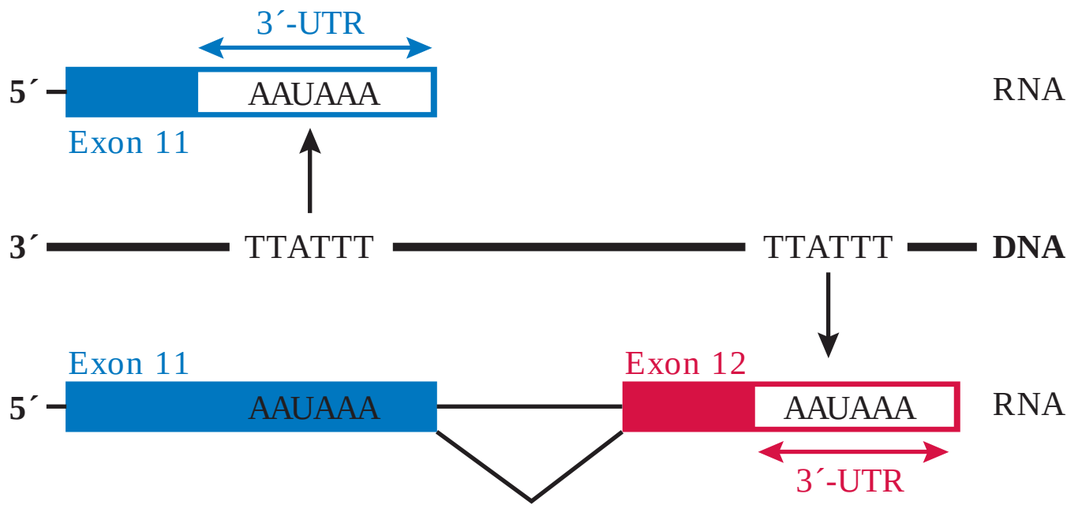


Fig. 6.11 Use of upstream poly(A) site leads to termination of transcription and a reduced number of exons, altering protein structure. Filled boxes indicate protein-coding regions. A similar result is obtained if the upstream poly(A) site lies within an intron. Exons and introns are explained in Section 3.3.

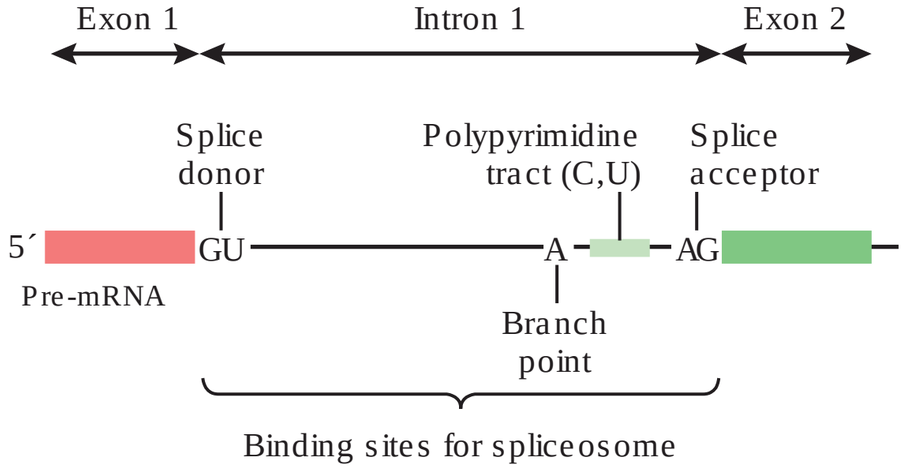


Fig. 6.12 Basic requirements for splicing of pre-mRNA. The figure shows the 5′ part of a pre-mRNA.

3.3. Splicing of Pre-mRNA

Splicing is part of the processing of pre-mRNA that gives rise to mature mRNA. Spliceosomes remove **introns** and join the remaining **exons**. A typical human pre-mRNA contains approximately 25 exons of ~150 nucleotides each as well as 24 introns of ~2,000 nucleotides each (total length ~50,000 nucleotides).

Splicing of pre-mRNA starts during transcription. Some splicing proteins can bind to RNA polymerase, but much of the splicing takes place after the RNA polymerase has disengaged from the DNA.

Splicing is determined by the following elements of the pre-mRNA (Fig. 6.12): splice site donor (5′), splice site acceptor (3′), polypyrimidine tract upstream of the splice acceptor, splicing branch point, splicing enhancers, and splicing silencers.

T e consensus **splice donor** sequence is **GU**, and the consensus **splice acceptor** sequence is **AG**. Nucleotides flanking these sequences af ect the choice of splice site.

T e core spliceosome, which performs almost all RNA splicing, consists of a dynamic complex that, over the course of splicing, involves five **snRNAs** and more than 300 proteins. Since the snRNAs of the spliceosome catalyze the actual splicing, the spliceosome (like the ribosome) is a **ribozyme**.

In addition to splice donors, splice acceptors, and spliceosomes, splicing is directed by sequences called exonic and intronic **splicing enhancers**, as well as exonic and intronic **splicing silencers**, respectively. SR proteins recognize and bind to splicing enhancers and favor splicing. Conversely, hnRNPs (RNA-protein complexes) bind to splicing silencers and block splicing.

Exons are spliced together only in the sequence in which they occur in pre-mRNA, though it is possible to skip one or more exons. During splicing, the ends of exons are always bound to the spliceosome; that is, they are never free.

During splicing, a multiprotein **exon junction complex** (**EJC**) is deposited on the spliced mRNA upstream of each splice junction. EJCs are required for the export of mRNA from the nucleus. In the cytosol, EJCs are removed during the first round of translation, but if a stop codon is close to an EJC, the mRNA enters nonsense-mediated RNA decay (see Section 3 in Chapter 7).

Acquired mutations in splicing factors (proteins) occur especially frequently in **myelodysplastic syndrome** (**MDS**). MDS is characterized by impaired production of blood cells and an increased risk of development of acute leukemia.

Mutations of splice donor or splice acceptor sites can abolish correct splicing and may also induce splicing at **cryptic splice sites**, which are splice sites that are normally not used. Furthermore, a mutation may create a new splice site.

Mutations frequently cause only a partial shift in splicing, thereby generating two or more mRNAs. An example of a partial shift to use a new splice acceptor site is a form of **β-thalassemia** that originated in the Mediterranean basin (see Fig. 17.3).

3.4. Alternative Splicing of Pre-mRNA

Most RNAs that contain two or more exons undergo alternative splicing, which greatly increases the diversity of proteins that can be synthesized. T rough alternative splicing, parts of a protein can be exchanged, added, or deleted.

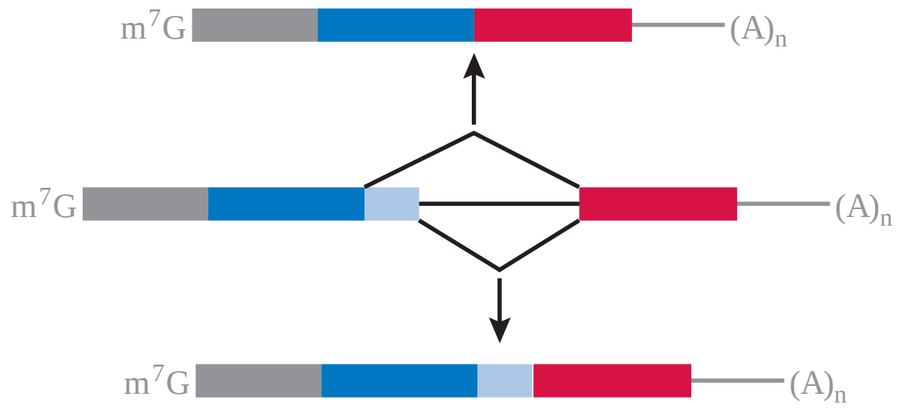
Four essential types of alternative splicing are available: use of an alternative 5′ splice site, use of an alternative 3′ splice site, cassette exon inclusion/skipping, and intron retention (Fig. 6.13).

T e splicing of the RNA transcript from the gene for glucocorticoid receptors is an example of the use of an alternative splice acceptor (Fig. 6.14). In this case, alternative splicing leads to receptors that do or do not bind to a glucocorticoid.

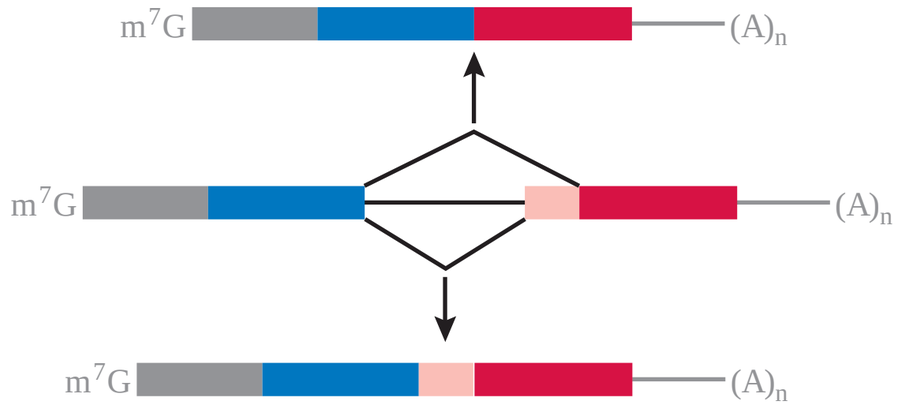
In summary, a gene is a segment of DNA that is transcribed into RNA, and after transcription, introns are removed from the RNA, while exons are retained (Fig. 6.15). As explained in Chapter 7, an mRNA is translated into protein, but the 5′ end, 3′ end, and poly(A) tail are not translated. T e untranslated ends are called the **5′-untranslated region** (**5′-UTR**) and the **3′-untranslated region** (**3′-UTR**).

A single gene can give rise to diverse proteins thanks to alternative transcription start sites and polyadenylation sites, alternative splicing (see Sections 2 and 3), translation initiation, and posttranslational modifications (see Sections 3 and 4 in Chapter 7).

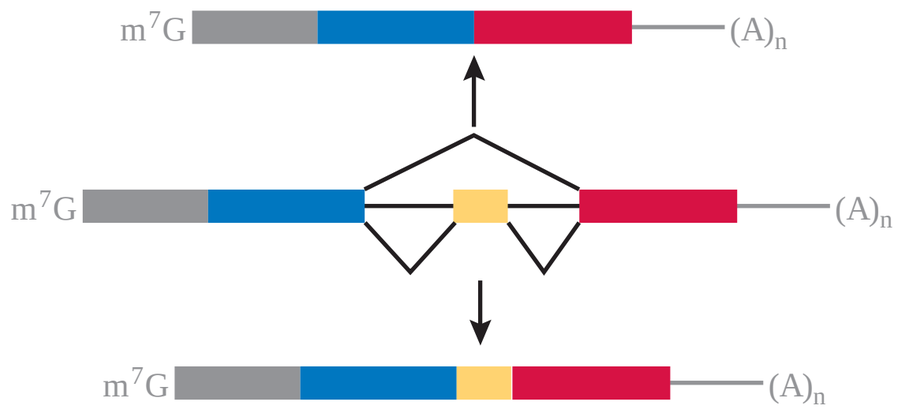
Alternative splice donor:



Alternative splice acceptor:



Exon inclusion or skipping (cassette exon):



Intron splicing or retention:

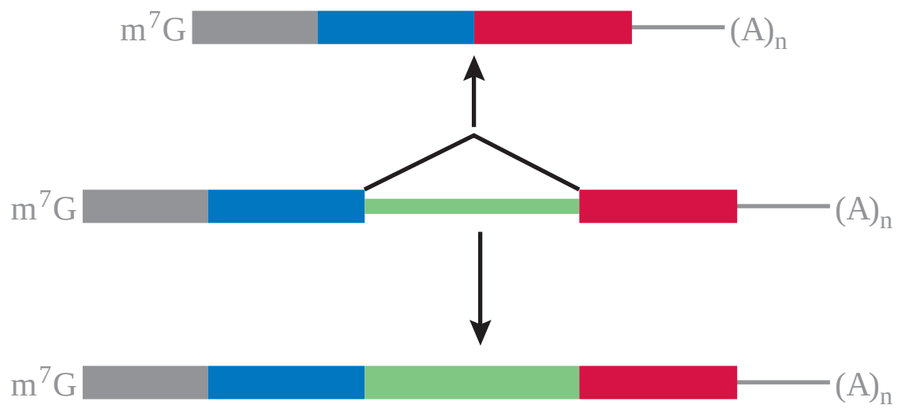


Fig. 6.13 **Basic modes of alternative splicing.**

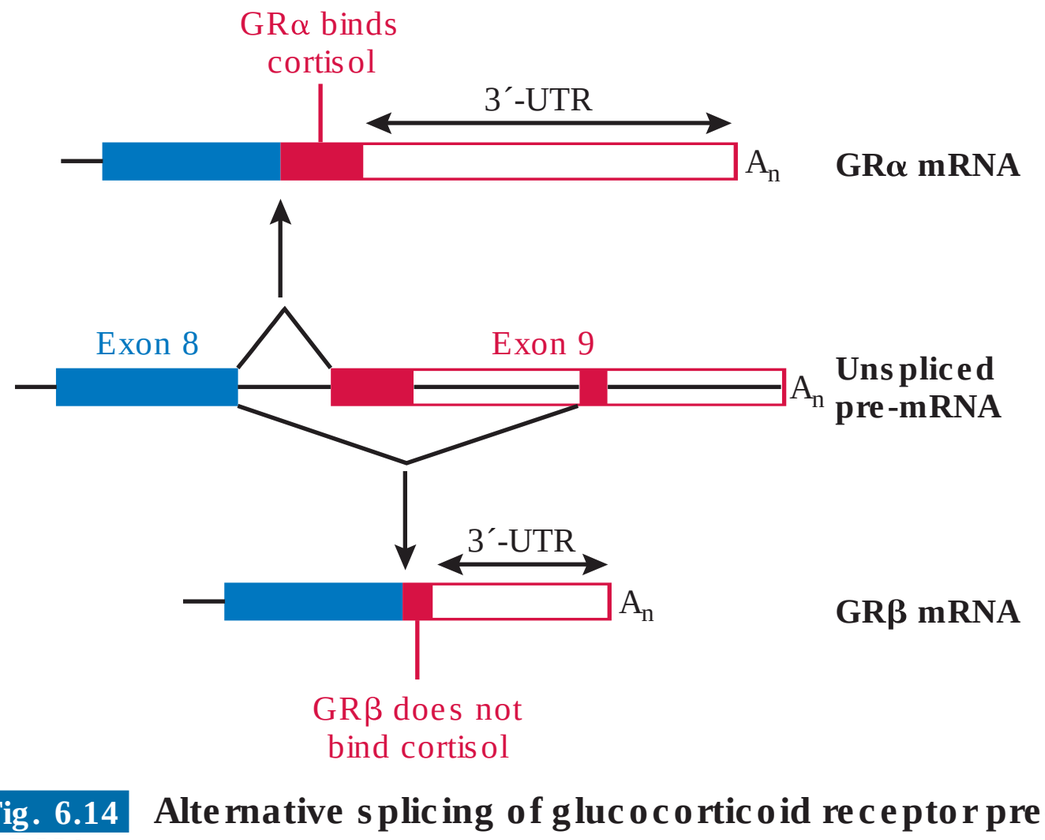


Fig. 6.14 **Alternative splicing of glucocorticoid receptor pre-mRNA.** Filled boxes indicate translated sequences. Exon 9 encodes the glucocorticoid binding domain. Exons 1–7 are not shown. (There is an additional form of GRα mRNA with a shorter 3′-untranslated region due to use of an upstream alternative polyadenylation site that is not shown.)

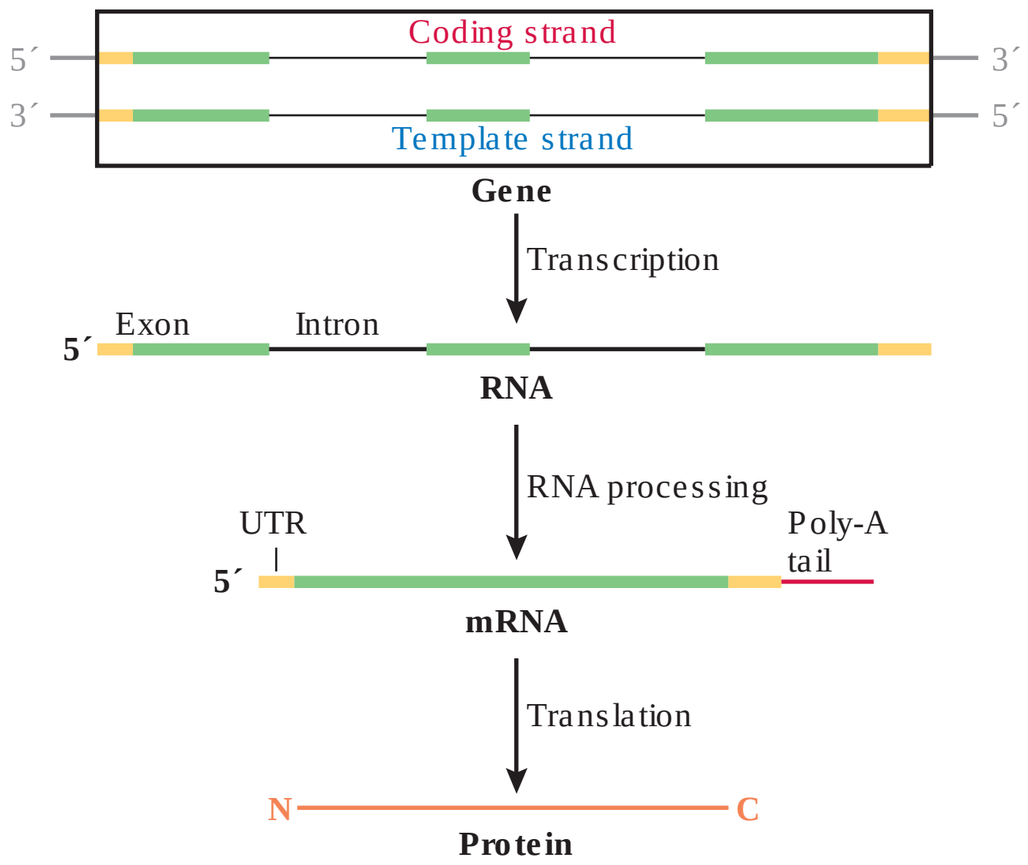


Fig. 6.15 **From gene to RNA to protein.**

3.5. Export of mRNA Into the Cytosol

Messenger ribonucleoprotein particles (complexes of mRNA and proteins) are exported from the nucleus through **nuclear pore complexes** (**NPC**s), which dot a layer of endoplasmic reticulum around the nucleus (Fig. 6.16). For export through the NPCs, RNA **export factors** need to be bound to the complex of mRNA and its associated proteins (e.g., the cap-binding complex, poly(A)-binding protein, and EJCs).

3.6. Degradation of mRNA

T e quality of pre-mRNA and mRNA is controlled extensively. RNAs that do not meet requirements are degraded, and there is ongoing turnover of all mRNAs. In the cytosol, the 5′ cap and 3′ poly(A) tail, bound to proteins, allow mRNA to survive. mRNA is degraded first by shortening of the poly(A) tail by deadenylases, producing adenosine monophosphate. T e resulting RNA can then be degraded in a 3′→5′ direction by the **exosome**, a large protein complex, until only the m7GpppN

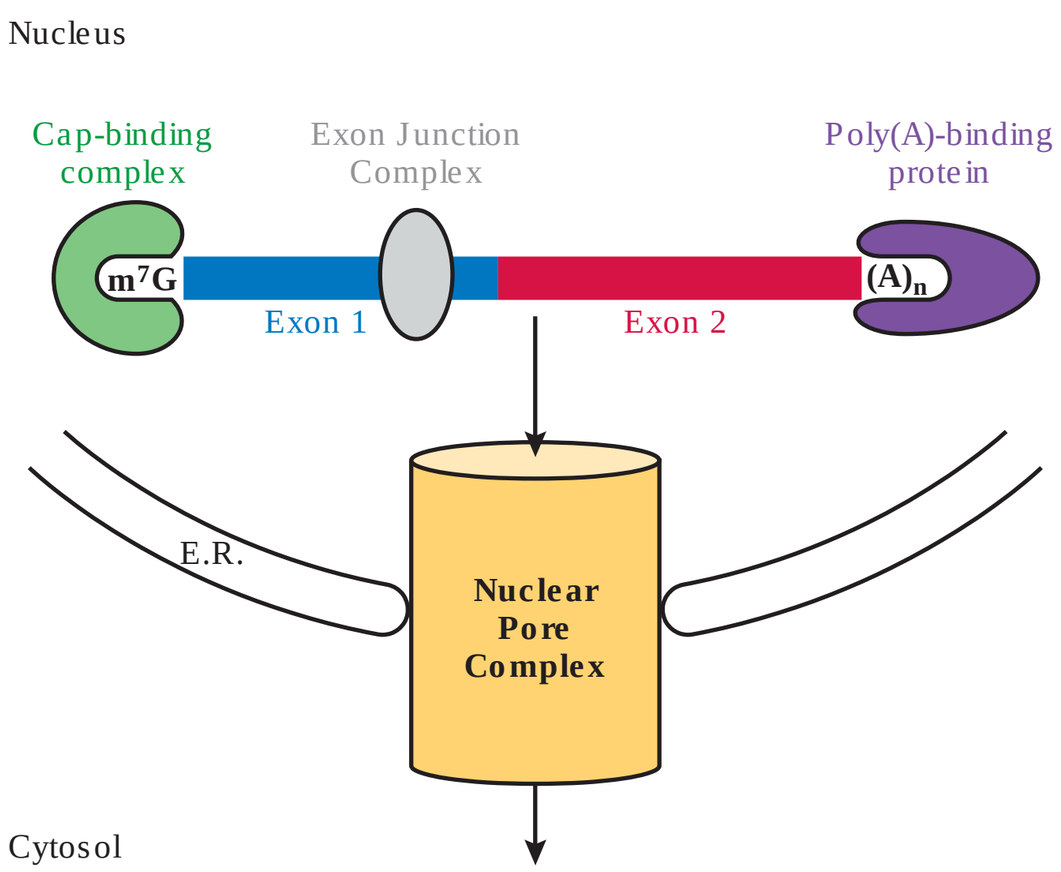


Fig. 6.16 Export of messenger ribonucleoprotein particle (mRNP) complexes from the nucleus. Additional proteins are needed to shepherd the mRNP complex through the nuclear pore complex.

cap remains, which is degraded by a dedicated enzyme. Alternatively, after the removal of the poly(A) tail, the RNA can be degraded first by 5′ **decapping** and then by a **5′→3′ exonuclease** (Xrn1). Both pathways of RNA degradation are under the control of many proteins that activate or inhibit.

SUMMARY

- DNA in heterochromatin is tightly packed and is not transcribed. DNA in euchromatin can be transcribed.
- Methylation of DNA on C's of CpGs, recognition of methylated CpGs, and methylation of histones favor the formation of heterochromatin. DNA methylation is the basis of epigenetic inheritance. CpGs are methylated as part of development, dif erentiation, and inactivation of X chromosomes when two or more X chromosomes are present. DNA segments that contain a relatively high fraction of CpGs are called CpG islands. Most CpG islands are unmethylated.
- In tumor cells, excessive DNA methylation is often responsible for decreased transcription of genes that encode tumor suppressors.
- Acetylation of histones, catalyzed by lysine acetyltransferases, makes DNA more amenable to transcription. A similar ef ect is achieved clinically with inhibitors of histone deacetylases (HDACs). Among these inhibitors, valproic acid is used to treat seizures, while vorinostat, romidepsin, belinostat, and panabinostat are used to treat certain forms of lymphoma.
- A gene is commonly defined as a segment of DNA that is transcribed. T e template strand of DNA is transcribed in a 3′→5′ direction so that RNA is synthesized in a 5′→3′ direction. T e resulting RNA has the same sequence as the coding strand, except for U in place of T.
- Neighboring genes can be oriented in opposite directions. Of the two complementary DNA strands that make up a chromosome, one DNA strand is the coding strand of only the genes that are oriented in one particular direction, while the other strand contains the coding strands for genes that are oriented in the opposite direction.
- Upstream of the gene is a promoter region that contains a core promoter. For transcription to take place, transcription factors need to bind to promoter elements in the promoter region, and general transcription factors (GTFs) must bind to the core promoter. Some transcription factors are active only when bound to a ligand, such as a steroid. Once the initiation complex is assembled, RNA polymerase synthesizes RNA from the DNA template.
- Some drugs that are in clinical use, such as glucocorticoids, estrogens, progestins, fibrates, and retinoic acid, activate transcription factors.
- During transcription, transcribed RNA receives a 7-methylguanosine triphosphate cap and a 50- to 100-nucleotide poly(A) tail.
- Both during and after transcription, spliceosomes remove introns and link exons. T e first exon contains the 5′-UTR and the last exon contains the 3′-UTR. Alternative splicing is due to the use of an alternate splice donor or acceptor, exon skipping, or intron retention. A cryptic splice site is a site that is not normally used for splicing but is used under special conditions, such as when a new mutation is present.
- Mature mRNA with cap, cap-binding complex, exon junction complexes (EJCs), poly(A) tail, and poly(A)-binding protein binds additional proteins and is then exported into the cytosol via the nuclear pore complex (NPC).

FURTHER READING

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Review Questions

1. For a particular protein, the template strand contains the sequence 5′-ACCGT. After transcription into RNA, the RNA contains which one of the following sequences?

   A. 5′-ACCGU
   B. 5′-ACGGU
   C. 5′-UGCCA
   D. 5′-UGGCA

2. A couple had a son who had β-thalassemia (i.e., the child made only a small amount of β-globin). T e couple sought genetic counseling. T e father was found to have one normal and one mutant β-globin allele. T e mutant allele contained an A→G mutation at position −30 of the β-globin promoter. T e A nucleotide is most likely in which of the following?

   A. Downstream promoter element
   B. Enhancer
   C. Initiator motif
   D. Nuclear receptor
   E. TATA box

3. Rett syndrome in a 4-year-old girl is caused by a hereditary deficiency in which one of the following processes?

   A. DNA methyltransferase
   B. Histone acetyltransferase
   C. Histone methyltransferase
   D. Methyl CpG-binding protein

4. Most nuclear receptors bind to which of the following?

   A. Both the template and the coding strand
   B. Coding strand only
   C. Template strand only

5. Which one of the following processes generally favors transcription of a gene?

   A. Lysine acetylation of histones
   B. Lysine methylation of histones
   C. Methylation of CpG islands

6. Given the following data, what is the length of the mature mRNA?

   5′-UTR: 20 nt (nucleotides), 3′-UTR: 50 nt without the poly(A) tail, exon 1: 150 nt, exon 2: 150 nt, intron 1: 2000 nt, and poly(A) tail: 100 nt

   A. 231 nt
   B. 331 nt
   C. 401 nt
   D. 471 nt
   E. 2301 nt
   F. 2401 nt

Chapter 7 Translation and Posttranslational Protein Processing

SYNOPSIS

- As detailed in Chapter 6, the nucleotide sequence of DNA gives rise to an mRNA of essentially complementary sequence.
- In translation, ribosomes synthesize proteins according to the rules of the genetic code and the genetic information contained in mRNA. The mRNA sequence is read in units of three nucleotides, called codons. Each codon specifies either a particular amino acid or a signal to terminate the synthesis of the protein.
- Aminoacyl-tRNA synthetases charge each tRNA with an appropriate amino acid, thus generating aminoacyl-tRNAs.
- Ribosomes scan mRNA for a start codon to initiate protein synthesis. At each codon, the translating ribosome matches the codon sequence with a specific aminoacyl-tRNA and catalyzes peptide bond formation.
- In humans, if a newly synthesized peptide contains a signal sequence, ribosomes dock to the endoplasmic reticulum (ER) and feed the growing peptide chain into the ER through a peptide channel. As soon as certain asparagine side chains reach the lumen of the ER, they are glycosylated. Glycosylation can influence protein folding and later also protein sorting. Bacteria do not have an ER.
- Proteins fold mostly in the ER. Chaperones detect misfolded proteins, refold them, or direct them to degradation.
- Proteins travel from the ER in vesicles to the Golgi. In the Golgi, proteins can be extensively modified and are further sorted for transport inside vesicles to specific parts of the cell.
- Many commonly used antibiotics selectively impair translation in bacteria.

LEARNING OBJECTIVES

For mastery *of* this topic, you should be able to do the *following*:

- Describe the factors that determine the start, continuation, and end of translation.
- Explain why some DNA mutations in the coding sequence do not result in a mutant protein.
- Explain why a one-base or two-base insertion or deletion is usually a loss-of-function mutation and why no mutant protein accumulates in the tissue.
- List factors that set the gross rate of protein synthesis.
- List antibiotics that interfere with protein synthesis and describe their mechanism of action.
- Describe the synthesis of membrane proteins.
- Describe the factors that influence sorting of newly synthesized proteins and their transport to different subcellular compartments.
- Describe common posttranslational modifications.
- Discuss how cells control the quality of newly synthesized proteins and degrade these proteins if needed.

1. CODONS AND THE GENETIC CODE

T e amino acid sequence o*f* a protein is encoded in messenger RNA (mRNA) in the *f*orm o*f* a sequence o*f* codons. Each codon consists o*f* three nucleotides and encodes one amino acid or a signal to stop translation. T e genetic code describes the correlation between codons and amino acids; it allows one to predict the e*ff*ects o*f* some mutations on the amino acid sequence o*f* a protein.

Translation is the process of synthesizing proteins in ribosomes according to the nucleotide sequence of mRNA.

T ere are 20 dif erent amino acids that are incorporated into proteins, and their sequence is encoded in mRNA as **codons** that consist of three nucleotides. Since there are four dif erent bases, 3-nucleotide codons can have a total of $4^3 = 64$ dif erent sequences, enough to encode 20 amino acids (2-nucleotide codons would of er only $4^2 = 16$ options).

T e **genetic code** is a list of codon sequences and the corresponding amino acids that are used in translation (Table 7.1). T e AUG codon codes for Met and signals the **start** of translation. T e codons UAA, UAG, and UGA are **stop codons** (**nonsense codons**, **termination codons**) and usually end translation.

Since all possible codon sequences are used, multiple different codons may code for the same amino acid; that is, the genetic code is **degenerate**.

T e **coding strand** of DNA has the same sequence as the mRNA that is generated from the template strand, except that it contains T in place of U. Both the coding strand and the mRNA are complementary to the template strand (see Section 2 in Chapter 6).

Silent mutations (**synonymous substitutions**) are mutations that do not lead to a change in amino acid sequence because the genetic code is degenerate. However, silent mutations can af ect mRNA splicing or mRNA stability and may therefore be pathogenic.

Nonsense mutations are mutations that lead to the formation of a stop codon. Only a small amount of the altered protein may be produced because the premature stop codon causes destruction of the mRNA by **nonsense-mediated mRNA decay** (see Section 3).

Missense mutations are mutations that lead to the substitution of an amino acid with another amino acid. Substitutions of amino acids with very dif erent properties (nonconservative substitutions; e.g., substitution of a hydrophobic amino acid with a charged amino acid) have more severe ef ects than substitutions of amino acids with similar properties (conservative substitutions).

Table 7.1 Genetic Code for Information Stored in Chromosomes

	Second Base				
First Base	**U**	**C**	**A**	**G**	**Third Base**
U	Phe	Ser	Tyr	Cys	U
					C
	Leu		Stop	Stop	A
				Trp	G
C		Pro	His	Arg	U
					C
			Gln		A
					G
A	Ile	Thr	Asn	Ser	U
					C
			Lys	Arg	A
	Met				G
G	Val	Ala	Asp	Gly	U
					C
			Glu		A
					G

To decode the coding strand of DNA, replace U with T. The genetic code for mitochondria differs in that AGA and AGG are additional stop codons, UGA codes for Trp, and both AUU and AUA code for Met.

Since each codon contains three nucleotides, there are theoretically three different **reading frames** for mRNA. The actual reading frame is usually determined by the first occurrence of the nucleotide sequence AUG (encoding Met) in a particular sequence context (see Section 3). The alternate reading frames usually contain relatively frequent stop codons (statistically, ~1:20 random codons is a stop codon). Since the physiologically used reading frame has fewer stop codons than the other two reading frames, it is sometimes called the **open reading frame**.

Frame-shift mutations are nucleotide insertions or deletions that are not divisible by 3 and thus change the reading frame for part of the mRNA. Because the alternate reading frames have frequent stop codons, a frameshift mutation generally leads to protein truncation and to degradation of the mRNA by **nonsense-mediated mRNA decay** (see Section 3).

With the exception of **mitochondria**, which have their own translation machinery and employ a modified form of the genetic code (see Table 7.1), all organisms use the same genetic code (i.e., the code is universal). Hence, genetically modified bacteria, for instance, can be used in a straightforward manner to manufacture human proteins (**recombinant proteins**) for use as biologicals (e.g., insulin, erythropoietin). However, posttranslational processing of eukaryotic proteins in prokaryotes may be incomplete or incorrect and may thus require further manipulation.

2. TRANSFER RNAs

In preparation for protein synthesis, aminoacyl-tRNA (transfer RNA) synthetases charge their cognate tRNAs with the cognate amino acids to form aminoacyl-tRNAs. Each tRNA contains an anticodon that will read the corresponding codon of an mRNA. The anticodon of each tRNA must match the first two nucleotides of a codon according to the common (Watson-Crick) base pairing rule; however, there is flexibility in matching the third nucleotide because of wobble base pairing.

tRNAs are bifunctional molecules that read selected codons and carry a matching amino acid. While there are 20 different amino acids that are incorporated into proteins and 61 codons that code for these amino acids, there are more than 500 different tRNAs.

tRNAs consist of ~80 nucleotides, and they all fold into an L-shaped tertiary structure (Fig. 7.1). The sequence of each tRNA is encoded in chromosomes and in mitochondrial DNA (see Fig. 23.8). Each tRNA is synthesized by transcription of a gene and then processed. The 3′ end of all tRNAs is extended with the sequence CCA-3′. Nucleotides of tRNAs are extensively modified, most often by methylation. A few adenosines can be edited by deamination to give rise to inosines. In the L-shaped tRNA, one end contains three nucleotides that are complementary to the three nucleotides in the codon; this region is called the **anticodon**.

Each tRNA anticodon can often read multiple different codons due to **wobble base pairing**. An anticodon has to match the first two nucleotides of each mRNA codon according to Watson-Crick base-pairing rules (A-U, C-G), but there is flexibility with the third nucleotide. The third nucleotide of a codon can be A, C, or U and still bind sufficiently well to inosine (I, an edited A; see above) in the anticodon; furthermore, G and U can form a wobble base pair. As an example of wobble base pairing, the anticodon of the glycine-tRNA has the sequence 5′-ICC, which can pair with three codons: GGA, GGC, and GGU.

tRNAs are named for the amino acid they are charged with and the codons that are assigned to this amino acid. For instance, $tRNA^{Cys}$ is charged with cysteine and recognizes the cysteine codons. The initiator $tRNA_i^{Met}$ is used selectively for the initiation of protein synthesis, and the elongator $tRNA^{Met}$ is used during chain elongation. Although there are usually multiple tRNAs that code for the same amino acid, these tRNAs are not further distinguished in this book.

Aminoacyl-tRNA synthetases use ATP to couple the CCA-3′ end of a tRNA to the appropriate amino acid. For each amino acid, there is one aminoacyl-tRNA synthetase. The

Fig. 7.1 Structure of human $tRNA^{Sec}$. (Based on Protein Data Bank file 3A3A from Itoh Y, Chiba S, Sekine SI, Yokoyama S. Crystal structure of human selenocysteine tRNA. *Nucleic Acids Res*. 2009; 37:6259-6268.)

aminoacyl-tRNA synthetases recognize tRNAs in varied ways but mostly via the nucleotide sequence in the amino acid acceptor stem (colored red in Fig. 7.1) and in the anticodon region.

For some amino acids, such as Leu, multiple tRNAs are needed to read all Leu codons. In such circumstances, the aminoacyl-tRNA synthetase (in this case, leucyl-tRNA synthetase) accepts multiple $tRNAs^{Leu}$ as substrates. These isoacceptor tRNAs differ in nucleotides in the anticodon and in other regions of the L-shaped structure.

The aminoacyl-tRNA synthetases for leucine, isoleucine, valine, threonine, alanine, and phenylalanine contain an **editing** (**proofreading**) function. These synthetases can acylate their tRNA with the wrong amino acid. To compensate, these enzymes use a second catalytic site to remove incorrect amino acids.

Selenocysteine (see Fig. 9.2), an amino acid contained in a few proteins, is synthesized on $tRNA^{Sec}$ (Sec = selenocysteine). First, seryl-tRNA synthetase aminoacylates $tRNA^{Sec}$ with serine. An enzyme then phosphorylates the serine. A second enzyme exchanges the phosphate for a selenol (–SeH) group to yield selenocysteinyl-$tRNA^{Sec}$. $tRNA^{Sec}$ is unique in that it carries the anticodon 5′-UCA-3′, which is complementary to the UGA stop codon. Selenocysteine is encoded in mRNA via a combination of a UGA stop codon and a downstream selenocysteine insertion sequence (SECIS).

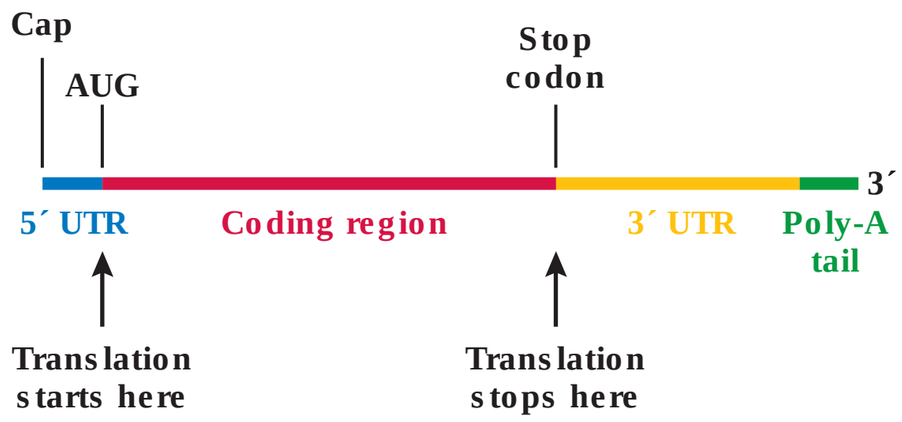

Fig. 7.2 Structure of a typical mRNA.

The antibiotic **pseudomonic acid** (**mupirocin**) inhibits protein synthesis by inhibiting isoleucyl-tRNA synthetase in bacteria. It is often used to treat methicillin-resistant Staphylococcus aureus.

3. RIBOSOMES TRANSLATE mRNA INTO PROTEIN

Ribosomes are large RNA-protein complexes that bind the mRNA, read the mRNA, accept tRNAs that match the codons of the mRNA, and catalyze peptide bond formation between amino acids attached to tRNAs. The first amino acid of a protein is usually methionine; however, it is typically removed soon after translation. Proteins that contain a signal sequence are transferred into the ER during synthesis. Nonsense-mediated RNA decay ensures that mRNAs that contain premature stop codons are not translated. Micro RNAs interfere with translation. Protein synthesis increases after a mixed meal and decreases during times of stress, viral infection, or nutrient deprivation. Several antibiotics used in clinical practice block translation in bacteria but not appreciably in humans.

The production of mRNA, its 5′ cap structure, and its poly-A tail are described in Sections 2 and 3 in Chapter 6. The features of mRNA that are important to translation are shown in Fig. 7.2.

Each human ribosome is made up of four **ribosomal RNA**s (**rRNA**s) and ~80 proteins. The largest rRNA is a **ribozyme** that catalyzes peptide bond formation. A ribosome consists of a small subunit and a large subunit.

In the cytosol, a complex of translation **initiation factors**, $Met\text{-}tRNA_i^{Met}$ (methionine initiator tRNA acylated with methionine), and the small subunit of a ribosome binds to the **5′ cap** of an mRNA, checks for a poly-A **tail**, scans the mRNA from the 5′-cap, finds the **start codon** (almost always **AUG**), and starts translation. This AUG is often flanked by ACC on the 5′ side and by G on the 3′ side. If the flanking nucleotides of the AUG do not match sufficiently well, translation starts at a suitable AUG that is further away from the cap. **Insulin** and other **growth factors** increase protein synthesis in part by leading to increased binding of an initiation factor to the 5′ cap of mRNA.

The sequence between the 5′ cap and the start codon of the mRNA is called the **5′ untranslated region** (**5′ UTR**; see

Fig. 7.2). Occasionally, the 5′ UTR contains a regulatory element, such as an iron response element (see Section 3 in Chapter 15).

Once the start codon has been found, the large ribosome subunit binds to the mRNA. An **elongation factor** (eIF5A) brings an aminoacylated **tRNA** to the codon that follows the start codon. If the codon and anticodon match, guanosine triphosphate (GTP) is hydrolyzed and the aminoacylated tRNA undergoes a conformational change called accommodation. This change in structure moves the amino acid on the tRNA into the peptidyl transferase catalytic site of the ribosome and aligns it with the Met on the initiator tRNA. A new peptide bond forms via a nucleophilic attack of the amino group of the incoming amino acid on the carbonyl group of methionine on the initiator tRNA (Fig. 7.3). The new peptide bond forms such that Met is now linked to the amino acid on the newly added tRNA.

Next, a different **elongation factor** (EF-G) binds to the ribosome, GTP is hydrolyzed, and the ribosome moves on the mRNA by one codon in the 5′→3′ direction. The tRNA that now carries a dipeptide also moves in the ribosome. A new matching aminoacylated tRNA binds to the ribosome, and the deacylated ("empty") initiator tRNA leaves the ribosome. Repetition of the aforementioned processes leads to further elongation of the peptide chain according to the coding information in the mRNA. Each ribosome forms about one to two peptide bonds per second. **Insulin** favors protein synthesis in part by increasing the activity of the elongation factor.

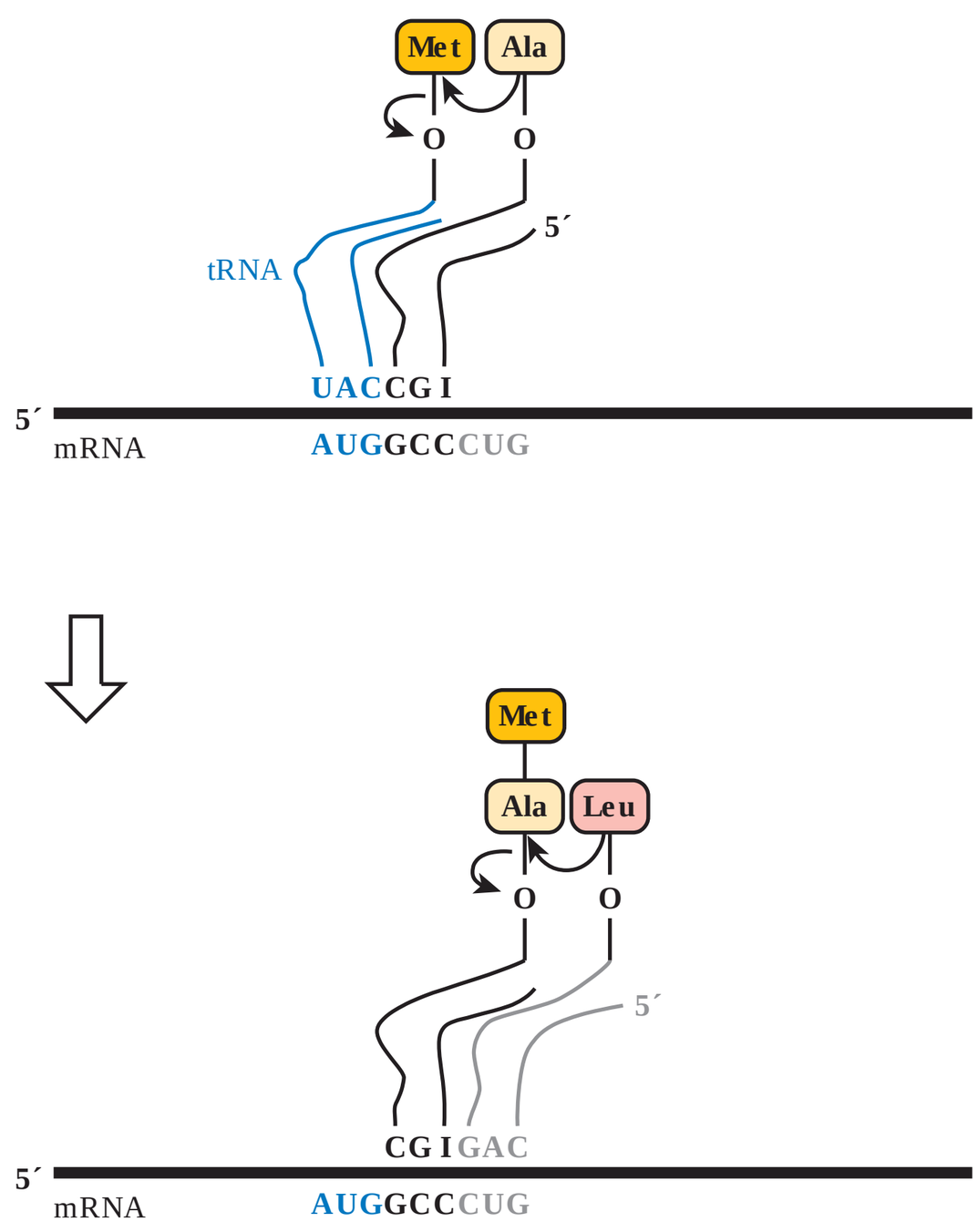

Fig. 7.3 Peptide synthesis in ribosomes. The hydroxyl group at the 3′ end of the incoming tRNA is esterified with the carboxyl group of its cognate amino acid. The free amino group of this amino acid mounts a nucleophilic attack on the carboxyl group of the preceding amino acid of the growing peptide chain.

Once the ribosome encounters a **stop** codon (**termination** codon), a **release factor** (a protein) recognizes the stop codon. Together with another release factor, it releases the peptide chain from the ribosome. With the help of a ribosome recycling factor and other proteins, the ribosome then dissociates into the small subunit and the large subunit.

The segment of mRNA between the stop codon and the poly-A tail is called the **3′ UTR**. Just like the 5′ UTR, the 3′ UTR also may contain regulatory sequences that affect the rate of mRNA translation. This is again applies to iron response elements. The 3′ UTR may also contain a sequence that leads to rapid degradation of the mRNA, which allows cells to express certain proteins for only a brief time.

mRNAs that contain **premature stop** (termination) codons may be degraded by the classic **nonsense-mediated decay** (**NMD**) pathway. When mRNA first reaches the cytosol, every exon-exon splice site is marked ~22 nucleotides upstream by exon junction complexes. If a ribosome stalls more than ~50 nucleotides upstream of an exon-exon junction, the mRNA enters NMD. In NMD, the 5′ cap and the 3′ poly-A tail are removed, and exonucleases degrade the mRNA from both ends. By contrast, if a ribosome moves smoothly along the mRNA without stalling, the exon junction complexes are removed, and the mRNA is now a bona fide template for translation. There are several other pathways that recognize abnormal mRNAs that are not discussed here. For the synthesis of many proteins, loss of abnormal mRNA, which may lead to haploinsufficiency, is the lesser evil compared to production of a truncated protein, which may have a dominant-negative effect (see Chapter 5). It is estimated that despite the degradation of mRNAs with premature stop codons, ~10% of all heritable diseases are caused by nonsense mutations.

Two different **methionine aminopeptidases** remove the N-terminal **Met** from most nascent proteins as they emerge from the ribosome (i.e., before the proteins fold). For these aminopeptidases to cleave the Met residue, the second amino acid of the nascent protein must not be bulky. Proteins start folding into secondary structures during translation. α-Helices are small enough to start forming in the exit tunnel of ribosomes. However, proteins can acquire a tertiary structure (see Chapter 9) only after leaving the ribosomes.

A single mRNA often contains many ribosomes that travel on it. Furthermore, the 5′ cap and the 3′ poly-A tail of an mRNA are held together by initiation factors. This is facilitates recognition of the two mRNA ends and reassembly of ribosomes on the mRNA.

Micro RNAs (**miRNAs**) are RNAs that are ~22 nucleotides long, favor degradation of mRNA, and inhibit translation. miRNAs bind to complementary segments of mRNA. Humans produce more than 1000 different miRNAs. Each miRNA can bind to several different mRNAs, in part because a miRNA does not have to match exactly the sequence of the mRNA. About half of all mRNAs are subject to regulation by a miRNA.

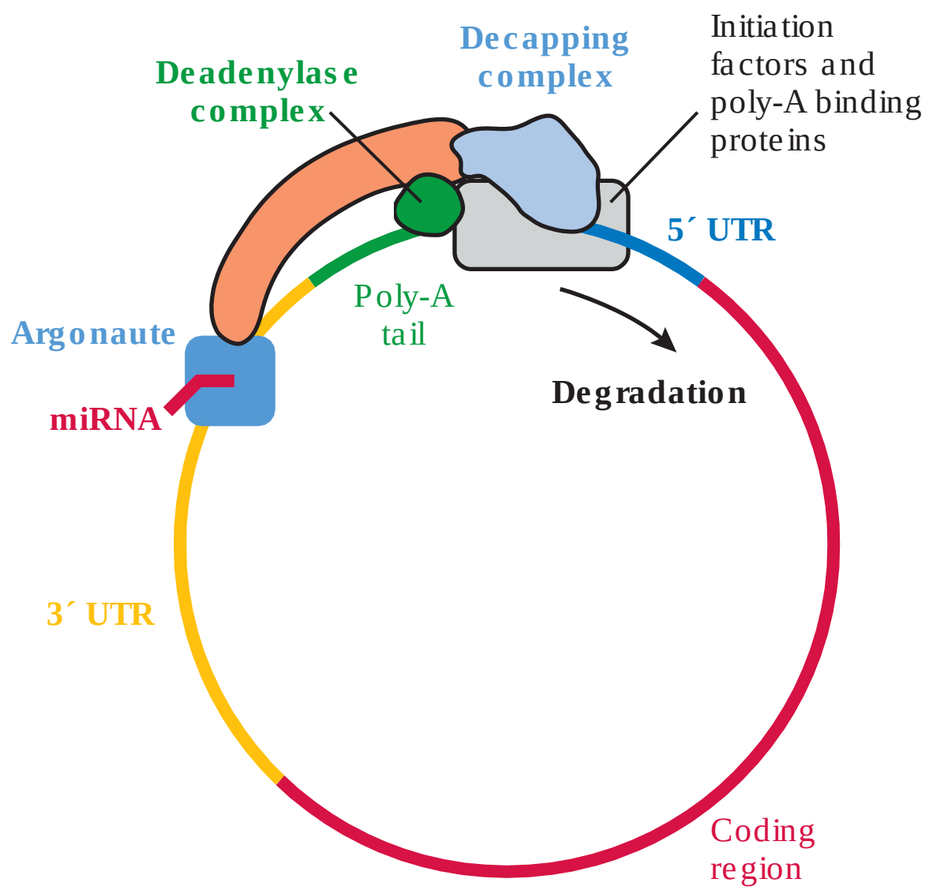

Fig. 7.4 miRNA-induced degradation of mRNA.

miRNAs combine with the protein **Argonaute** to form the core of a **miRNA-induced silencing complex** miRISC (Fig. 7.4). miRNAs reduce protein synthesis mainly by favoring degradation of mRNA. miRNA and Argonaute first bind to the 3′ UTR of an mRNA and then recruit a scaf old protein, which in turn binds enzymes that remove the poly-A tail and the 5′ cap from the mRNA. Subsequently, a 5′ exonuclease in the cytosol degrades the decapped and deadenylated mRNA. Direct inhibition of translation might occur by interference with initiation factors.

Protein synthesis slows dramatically in response to conditions of **nutrient deprivation** (especially of amino acids and glucose), viral **infection**, and various forms of cellular **stress**. T ese conditions slow translation by lowering the concentration of aminoacyl-tRNAs available to ribosomes. T e following three examples illustrate how this regulation works:

- Cells that are starved for a single **amino acid** stop protein synthesis.
- Red blood cell precursors that are actively synthesizing hemoglobin (see Chapter 16) devote almost all of their protein synthesis to the production of **globins**. If **heme** (the prosthetic group of globins) becomes scarce (e.g., due to iron deficiency), the rate of protein synthesis decreases and becomes limited by heme production.
- Many **viruses** that infect human cells produce large quantities of double-stranded **RNA**. Most human cells contain a kinase that recognizes double-stranded RNA and inactivates an initiation factor for protein synthesis. T us translation is shut down in a virus-infected cell. T is prevents the synthesis of viral proteins. Type I **interferons**, which are proteins produced endogenously in response to virus infection or given as a drug, can stimulate the same double-stranded RNA-dependent kinase. Some viruses, including influenza, herpes simplex, and human immunodeficiency virus, have evolved mechanisms for antagonizing the kinase-directed shutdown of protein synthesis.

Some mRNAs contain **CUG** codons for **Leu,** which are used to initiate protein synthesis when translation initiation via AUG codons for Met is shut down due to stress or infection. T is applies to mRNAs for major histocompatibility complex class I proteins, which present short peptides, including peptides of pathogens, to cytotoxic T-cells.

Proteins synthesized on ribosomes in the **cytosol** can be relocated to various subcellular compartments based on specific peptide sequences. For example, the presence of a **nuclear localization sequence** facilitates translocation of the protein into the nucleus; a **mitochondrial localization sequence** facilitates translocation of the protein into the mitochondria; and a **peroxisome-targeting sequence** sends the protein into peroxisomes.

T e protein-transporting **translocase of the outer membrane** (TOM) and **translocase of the inner membrane** (TIM) complexes control the import of more than 1000 proteins into mitochondria across the outer and inner membrane. T e electrochemical protein gradient of mitochondria (see Section 1.2 in Chapter 23) provides the energy for protein translocation.

About one-third of all proteins contain a **signal sequence** (leader sequence, preprosequence) and are therefore synthesized by transfer of the growing peptide chain into the **ER** (Fig. 7.5). T is group encompasses proteins that end up in the plasma membrane, in the lysosomes, or in secretory vesicles. Examples include the following: G protein–coupled receptors in the plasma membrane (see Chapter 33), acid maltase in lysosomes (see Chapter 24), and insulin in secretory vesicles (see Chapter 26).

When a growing peptide chain emerges from the ribosome and contains a signal sequence, a **signal recognition particle** (**SRP**) binds to it (Fig. 7.6). T e SRP consists of one RNA and six proteins. A part of the SRP reaches into the ribosome at the elongation factor binding site, pairs with rRNA, and temporarily halts translation. T e SRP (with the ribosome bound to it) then binds to the **SRP receptor** on the surface of the ER. T is facilitates binding of the ribosome to a channel (**translocon**) that allows the growing peptide chain to be transferred into the ER once translation resumes due to departure of the SRP.

Proteins that are synthesized via transfer of the growing peptide chain into the ER can be transferred entirely into the ER or partially inserted into the ER membrane. Proteins destined for secretion are entirely transferred into the ER, and the signal peptide is generally cleaved by **signal peptidase**. Because of the presence of internal start-transfer and stop-transfer sequences, multiple transmembrane sequences of a membrane protein can be inserted into the ER membrane. For example, G protein–coupled receptors cross the plasma membrane seven times (see Chapter 33). (In the ER membrane, the signal peptide is degraded by signal peptide peptidase.)

Ribosomes in **mitochondria** (**mitoribosomes**) dif er appreciably from ribosomes in the cytosol (and also from those in bacteria). Mitoribosomes translate only the 13 mRNAs that are encoded by mitochondrial DNA (mtDNA; see Fig. 23.8). Mitoribosomes contain only two rRNAs (both encoded by mtDNA). Although all ~80 proteins of mitoribosomes are

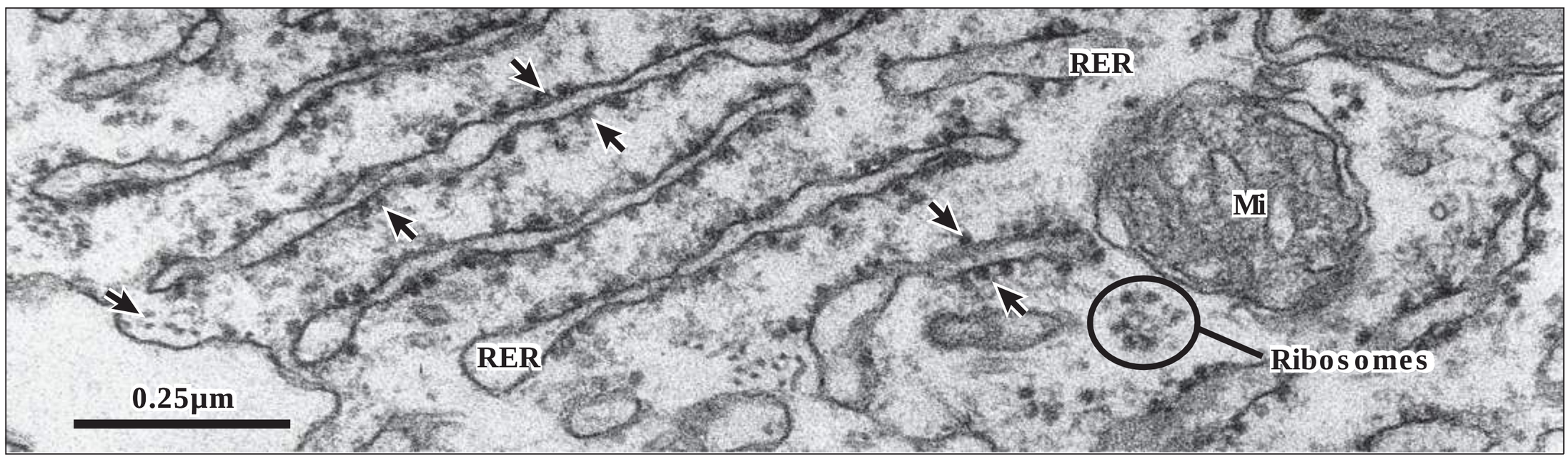

Fig. 7.5 Some ribosomes attach to the endoplasmic reticulum. Transmission electron microscopic view of part of a fibroblast. *Arrows* show ribosomes that are attached to the ER membrane. *Mi*, mitochondrion; *RER*, rough endoplasmic reticulum. The *circle* denotes free ribosomes. (Courtesy Dr. B.J. Crawford.)

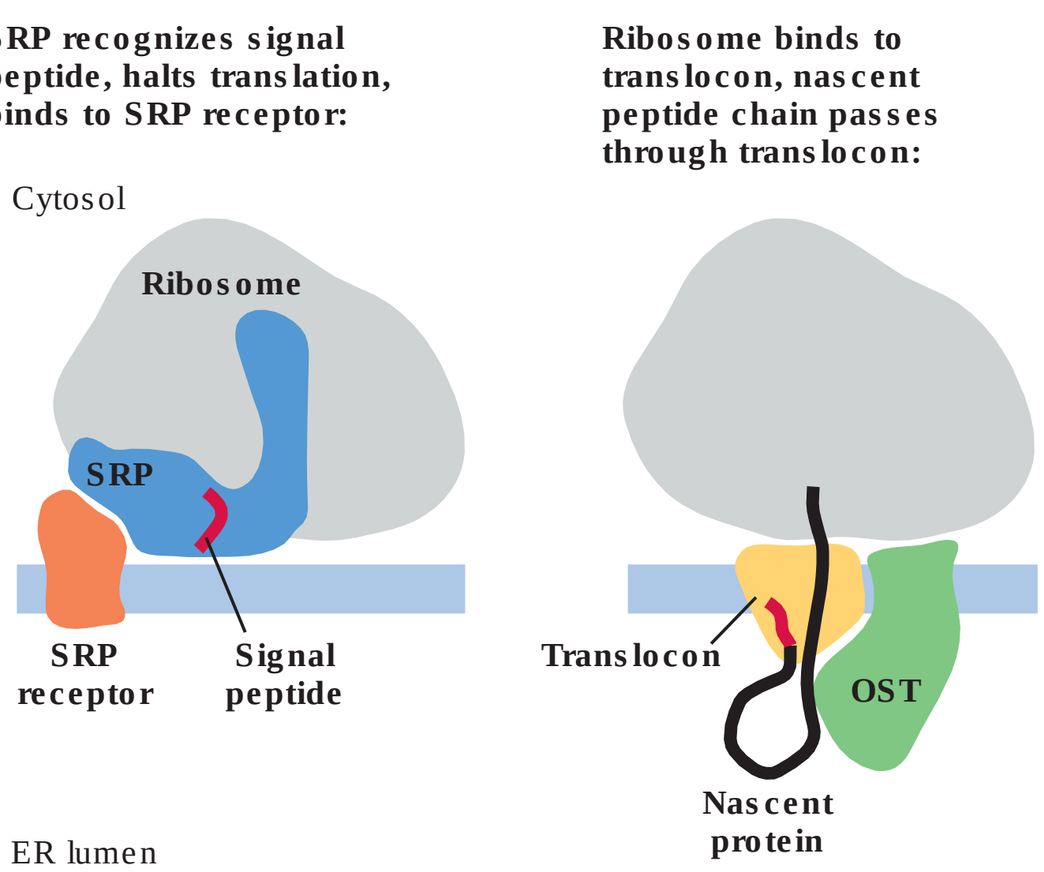

Fig. 7.6 Transfer of a growing peptide chain into the endoplasmic reticulum. *ER*, endoplasmic reticulum; *OST*, oligosaccharyltransferase; *SRP*, signal recognition particle, which adds a glycan (see Section 4.2).

encoded in the nucleus, synthesized in the cytosol, and then imported into mitochondria, only about half of these proteins are the same as in ribosomes in the cytosol.

Mitoribosomes use a **genetic code** that dif ers somewhat from the one cytosolic ribosomes use (see Table 7.1). Mitochondrial mRNAs contain no 5′ cap and essentially no 5′ UTR. T e start codon is AUG for 9 of the 13 proteins.

In mitochondria, membrane proteins can also be inserted into the inner membrane during translation. Few details are known about this process.

Translation of mRNA in **prokaryotes** dif ers from translation in the cytosol of humans in important ways. In prokaryotes, the small subunit of a ribosome binds to a conserved Shine-Dalgarno sequence, which is 5′ to the AUG start codon. T e methionine on the initiation tRNA is formylated (a formyl group, –CHO, is added; this also occurs in human mitochondria), and the formyl group is then removed while the first methionine residue is left in place. T e initiation factors, proteins in the ribosome, elongation factors, and release factors dif er between prokaryotes and humans.

A number of important classes of **antibiotics** inhibit translation by bacteria but not by human cells:

- **Aminoglycosides**, such as **streptomycin**, **gentamycin**, **tobramycin**, and **amikacin**, interfere with the decoding of mRNA and the accommodation of incoming tRNA. As a result, erroneous amino acids are inserted, and elongation of the peptide chain is inhibited. Aminoglycosides have toxic side ef ects on tubule function in the kidneys and also on hearing and equilibrium.
- **Chloramphenicol** inhibits protein chain elongation by interfering with the peptidyl transferase activity of ribosomes.
- **Macrolides** such as **erythromycin**, **telithromycin**, **azithromycin**, and **clarithromycin** interfere with peptide synthesis by blocking the exit tunnel of the nascent peptide chain from the ribosome.

4. POSTTRANSLATIONAL MODIFICATION

During and a*ft*er synthesis, proteins *f*old and undergo processing. Protein *f*olding is sometimes aided by chaperones. Proteins can be modif ed by proteolysis, by attachment o*f* carbohydrates, lipids, or isoprenes, or by redox reactions. Mis*f*olded or mutant proteins may be recognized and re*f*olded or degraded. T is quality control plays a major role in a variety o*f* heritable diseases.

4.1. General Comments

In the ER, as proteins fold, **protein disulfide isomerase** catalyzes the formation of **disulfide bonds** between cysteine residues.

Peptidylprolyl isomerase isomerizes cis and trans peptide bonds that involve **proline** (see Fig. 9.3).

Chaperones are proteins that help fold other proteins. They are found in the nucleus, cytosol, ER, mitochondria, cell surface, and extracellular space. Most chaperones are **heat shock proteins**, which were originally discovered as being expressed in increased amounts when cells are exposed to heat.

Most chaperones use ATP to facilitate folding and bind to misfolded proteins to prevent aggregation, as well as unfold and refold misfolded proteins. Chaperones often receive assistance from multiple cochaperones. Many tumors overexpress chaperones, thus favoring tumor progression and metastasis.

Chaperones recognize their targets by various means, including certain short peptide consensus sequences and surface hydrophobicity (hydrophobic residues are supposed to be buried; see Chapter 9).

Glycosylation (see below) can guide protein folding by preventing access to certain conformations.

4.2. Glycosylation

N-linked glycosylation occurs inside the ER on select asparagine residues of nascent proteins during translation. Oligosaccharyltransferase (**dolichyl-diphosphooligosaccharide-protein glycotransferase**) transfers a 14-residue preformed glycan to the nitrogen on the side chain of an **asparagine** in a consensus sequence (Fig. 7.7). The oligosacharyltransferase complex is bound to the translocon that transfers a nascent protein into the ER. For some proteins, the addition of a bulky, hydrophilic glycan influences protein folding.

The synthesis of the 14-residue glycan for N-linked glycosylation starts on the cytosolic side and ends on the luminal side of the ER (Fig. 7.8). On the outer surface of the ER, two N-acetyl glucosamine residues (activated by UDP) and five mannose residues (activated by GDP) are attached to dolichol phosphate. Dolichol is a lipid that stems from the same isoprene-producing pathway as farnesyl-PP, geranylgeranyl-PP, ubiquinone, and cholesterol (see Fig. 29.4). The resulting glycan is flipped to the luminal side of the ER membrane. There, four more mannose and three glucose residues (all activated by dolichol-P) are attached to generate the 14-residue glycan that contains three branches (all at mannose residues).

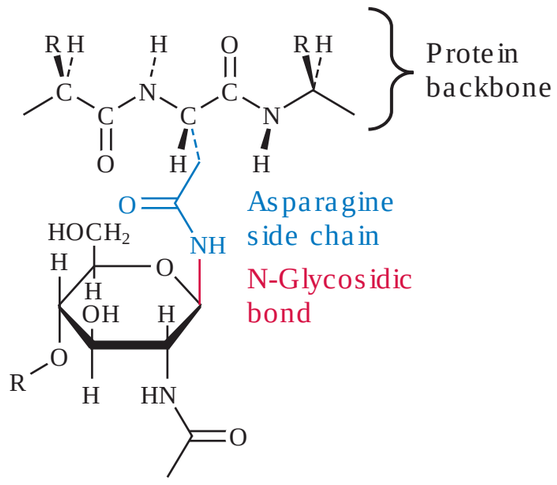

Fig. 7.7 N-glycosylation of the asparagine side chain of a protein.

In the **Golgi**, protein-linked glycans are extensively modified by a combination of trimming of glucose and mannose residues; phosphorylation, sulfation, and acetylation of existing sugar residues; and addition of new sugar residues such as N-acetylglucosamine, galactose, fructose, and neuraminic acid. The trimming of residues depends on a proper protein conformation as sensed by chaperones.

One example of a secreted protein that must be glycosylated is **erythropoietin**, which stimulates red blood cell synthesis (see Chapter 16). For the production of **recombinant** human erythropoietin (**epoetin alfa**), mammalian cells are typically engineered to both produce human erythropoietin protein and glycosylate this protein so that a pharmacologically useful half-life can be attained. **Darbepoietin** is an engineered analog of erythropoietin that contains five instead of three N-linked glycans and has a threefold longer half-life than recombinant human erythropoietin when given intravenously.

Abnormal N-linked glycosylation is observed in certain **congenital disorders of glycosylation** (**CDG**). A CDG may be due to reduced glycosylation in the ER or abnormal

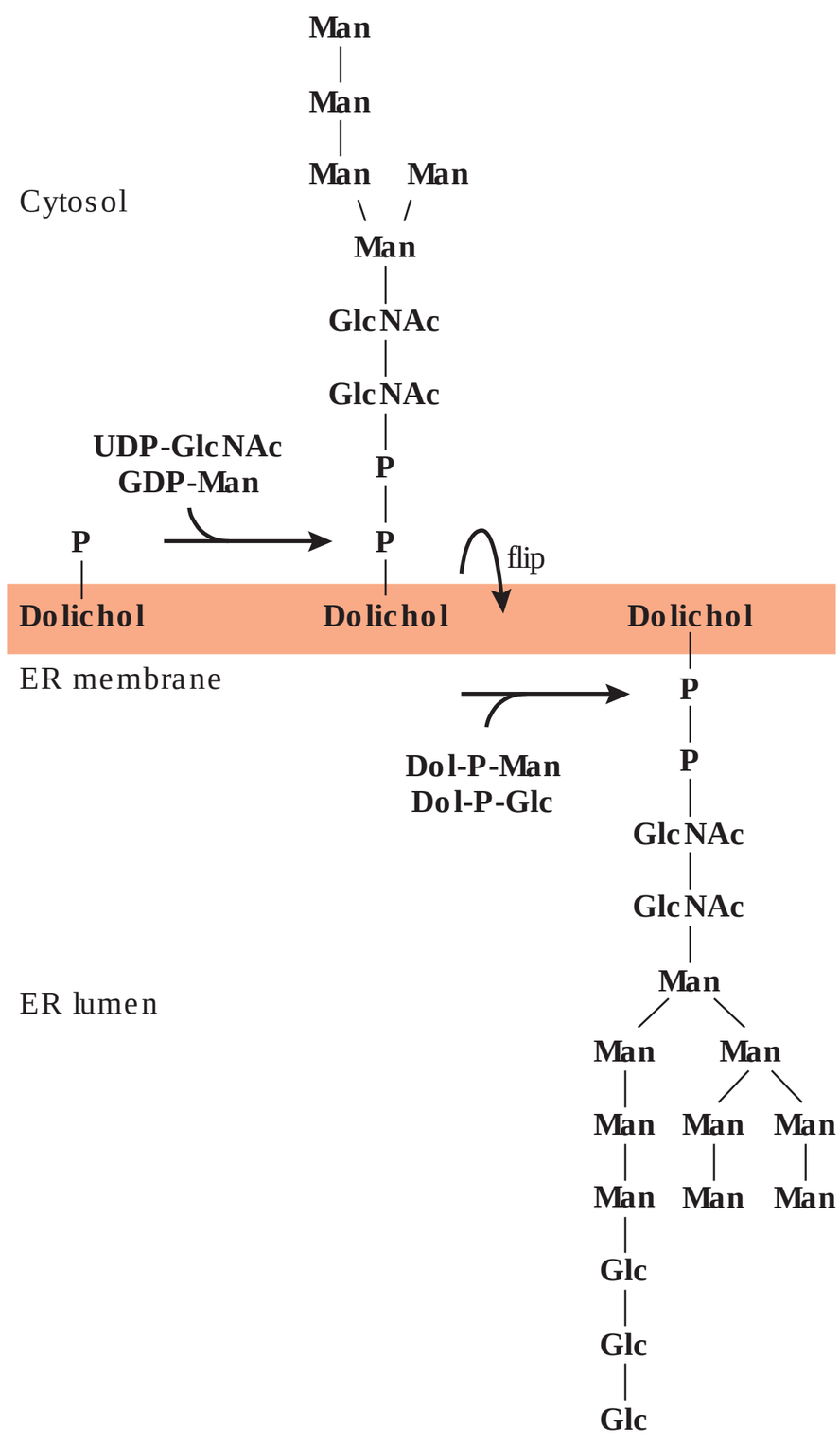

Fig. 7.8 Synthesis of a 14-residue glycan for N-linked glycosylation in the endoplasmic reticulum. *Dol*, dolichol; *Glc*, glucose; *GlcNAc*, N-acetyl-glucosamine, *Man*, mannose; *P*, phosphate.

processing in the Golgi. CDGs cause a wide variety of clinical symptoms.

At a prevalence of ~1 in 20,000, **PMM2-CDG** (formerly **CDG-Ia**) is the most prevalent CDG. It is due to homozygosity or compound heterozygosity for a defective PMM2 allele. The PMM2 gene encodes **phosphomannomutase 2**, which catalyzes the following reaction:

Mannose 6-phosphate ↔ mannose 1-phosphate

Mannose 1-phosphate is normally activated with GDP and then added to the growing dolichol-PP-glycan toward the production of a 14-residue glycan in the lumen of the ER. Affected patients show a wide spectrum of disease and age of onset. The patients generally have some intellectual disability, impaired vision, peripheral neuropathy, and cerebellar ataxia that make it difficult for them to walk independently. Diagnosis is often based on finding abnormal glycosylation of transferrin by isoelectric focusing (transferrin is a protein that carries iron in blood; see Section 4 in Chapter 15). If positive, this test is followed with a measurement of the phosphomannomutase activity in leukocytes or fibroblasts.

The only CDG for which there is currently effective treatment is **MPI-CDG** (formerly **CDG-Ib**), which is treated with supplements of mannose. MPI-CDG is due to a deficiency of mannose 6-phosphate isomerase, which catalyzes the following reaction:

Fructose 6-phosphate ↔ mannose 6-phosphate

This reaction is a prerequisite for the synthesis of GDP-mannose.

The most commonly performed laboratory test for a type I CDG (which affects N-glycosylation) is a test of the glycosylation of **transferrin**.

Known CDGs are caused by mutations in more than 50 different genes, and sequences of all exons of thousands of persons (~20% of whom turned out to be carriers of a CDG) suggest that the overall prevalence of autosomal recessively inherited CDGs is perhaps ~1 in 1,000. This is number implies that most affected patients are currently not diagnosed as having a CDG; because the field is young and some affected fetuses may not be viable, this seems possible.

O-linked glycosylation occurs in the Golgi apparatus on the –OH groups of **Ser** and **Thr** and starts with the addition of **N-acetyl-galactosamine** (**GalNAc**), which can be extended with N-acetyl-glucosamine, N-acetyl-neuraminic acid, or galactose, and subsequently with these and other sugars.

Proteoglycans and mucins are examples of proteins that undergo O-linked glycosylation. **Proteoglycans** are abundant in the extracellular matrix, where they absorb and distribute compressive forces, store growth factors, and bind to coagulation factors (see Section 2 in Chapter 13). **Mucins**, which are produced by many types of epithelial cells and are often secreted to give rise to mucus (e.g., in the digestive tract and in the airways), contain an abundance of Ser and Thr residues that can undergo O-linked glycosylation.

O-linked glycosylation is much less common than N-linked glycosylation, and the components of O-linked glycosylation have greater redundancy than the components of N-linked glycosylation. Accordingly, disorders of O-linked glycosylation are less common than disorders of N-linked glycosylation.

Disorders of O-linked glycosylation are typically tissue or organ specific. An example of such a disorder is **paroxysmal nocturnal hemoglobinuria** (see Section 1.5 in Chapter 11).

4.3. Acylation With Fatty Acids and Prenylation

The addition of a fatty acid to a protein can influence the association of the protein with a membrane or other protein and thus frequently plays a role in signaling. Fatty acylation of the –SH group of cysteine is reversible, whereas fatty acylation of the $-NH_3^+$ group of an N-terminal amino acid is irreversible.

A protein acylated with a single fatty acid has an increased affinity for membranes, but it resides in a membrane for only minutes. A second interaction in the form of an additional fatty acid, a prenyl group (see below), positively charged amino acids, or hydrophobic amino acids is generally needed to increase the residence time in membranes to a time scale of hours.

Palmitoylation, the addition of a 16-carbon fatty acid, occurs mostly on the –SH group of the side chain of cysteine, forming a thioester bond that can be cleaved again. Addition of palmitate by more than 20 different palmitoyltransferases occurs predominantly in the Golgi, whereas removal of palmitate by acyl protein thioesterases occurs throughout a cell, thereby affecting membrane association and traffic between membranes. Several palmitoyltransferases in the ER and Golgi as well as several acyl protein thioesterases at the plasma membrane can catalyze the addition and removal of palmitate to hundreds of different proteins.

Myristoylation, the addition of a 14-carbon fatty acid, occurs mostly on N-terminal glycine and is therefore irreversible. Whereas almost all newly synthesized proteins contain an N-terminal methionine, a methionine aminopeptidase commonly removes this residue, such that a glycine in second position may now be the N-terminal amino acid. Because of the substrate specificity of the myristoyl-CoA:protein N-myristoyl transferases, only some of the proteins that have an N-terminal Gly are myristoylated. During apoptosis, caspases (which are proteases) cleave proteins and thereby generate a fragment that often contains an N-terminal Gly residue, which may then be myristoylated.

On the cytosolic face of the ER, proteins can be irreversibly prenylated with **farnesyl pyrophosphate** or **geranylgeranyl pyrophosphate**. The synthesis of farnesyl pyrophosphate and geranylgeranyl-phosphate is shown in Fig. 29.4. Prenyl anchors are shown in Fig. 11.9. Prenylation occurs on the side chain of a cysteine residue within a consensus sequence near the C-terminus of a protein. The particular amino acid sequence determines whether farnesylation or geranylgeranylation occurs. Some proteins have a consensus sequence that

specifies geranylgeranylation on two closely spaced cysteine side chains. After prenylation, the C-terminal amino acids are removed, such that the prenylated cysteine residue forms the new C-terminus. T is C-terminus is then methylated, which renders it less hydrophilic. Prenylation makes a protein more hydrophobic, but for stable association with a membrane, a prenylated protein also needs to acquire a fatty-acid anchor (see above) or contain a series of positively charged amino acids that bind to the negative surface charge of a phospholipid-containing membrane. Prenylation favors a membrane-based interaction of proteins, such as in Ras protein signaling in the mitogen-activated protein kinase pathway (see Chapter 33).

4.4. Phosphorylation, Sulfation, and Nitrosylation

Protein phosphorylation, the addition of a phosphate group to the side chain of serine, threonine, or tyrosine, is a widespread means of regulating protein function. Humans have more than 500 kinases that catalyze phosphorylation and well over 100 phosphatases that dephosphorylate proteins.

For **sulfation**, protein-tyrosine sulfotransferase in the trans-Golgi network (TGN) uses 3′-phosphoadenosine-5′-phosphosulfate (PAPS; for synthesis see Fig. 36.16) to sulfate a tyrosine residue on some secreted proteins and some transmembrane proteins.

S-**Nitrosylation** by nitric oxide (NO) of cysteine residues in proteins gives rise to SNO-proteins (S-nitrosylated proteins). NO is formed by NO synthases and serves as a signaling molecule. Nitrosylation can be reversed by reduced glutathione (see Fig. 21.5) such that the original cysteine –SH group is restored.

4.5. Ubiquitylation and SUMOylation

Ubiquitylation, the conjugation of a protein with ubiquitin, a 76-amino acid polypeptide, is reversible, occurs in many different fashions, and serves a variety of roles. Humans have more than 100 deubiquitylases that can remove ubiquitin from ubiquitylated proteins. **Monoubiquitylation** plays a role in signaling, such as in the coordination of DNA repair or in silencing gene expression through histone modification. **Polyubiquitylation** via Lys-48 of ubiquitin is a signal for degradation of a protein in proteasomes (see Fig. 35.1 and Section 1.2 in Chapter 35). E3 ubiquitin-protein ligases (of which there are more than 600) play a crucial role in binding to proteins and initiating ubiquitylation. Misfolded proteins may be polyubiquitylated because they display excessive hydrophobicity or a normally hidden sequence that is recognized as a signal for degradation. Parkin is an E3 enzyme that plays a major role in protein quality control in mitochondria, as well as in the removal of mitochondria by autophagy. Mutant parkin gives rise to a form of **hereditary Parkinson disease** (see Section 8.5 in Chapter 9).

Conjugation of proteins with small ubiquitin-like modifier (**SUMO)** proteins plays a role in signaling (but not in protein degradation). Humans make three (possibly four) physiologically relevant SUMO proteins. At ~100 amino acids, SUMO proteins are slightly larger than ubiquitin. T e set of enzyme activities required for SUMOylation resembles the set required for ubiquitylation. However, there are many fewer enzymes that play a role in selecting proteins for SUMOylation than those for ubiquitylation, in part because SUMOylation occurs on lysine side chains in a consensus sequence.

SUMO plays a role in chromatin organization, transcription, DNA repair, and the production of ribosomes. SUMOylation can prevent the formation of dimers or multimers by steric hindrance, or it can promote complex formation, whereby it is usually helped by proteins that contain a SUMO-interacting motif. T ere are many SUMO-specific hydrolases that can deSUMOylate a protein.

5. PROTEIN SORTING AND QUALITY CONTROL

About one-third o*f* all proteins are translated into the ER and end up in the membrane or lumen o*f* the ER. Chaperone proteins recognize abnormally *f*olded proteins *f*oster re*f*olding and guide de*f*ective proteins to degradation. Coated vesicles transport proteins *f*rom the ER to the Golgi. At the trans end o*f* the Golgi, proteins are sorted according to destination, such as lysosomes, secretory vesicles, or plasma membrane.

Generally, vesicles coated with clathrin, coat protein I (COPI), or coat protein II (COPII) transport proteins (and lipids) between subcellular compartments (Fig. 7.9). Coat proteins, along with cargo adaptor proteins, bind to the cytosolic face of a membrane, bend the membrane, bind cargo, and give rise to a vesicle (Fig. 7.10). After budding of , the vesicle is uncoated; that is, the coat proteins and the cargo adaptor proteins are removed (they are reused). Depending on the particular cargo adaptor proteins on the surface of vesicles (or cytosolic proteins that bind to cargo adaptors), the uncoated vesicles fuse with a target membrane with the help of SNARE complexes (see Fig. 9.7).

In the ER, newly synthesized, folded proteins enter budding coated vesicles at specific ER exit sites. Most proteins leave the ER in COPII-coated vesicles and then enter the Golgi at its cis face, where they start their migration through the Golgi.

Protein sorting takes place in the trans-Golgi network (TGN). About one-third of all newly synthesized proteins pass through the TGN. On the cytosolic side of the TGN, cargo adapters recognize amino acid sequences of transmembrane proteins. Such cargo adapters also recognize cargo receptors,

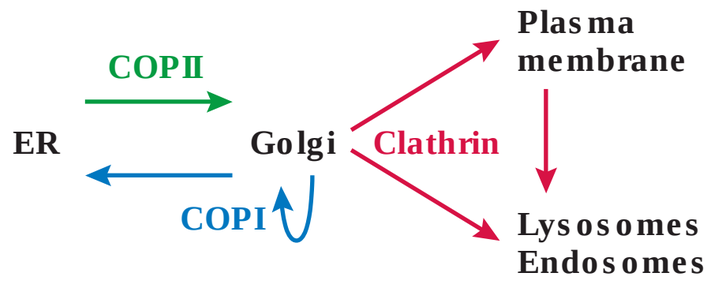

Fig. 7.9 Transport of proteins among intracellular compartments by coated vesicles. *COP*, coat protein; *ER*, endoplasmic reticulum.

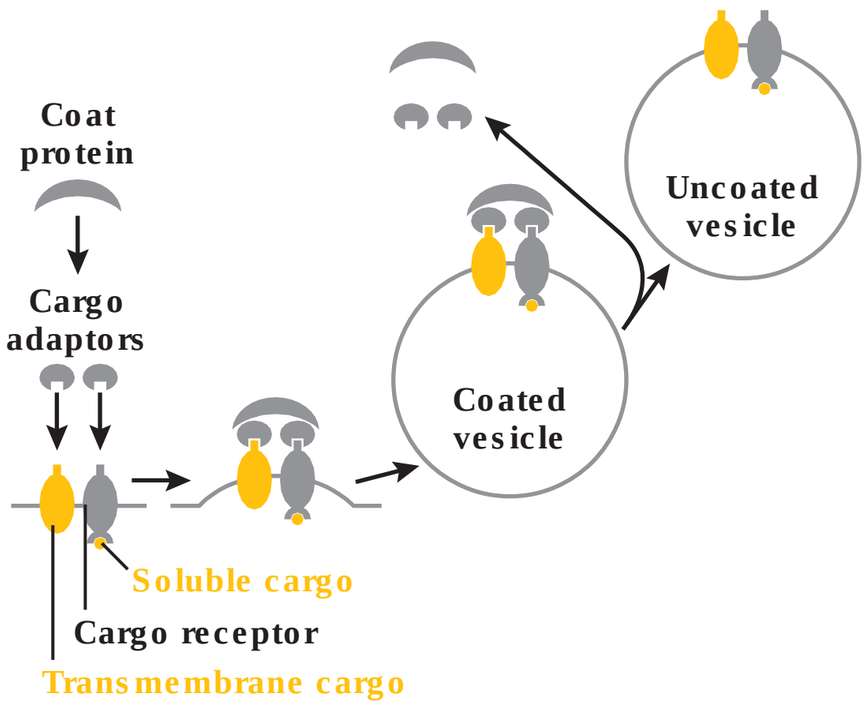

Fig. 7.10 Basic principle of vesicle formation and protein sorting in the endoplasmic reticulum and Golgi.

which are proteins that in turn bind soluble proteins inside the TGN.

At the TGN, proteins can be sorted to end up in endosomes, lysosomes, secretory granules, or in the plasma membrane (in polarized epithelial cells, some proteins can be sorted so that they selectively end up in the apical or basolateral plasma membrane). Cargo adaptor proteins bind to transmembrane proteins that contain a matching localization sequence. At their destination, vesicles fuse with the new membrane and empty their soluble contents into the target compartment.

T e cargo adaptor proteins can also bind cargo receptor proteins that in turn bind soluble cargo, such as **mannose 6-phosphate**–labeled enzymes destined for **lysosomes**. In the ER, most nascent hydrolases destined for lysosomes are conjugated with a 14-residue glycan. In the Golgi, some mannose residues in the 14-residue glycan are phosphorylated to form mannose 6-phosphate (this particular enzyme is missing in the very rare disease **I-cell disease**). Uncovering enzyme (N-acetylglucosamine-1-phosphodiester α-N-acetylglucosaminidase) removes terminal sugar residues to expose mannose 6-phosphate. In the TGN membrane, the mannose 6-phosphate receptor, a cargo receptor, binds an enzyme destined for lysosomes by virtue of its mannose 6-phosphate. On the cytosolic face of the TGN membrane, a cargo adapter binds to this mannose 6-phosphate receptor, resulting in the transport of the enzyme to lysosomes.

Posttranslational quality control takes place at several levels. In the ER, where protein folding predominantly takes place, chaperones are the main sensors of inappropriate protein folding, and they are involved in directing proteins toward refolding or degradation. For degradation, proteins are exported to the cytosol. T ere, protein monomers can be conjugated with ubiquitin and degraded inside proteasomes (see Fig. 35.1 and Section 1.2 in Chapter 35), or they can be recognized by a chaperone by virtue of their KFERQ motif (the sequence Lys-Phe-Glu-Arg-Gln, which is present in ~30% of all proteins in the cytosol) and be delivered to a lysosome for degradation. Protein aggregates in the cytosol are collected in a central location and can enter macroautophagy, a process by which the aggregates are enveloped by an autophagosome that then fuses with a lysosome for protein degradation (macroautophagy also envelopes defective organelles).

Improperly folded, damaged, or defective proteins in the Golgi and in the plasma membrane are preferentially delivered to lysosomes for degradation.

SUMMARY

- tRNAs can read multiple codons thanks to wobble base pairing at the third position of a codon.
- Silent mutations change the codon but not the amino acid residue, nonsense mutations create a stop codon, and missense mutations encode a dif erent amino acid.
- Frameshift mutations change the reading frame of part of an mRNA.
- T e most common start codon is AUG in an appropriate sequence context. AUG codes for Met, which is removed soon after protein synthesis.
- During the first round of translation, mRNAs with premature stop codons are degraded by nonsense-mediated decay (NMD).
- Translation in mitochondria dif ers from translation in the cytosol with regard to ribosome composition, tRNAs, and genetic code.
- T ere are more than 500 tRNAs that are encoded in the DNA of the nucleus. During processing, the tRNAs are extended by CCA, and many nucleotides are modified. Aminoacyl-tRNA synthetases charge these tRNAs with their cognate amino acid.
- Pseudomonic acid (mupirocin), which is used to treat methicillin-resistant Staphylococcus aureus, inhibits isoleucyl-tRNA synthetase in bacteria.
- miRNAs bind to mRNAs and thereby impair translation directly or induce mRNA degradation.
- Stress, infection, and nutrient deprivation inhibit translation and may lead to use of alternate start codons.
- Aminoglycosides, chloramphenicol, and macrolides are used clinically to impair translation in bacteria.
- Proteins that are destined for the cytosol, nucleus, mitochondria, or peroxisomes are synthesized by ribosomes in the cytosol. If needed, these proteins contain organelle-specific localization sequences.
- Proteins that are destined for the endoplasmic reticulum (ER), Golgi, secretory vesicles, plasma membrane, or lysosomes have a signal sequence. Signal recognition particles (SRPs) recognize the signal sequence on a nascent protein, bind to the SRP receptor, and set up the ribosome on the translocon to move the growing peptide chain into the ER.
- In the ER, some nascent proteins are glycosylated with a 14-residue glycan at the amino group of the side chain of an asparagine. Subsequently, in the Golgi, the glycan is often greatly modified. T e Golgi can also glycosylate proteins on serine and threonine residues. Congenital disorders of glycosylation (CDGs) are a class of disorders that

can be due to a lack of a glycan addition in the ER or faulty processing in the Golgi.

- Chaperones can help fold proteins, bind to aggregated proteins, unfold and refold misfolded proteins, and direct proteins to degradation in proteasomes or lysosomes.
- Acylation with palmitate or myristate, or prenylation with a farnesyl or geranylgeranyl group increases the likelihood that a protein resides in a membrane. Long-term association with a membrane requires two or more such modifications or additional positively charged or hydrophobic amino acid sequences.
- Ubiquitylation of proteins can lead to protein degradation or play a role in a variety of processes, such as DNA repair and signaling. SUMOylation plays a role in signaling, often by creating steric hindrance or by inducing binding to proteins that contain SUMO-interacting motifs.
- Coated vesicles transport proteins between the ER, Golgi, plasma membrane, and lysosomes.

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Review Questions

1. Which of the following most likely occurs when a ribosome encounters a UAG codon of an mRNA that derives from a normal, nonpathogenic allele?

   A. Eukaryotic release factor (a protein) binds to UAG
   B. Met is incorporated into the nascent peptide
   C. The ribosome binds to the SRP
   D. The ribosome stalls and the mRNA is degraded by NMD

2. The coding strand of a particular gene contains a codon that reads 5′-CAT. Based on the genetic code shown in Table 7.1, which amino acid is added to the nascent protein chain as a consequence of the aforementioned sequence?

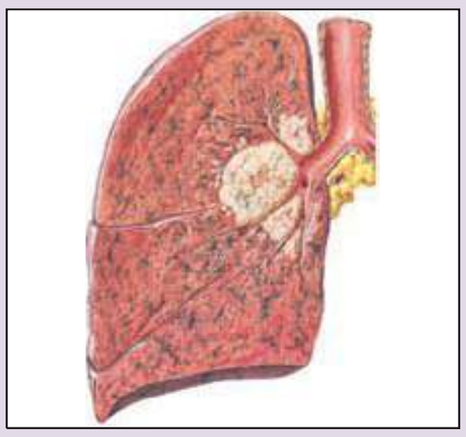
   A. Gln
   B. His
   C. Met
   D. Tyr
   E. Val

3. The unprocessed transcript of a gene contains the following number of nucleotides:

   3′-UTR: 45
   5′-UTR: 15
   Exon 1: 120
   Exon 2: 120
   Intron 1: 240

   How many amino acid residues will the protein product of translation have?

   A. 60
   B. 80
   C. 100
   D. 180
   E. 300



Chapter 8 Cell Cycle and Cancer

SYNOPSIS

- The activity of cyclin-dependent kinases (CDKs) is a key regulator of the cell cycle. Cyclins increase CDK activity, whereas CDK inhibitor proteins decrease it.
- From a quiescent state, cells enter the cell cycle in response to growth factors. Growth factor stimulation activates CDKs, which in turn inhibit the activity of a protein called retinoblastoma (RB). RB then no longer binds to E2F transcription factors. Free E2Fs alter the expression of proteins to favor progress in the cell cycle.
- In the presence of DNA damage, the p53 pathway halts the cell cycle before the DNA is replicated.
- In tumor cells, the RB protein, which prevents cell cycle progression in normal cells, and the p53 protein, which arrests the cell cycle in response to DNA damage, are often nonfunctional.
- The WNT/β-catenin pathway plays a role in development and activates transcription of certain genes, such as the gene for cyclin D and MYC, a transcription factor. In tumors, this pathway is often overly active.
- Over time, all cells acquire mutations. A small fraction of these mutations is tumorigenic.
- Old age, a long history of smoking, obesity, and excessive alcohol consumption are major risk factors for cancer.
- Genes that drive the formation of tumors are divided into oncogenes and tumor suppressor genes. A single allele of an oncogene is sufficient to drive tumorigenesis, but both alleles of a tumor suppressor gene need to be nonfunctional to permit tumorigenesis. A typical tumor cell contains one oncogene and has lost the function of several tumor suppressors.
- Patients with an inherited cancer syndrome are at an increased risk of neoplasia at an unusually early age. Hereditary cancer syndromes are typically due to heterozygosity for a pathogenic tumor suppressor allele. Some somatic cells then acquire a genetic alteration that abolishes the function of the remaining, previously normal allele.
- About half of the cases of familial breast and ovarian cancer syndromes are due to inheritance of a mutation in the BRCA gene, which encodes a protein that plays a role in DNA repair. Similarly, about half of the cases of familial melanoma are due to an inherited mutation in the CDKN2A gene, which encodes an inhibitor of the cell cycle. Most cases of hereditary colon cancer are due to Lynch syndrome, which is caused by a DNA mismatch repair deficiency. A small fraction of hereditary colon cancer is due to familial adenomatous polyposis (FAP), which is caused by a mutation in the APC gene.
- Pharmacological treatment of metastatic cancer often involves drugs that induce DNA crosslinks, as well as drugs that inhibit deoxythymidine monophosphate synthesis, topoisomerases, or the rearrangement of microtubules before cell division.
- For the treatment of certain forms of breast cancer, lung cancer, colorectal cancer, and melanoma, there are several inhibitors of kinases in growth-promoting signaling pathways.
- Patients with prostate cancer are generally given androgen deprivation therapy, regardless of the genetic makeup of tumor cells.
- Tumor cells use considerably more glucose than normal cells. This makes it possible to locate metastases after radioactive fluorodeoxyglucose has been infused into a patient.

LEARNING OBJECTIVES

For mastery of this topic, you should be able to do the following:

- Compare and contrast the RB and p53 pathways.
- Explain how DNA damage in the G1 phase normally leads to cell cycle arrest and possibly apoptosis.
- Compare and contrast an oncoprotein and a tumor suppressor.
- Describe the genetic makeup of a typical tumor.
- Describe lifestyle choices that can help patients minimize their cancer risk.
- Compare and contrast the major genetic causes of the hereditary breast and ovarian cancer syndrome, FAP, Lynch syndrome, and familial melanoma.
- Use patient history, clinical findings, and lab tests to determine whether a patient with colorectal cancer has sporadic cancer, FAP, or Lynch syndrome.
- Given an abnormality in a growth-promoting signaling pathway in a breast carcinoma, lung carcinoma, colorectal cancer, or melanoma, list drugs that target these signaling pathways and can potentially be used for treatment.

1. CELL CYCLE AND ITS REGULATION

The cell cycle consists of phases G1, S (for DNA synthesis), G2, and M (for mitosis). Cyclin activation of cyclin-dependent kinases (CDKs) is essential to moving cells through the cell cycle. When growth factors stimulate quiescent cells to enter the G1 phase, cyclin-activated CDKs phosphorylate the retinoblastoma (RB) protein, which then no longer binds to E2F transcription factors. E2Fs alter transcription in cells to fit the needs of the cell cycle—for instance, the needs of DNA replication. If the protein p53 receives information about DNA damage, it prevents entry of the cell into S phase, and it may even induce apoptosis (self-destruction of the cell). In apoptosis, DNA and many cellular proteins are degraded in a regulated fashion.

1.1. Cell Cycle and the Retinoblastoma Pathway

The cell cycle is commonly divided into the following **phases**: **G0** (quiescence), **G1** (gap 1), **S** (DNA synthesis), **G2** (gap 2), and **M** (mitosis). In adults, most cells are in a quiescent state (Fig. 8.1).

During the G0 phase, **RB** binds to **E2F** transcription factors (Fig. 8.2). E2Fs bind to promoter elements upstream of

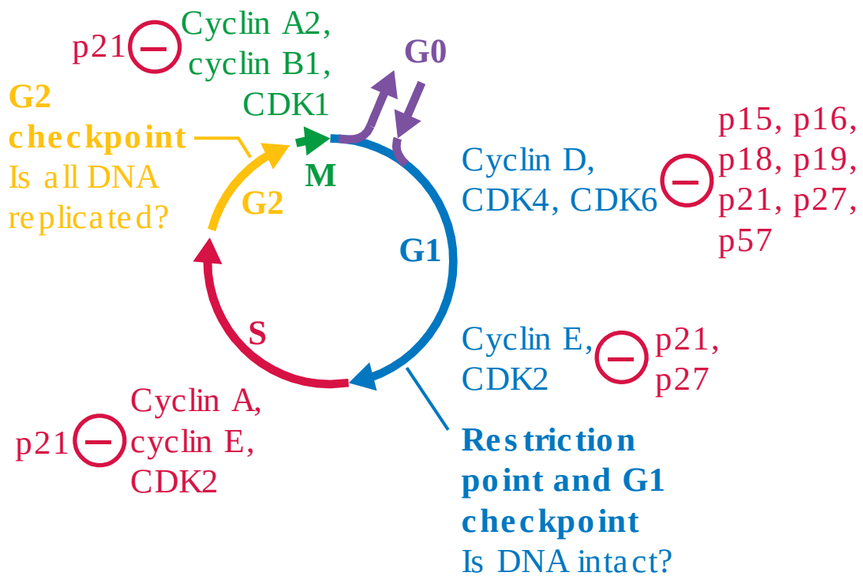


Fig. 8.1 **Cell cycle and its phase-specific kinase activities.**

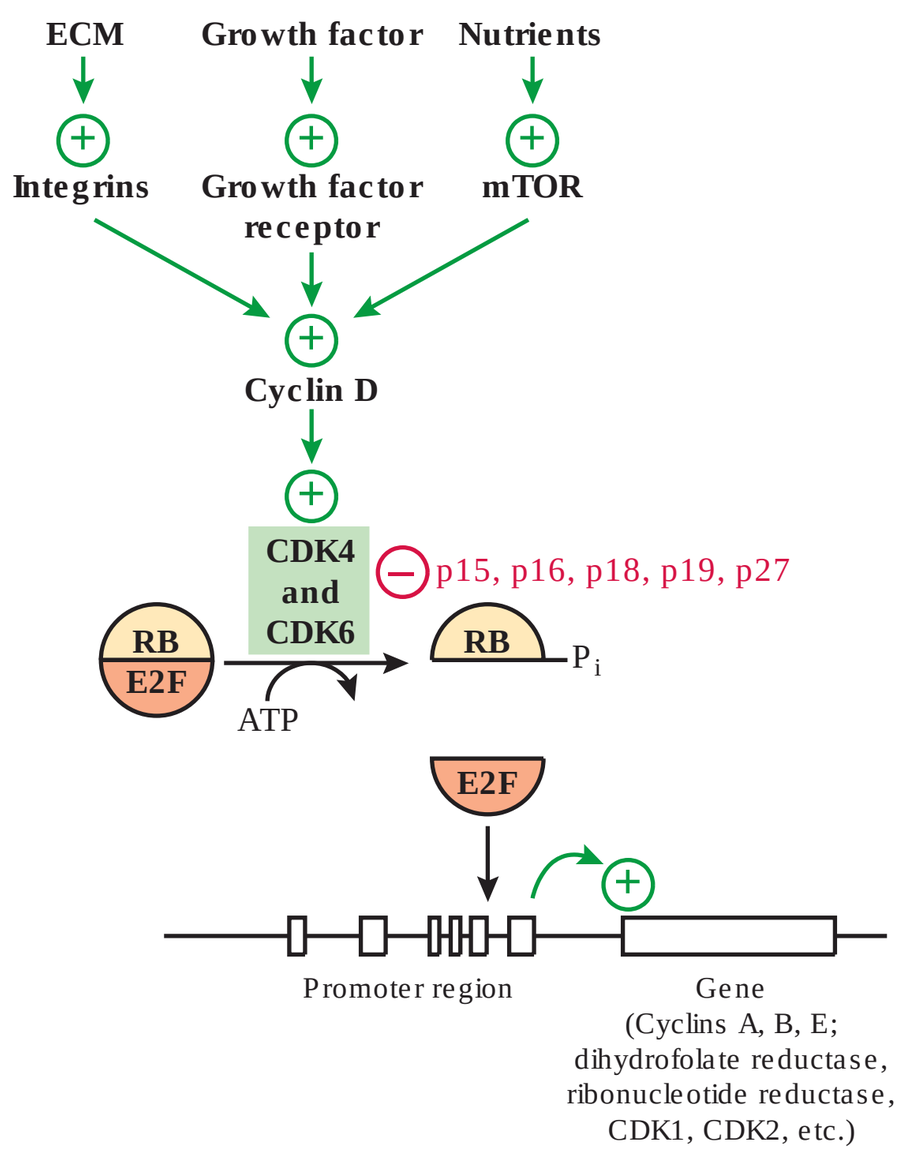


Fig. 8.2 **Phosphorylation of the retinoblastoma (RB) protein by cyclin-dependent kinases (CDKs) releases the transcription factor E2F.**

genes (see Chapter 6). Binding of E2Fs to RB prevents the transcription of genes, the products of which play a role in cell proliferation.

To leave the G0 phase and enter into the G1 phase, cells need to sense a sufficiently high concentration of extracellular **growth factors** (Fig. 8.3); epithelial cells also need to sense **adhesion** to the extracellular matrix (see Fig. 8.2). Examples of growth factors are insulin, insulin-like growth factor 1, epidermal growth factor (EGF), fibroblast growth factor, transforming growth factors α and β (see Chapter 13), and erythropoietin (see Section 1.3 in Chapter 16). The receptors of these growth factors signal to the cytosol and the nucleus of cells (see Fig. 8.3). **Integrins** are membrane proteins that participate in linking actin filaments and intermediate filaments (part of the cytoskeleton) inside cells to the

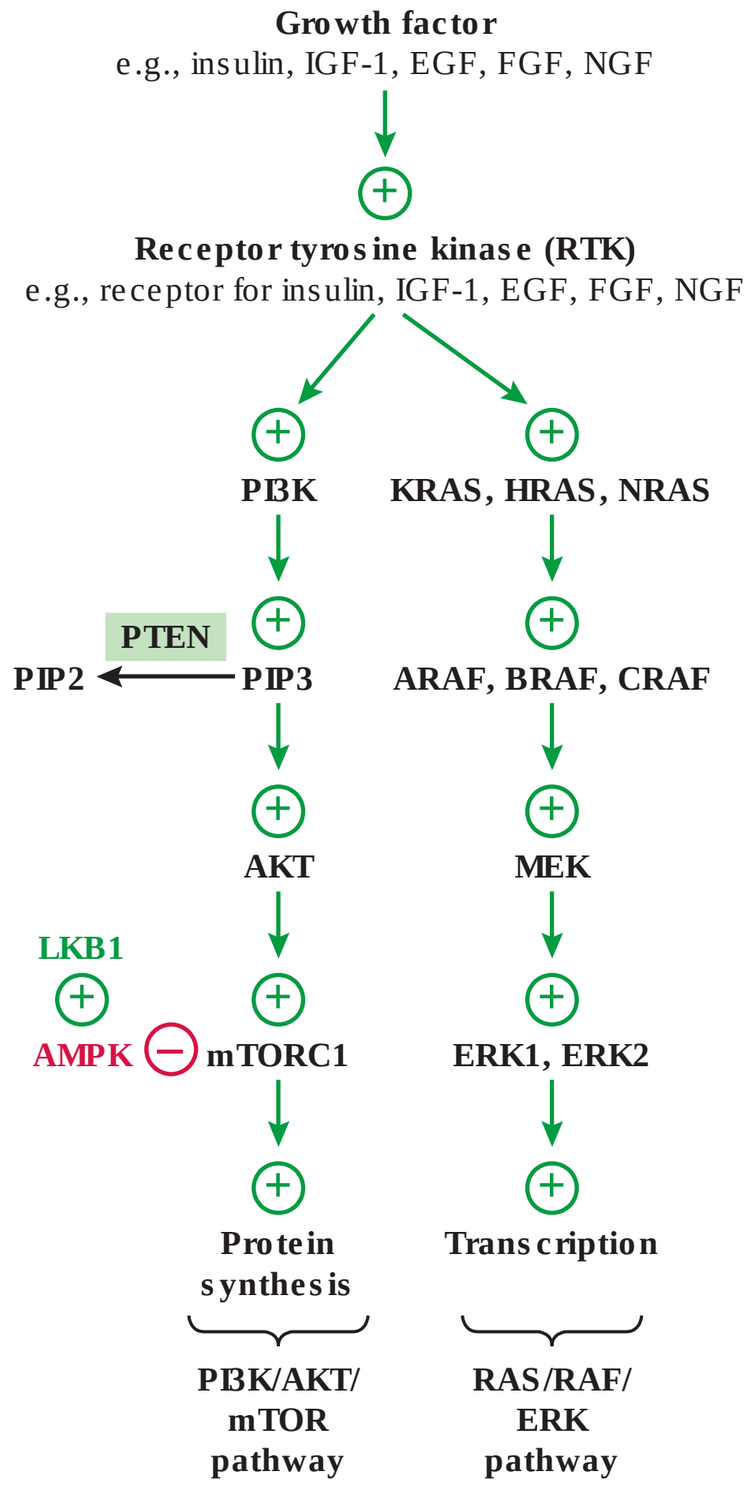


Fig. 8.3 **Growth factor signaling.** IGF-1, insulin-like growth factor 1; EGF, epidermal growth factor; FGF, fibroblast growth factor; NGF, nerve growth factor.

extracellular matrix. Integrins can accomplish this link in response to intracellular signals, and they can also signal to the inside of a cell that attachment to the extracellular matrix has taken place.

Growth factor receptors that are tyrosine kinases signal via both the **PI3K/AKT/mTOR pathway** and via the **RAS/RAF/ERK pathway** (**MAPK pathway**). In the PI3K/AKT/mTOR pathway, phosphatidylinositol (3,4,5)-trisphosphate (PIP3) is a phospholipid in the plasma membrane that activates the kinase AKT. The phosphatase **PTEN** dephosphorylates PIP3 to PIP2, thereby inhibiting signaling in the PI3K/AKT/mTOR pathway.

An acquired loss of one or two alleles of PTEN is frequently seen in sporadic tumors, and an inherited loss of one allele for PTEN gives rise to **Cowden** syndrome, a heritable cancer syndrome associated with an increased likelihood of neoplasms in the thyroid gland, breast, endometrium, kidneys, colon, and rectum.

Adenosine monophosphate–activated protein kinase (AMPK) phosphorylates and thereby inhibits mTORC1 when energy production is impaired (see Fig. 8.3). LKB1 (encoded by the *STK11* gene) is a protein kinase that activates AMPK.

Peutz-Jeghers syndrome is most often caused by loss-of-function mutations in *STK11*, the gene that encodes LKB1 (see Fig. 8.3). Affected persons are at an increased risk of a variety of tumors, such as in the gastrointestinal system and breasts.

Progression through the cell cycle is promoted by **CDK**s, which in turn are activated by **cyclins** (see Figs. 8.1 and 8.2). The cyclins are present only during certain parts of the cell cycle, whereas the amount of CDK protein is much less variable. The major CDKs are CDK1, CDK2, CDK4, and CDK6. The major cyclins are A, B, D, and E. Cyclins are often overexpressed in tumor cells.

Stimuli from growth factors and cell adhesion lead to an increased production of **cyclin D**, which activates **CDK4** and **CDK6** (see Figs. 8.1 to 8.3). CDK4 and CDK6 in turn phosphorylate the **RB protein**, which then releases **E2F** transcription factors. Some E2Fs bound to promoter elements increase transcription, whereas others decrease it. As a result, the cell's transcription program is modified to fit the needs of the cell cycle. E2F leads to an increased transcription of the genes that encode CDK2, cyclins A and E, dihydrofolate reductase, and ribonucleotide reductase (all needed for the S phase), as well as CDK1 and cyclin B (needed for mitosis). Cyclin E–activated CDK2 further increases the phosphorylation of RB. Dihydrofolate reductase is needed for the synthesis of thymidine triphosphate (see Fig. 37.6), and ribonucleotide reductase is needed for the reduction of ribonucleotides to deoxyribonucleotides (see Fig. 37.5), which are needed for DNA replication (see Chapter 3).

CDK inhibitor proteins (**CKI**s) bind to CDKs and inhibit them. CKIs inhibit the binding of cyclins to CDKs and also impair catalysis in preformed cyclin/CDK complexes. The **Ink4** inhibitors (inhibitors of CDK4) inhibit only CDK4 and CDK6. The Ink4 family consists of **p15^{Ink4b}**, **p16^{Ink4a}**, **p18^{Ink4c}**, and **p19^{Ink4d}** (in short: p15, p16, p18, and p19; see Figs. 8.1 and 8.2).

The RB pathway is important for cells to enter the G1 phase; this pathway is abnormally active in most tumor cells (discussed later in this chapter). Common tumorigenic events include overexpression of cyclin D, loss of RB function, and loss of p16^{Ink4a} function.

Children who inherited only one functional copy of the RB1 gene develop **retinoblastoma**, often bilaterally and before 2 years of age. These children have an ~30% risk of developing bone cancer in their teenage years or a soft tissue sarcoma or skin melanoma at ~30 years of age.

After reaching the **restriction point** (see Fig. 8.1), a cell goes through the cell cycle independent of growth factors. This is achieved by phosphorylation of RB, liberation of E2F, an E2F-induced increase in the amount of cyclin E, and cyclin E/CDK2-induced degradation of the CDK inhibitor p27^{Kip1}.

During the G1 phase, **origin of replication complexes** bind to **origins of replication** (see Fig. 3.3) of DNA.

1.2. The p53 Tumor Suppressor Pathway

The **checkpoint** for DNA integrity depends primarily on **p53,** a protein that senses DNA damage signals (Fig. 8.4).

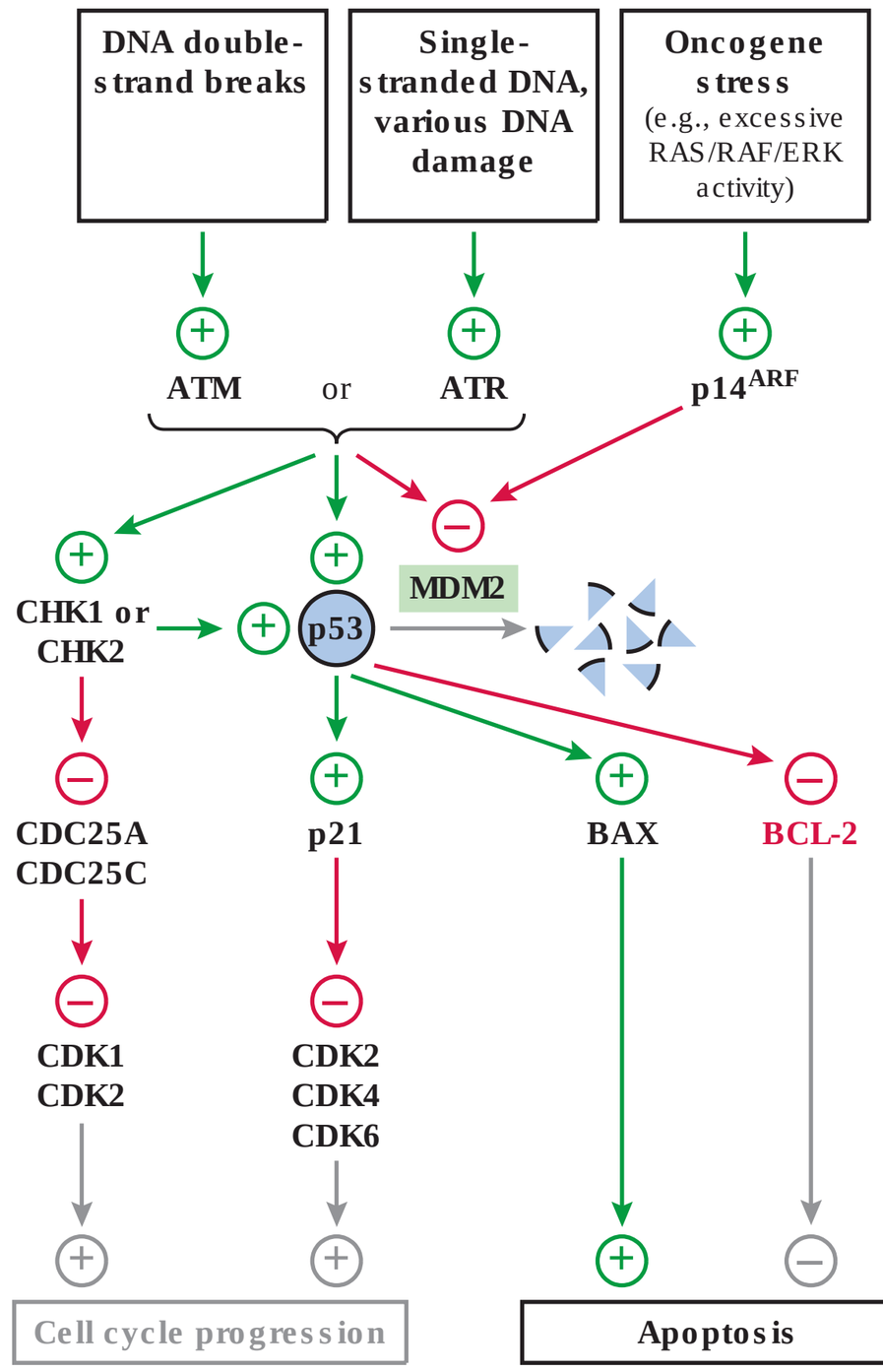


Fig. 8.4 DNA damage and the p53 pathway.

In the absence of DNA damage, the enzyme **MDM2** ubiquitylates p53 so that p53 is degraded inside proteasomes (ubiquitylation and proteasomes are described in Section 1.2 in Chapter 35).

In the presence of DNA damage, the protein kinases **ATM** and **ATR** phosphorylate p53 and MDM2; this leads to an increase in the concentration of p53 (see Fig. 8.4). p53 acts as a transcription factor and increases the concentration of the CKI **p21**. p21 then inhibits **CDK4/6** and **CDK2**, leading to cell cycle arrest (see also Fig. 8.1). The DNA damage–induced arrest of the cell cycle is redundant in that ATM also activates **checkpoint kinase 2** (**CHK2**) and ATR activates **checkpoint kinase 1** (**CHK1**; see also Section 5 in Chapter 2). These checkpoint kinases also detect DNA damage and then prevent the activation of the cyclin B/CDK1 complex that is needed for mitosis.

Besides DNA damage, **oncogenes** can also increase the activity of p53 (see Fig. 8.4). Oncogenic mutations of RAS, RAF, or ERK genes lead to increased synthesis of p14^{ARF}. p14^{ARF} then sequesters MDM2 into the nucleolus, thereby increasing the survival of p53.

In neoplasms, sensing of DNA damage or oncogenic stress by p53, followed by cell cycle arrest, is often impaired. Most tumors contain p53 mutations, and most of these mutations impair the binding of p53 to DNA. That is, the mutations make it impossible for p53 to act as a transcription factor that

produces mRNAs for proteins that arrest the cell cycle or promote apoptosis. Tumorigenic mutations that impair the p53 tumor suppressor pathway are frequently found in the genes *ATM*, CDKN2A (encoding $p14^{ARF}$ and $p16^{Ink4a}$), and MDM2.

Li-Fraumeni syndrome is an inherited cancer syndrome caused by heterozygosity for a mutant *TP53* gene, which encodes p53. In neoplasms, the second *TP53* allele is mutated or otherwise made nonfunctional. All af ected patients develop tumors before age 70 years; the first tumor is generally diagnosed at age ~30 years in women and ~40 years in men. T e tumors can occur in a number of tissues, such as bone, hematopoietic tissue, breast, brain, and adrenal glands.

Several **cell cycle checkpoints** exist: one for intact DNA in late G1, one for completeness of DNA replication in G2, and another for proper alignment of chromosomes in M phase. T e G1 checkpoint depends on both the p53 and RB pathways: p53 enhances the transcription of inhibitors of cyclin D/CDK4 and cyclin D/CDK6; less RB is phosphorylated and more RB binds to E2F; and E2F is no longer free to stimulate gene transcription that would favor progress in the cell cycle. T e restriction point is considered to be a separate process from the G1 checkpoint (independence from growth factors vs. integrity of DNA), but the two checkpoints depend on each other through the intertwining of RB and p53 pathways.

1.3. The WNT/β-Catenin Pathway

WNT signaling pathways play roles in development, establishment of cell polarity, and stem cell maintenance. WNT signaling is divided into **canonical** and noncanonical signaling, which work with and without **β-catenin**, respectively. Only the canonical pathway with β-catenin is presented here.

Humans make 19 dif erent **WNT** proteins, which are palmitoylated (see Fig. 11.9 and Section 1.5 in Chapter 11) and glycosylated (see Figs. 7.7 and 7.8 and Section 4.2 in Chapter 7). After a cell secretes a WNT protein, WNT acts either on the same cell or a nearby cell.

Humans make 10 dif erent **Frizzled** proteins, which are the receptors for WNT and are also G protein–coupled receptors.

In the absence of a WNT signal, free **β-catenin** is phosphorylated and ubiquitylated by a protein complex that includes the adenomatous polyposis coli **(APC)** protein; once ubiquitylated, APC is degraded inside proteasomes (see Fig. 35.1 and Section 1.2 in Chapter 35).

In the presence of a WNT signal, β-catenin is no longer phosphorylated, ubiquitylated, and degraded. Instead, β-catenin binds to a transcription factor of the T-cell factor/lymphoid enhancer factor (TCF/LEF) family (i.e., TCF7, TCF7L1, TCF7L2, or LEF1), which then moves into the nucleus and increases the transcription of certain genes that encode proteins that favor cell proliferation, such as the gene for cyclin D1.

β-Catenin also plays a role in connecting **actin filaments** of neighboring cells via adherens junctions (Fig. 8.5). Actin filaments are part of the cytoskeleton and help determine and

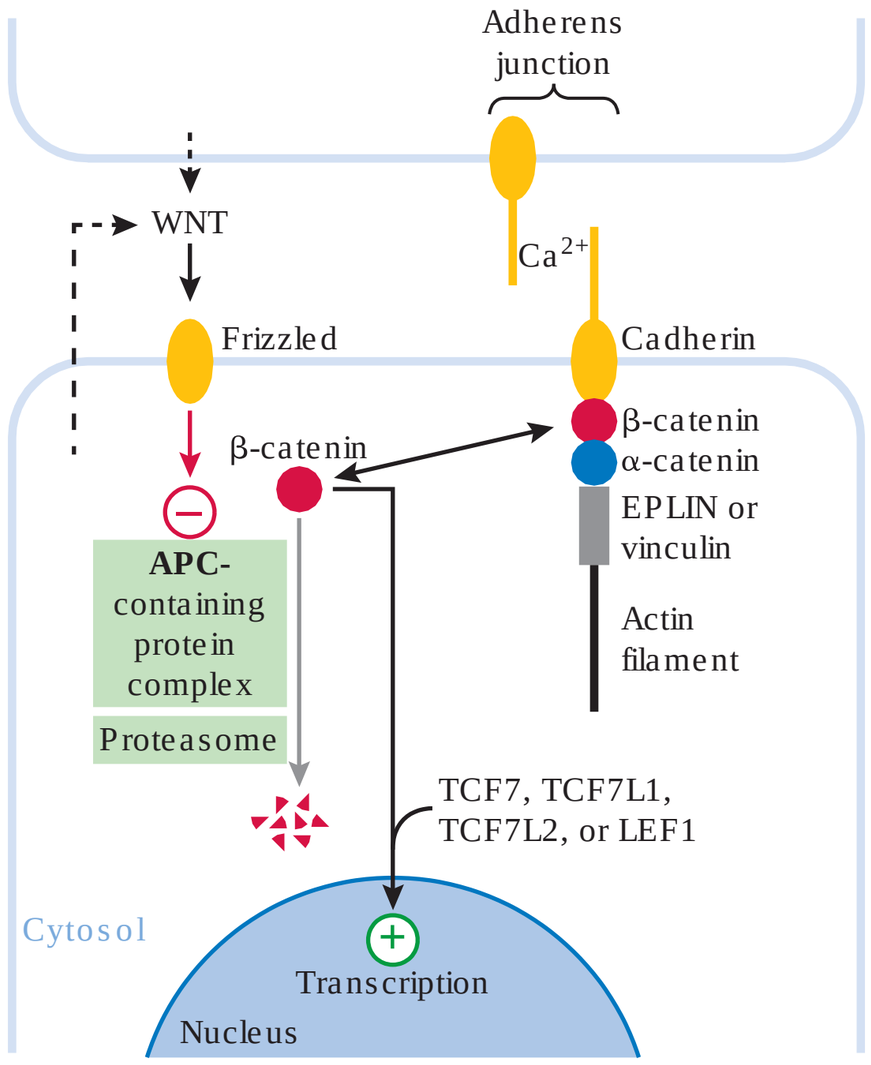


Fig. 8.5 **The WNT/β-catenin pathway.**

maintain cell shape. T e adherens junctions consist of membrane-anchored **cadherins** (in two dif erent cells) that bind to each other in the presence of Ca^{2+}, as well as β-catenin, α-catenin, and EPLIN or vinculin that connect cadherin to the actin filaments.

During the G0 phase, β-catenin is mostly at the cell membrane in adherens junctions, and its concentration in the cytosol is very low due to degradation in proteasomes (see Fig. 8.5). During the S and G2 phases, the concentration of soluble β-catenin increases; then, as cells enter the G0 or G1 phase, the concentration decreases again.

In many **tumors**, particularly pancreatic adenomas and colorectal carcinomas, WNT signaling is active regardless of the presence of WNT. T is is commonly due to a genetic alteration that leads to loss of APC function (and hence survival of free β-catenin even in the absence of a WNT signal), but it can also be caused by the absence of cadherins.

1.4. Role of MYCs

T e **MYC** transcription factors (MYC or c-MYC, N-MYC, and L-MYC) regulate the transcription of ~15% of all genes, many of which encode proteins that play a role in cell growth or cell cycle progression.

Normally, signals from the **RAS/RAF/ERK pathway** (see Fig. 8.3) or the **WNT pathway** (see Fig. 8.5) enhance the rate of transcription of MYC genes (Fig. 8.6). In turn, the MYC proteins partner with the transcription factor MAX to bind to E-box promoter elements, which contain the sequence CANNTG (N, any nucleotide).

MYC favors transcription of the genes that encode CDK4, cyclins, or enzymes of nucleotide biosynthesis, but it represses

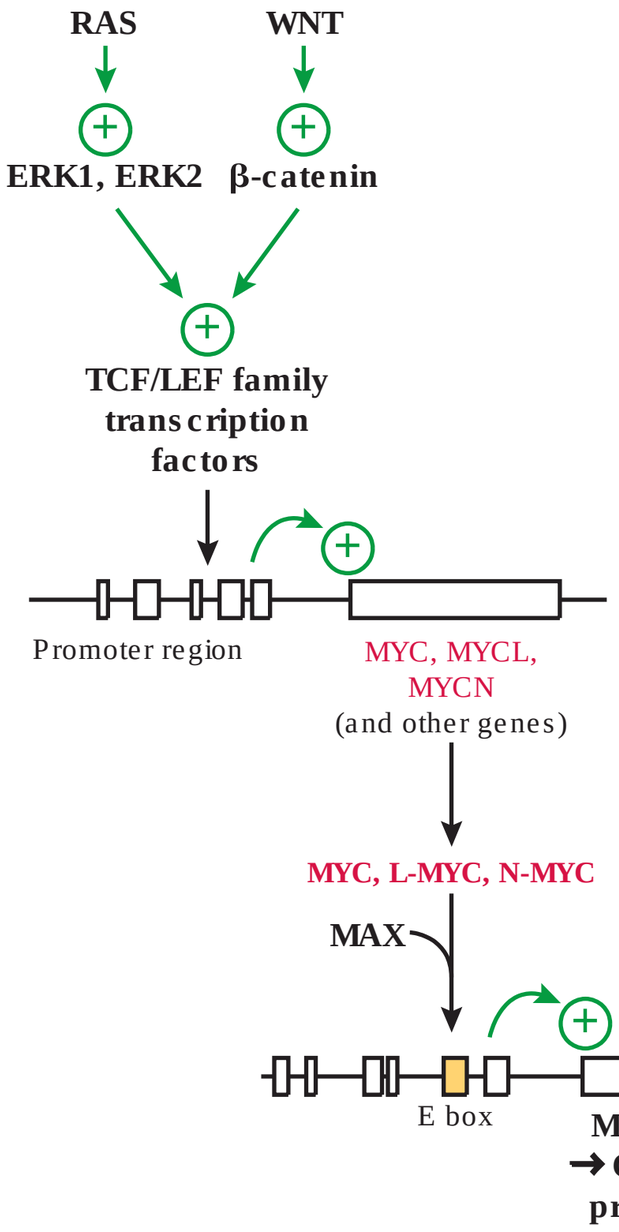


Fig. 8.6 **The role of MYC in the cell cycle.**

the transcription of the genes that encode the CDK inhibitors p21 and p27.

In tumors, excessive MYC activity can be due to translocation (joining a different promoter to a MYC gene), gene amplification, or mutation of one of the MYC genes. Furthermore, increased MYC activity may be due to increased upstream signals from the **WNT pathway** (see Fig. 8.5) or the **RAS/RAF/ERK pathway** (see Fig. 8.3). Among gene amplifications, amplification of the MYCN and MYCL genes (encoding N-MYC and L-MYC, respectively) is especially common. MYC-overexpressing tumors are generally very sensitive to nutrient deprivation.

1.5. Apoptosis

Apoptosis is a process of regulated cell suicide that can be initiated by extracellular or intracellular signals. Extracellular signals for apoptosis are particularly important in development and function of the immune system. Intracellular signals that induce apoptosis derive from **DNA damage response, hypoxia**, or **oxidative stress**. Apoptosis is the net result of an interplay of proapoptotic and antiapoptotic factors. Apoptosis is an important defense against the formation of a neoplasm.

During apoptosis, intracellular **caspases** become active, degrade proteins, and activate DNA degradation. Caspases contain a cysteine (C) residue in their catalytic site, and they cleave substrates that contain an aspartate (Asp) residue. Humans express 11 different caspases, always as inactive precursors (i.e., zymogens, proenzymes, or procaspases) that are activated through proteolysis. Caspases are organized into cascades in which one caspase activates another caspase, thereby greatly amplifying the initial enzyme activity.

Among the caspases, **initiator caspases** (caspases 2, 8, 9, and 10) play a role early in the signaling pathway. **Effector caspases (executioner caspases**; caspases 3 and 7) catalyze terminal steps and degrade several hundred different proteins that contain an N-Asp-x-x-Asp sequence by hydrolyzing the protein after the second Asp.

Apoptosis can be divided into an **extrinsic pathway** that depends on plasma membrane receptors and an **intrinsic pathway** that depends on mitochondria. Intrinsic and extrinsic pathways use different initiator caspases, but they have common effector caspases.

The intrinsic pathway depends on the **permeabilization** of the outer mitochondrial membrane and the release of **cytochrome C** from the intermembrane space of mitochondria. The permeabilization of the outer mitochondrial membrane in turn depends on the balance of proapoptotic proteins, such as **BAX**, versus antiapoptotic proteins, such as **BCL2**. In a perfectly healthy cell, the antiapoptotic BCL2 prevails over the proapoptotic BAX, in part by forming an inactive BCL2-BAX dimer. When proapoptotic signals prevail (described later in this chapter), mitochondria release cytochrome C (in oxidative phosphorylation, cytochrome C normally carries electrons from complex III to complex IV; see Fig. 23.3 and Section 1.2 in Chapter 23).

In the presence of DNA double-strand breaks, **p53** becomes active and favors apoptosis via both the extrinsic and intrinsic pathways (see Figs. 8.4 and 8.7). In response to DNA damage, ATM, ATR, CHK1, and CHK2 increase p53 activity. p53 then activates the extrinsic pathway by favoring the transcription of the FAS ligand and the FAS receptor, which in turn leads to the activation of initiator caspases (caspases 8 or 10) and effector caspases (caspases 3 or 7). These effector caspases not only degrade proteins but also activate a **DNase** that cuts DNA in the linker regions between nucleosomes, generating fragments of ~180 base pairs. p53 activates the intrinsic pathway of apoptosis by stimulating the transcription of the BAX gene and the BBC3 gene, which encodes the protein **PUMA**. PUMA neutralizes BCL2 and activates the proapoptotic BAX. BAX moves into the outer membrane of mitochondria, rendering the membranes permeable to cytochrome C. Cytochrome C in the cytosol then favors the formation of an **apoptosome**, activation of an initiator caspase, and activation of effector caspases (see Fig. 8.7).

Apoptosis results in the fragmentation of a cell into numerous membrane-enclosed vesicles that are phagocytosed by neutrophils and macrophages.

In neoplasms, genetic and epigenetic alterations frequently lead to a loss of proapoptotic factors and a gain in antiapoptotic factors to favor cell survival despite abnormal DNA. These alterations also render tumor cells resistant to chemotherapy-induced apoptosis.

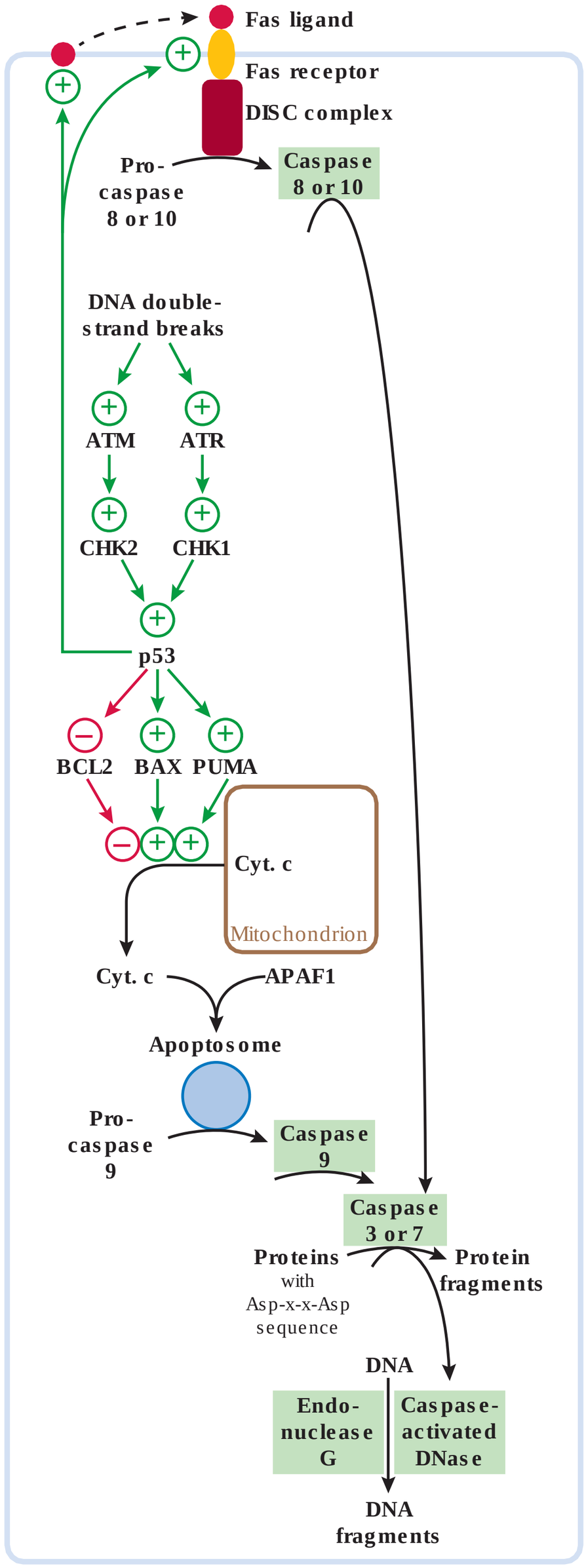


Fig. 8.7 Apoptosis. *APAF*, Apoptotic protease-activating factor 1; *DISC*, death-inducing signaling complex.

1.6. Control of S, G2, and M Phases of the Cell Cycle

During S phase, DNA is replicated, cyclin D is degraded, and **cyclin E/CDK2** and later **cyclin A**/CDK2 complexes provide the main CDK activity (see Fig. 8.1). Overexpression of cyclins E1 and E2 is tumorigenic and is observed in tumors of the uterus or ovaries.

The **Cip/Kip** family proteins inhibit CDK2, as well as other CDKs. The Cip/Kip family includes **$p21^{Cip1}$**, **$p27^{Kip1}$**, and **$p57^{Kip2}$** (noted as p21, p27, and p57). The DNA damage sensed by p53 leads to increased synthesis of $p21^{Cip1}$ (see Fig. 8.4). The removal of a growth factor stimulus induces the synthesis of $p27^{Kip1}$, which is responsible for inducing and maintaining quiescence (G0; see Fig. 8.1).

The main CDK for transition from G2 to M phase is the **cyclin B/CDK1** complex. The cyclin B/CDK1 complex already forms during S phase, but it is inactive because of inhibitory phosphorylation by kinases named WEE1-like protein kinase 1 and 2, and by translocation to the cytosol. In late G2 phase, a **CDC25 phosphatase** activates CDK1 by dephosphorylating it, and the cyclin B/CDK1 complex moves to the nucleus.

In the presence of DNA damage, CHK1 phosphorylates and inhibits CDC25, which in turn keeps CDK1 inactive and makes entry into M phase impossible.

2. GENETIC ALTERATIONS IN CANCER CELLS

*T*umors and normal cells contain many mutations; a relatively small number o*f* driver mutations among them is responsible *f*or tumorigenesis. A single allele o*f* an oncogene (representing a gain o*f f*unction) is su*ffi*cient to *f*avor tumorigenesis, but both alleles o*f* a tumor suppressor must usually be altered to lose tumor suppressor *f*unction and *f*avor tumorigenesis. *T*umor cells typically contain approximately two to eight driver mutations, o*f* which one is an oncogene and the rest are tumor suppressors. Age is a major risk *f*actor *f*or cancer. Smoking *f*urther increases cancer risk via the *f*ormation o*f* mutagenic DNA adducts. Obesity is a risk *f*actor *f*or a limited number o*f* cancers, notably cancer o*f* the endometrium. Isolation and analysis o*f* circulating tumor cells hold promise in making a prognosis and determining the optimal treatment.

2.1. Genetic Alterations That Favor Neoplasia

Cancer is the consequence of multiple abnormalities of the genome of somatic cells. This damage is caused by inadequate or faulty repair of DNA damage and replication errors. Most of the mutations seen in tumors are single nucleotide substitutions, and most of these are missense mutations. In adults, solid tumors typically contain ~50 mutations that alter the amino acid sequence of proteins (when DNA repair is impaired, tumors show an even larger number of mutations). Some of the clinically significant mutations increase proliferation, and others inhibit apoptosis.

In a simple binary classification, **driver mutations** are key to neoplasia, whereas **passenger mutations** have few consequences. Currently, ~150 known driver mutations exist. A typical tumor contains two to eight driver mutations. For a type of cancer (e.g., colon cancer), tumors differ appreciably in their genetic abnormalities.

Compared with nonmalignant cells, tumors often show **aneuploidy** (i.e., a number of chromosomes that is not divisible by 23) and other **chromosome alterations**, such as partial deletions, translocations, and gene amplification. Most of the chromosome alterations are likely benign.

Oncoproteins from **oncogenes** favor the formation of neoplasms. Oncoproteins may be normal proteins that are expressed in unusual abundance because of **gene amplification** or a chromosome **translocation** that links the gene to a promoter that results in an increased rate of transcription. Alternatively, oncoproteins may be mutant proteins encoded by oncogenes that in turn derive from **proto-oncogenes**; for example, the gene for a growth factor receptor (a proto-oncogene) may be mutated to an oncogene so that the mutant receptor signals even in the absence of a growth factor. A single copy of an oncogene is sufficient to be tumorigenic.

Tumor suppressors act against tumorigenesis. In neoplasms, the tumor suppressor function can be lost by mutation or epigenetic ef ects. Mutations may cause a loss of function because of missense mutation, loss of heterozygosity (see Section 4.2 in Chapter 2), truncation, or aberrant splicing. For example, mutations in the *T*P53 gene, which encodes p53, often impair DNA binding by p53. Epigenetic silencing of tumor suppressor expression may be due to DNA methylation, histone modification, or micro-RNAs (miRNAs; see Section 3 in Chapter 7). Common targets of promoter methylation are the genes CDKN2A (encoding the CKIs p14 and p16), MLH1 (encoding a DNA mismatch repair protein), and BRCA1 (encoding a homologous recombination repair protein).

Most often, the function of both alleles of a tumor suppressor needs to be lost for tumorigenesis to occur. T e function of the two alleles may be lost by dif erent mechanisms. Sometimes, only one allele of a tumor suppressor gene needs to be mutated for a tumor to develop, because the mutant allele shows a dominant negative ef ect or because there is haploinsufficiency when only a single functional allele is present.

Oncogenic miRNAs are miRNAs that degrade tumor suppressor mRNAs, and **tumor suppressor miRNAs** are miRNAs that degrade oncogene mRNAs. Because miRNAs enhance the degradation of multiple mRNAs, their ef ects may be tissue specific and difficult to predict.

Mutations in tumor suppressors, proto-oncogenes, or oncogenes can each be driver mutations.

Tumors typically have only about one oncogene mutation, whereas the remaining one to seven driver mutations concern the loss of tumor suppressor function.

Most of the major cancer-causing mutations af ect the function of one out of about a dozen **pathways.** T e most commonly af ected pathways in cancer are the RB pathway (see Fig. 8.2 and Section 1.1) and the p53 pathway (see Fig. 8.4 and Section 1.2).

T e function of **RB** is lost in almost all tumors. RB controls the G1/S phase transition (Figs. 8.1 and 8.2). RB function can be lost via mutations that abolish the interaction of RB with E2F (e.g., point mutations that generate a truncated protein, deletion of a DNA segment that includes the RB1 gene), via inhibition by a protein from a virus (e.g., papillomavirus protein E7), or via inappropriate phosphorylation of RB due to excessively high activity of CDK2, CDK4, or CDK6 (due to overexpression of cyclin D or cyclin E or loss of the inhibitory activity of p16 or p27).

T e function of **p53** is also frequently lost in tumors. In the presence of DNA damage, p53 halts the cell cycle at the G1/S cell cycle checkpoint (see Figs. 8.1 and Fig. 8.4). T e function of p53 may be lost due to a mutation in the DNA-binding domain, sequestration in the cytosol, degradation by increased MDM2 activity, degradation induced by the papilloma virus protein E6, or loss of the MDM2 inhibitor $p14^{ARF}$. With reduced p53 function, apoptosis is also reduced.

T e PI3K/AKT pathway (see Fig. 8.3) is often activated, sometimes by the loss of PTEN activity.

Most of the targeted **antineoplastic agents** on the market today are inhibitors. Some of these inhibitors are used to impair the function of oncoproteins, such as protein kinases. For the most part, no drugs are available to remedy loss of tumor suppressor function directly. However, the loss of tumor suppressor activity generally results in increased activity of another protein downstream in the pathway that can sometimes be inhibited with a drug.

Many **heritable cancer syndromes** are inherited in an **autosomal dominant** fashion and are due to the inheritance of only one functional copy of a tumor suppressor allele. T e mode of inheritance of any disease depends on the definition of the disorder. In heritable cancer syndromes, the disease is defined as an unusually early onset and an unusually high probability of developing a particular neoplasm. For a neoplasm to form, the function of both alleles of a tumor suppressor gene generally needs to be lost. T e occurrence of an inactivating mutation depends on time. In a person who has only one allele to lose to inactivation, cancer occurs sooner than in a person who has two alleles to lose; hence the dominant pattern of inheritance.

2.2. Effect of Age on Tumorigenesis

T e risk of being diagnosed with cancer greatly increases with age (Fig. 8.8), presumably because mutations accumulate with time. In fact, tumors in children generally contain fewer mutations than those in older adults. Furthermore, even normal, aged skin contains a significant fraction of tumorigenic driver mutations. By 85 years of age, ~50% of individuals develop cancer. One could surmise that if a person becomes old enough, he/she will die of cancer, but it turns out that according to mathematical predictions, even a 120-year-old person still has a ~20% chance of not dying of cancer.

A large portion of the age-dependent cancer risk is associated with the number of stem cell divisions, presumably because of associated replication errors. Cancer is thus a result of chance, and additional mutations due to smoking, exposure to ultraviolet (UV) radiation, excessive alcohol consumption, or obesity (see the following text) further increase this chance.

Tumors that are thought to be promoted by **mutagens**, such as smoking-induced lung cancer or UV light–induced

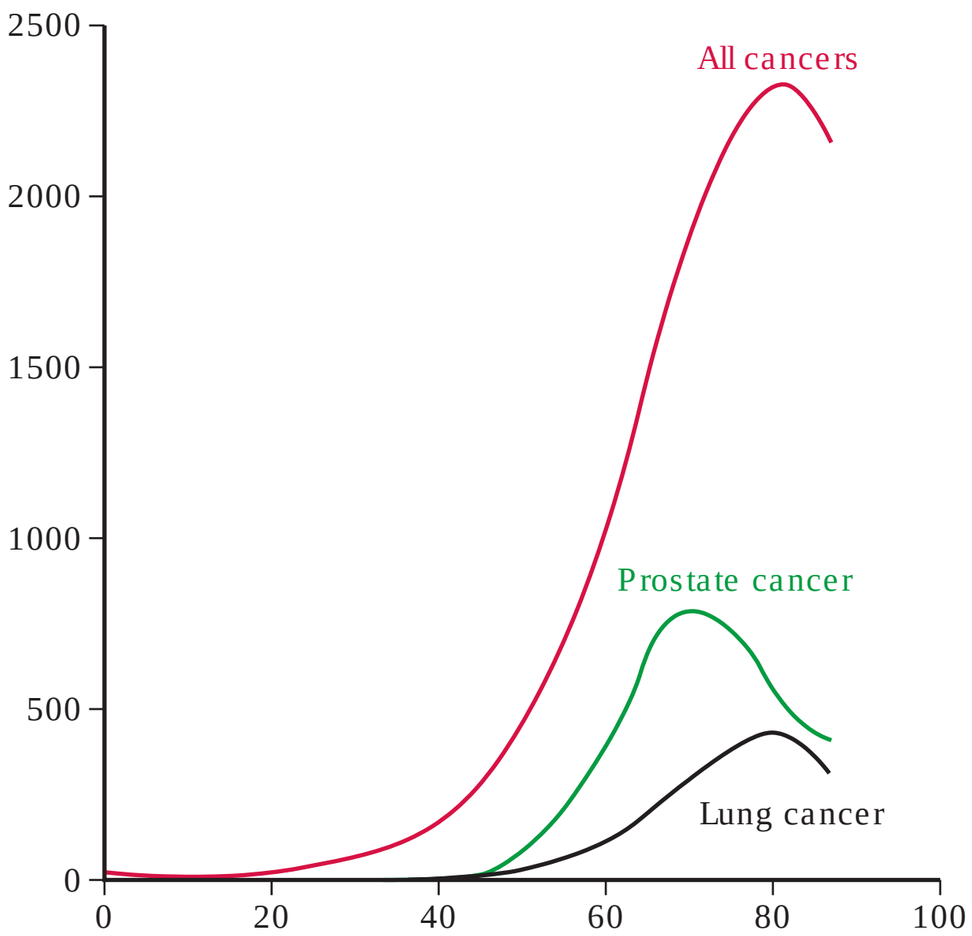


Fig. 8.8 Age as a key risk factor for cancer. Age-specific Surveillance, Epidemiology, and End Results (SEER Program) incidence rates in the United States, 2009–2013. (Data from Howlader N, Noone AM, Krapcho M, et al, eds. SEER Cancer Statistics Review, 1975-2013, National Cancer Institute. Bethesda, MD. Available at http://seer.cancer.gov/csr/1975_2013.)

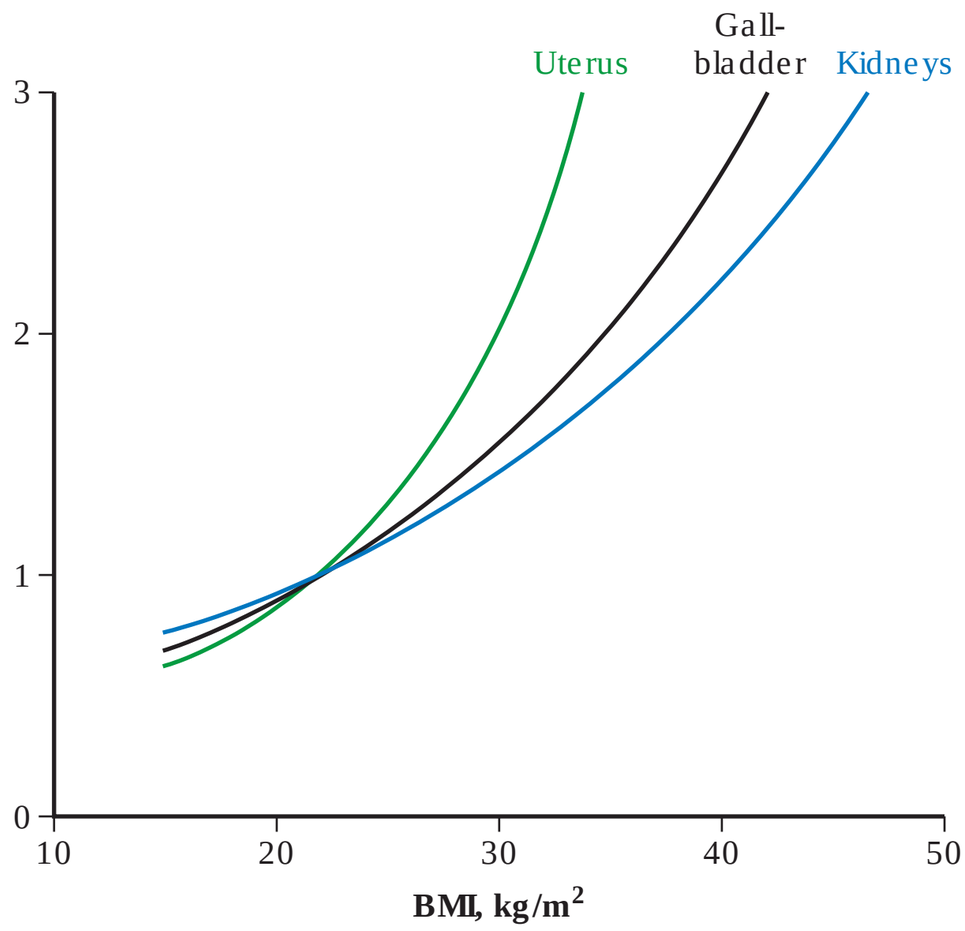


Fig. 8.9 Relative risk for cancer in select organs. Records of ~5 million people in the United Kingdom (~9% of all inhabitants) from 1987–2012 were analyzed. (Data from Bhaskaran K, Douglas I, Forbes H, dos-Santos-Silva I, Leon DA, Smeeth L. Body-mass index and risk of 22 specific cancers: a population-based cohort study of 5 24 million UK adults. *Lancet.* 2014;384:755-765.)

melanoma, contain an especially high number of mutations. For instance, non-small-cell lung carcinomas from smokers have ~10 times the number of mutations found in the same tumors from nonsmokers.

Although clinical signs of a malignancy appear within a short time span, tumors are years or decades in the making.

2.3. Smoking and Cancer

Smoking increases a person's chance of developing cancer of the oral and nasal cavities, lungs, esophagus, pancreas, bladder, and other organs. Worldwide, ~20% of all cancer deaths are attributed to smoking tobacco.

Tobacco smoke contains some 20 known carcinogens, which can be grouped into **polycyclic aromatic hydrocarbons** (**PAH**) and the **nitrosamine** 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone. T e most studied PAH is the highly carcinogenic **benzo[a]pyrene** (see Chapter 2). T e carcinogens are modified in the body and then exert their toxic ef ect by forming adducts with DNA, predominantly at G or A. Repair of these adducts occurs via nucleotide excision repair (NER; see Fig. 2.7 and Section 3 in Chapter 2). If the adducts escape proper DNA repair, they may lead to a permanent mutation. Among the many permanent mutations, some lead to the formation of an oncogene and others lead to the loss of tumor suppressor function.

Smoke from a **water pipe** (**shisha**, **hookah**) is a major health hazard because significant amounts of carcinogens emanate from the charcoal that is used to heat the tobacco, and these carcinogens are inhaled with the smoke. For a comparable amount of nicotine consumed, smoke from a water pipe contains ~10 times more benzo[a]pyrene than smoke from a cigarette.

Despite the fact that smoking vastly increases a person's risk for **lung cancer**, no more than ~20% of smokers develop lung cancer.

Chewing tobacco contains N-nitrosamines that give rise to cancer of the oral cavity.

2.4. Obesity, Alcohol, and Cancer

Obesity is a gender-specific risk factor that af ects a person's risk for certain neoplasms, especially in the uterus, gallbladder, kidneys, and liver (Fig. 8.9; calculation of body mass index [BMI] is explained in Section 5.1 in Chapter 39; a BMI >30 kg/m^2 indicates obesity). In contrast, the incidence of melanoma or bladder cancer does not markedly depend on adiposity.

T e most thoroughly tested explanation of a link between obesity and cancer exists for cancer of the endometrium. T e main dif erence between an obese and a lean person is the mass of adipose tissue. Adipose tissue converts circulating androstenedione into **estrone** (see Fig. 31.5 and Section 2.4 in Chapter 31), which constitutes the biggest source of estrogens after menopause. T e obesity-induced, elevated concentration of estrone favors the growth of estrogen-dependent tumors.

Other proposed explanations for an obesity-induced increase in cancer risk state that the obesity-induced increased concentration of **insulin** or **hormones** released from the adipose tissue (e.g., more **leptin**, less **adiponectin**) favor

growth-promoting signaling pathways, such as PI3K/AKT and RAS/RAF/ERK (see Fig. 8.3).

The effect of ethanol (alcohol) consumption alone (and in combination with smoking) on the risk of cancer in the mouth, pharynx, larynx, and esophagus is described in Section 4.5 in Chapter 30.

From the above, it is evident that counseling of patients regarding smoking, BMI, and alcohol consumption should be an integral part of health care.

2.5. Circulating Tumor Cells

Circulating tumor cells (**CTC**s) are cells that have been shed by a tumor or its metastases and entered the bloodstream. CTCs are of interest with regard to prognosis and monitoring therapy. After the removal of a primary tumor, CTCs are an indicator of the abundance of residual tumor cells.

In the laboratory, CTCs are enriched and identified on the basis of the presence and absence of certain cell surface proteins. CTCs are very rare. A blood sample that tests positive for CTCs typically contains more than four cells per 7.5 mL of blood in a blood collection tube. It is now possible to analyze the DNA in a single CTC.

Currently, CTCs can be used to make a prognosis and choose a treatment for cancer of the breast, prostate, or colon.

3. EXAMPLES OF COMMON NEOPLASMS

Inherited **BRCA** mutations are one cause of hereditary breast and ovarian cancer (HBOC) syndrome. The most common targeted treatments of breast cancer involve the use of a selective estrogen receptor modulator, an aromatase inhibitor, or inhibition of the human epidermal growth factor receptor 2 (HER2). Many tumors of the lung test positive for the overexpression of kinases that can be inhibited pharmacologically. Prostate tumors are genetically very diverse and often contain a translocation that places a promoter with an androgen response element next to a transcription factor. Accordingly, patients with advanced prostate cancer are commonly given androgen deprivation therapy. Sporadic colorectal cancers frequently lack a functional APC protein and thus resemble tumors in patients with familial adenomatous polyposis (FAP; caused by a heritable monoallelic loss of APC function). Alternatively, sporadic colorectal cancers lack a functional DNA mismatch repair pathway and thus resemble tumors in patients with Lynch syndrome (caused by monoallelic inherited loss of a mismatch repair gene). Metastatic melanoma is commonly treated with immunotherapy. The growth of tumors that test positive for a RAF mutation can transiently be impeded with kinase-specific inhibitors. About half of the cases of familial melanoma are caused by mutations in a gene that encodes an inhibitor of cell cycle progression.

Much of the detail we know about the genetic changes that occur in various tumors is based on massive parallel sequencing (next generation sequencing; see Section 5.2 in Chapter 4). Such sequencing is now gaining entry into clinical use, most often in the form of select cancer gene panels. The major current challenges are to learn to interpret the implications of observed mutations and to determine the most effective treatments. Health care providers will have to know the meaning and limitations of past and current tests, particularly when dealing with patients who have a hereditary cancer syndrome.

It is now apparent that there are about a dozen pathways that consistently function abnormally in cancer (see Section 2.1). Patients who have a hereditary mutation in one of these pathways commonly have an increased chance of developing tumors in multiple organs. Hence, a family history of cancer in one organ can be caused by several different heritable cancer syndromes; affected family members therefore need to be tested for mutations in multiple pathways. As an example, a patient who seems to have Lynch syndrome frequently also meets the criteria for testing for HBOC. Increasingly, gene panels and massive parallel sequencing are used to screen for multiple genetic alterations (see Section 6 in Chapter 4).

Currently, the most successful treatment of cancer is removal of the tumor by **surgery**. However, metastases are often in sites that cannot readily be accessed by surgery. At present, there are not sufficient means to detect small groups of precursor cells before they give rise to cancer. However, some premalignant conditions can successfully be detected, such as adenomatous colon polyps by colonoscopy and cervical dysplasia by colposcopy.

3.1. Breast Cancer

By histology, breast tumors are commonly divided into **ductal carcinomas** and **lobular carcinomas** (Figs. 8.10 and

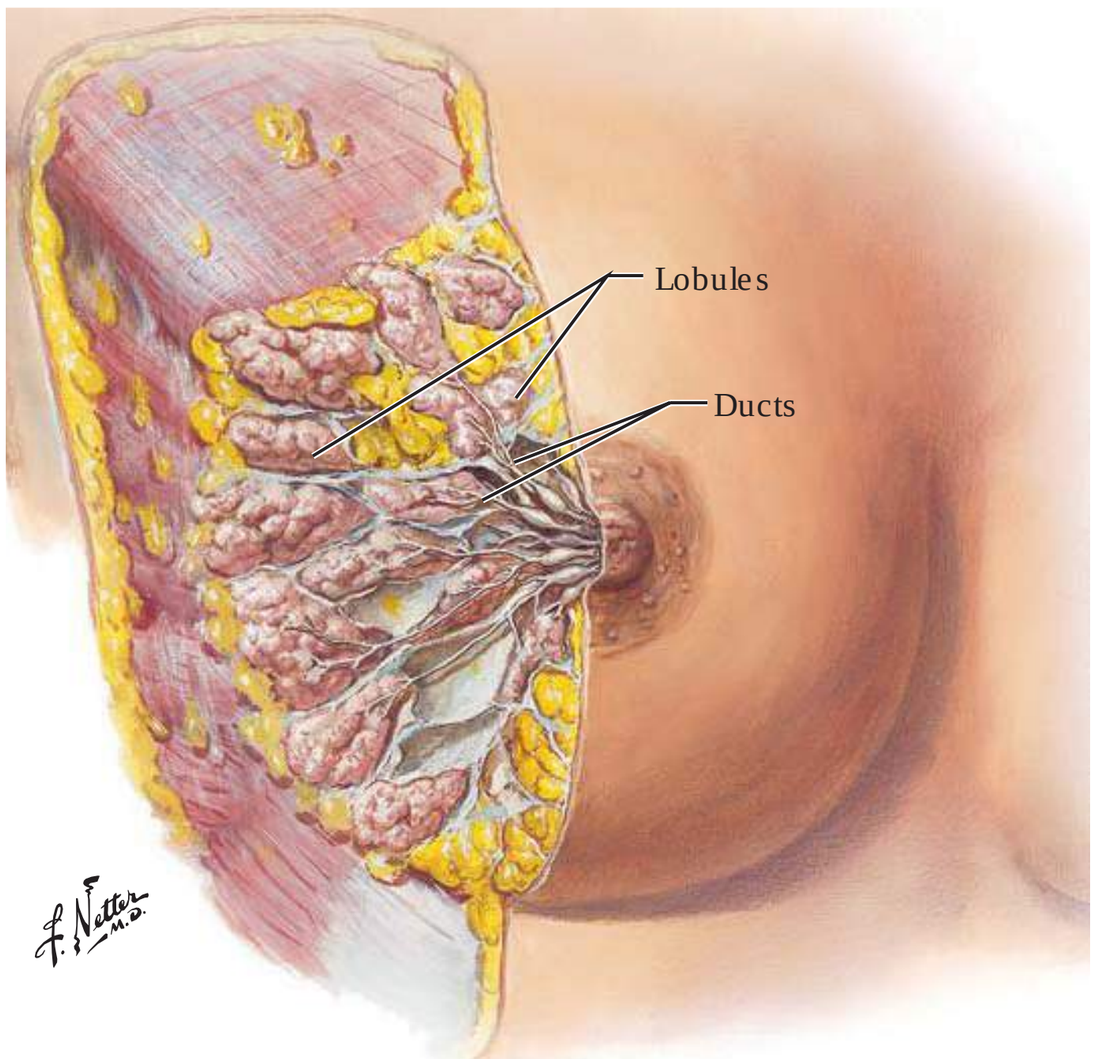


Fig. 8.10 **Structure of the female breast.**

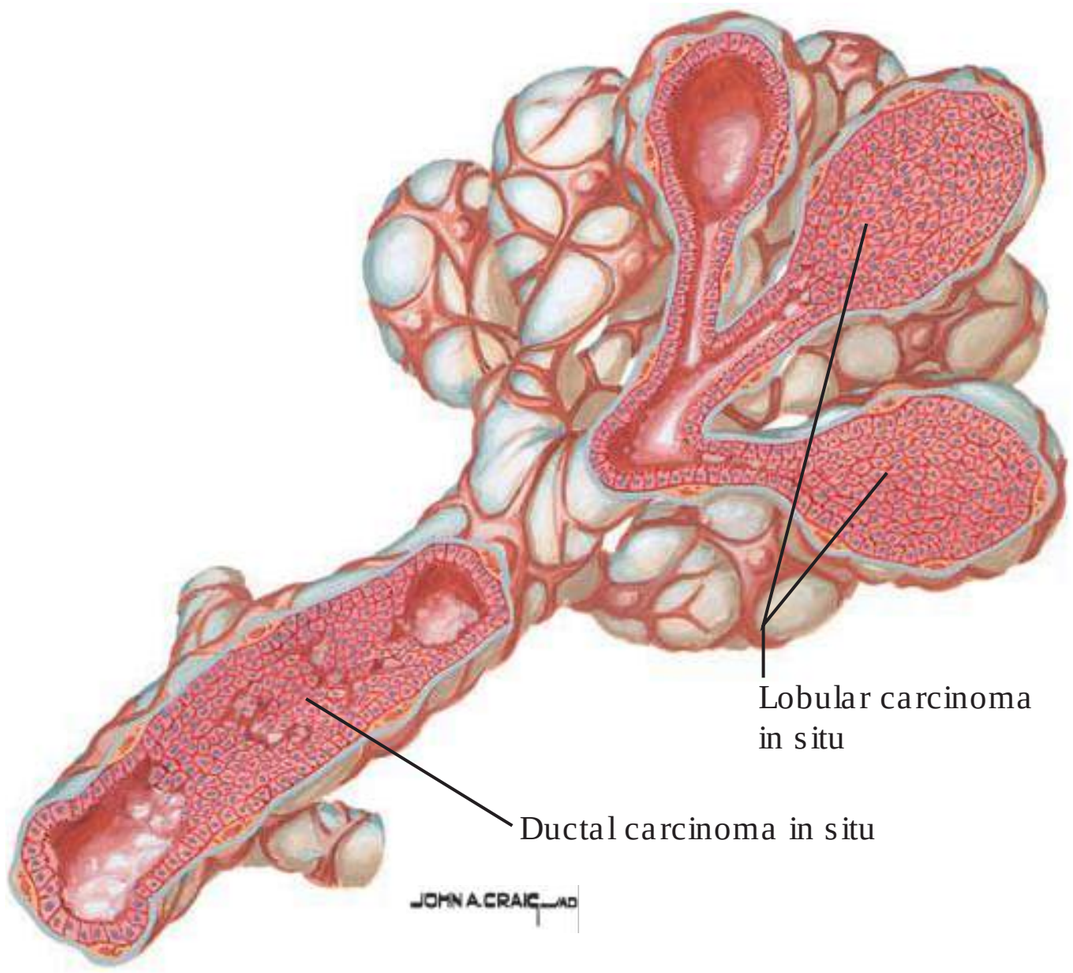


Fig. 8.11 **Ductal and lobular carcinoma.**

8.11), implying that the tumor cells arise from the epithelium of either ducts or lobules. Sometimes, tumors are designated as **mixed** ductal and lobular carcinoma. Furthermore, tumors are divided into **in situ carcinomas**, which are limited to growth within the epithelium of the ducts or lobules, and **invasive carcinomas** (**infiltrative carcinomas**), which have grown beyond the confines of the ducts and lobules into connective tissue of the breast. About 25% of all breast tumors are ductal carcinomas in situ, ~60% are invasive ductal carcinomas, and ~5% are invasive lobular carcinomas.

In the United States, ~12% of all women eventually develop invasive breast cancer. About 90% of these patients have **sporadic** breast cancer, whereas ~10% have an **inherited** mutation that is known to be associated with an increased cancer risk.

Mutations in the BRCA1 and BRCA2 genes are the main contributors to **HBOC** syndrome, accounting for ~50% of cases. The BRCA1 and BRCA2 proteins play a role in homologous recombination repair of double-strand breaks (see Section 4.2 of Chapter 2). Another ~8% of HBOC is due to mutations in other genes, such as PALB2, CHEK2, and the mutated genes that cause other known hereditary cancer syndromes, such as *T*P53 (Li-Fraumeni syndrome), P*T*EN (Cowden syndrome), S*T*K11 (Peutz-Jeghers syndrome), or A*T*M. This leaves many more genetic causes of HBOC to be discovered. In the United States, at least 1 in 400 persons has inherited a pathogenic BRCA1 or BRCA2 mutation (among Ashkenazy Jews, the incidence is ~1:40). Among women with breast cancer, the prevalence of a heritable BRCA mutation is ~2%.

By 70 years of age, a woman who has inherited a BRCA1 or BRCA2 mutation has an ~55% chance of developing breast cancer and an ~25% chance of developing ovarian cancer (rates are somewhat higher for BRCA1 and lower for BRCA2 mutations).

Clinically, the importance of diagnosing HBOC lies in increased surveillance, testing of relatives, and adjustments to therapy.

The most common mutations in sporadic breast tumors as determined by massive parallel sequencing (see Section 5.2 in Chapter 4) are in the *T*P53 and PIK3CA genes. p53 becomes active in response to DNA damage and then arrests the cell cycle via an increased expression of p21 (see Fig. 8.4). The PIK3CA gene encodes the catalytic subunit of PI3K (see Fig. 8.3). Mutations in PIK3CA generally lead to increased activity in the PI3K/AKT/mTOR pathway, which inhibits apoptosis and promotes protein synthesis.

Breast tumors of patients with a germline nonfunctional BRCA allele generally show loss of BRCA function via a somatic alteration.

The primary treatment of breast cancer is **surgical** removal of the lesion; depending on the nature of the tumor, **adjuvant radiation therapy** and **adjuvant systemic therapy** are added to destroy any remaining tumor cells. Radiation therapy uses ionizing radiation, which induces single- and double-strand breaks (see Section 4 in Chapter 2). Systemic therapy often includes an anthracycline and cyclophosphamide regimen, followed by taxane treatment (abbreviated **AC-T regimen**). **Anthracyclines** are DNA intercalators, and the most commonly used drug is doxorubicin, which also inhibits topoisomerase II (see Section 5 in Chapter 1). **Cyclophosphamide** is a nitrogen mustard that induces intrastrand and interstrand DNA crosslinks (see Section 4.2 in Chapter 2). The **taxanes** inhibit the degradation of microtubules, which are part of the cytoskeleton; microtubules serve as lines for intracellular cargo transport, are degraded in preparation for prophase, are attached to kinetochores during prometaphase, and then serve as tracks for motor proteins that pull apart chromatids during anaphase. Tumor tissue is commonly tested for **estrogen receptors** (**ER**), **progesterone receptors** (**PR**), and **HER2**, because the results predict the effectiveness of targeted therapies. The physiological roles of estrogens and progesterone are described in Section 2.4 of Chapter 31. HER2 forms a heterodimer with an epidermal growth factor–stimulated **epidermal growth factor receptor** (**EGFR**) and then activates the RAS/RAF/ERK and PI3K/AKT/mTOR pathways (see Fig. 8.3). If the tumor is ER positive and PR positive, hormone therapy most often involves the use of a **selective estrogen receptor modulator**, such as **tamoxifen**; sometimes, an **aromatase inhibitor** is used (aromatase is needed for the synthesis of estrogens; see Fig. 31.5 and Section 2.4 in Chapter 31). If a tumor is HER2 positive, treatment with one or two different monoclonal **HER2 antibodies** (**trastuzumab**, **pertuzumab**) or with **lapatinib,** an inhibitor of the tyrosine kinase activity of both **HER2** and **EGFR,** is common. Tumors that test negative for ER, PR, and HER2 are called **triple negative**.

In clinical trials, perhaps half of all patients who have a germline BRCA mutation and then develop breast cancer benefit from using a poly (ADP-ribose) polymerase **(PARP)**

inhibitor. PARP inhibition impairs the base excision DNA repair pathway (BER; see Section 1 in Chapter 2). Because of redundancy in DNA repair pathways, cells with functional BRCA1 and BRCA2 survive in the presence of PARP inhibitors. However, cells without functional BRCA1 and BRCA2 undergo apoptosis when a PARP inhibitor inhibits BER (this finding is sometimes referred to as an example of **synthetic lethality**).

3.2. Lung Cancer

Smoking increases a person's risk for lung cancer 15- to 30-fold. In the United Kingdom, by the age of 75 years ~13% of all lifelong smokers die of lung cancer. Smokers account for ~90% of all lung cancers. Quitting smoking (compared with continued smoking) cuts the smoking-induced risk for lung cancer in half every 10 years.

In the absence of a clear driver mutation that can be targeted pharmacologically, lung cancer is treated with **platinum drugs** and a **topoisomerase inhibitor**. T is is sometimes followed by radiation therapy.

Tumors that contain certain tyrosine kinase driver mutations can be treated with **tyrosine kinase inhibitors**. T us, patients with an EGFR driver mutation can be given erlotinib or gefitinib (which reversibly inhibit the tyrosine kinase activity of the EGFR), or they can be treated with afatinib (which irreversibly inhibits the tyrosine kinase activities of EGFR and HER2). Patients with an anaplastic lymphoma kinase (ALK, a receptor tyrosine kinase) fusion oncogene can be treated with the protein kinase inhibitors crizotinib and ceritinib.

Bronchogenic carcinoma accounts for ~95% of all lung cancer and is subdivided as follows.

Small-cell lung carcinoma (**SCLC**, oat cell carcinoma) makes up ~20% of all lung cancer and is mostly related to smoking (see Fig. 1.11). Small-cell tumors show a high rate of mitosis and are also very sensitive to systemic chemotherapy, but they still have the lowest rate of 5-year survival among all patients with lung cancers. SCLC is commonly treated with platinum drugs, which generate intrastrand and interstrand adducts that are repaired by NER and by homologous recombination repair, respectively (see Figs. 2.8 and 2.10 and Sections 3 and 4.2 in Chapter 2).

Non-small-cell lung carcinoma (**NSCLC**) makes up ~70% of all lung cancers and is further subdivided into the following three types:

1. **Squamous cell carcinoma** (epidermoid carcinoma; Fig. 8.12) accounts for ~35% of all lung cancers and is related to smoking. About 80% of the tumors contain *T*P53 mutations; ~70% contain deletions, promoter methylation, or mutations of the CDKN2A gene (encoding the CDK inhibitors $p16^{Ink4a}$ and $p14^{Arf}$); ~50% contain alterations of the PI3K/AKT pathway; and ~35% contain alterations of the RTK/RAS/RAF/ERK pathway.
2. **Adenocarcinoma** (**AC**) occurs in ~25% of all lung cancers and is typically near the periphery of the lung (Fig. 8.13). About 80% of these tumors occur in smokers and ~20% in nonsmokers. AC is the most common form of lung cancer in nonsmokers. Lung adenocarcinomas in smokers have about 10-fold the number of mutations in never-smokers. AC is genetically very diverse and difficult to treat successfully. Individualized treatment may be more successful than the standard chemotherapy. Genetic analysis for selected key pathogenic mutations and mutation-matched treatment is now the standard of care. About 75% of the ACs show alterations in the RTK/RAS/RAF pathway (whereby EGFR and KRAS are key contributors), ~65% have an abnormality in the p53 pathway (almost all attributable to *T*P53 mutations), and ~65% have an altered regulation of the cell cycle (e.g., due to a CDKN2A alteration). A small fraction of tumors contains a translocation that generates an **ALK** fusion oncogene (for treatment, see earlier discussion).
3. **Large-cell anaplastic carcinoma** (Fig. 8.14) accounts for ~10% of all lung cancers and is usually related to smoking. It has a 5-year survival rate of ~10%, which is the lowest among all NSCLCs.

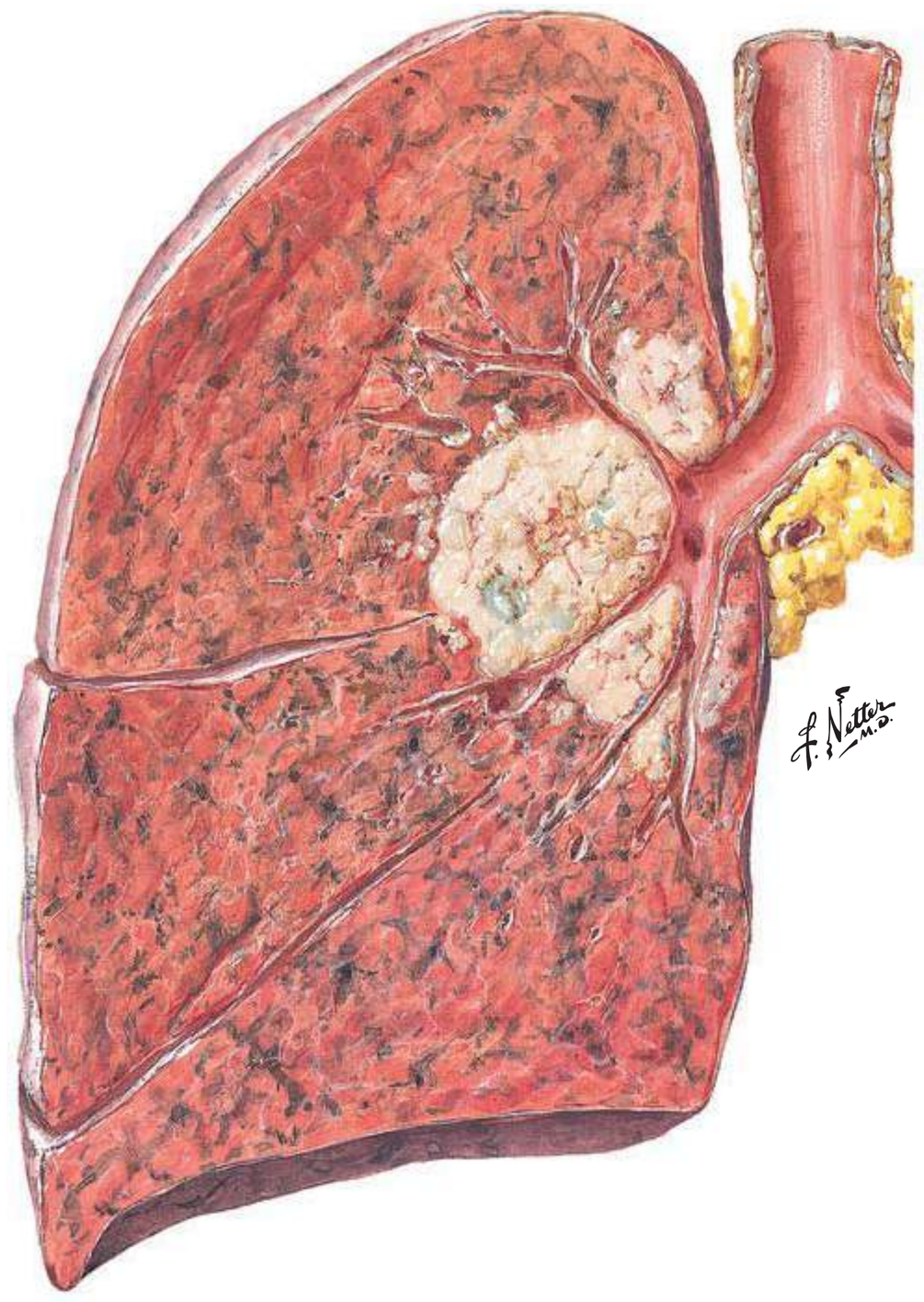


Fig. 8.12 **Squamous cell lung carcinoma.**

3.3. Prostate Cancer

In the United States, the lifetime risk for prostate cancer in men (Fig. 8.15) is ~14%, which is slightly higher than the lifetime risk for breast cancer in women.

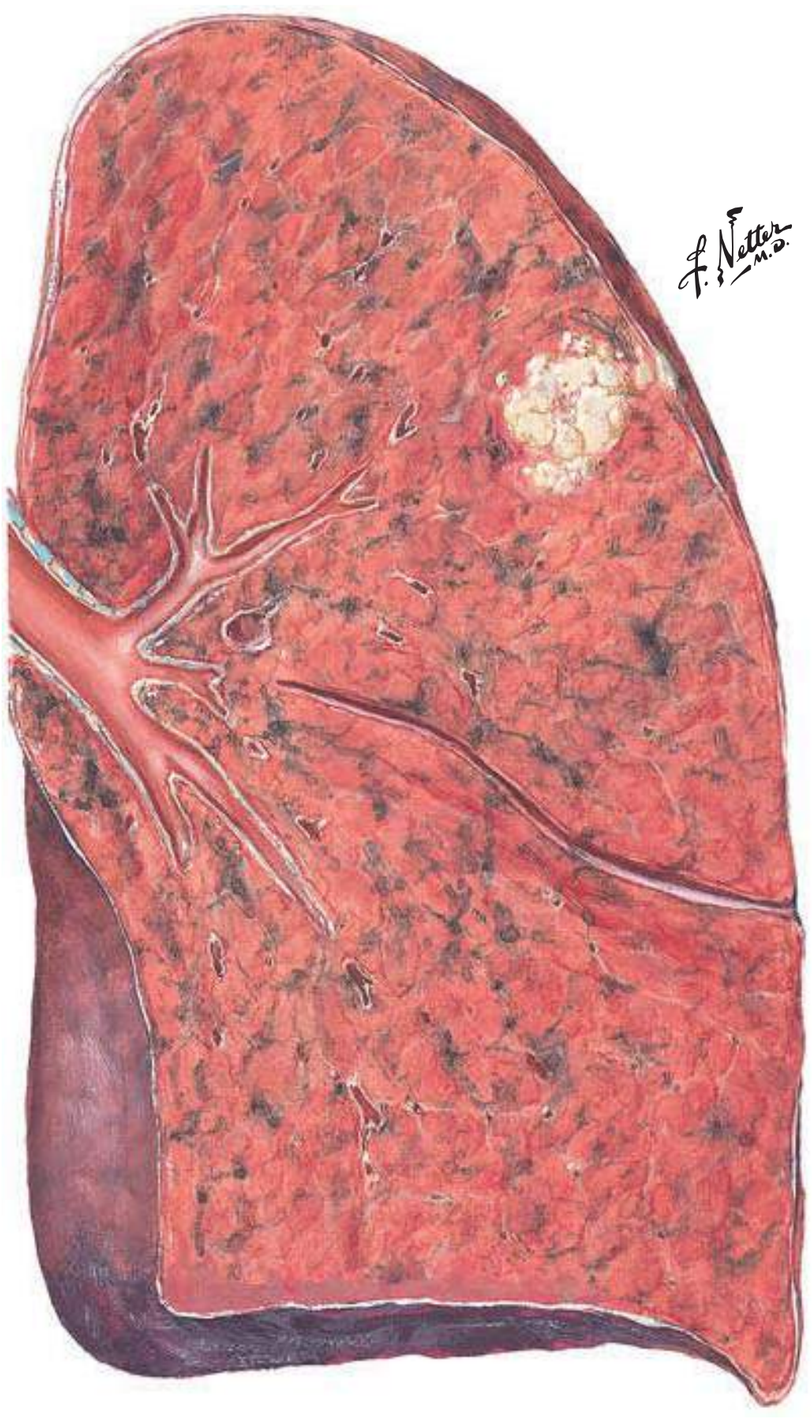


Fig. 8.13 **Adenocarcinoma of the lung.**

Within the prostate gland, prostate cancer often exists in several distinct foci with the largest focus called the index tumor. The size of the tumor and a histology-derived Gleason grade determine the prognosis.

The main genetic alterations of prostate tumors are **translocations** that place an androgen-regulated promoter next to the gene for the transcription factor ERG or other members of this family. A typical prostate tumor has ~90 chromosome alterations. The genetic makeup of these tumors is very diverse, giving rise to numerous subtypes. Compared with other tumors, prostate tumors have relatively few point mutations.

Aside from surgery and radiation, the treatment of advanced prostate cancer often involves **androgen deprivation therapy**. The concentration of testosterone can be greatly lowered by either surgical or chemical castration (see Section 2.2 in Chapter 31). Taxanes (inhibitors of microtubule degradation; see Section 3.1) are often used as well.

3.4. Colorectal Cancer

In the United States, the lifetime risk for colorectal cancer is ~5%.

Colorectal carcinomas arise from adenomatous **polyps** that in turn give rise to **adenomas** that show some dysplasia and increased proliferation. Colonoscopies with the removal of polyps and adenomas can significantly reduce the incidence of colorectal cancer.

Most patients with colorectal cancer have the sporadic form, but ~5% of all patients with colorectal cancer have the heritable cancer syndromes Lynch syndrome, MUTYH-associated polyposis (MAP), or FAP.

Patients with **FAP** have a nonfunctional APC allele in their germline and have lost the function of the second allele in neoplasms. In the United States, FAP affects ~1 in 8000 people. Of all patients with colorectal cancer, ~0.5% have FAP. About 70% of persons have a parent with FAP, and ~30% have FAP due to a de novo mutation, often in the germline of a parent; either way, each offspring of an affected patient has a 50% risk of receiving the faulty APC allele. Almost all pathogenic APC alleles lead to truncation of the expressed APC protein.

APC is a tumor suppressor that plays a role in the degradation of **β-catenin** (see Fig. 8.5, Section 1.3). In the absence of a WNT signal, β-catenin is degraded. In the presence of a WNT signal, such as during development, APC no longer degrades β-catenin; β-catenin then binds to TCF/LEF family transcription factors, moves to the nucleus, and stimulates the transcription of certain genes that help advance the cell cycle, including MYC (see Fig. 8.6). When mutant APC is no longer able to guide the degradation of β-catenin, the cell behaves as if a WNT signal were present that favors cell proliferation.

Patients who have FAP are born with only one functional APC allele. Somatic alteration of the second allele (the only functional one) leads to numerous polyps, and if these are not removed, colorectal cancer develops earlier than in persons with two functional APC alleles. Systemic therapy of polyps with the nonsteroidal antiinflammatory drug **sulindac** reduces the average number and size of colorectal polyps.

For patients who have FAP, prophylactic removal of the colon, typically in the patient's 20s, is common. Without colectomy, patients develop hundreds to thousands of adenomas in their colon, and their risk of colorectal cancer is close to 100% by the age of 40 years (Fig. 8.16). Depending on the specific mutation in the APC gene, FAP is also associated with polyps in the upper gastrointestinal tract, tumors in the brain (especially medulloblastoma), epidermoid cysts, desmoid tumors, and osteomas (most often in the face).

About 85% of **sporadic** colorectal tumors (Fig. 8.17) have lost the function of APC (as in FAP). Most sporadic colorectal tumors have ~60 mutations that affect the amino acid sequence of a protein. Most of these colorectal tumors have also lost the function of p53 (see Fig. 8.4), and ~40% of the tumors show an increased activity of KRAS (see Fig. 8.3).

Approximately 1% of patients with colorectal cancer have the heritable disorder **MAP** caused by inherited homozygosity or compound heterozygosity for a loss-of-function mutation in the MUTYH gene. This is hereditary cancer syndrome is unusual in that it shows an autosomal recessive pattern of inheritance. The MUTYH gene encodes the **DNA MYH glycosylase,** which is one of the many DNA glycosylases that contribute to BER of DNA (see Section 1 in Chapter 2). The phenotype of MAP is generally milder than that of FAP, although there is great variability.

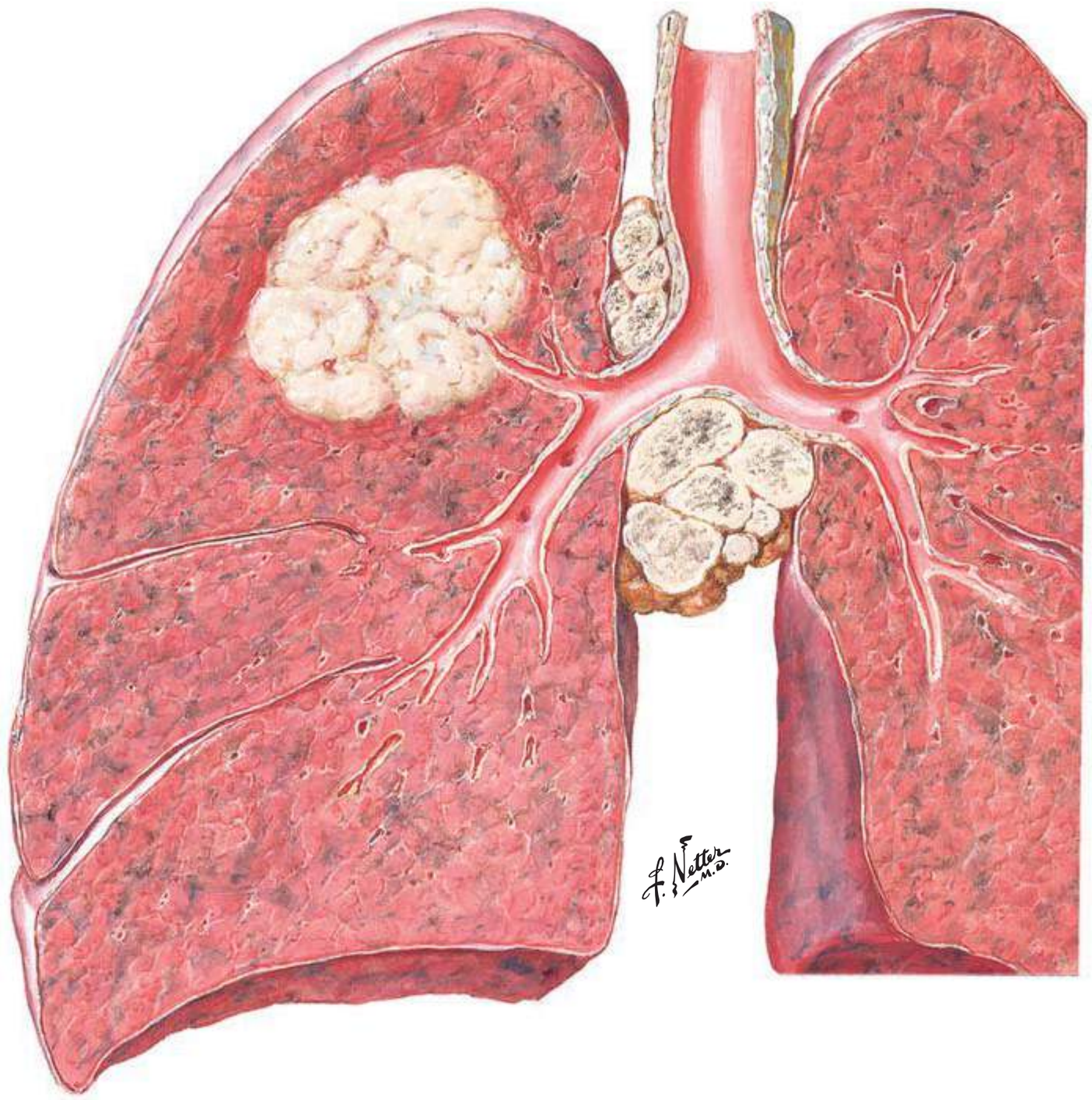


Fig. 8.14 **Large-cell anaplastic carcinoma of the lung.**

Lynch syndrome (**hereditary nonpolyposis colon cancer**) is caused by a problem with DNA mismatch repair (see Section 2 in Chapter 2). T is hereditary cancer syndrome accounts for ~3% of all colorectal tumors and is thus considerably more common than FAP. About 70% of patients who have Lynch syndrome inherit a mutant MLH1 or MSH2 allele, and the remainder have mutant alleles of the PMS2 or MSH6 genes (see Table 2.1 in Chapter 2). With time, some somatic cells, for instance in the colon, acquire a genetic alteration that leads to loss of the remaining functional allele, which impairs mismatch repair. T is leads to replication errors throughout the genome, but especially in **short tandem repeats** (**microsatellites**; e.g., A_{26} or C_8).

Besides colorectal cancer (see Fig. 2.5), patients with Lynch syndrome also have a much higher risk of developing other cancers, notably cancer of the endometrium, upper urinary tract, stomach, and small intestine.

About 15% of **sporadic** colon cancers show impaired mismatch repair (as in Lynch syndrome) and as a result contain ~700 mutations that change an amino acid sequence (this is ~10 times the number of mutations in mismatch repair competent cells; see earlier discussion). T is type of sporadic colon cancer, as well as colorectal tumors in patients with Lynch syndrome, is sometimes called the **mutator phenotype**. Besides the loss of mismatch repair, WNT signaling (see Fig. 8.5) is almost always activated (mostly via loss of APC function), and the RAS/RAF/ERK pathway (see Fig. 8.3) is overly active (mostly due to activating mutations in the BRAF, KRAS, and NRAS proto-oncogenes).

Screening for defective mismatch repair is typically performed with **microsatellite analysis** or **immunohistochemical staining** for mismatch repair proteins, as described in Section 2 in Chapter 2.

Massive parallel sequencing (see Chapter 4) of a panel of genes known to cause hereditary colon cancer is now an option in genetic counseling.

Systemic treatment for metastatic colorectal cancer typically involves a cocktail of chemotherapeutic drugs, such as **fluorouracil** (see Fig. 37.7 and Section 5.1 in Chapter 37), **leucovorin** (to amplify the ef ect of fluorouracil; see Fig. 37.7 and Section 5.1 in Chapter 37), and either the platinum drug **oxaliplatin** (a regimen known as FOLFOX; see also Fig. 2.8 and Section 3 in Chapter 2) or the topoisomerase I inhibitor **irinotecan** (a regimen known as FOLFIRI; see Section 5 in Chapter 1). Patients whose tumors have a mismatch repair deficiency are not treated with fluorouracil because of a lack of benefit (the mechanism is unclear).

T erapy of metastatic colon cancer is often amplified with drugs that target the **EGF receptor** or vascular endothelial growth factor **(VEGF)** signaling. VEGF leads to growth of

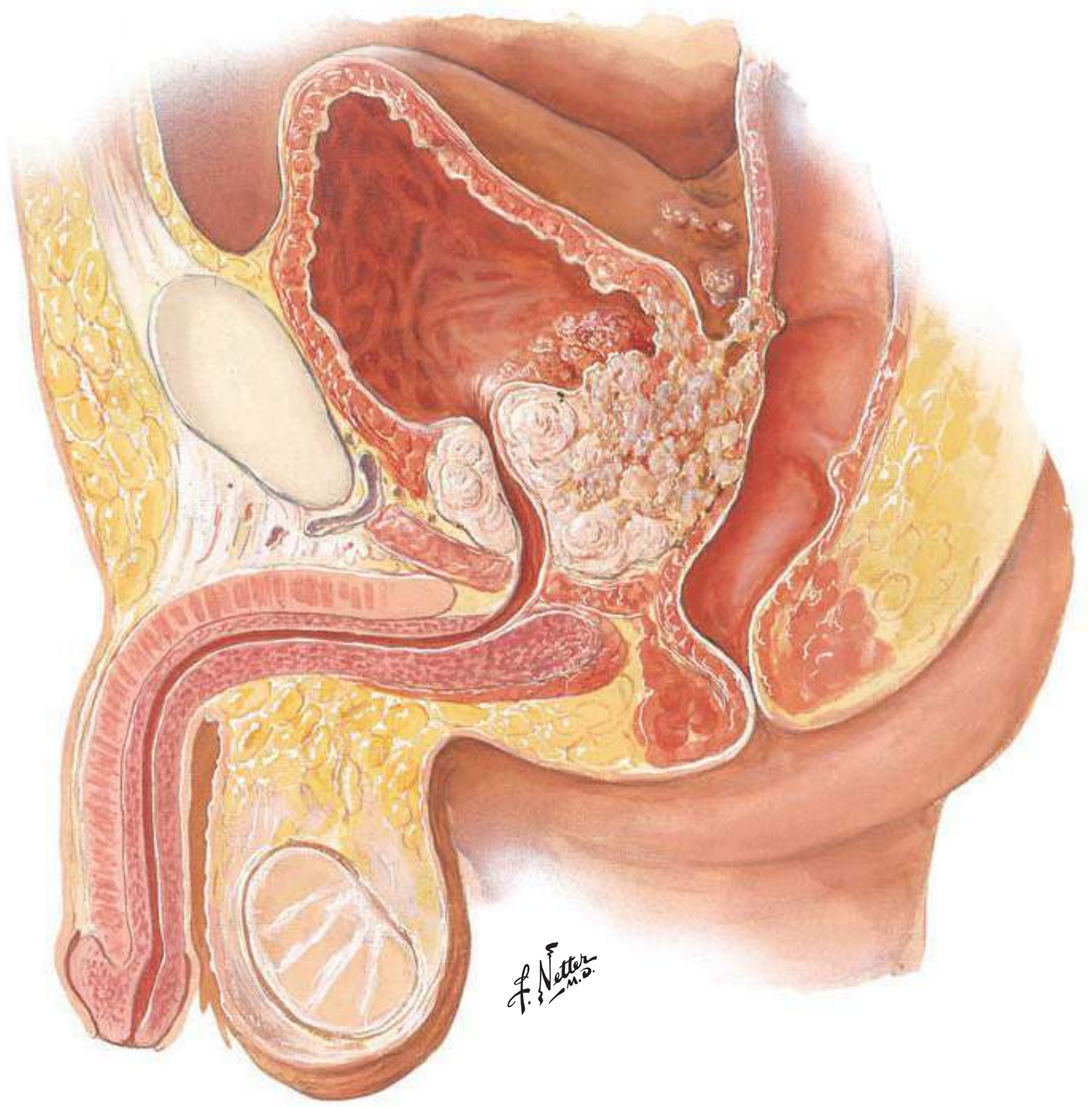


Fig. 8.15 **Prostate cancer.**

new blood vessels, such as into tumor tissue. Antibodies that target the EGFR are most effective in the setting of wild-type RAS genes (RAS is downstream of the EGFR; see Fig. 8.3).

3.5. Melanoma

In the United States, a person's lifetime risk for melanoma of the skin is ~2%.

Melanoma of the skin originates from **melanocytes** in the epidermis, which produce melanin pigments (see also Figs. 35.14 and 35.15). Melanomas (Fig. 8.18) are often recognized by their irregular borders, size (>6 mm in diameter), varied color, and change in appearance over time.

UV radiation, low-level skin **pigmentation, freckling**, and a large number of **nevi** are significant risk factors for cutaneous malignant melanoma. Clothing and sunscreen can significantly attenuate outdoor UV irradiation and reduce melanoma risk, as can self-examination of the skin for lesions.

Melanomas are classified on the basis of their shape, size, and invasion of other tissue layers. Locally confined melanomas are readily amenable to surgery, whereas highly metastasized melanomas have a poor prognosis.

Many patients with metastatic melanoma are treated with **immunotherapy**. Pharmacological immunotherapy favors the recognition of melanoma cells by T cells, followed by the destruction of tumor cells.

About 35% of patients with melanoma have a $BRAF^{V600}$ **mutation** that activates BRAF constitutively. **Vemurafenib** and **dabrafenib** inhibit $BRAF^{V600}$ and generally cause shrinkage of tumors, but this is unfortunately followed by relapse due to drug resistance. Similarly, the drug **trametinib**, which inhibits **MAP/ERK kinase** (downstream of BRAF; see Fig. 8.3), is initially effective but eventually becomes ineffective. A longer period of remission is obtained with the combination of a BRAF inhibitor and an ERK inhibitor.

About 25% of melanomas contain an activating mutation in the NRAS gene (NRAS is upstream of BRAF in the RTK/RAS/RAF/ERK pathway; see Fig. 8.3). Currently no approved drugs that inhibit mutant NRAS are available. Inhibition of the downstream kinase BRAF is (surprisingly) counterproductive.

Familial melanoma is a hereditary cancer syndrome that in ~50% of patients is due to an inherited mutant CDKN2A gene, which encodes **$p16^{Ink4a}$** and p14ARF. The prevalence of familial melanoma is ~4%. $p16^{Ink4a}$ normally inhibits cyclin D/CDK4 and cyclin D/CDK6 (see Figs. 8.1 and 8.2). $p14^{ARF}$ normally inactivates MDM2 and thus stabilizes p53 (see Fig. 8.4). Most pathogenic mutations of the CDKN2A gene lead to loss of only $p16^{Ink4a}$ function, but patients with these mutations also have an increased risk for **pancreatic cancer**.

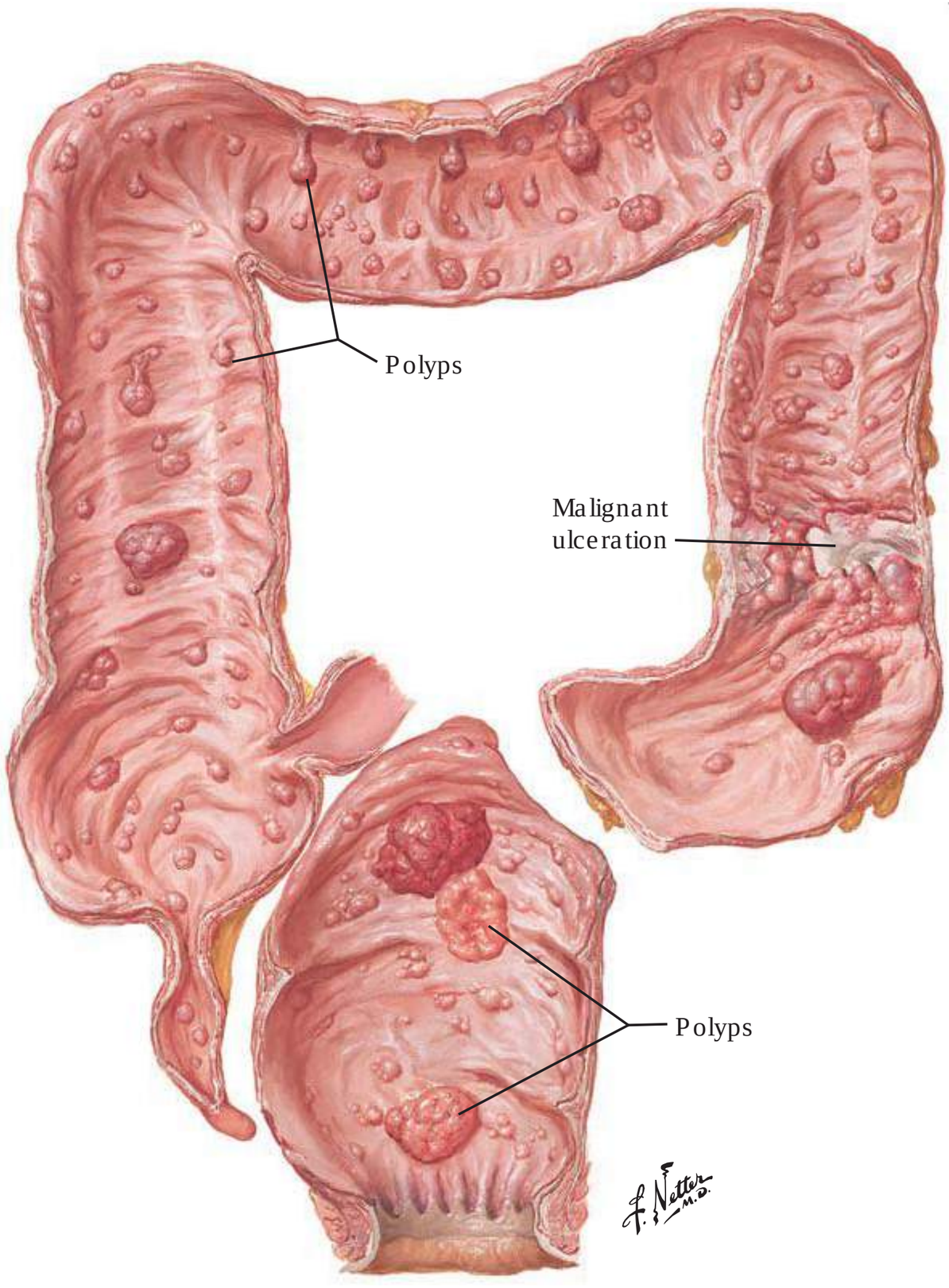


Fig. 8.16 **Familial adenomatous polyposis of the large intestine.**

4. GLUCOSE USE BY TUMORS

*T*umors use considerably more glucose than normal cells. Positron emission tomography (PE*T*) scanning o*f* patients a*ft*er the in*f*usion o*f* a radioactive glucose analog helps locate metastases.

Even well-oxygenated tumor cells generate comparable amounts of adenosine triphosphate (ATP) via anaerobic and aerobic glycolysis, whereas normal cells produce almost all of their ATP via aerobic glycolysis. T is observation is commonly referred to as the **Warburg ef ect**. No widely accepted explanation is yet found for the Warburg ef ect.

PET with radioactively labeled **2-fluoro-deoxyglucose** (2FDG) takes advantage of the increased glucose use of tumor cells (Fig. 8.19; 2FDG uptake is described in Section 6.3 in Chapter 19). PET studies are commonly used to reveal metastases.

SUMMARY

- Quiescent cells are in the G0 phase. T e activation of growth factor receptors induces a transition from the G0 to the G1 phase via synthesis of cyclin D, activation of CDK4 and CDK6, and phosphorylation of RB, which in turn releases E2Fs that alter transcription such that more cyclin D and E are expressed.
- p52 becomes active in response to phosphorylation by the kinases ATM, ATR, CHK1, or CHK2, which in turn become active in response to DNA damage. Activated p52 acts as a transcription factor that increases the transcription of genes that encode the CDK inhibitor protein (CKI) p21 and the proapoptotic BAX.
- WNT signaling via Frizzled receptors leads to an increase in the concentration of β-catenin, which in turn activates transcription factors of the TCF/LEF family. T e TCF/LEF transcription factors enhance the transcription of the MYC gene and many other genes. MYC in turn is a transcription factor that af ects the transcription of a large number of genes.
- About half of the patients with hereditary breast and ovarian cancer syndrome have inherited a defective BRCA1 or BRCA2 allele. Genetic alterations in cells of the breast and in other tissues that lead to the loss of function of the remaining BRCA allele are tumorigenic. T rough

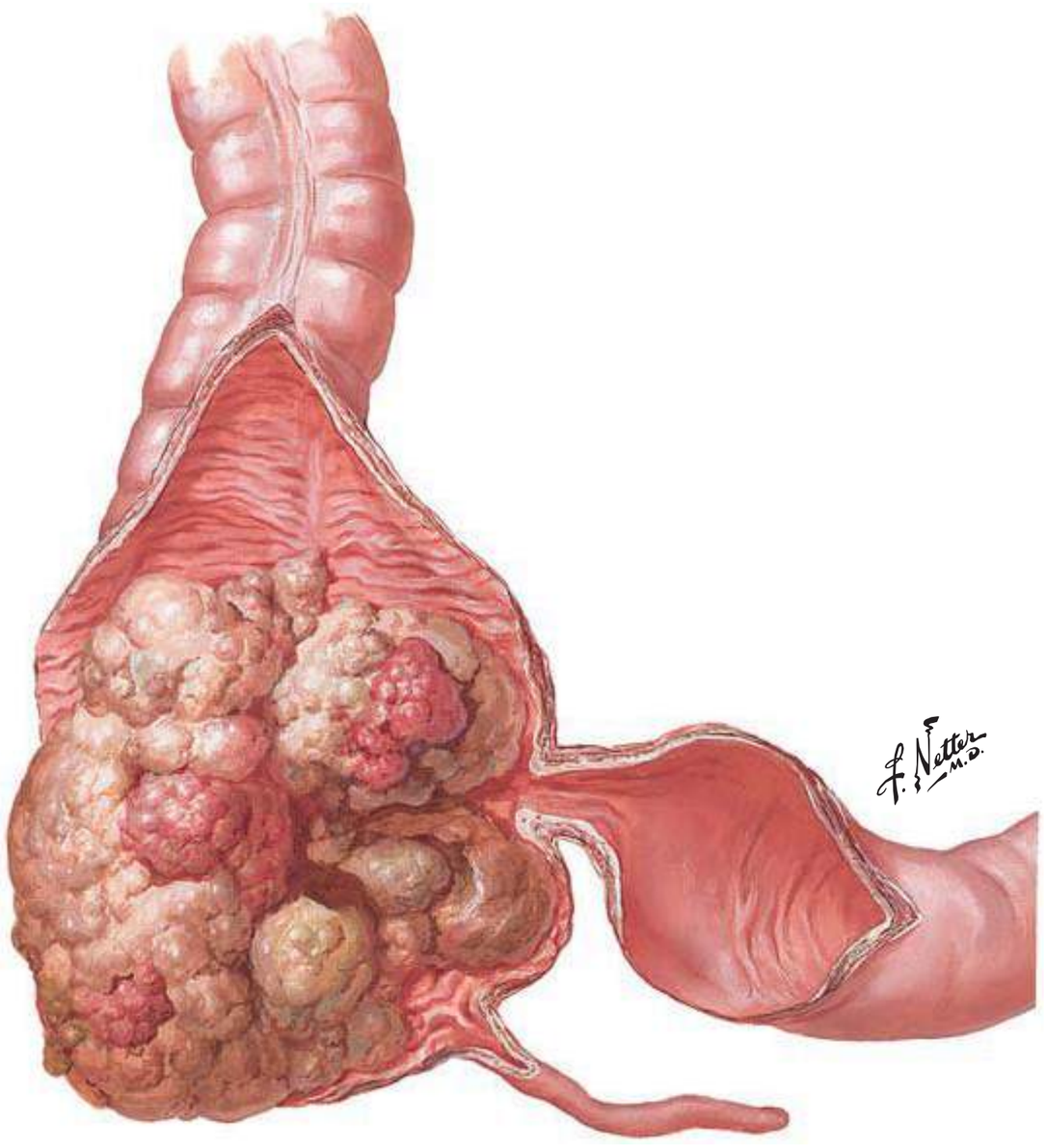


Fig. 8.17 **Carcinoma of the cecum.**

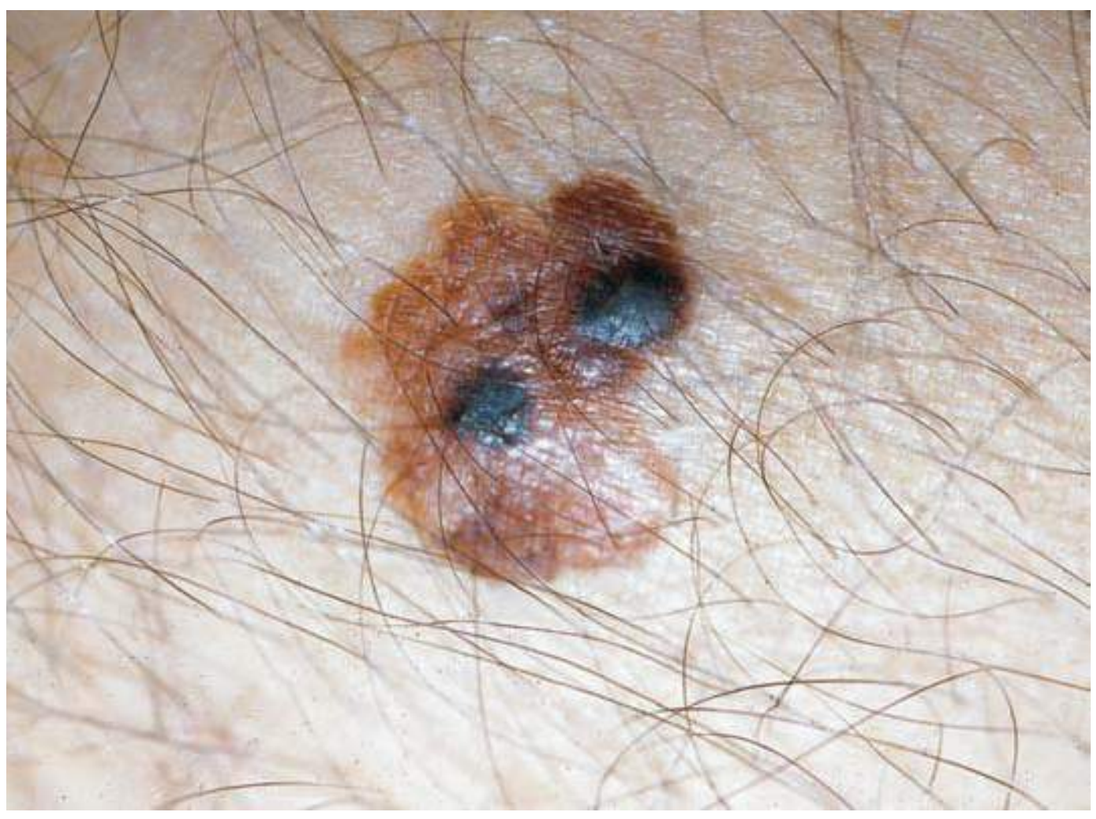


Fig. 8.18 **Melanoma.**

synthetic lethality, PARP inhibitors are especially toxic to tumor cells that lack functional BRCA1 or BRCA2.

- Lung cancers of smokers contain many more mutations than cancers of nonsmokers. Small-cell lung cancer develops mostly in smokers, is treated with platinum drugs, and is associated with a low rate of survival. Squamous cell carcinomas are also related to smoking and frequently show mutations in *T*P53 and CDKN2A. Lung adenocarcinomas (ACs), which occur in both smokers and nonsmokers, are genetically very diverse but often contain mutations in EGFR, KRAS, *T*P53, and CDKN2A.

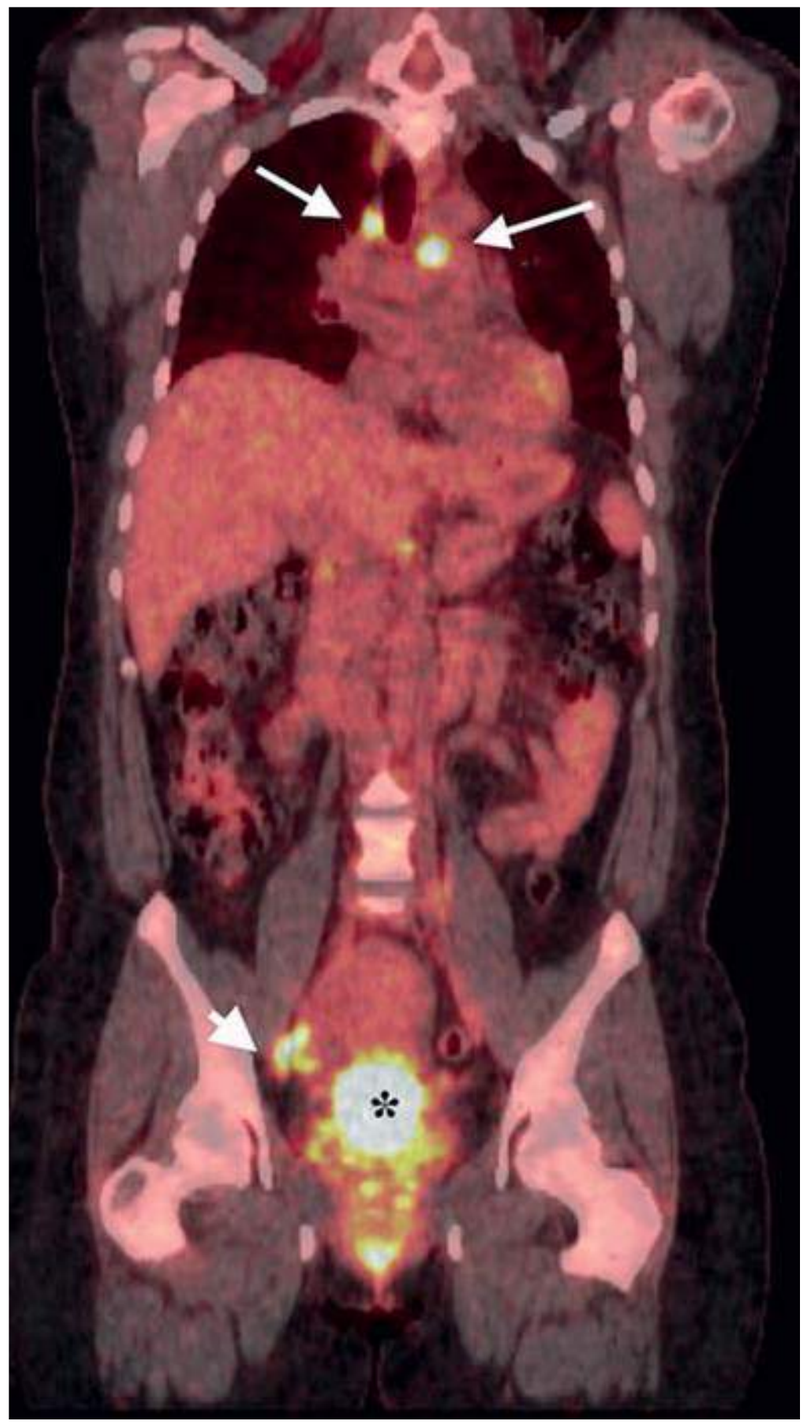


Fig. 8.19 **A coronal fused image of 2-^{18}F-deoxyglucose–based positron emission tomography and computed tomography (obtained with x-rays) of a patient with squamous cell carcinoma of the cervix.** The *asterisk* marks the primary tumor. The *short arrow* indicates a nearby adenoma, and the *long arrows* indicate lymph nodes in the mediastinum. (From Viswanathan C, Bhosale PR, Shah SN, Vikram R. Positron emission tomography-computed tomography imaging for malignancies in women. *Radiol Clin North Am*. 2013;51:1111-1125.)

- Tumors of the prostate usually contain a large number of translocations, and their growth is driven by androgens. T e tumors are genetically highly diverse. Pharmacological treatment of advanced prostate cancer often includes androgen deprivation and taxanes (inhibitors of microtubule depolymerization).
- Familial adenomatous polyposis (FAP) is caused by heterozygosity for a loss-of-function APC allele. APC plays a role in degrading β-catenin in the cytosol. Somatic loss of the second, functional APC allele is tumorigenic. Af ected patients have hundreds to thousands of polyps in their colon and develop colon cancer unless they have a colectomy. Most patients with sporadic colorectal cancer also have lost the function of their APC protein.
- MU*T*YH-associated polyposis (MAP) is due to an autosomal recessively inherited loss of DNA MYH glycosylase activity and resembles an attenuated form of FAP.
- Lynch syndrome is caused by heterozygosity for a loss-of-function allele for one of the mismatch repair proteins

(most often MLH1 or MSH2). Somatic loss of the second, functional allele is tumorigenic. T is causes microsatellite instability. A minority of patients with sporadic colorectal cancer also have lost the function of the mismatch repair pathway. Fluorouracil is inef ective in these patients.

- T e risk for malignant melanoma of the skin rises with freckling, number of nevi, and exposure to sunlight. Furthermore, ~2% of the population has a mutation in the CDKN2A gene that causes familial melanoma because of the loss of $p16^{Ink4a}$ function; p16 normally inhibits cyclin D–activated CDK4 and CDK6. Patients who are heterozygous for loss of p16 are also at an increased risk of pancreatic cancer. About one-third of patients with sporadic melanoma have constitutively active V600E mutant BRAF, and metastases of such tumors are best treated with a combination of a BRAF inhibitor and a MEK inhibitor.
- Tumor cells often use more glucose than normal cells, and 2-18F-deoxyglucose-based PET is used to locate metastases.

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Review Questions

1. Which one of the following alterations of the genome most favors a neoplasm?

   A. A mutation that gives rise to E2F that has increased affinity for RB
   B. Amplification of the CCND1 gene, which encodes cyclin D1
   C. Amplification of the CDKN2A gene
   D. Amplification of the *T*P53 gene
   E. Loss of the MDM2 gene

2. A 50-year-old patient with cancer of the colon and which one of the following characteristics is most likely to have Lynch syndrome?

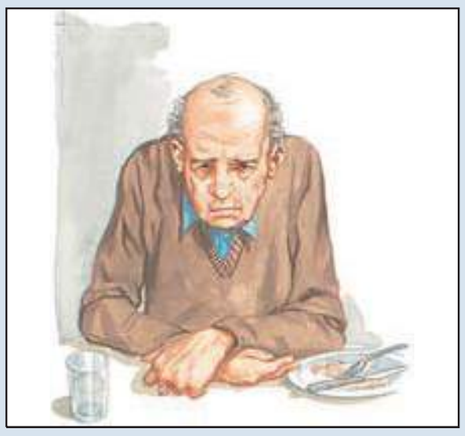
   A. Colon tumor that does not stain for MLH1
   B. Colon tumor that shows microsatellite instability
   C. Has a grandfather who died of colon cancer at age 70 years
   D. Heterozygosity for a loss-of-function mutation in the MSH2 gene in blood lymphocytes

3. Cowden syndrome is a heritable disorder that is caused by which of the following?

   A. Amplification of MYC
   B. Heterozygous deletion of CDKN2A
   C. Heterozygous loss-of-function mutation in P*T*EN
   D. Methylation of RB1 promoter
   E. Methylation of *T*P53 promoter

4. A 50-year-old patient underwent colonoscopy for the first time and was found to have colon cancer, as well as ~100 adenomas in the colon. Neither the parents nor the grandparents of the patient ever had colon cancer. T e most likely diagnosis for this patient is which of the following?

   A. FAP
   B. Lynch syndrome
   C. MU*T*YH-associated polyposis
   D. Sporadic colorectal cancer



Chapter

9 Structure of Proteins and Protein Aggregates in Degenerative Diseases

SYNOPSIS

- Proteins are made of linear chains of amino acids. During translation, 21 different amino acids can be incorporated into the nascent peptide chain. Many of these amino acids can be modified after translation.
- The sequence, posttranslational modification, and diversity of chemical properties of amino acids give rise to an amazing variety of proteins.
- Proteins are held in their three-dimensional shape through numerous means, such as hydrogen bonds, hydrophobic effects, electrostatic interactions, van der Waals interactions, and chemical crosslinks (e.g., disulfide bridges).
- The three-dimensional structures of proteins contain common elements; two such abundant elements are the α-helix and the β-sheet.
- In the course of evolution, mixing and matching let many motifs (e.g., a sequence of amino acids that binds a nucleotide) become part of proteins that now serve diverse functions.
- Proteins often contain domains that have a defined structure, regardless of flanking sequences. Each one of these domains has a function (e.g., binding DNA or holding and enclosing a substrate in an enzyme).
- Proteins fold during and after synthesis, sometimes with the assistance of chaperone proteins.
- The physiological, three-dimensional structure of proteins is only marginally stable. When this structure is lost, the protein no longer has the same function, and it is said to be denatured. By themselves, many denatured proteins cannot regain their original physiological shape.
- Some proteins or portions of proteins normally have no discernible higher-order structure.
- Denaturation of proteins in the stomach helps their digestion in the stomach and intestine, and it also kills pathogens. Similarly, in health care, denaturation and modification of proteins are used extensively to destroy pathogens.
- Pathologic intracellular or extracellular aggregation of proteins is found in a number of diseases, for instance in Alzheimer disease, Parkinson disease, and amyloidosis due to chronic dialysis.

LEARNING OBJECTIVES

For mastery *of* this topic, you should be able to do the *following*:

- Explain the forces that hold most proteins in their native shape.
- Draw a standard cartoon of an α-helix, a parallel β-sheet, and an antiparallel β-sheet. Indicate the location of the amino acid side chains relative to these images.
- Describe the forces and secondary structures that favor aggregation of proteins.
- Describe the changes in protein structure that occur when proteins are denatured.
- Describe the effects of commonly used disinfectants (e.g., iodine, alcohols, hydrogen peroxide, and heat) on proteins.
- Describe the nature and basic structure of amyloid, and indicate techniques to make amyloid visible.

1. AMINO ACIDS AS BUILDING BLOCKS OF PEPTIDES AND PROTEINS

Amino acids consist *of* a constant NH_3^+–CH–COO^- group and a variable side chain. T e diversity *of* chemical properties *of* side chains and the orientation *of* amino acids in space give rise to a great range *of functions of* peptides and proteins. Amino acids can be ordered into groups, the most important *of* which are nonpolar, polar uncharged, and polar charged.

1.1. General Comments About Amino Acids

Peptides, polypeptides, and proteins are polymers of amino acids, in which the amino acids are linked via peptide bonds (see Section 2). A dipeptide consists of two amino acids, a tripeptide of three, a tetrapeptide of four, etc. T e name **peptide** is commonly used for polymers that contain fewer than 50 amino acids, and the name **protein** is used for polymers that contain 50 or more amino acids. T e term **polypeptide** is used for peptides or proteins that contain more than ~15 amino acids. However, there are no generally agreed-upon definitions.

Amino acids have the general structure H_2N–CH(R)–COOH; R represents the so-called **side chain** (Fig. 9.1). Different amino acids have dif erent side chains. In solution, at a neutral pH, H_2N– becomes protonated to H_3N^+–, and –COOH is deprotonated to $–COO^-$ (see the discussion of charged amino acids below). In peptides and proteins, the structure H_2N–CH–COOH becomes part of the peptide backbone.

T ere are L- and D-isomers of chiral amino acids (see Fig. 9.1). Glycine is not chiral, because R=H so that the central C-atom has two identical substituents. T e "central" C-atom (called C_α) of the other amino acids has four dif erent substituents and is chiral. In the human body, practically all amino acids in peptides and proteins are of the **L-isomer**. In humans, the D-isomer has been found only in free amino acids to date (e.g., D-serine and D-aspartate, which play a role in signaling in the nervous system).

Messenger RNA encodes the synthesis of peptides and proteins from 21 dif erent amino acids. T e names of these amino acids, as well as their three- and one-letter abbreviations, are listed in Table 9.1. T e DNA trinucleotide sequences that code for these amino acids are shown in Table 8.1. **Selenocysteine** is encoded by a combination of a UGA stop codon and a

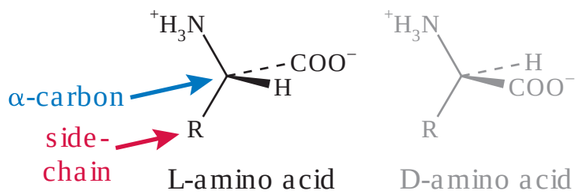


Fig. 9.1 **L- and D-amino acids.**

Table 9.1 **Names and Abbreviations of the Genetically Encoded Amino Acids**

1-Letter Abbreviation	Full Name	3-Letter Abbreviation
A	Alanine	Ala
C	Cysteine	Cys
D	Aspartate	Asp
E	Glutamate	Glu
F	Phenylalanine	Phe
G	Glycine	Gly
H	Histidine	His
I	Isoleucine	Ile
K	Lysine	Lys
L	Leucine	Leu
M	Methionine	Met
N	Asparagine	Asn
P	Proline	Pro
Q	Glutamine	Gln
R	Arginine	Arg
S	Serine	Ser
T	Threonine	Thr
U	Selenocysteine	Sec
V	Valine	Val
W	Tryptophan	Trp
Y	Tyrosine	Tyr

selenocysteine insertion sequence (SECIS) in the 3′-UTR of an mRNA. Examples of proteins that contain selenocysteine are glutathione peroxidase (see Chapter 21) and thioredoxin reductase (see Chapter 37).

1.2. Classification of Amino Acids

Fig. 9.2 shows the structures of the genetically encoded L-amino acids. Dif erences in the structure are confined to the side chain, except for proline, which also contains a bond between the main-chain amino group and the side chain (strictly speaking, it is therefore an imino acid).

T e group of **nonpolar amino acids** contains a variety of subgroups. **Glycine** is the smallest of all amino acids, as its side chain is –H. Glycine provides the greatest flexibility for the three-dimensional course of a protein backbone. **Aliphatic** amino acids (**alanine**, **valine**, **leucine**, **isoleucine**) are progressively more hydrophobic as the nonpolar surface area of their side chain increases. Aliphatic amino acid side chains commonly participate in hydrophobic ef ects and van der Waals interactions (see Section 3). T ese occur for instance within the protein, between membrane proteins and membrane lipids, between proteins and organic compounds that are important to protein function, and between enzymes and substrates. Amino acids with **sulfur** in their side chain include **methionine** and **cysteine**. Methionine is commonly the first (i.e., N-terminal) amino acid of a newly synthesized protein, but this residue is often removed during posttranslational processing. Two cysteine side chains can become oxidized to form a **disulfide bond** (–S–S–, also called a **disulfide bridge**). Such disulfide bonds are often an important determinant of the final structure of a protein. **Phenylalanine** and **tryptophan** are nonpolar amino acids that have hydrophobic **aromatic** side chains. T e aromatic side chains need a relatively large amount of space. T e benzene rings of Phe and Trp can hold a small ion or a methyl group or benzene ring of another molecule in place. T e side chain of **proline** is linked to the N-atom of the invariant NH_2^+–CH–COO^- group, creating a ring, which reduces the flexibility of the NH_2^+–CH–COO^- group and imposes additional steric constraints on the peptide backbone. For this reason, as detailed in Section 4.1, proline induces a kink into α-helices. On the other hand, proline, like glycine, can help form a sharp turn of the peptide chain, and it is therefore often found in such turns (see Section 4). **Selenocysteine** is found in a few enzymes that use this residue near their catalytic site. Examples are given above.

T e group of **polar, uncharged amino acids** includes the amino acids that have either a hydroxyl group or an amido group in their side chain. **Tyrosine**, like phenylalanine and tryptophan mentioned above, has an aromatic side chain, but its side chain is also polar due to the hydroxyl group. T e hydroxyl group can be phosphorylated or sulfated. Phosphorylation of tyrosine plays a prominent role in growth factor signaling (see Chapters 26 and 33). **Serine** and **threonine** also carry a hydroxyl group on their side chain, and this hydroxyl group can be conjugated with a phosphate, sulfate, or a polysaccharide. Phosphorylation of Ser or T r is a widespread regulatory means of altering the properties of a protein. **Glutamine** and **asparagine** contain amido groups (–CO–NH_2). T e NH_2 group can donate one or two hydrogen atoms while the C=O group can accept one or two hydrogen atoms to form one or more hydrogen bonds (see Section 3.2). T ese side chains are very hydrophilic because they can form hydrogen bonds with water.

T e group of **polar, charged amino acids** includes aspartate, glutamate, lysine, arginine, and histidine. T e side chains

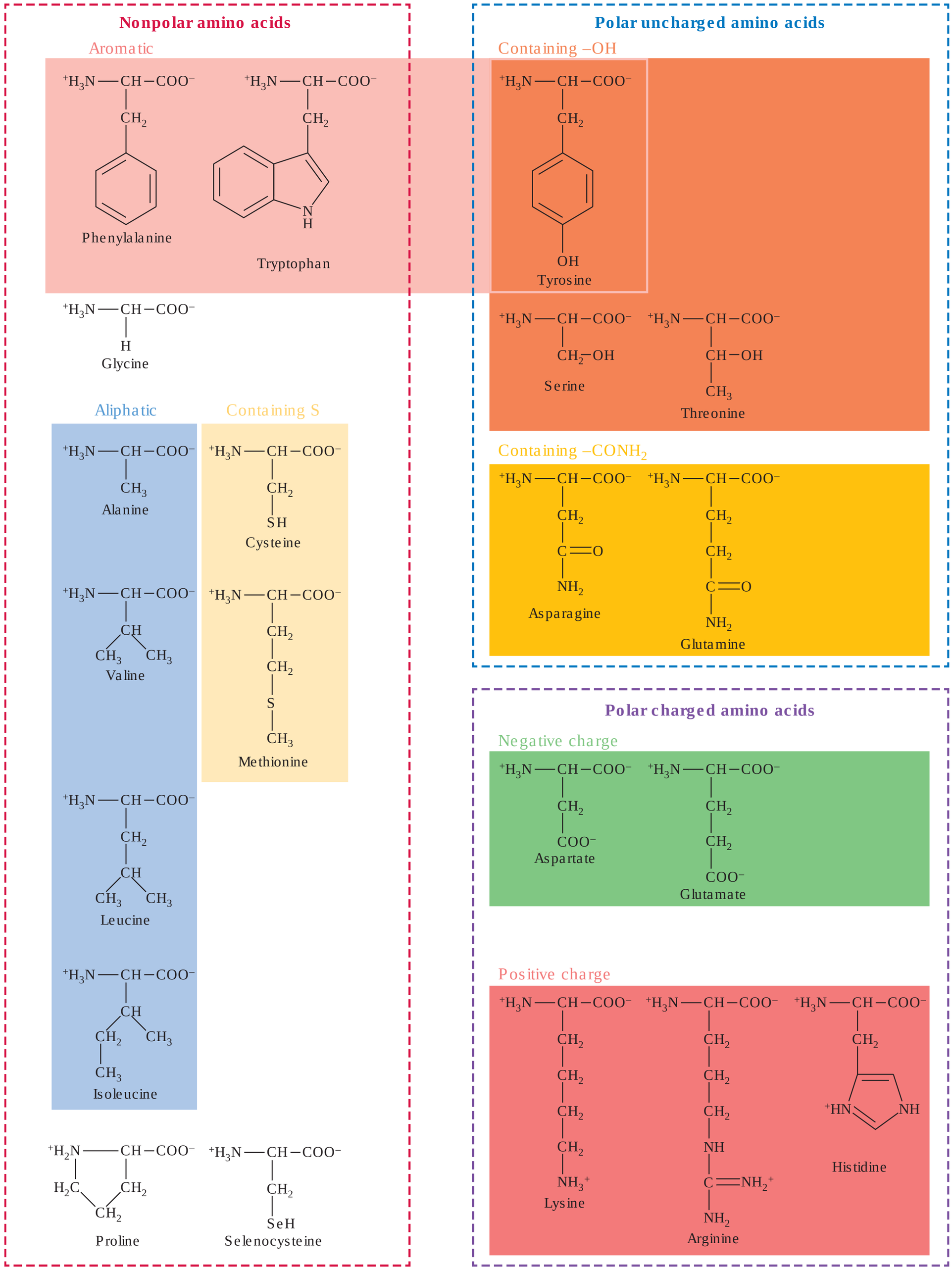


Fig. 9.2 **Structures of the amino acids that ribosomes incorporate into nascent peptide chains.**

of **aspartate** and **glutamate** end in $–COO^-$. The pK for the carboxyl group (–COOH) is in the range of 4 to 5. At a pH larger than the pK, most of the carboxyl groups lose a proton and are **negatively** charged ($–COO^-$). The negatively charged carboxyl groups of glutamate and aspartate often interact electrostatically with other groups. In addition, the carboxyl group also forms hydrogen bonds. Glutamate and aspartate are frequently found near the catalytic centers of enzymes or at sites of protein-protein interaction. **Lysine** and **arginine** have **positively** charged side chains. The pK for the amino group ($–NH_2$) of lysine is ~10, and the pK for =NH in the guanidino group [$–NH–C(NH)–NH_2$] of arginine is ~12. At a pH lower than these pK values, most of the amino and guanidino groups gain a proton and are positively charged ($–NH_3^+$, $–NH–C(NH_2^+)–NH_2$), as shown in Fig. 9.2. The side chains of lysine and arginine (like those of glutamate and aspartate mentioned above) form electrostatic interactions as well as hydrogen bonds. In DNA-binding proteins, lysine and arginine frequently coordinate the negatively charged phosphate moieties of the deoxyribonucleotides. In enzymes that require the prosthetic groups biotin or lipoic acid, the prosthetic group is covalently linked to the side chain amino group of a lysine near the catalytic site (see Chapters 10 and 22). In histones, the side chain of lysine is often modified by methyl or acetyl groups; these modifications affect DNA compaction and transcription (see Chapters 1 and 6). At a pH lower than ~6 to 7 (depending on the exact environment in a particular protein), most of the side chains of **histidine** are also positively charged. Histidine is often the donor and acceptor of H^+ in the active site of enzymes that catalyze H^+ transfer.

2. PEPTIDE BONDS, DISULFIDE BRIDGES, AND CROSSLINKS

A peptide bond covalently links the carboxyl group *of* one amino acid with the amino group *of* another amino acid, thus giving rise to peptides and proteins. The peptide bond (C–N in –CO–NH–) has partial double-bond character, thereby limiting the conformational *flexibility of* peptides and proteins. Peptides or proteins can be linked by disulfide bridges or crosslinks.

2.1. Peptide Bonds

In peptides, the carboxyl group of one amino acid (shown in black in Fig. 9.3) is linked to the amino group of another amino acid (shown in blue) via a **peptide bond** (an amide bond). The **peptide backbone** of peptides or proteins refers to the peptide-linked, repeated sequence –N–CH–CO– that is common to all amino acids.

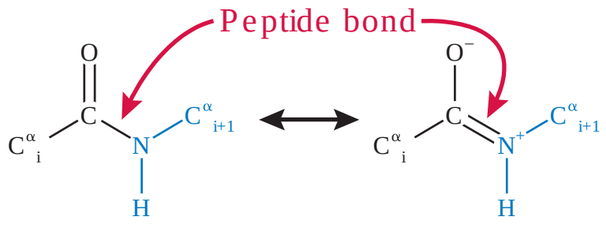


Fig. 9.3 Nature of the peptide bond. The peptide bond is shown in the trans configuration, which is the most common. A cis-configuration (180-degree rotation around C-N) occurs mostly with proline.

The peptide bond (C–N in –CO–NH–) has partial double-bond character and cannot rotate freely (see Fig. 9.3); the other bonds in the peptide backbone, N–C_α and C_α–C, are true single bonds, but their rotation is limited by **steric hindrance** (i.e., atoms are not allowed to encroach on each other's space). Nonetheless, there is sufficient flexibility that the polypeptide chain can assume a huge variety of conformations, anywhere from an unlikely, fully extended conformation to the common, folded, functional, energetically favorable, and compact structure (for protein folding, see Section 4).

Amino acids with small side chains (e.g., glycine) afford the peptide backbone greater conformational flexibility than amino acids with bulky side chains (e.g., tryptophan). Proline, which has a ring-locked imino group instead of a rotatable amino group, locally constrains the backbone to an especially limited range of conformations.

The **primary structure** of a peptide or protein is the linear **sequence** of amino acid residues from the N- to the C-terminus. At the **N-terminus**, a newly synthesized protein has an amino [$^+H_3N–$] group, and at the **C-terminus,** a carboxyl [$–COO^-$] group. As described in Chapter 1, a DNA sequence is written 5′→ 3′. The N-terminal amino acids of a protein are encoded on the 5′-side of the coding strand of a gene and the 5′-side of an mRNA (see Chapters 6 and 7). When ribosomes synthesize a protein, they start with the N-terminal amino acid.

2.2. Disulfide Bridges and Other Crosslinks

The side chain –S–H groups of two cysteine residues can be oxidized to form an –S–S– bond, which is called a **disulfide bridge** or a **disulfide bond**. A disulfide bridge can form both within a single polypeptide (e.g., human serum albumin; see Fig. 9.6), and also between subunits of a protein complex (e.g., between the subunits of the insulin receptor).

The side chains of lysine can be oxidized and then **crosslinked**; this happens with collagen and elastin in the extracellular matrix, for example, and gives rise to large and tough structures (see Chapters 12 and 13).

Besides an occasional covalent crosslinkage (sometimes between protein subunits), the three-dimensional structure of a folded, native protein is stabilized by scores of noncovalent interactions between atoms (see Sections 3 and 4).

3. FORCES THAT DETERMINE THE CONFORMATION OF PROTEINS AND PEPTIDES

Numerous interactions between atoms stabilize the three-dimensional structure *of* peptides and proteins. These interactions consist *of* hydrogen bonds, hydrophobic *effects*, electrostatic interactions, and van der Waals interactions.

3.1. Hydrophobic Effects

When hydrophobic molecules enter water, they disturb the normal, hydrogen-bonded structure of water. The tendency of

the overall system to assume the lowest possible energy state makes the hydrophobic molecules stick together when they face water. T is allows for maximal hydrogen bonding among water molecules. (Likewise, a few drops of cooking oil in water tend to coalesce into a single puddle.)

Hydrophobic ef ects amount to ~90% of the forces that keep a protein in the shape of a globule. T e remaining drive toward the normal physiological shape of a protein derives from hydrogen bonding, ionic interactions, and van der Waals forces (see below).

Hydrophobic ef ects are an important driving force not only for the conformation of each single protein but also for the interaction of proteins with other molecules. For instance, proteins can interact with other proteins via **leucine zippers**, which are often found in transcription factors (Fig. 6.6). A protein can be anchored in a lipid membrane by hydrophobic ef ects between amino acid side chains and fatty acid tails or cholesterol in the membrane. In proteins that are enzymes, hydrophobic amino acid side chains often form a water-free environment for the chemical reaction that the enzyme catalyzes. In yet other proteins, hydrophobic ef ects often contribute substantially to the binding of organic compounds, thereby influencing their characteristics (e.g., heme in hemoglobin; see Chapter 14).

Approximately equal amounts of hydrophobic amino acids are generally found on the surface and in the interior of proteins. It is often erroneously suggested that hydrophobic amino acids are found almost exclusively in the interior of proteins. However, even the partial burying of hydrophobic side chains is energetically favorable, and therefore hydrophobic side chains on the protein surface help a protein assume a compact, globular shape. Furthermore, the chemical properties of many hydrophobic amino acid side chains are quite complex. T us, the side chains of tryptophan and tyrosine have both a hydrophobic portion (the benzene ring) and a hydrophilic portion (the >N–H in the second ring of tryptophan, and the –OH on the benzene ring of tyrosine) that can participate in hydrogen bonding (>NH and –OH are hydrogen donors; –O– is a hydrogen acceptor). Hence, tryptophan and tyrosine are often found at the interface between the hydrophobic portion of the membrane lipid bilayer and the more hydrophilic phospholipid head groups (i.e., between the hydrophobic tails of the fatty acids in phospholipids and the carbonyl groups that are part of the –CO–O– ester link between fatty acids and the glycerol moiety of phospholipids; see Chapter 11).

3.2. Hydrogen Bonds

Hydrogen bonds form when two electronegative atoms each partially bind to a hydrogen atom. To this end, a **hydrogen donor**, such as –OH or –NH, must interact with a **hydrogen acceptor**, such as –O or –N. If a hydrogen bond forms between –N–H and O–, it is often schematically drawn as –N–H O–. Carbon atoms are not sufficiently electronegative to participate in hydrogen bonds.

T e peptide backbone contains hydrogen donors in the –N–H and hydrogen acceptors in the =O of each amide group (–CO–NH–; Fig. 9.4); however, for steric reasons the donor and acceptor must come from dif erent amino acid residues (see Sections 4.1 and 4.2).

T e amino acid side chains contain the hydrogen donors –O–H (in Ser, T r, Tyr), –N–H (in Trp, Asn, Gln, Lys, Arg, His),–S–H (in Cys), and –Se–H (in Sec), as well as the hydrogen acceptors =O (in Asn, Gln, Asp, Glu), –O– (in Ser, T r, Tyr), and –N= (in His). Water can act as both a hydrogen donor and hydrogen acceptor.

Formation of a hydrogen bond generally liberates ~6% as much energy as the formation of a covalent C–C bond. T e reaction of a hydrogen donor with an acceptor within a polypeptide is energetically more favorable than with surrounding water molecules. Hydrogen bonding between atoms of the peptide backbone stabilizes peptide α-helices and β-sheets (see Sections 4.1 and 4.2).

3.3. Electrostatic Interactions

Electrostatic interactions are the result of the attraction of unlike charges and the repulsion of like charges. Atoms of the peptide backbone are essentially uncharged, except for the amino and carboxyl termini. T e amino acid side chains of Asp and Glu are negatively charged (see Fig. 9.2), while those of Lys and Arg (and sometimes His) are positively charged. T e water molecule is a dipole: its O-atom carries approximately two-thirds of a negative charge, and its hydrogen atoms each carry approximately one-third of a positive charge. If a charged amino acid is brought into water, water molecules orient around the amino acid based on charge attraction and repulsion. For instance, if the $–COO^-$ group of a glutamate side chain faces water, water molecules order such that their H atoms are closest to the negative charge of $–COO^-$, while their O-atoms are farthest from the negative charge of $–COO^-$.

Removing a single charge from the protein surface and burying it inside a protein carries a very high energy penalty (~50% as much energy as the formation of a covalent C–C bond). T erefore, almost all charged residues are on the surface of a protein, and if a charged residue is in the protein interior, it almost always interacts with a nearby opposite charge.

Arginine and lysine carry their positively charged groups on the tip of a somewhat hydrophobic side chain. In membrane proteins, much of this chain can be located next to the hydrophobic portion of membrane lipids (see Chapter 11), and the positively charged portion can interact with the negatively charged phosphate groups of phospholipids (fittingly, arginine and lysine are said to “snorkel”).

3.4. Van der Waals Interactions

T e term van der Waals forces is used inconsistently. Originally, the term referred to all attractive forces between molecules that influence the behavior of gasses. Van der Waals forces in the original sense includes interactions between molecules that have a permanent, induced, or temporary **dipole**. A molecule has a dipole when the center of its positive charge

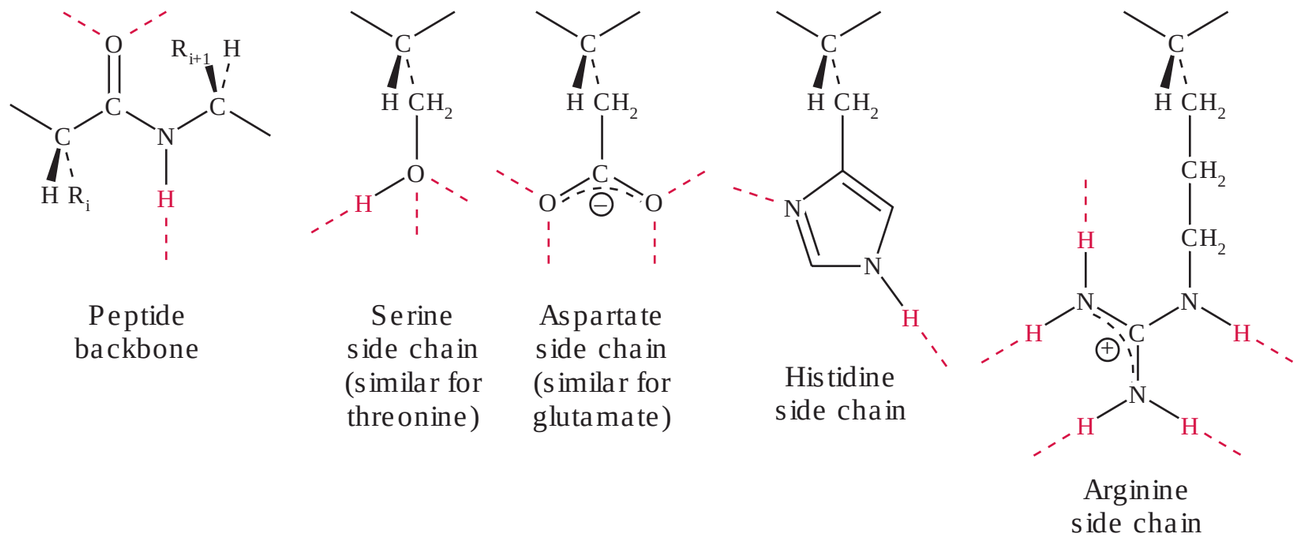


Fig. 9.4 Hydrogen bonding in proteins. The maximum number of hydrogen bonds that can be expected to form are shown in red. In nature, atoms that are shown here to form two hydrogen bonds often form only one hydrogen bond.

dif ers from the center of its negative charge (i.e., typically, when a more electronegative atom attracts electrons more than a less electronegative atom). In this case, the dipole is permanent (e.g., a carbonyl group, which carries a slight excess negative charge on O and a slight excess positive charge on C). A dipole is temporary when it is simply due to the movement of electrons around nuclei (an example would be a molecule of H_2, which has two nuclei and two electrons). A dipole is said to be induced if it occurs in response to the nearby appearance of a charge (e.g., the charge of an amino acid side chain such as glutamate or lysine).

In this book, the term van der Waals interactions is used to describe weak, short-range interactions that result from a combination of the following forces: attraction between the positively charged nucleus of one atom and the negatively charged electrons of another atom, electrostatic interactions between atoms that carry a slight charge due to the presence of a dipole moment, and the repulsion of electrons between filled electron orbitals.

Favorable van der Waals interactions can occur between all types of atoms, are extremely sensitive to the distance between atoms, and require close packing of atoms.

3.5. Coordination of Metal Ions

Metal ions (e.g., Ca^{2+}, Mn^{2+}, Fe^{2+}, Fe^{3+}, Zn^{2+}, or Cu^{2+}) often coordinate with amino acid side chains. A well-known example of this are the **Zn-fingers** of steroid hormone receptors, which bind to DNA, and in which four residues (≥2 Cys, remainder: His) coordinate Zn^{2+}. One α-helix (see Section 4.1) of a single Zn-finger commonly binds into the large groove of DNA and reads ~3 bases.

3.6. Entropy

Entropy is a force that works against the stability of the native conformation of proteins. All chemical systems tend to maximize their entropy. Proteins have the greatest amount of entropy when the constituent chemical groups move freely in space. However, in the native structure of proteins, atoms are packed closely together (driven by the forces described in Sections 3.1 to 3.5), and chemical groups thus have reduced freedom of motion. When a protein is heated to an unphysiologically high temperature, the protein gains entropy. T e increased motion annihilates noncovalent attractive forces (from hydrophobic ef ects to van der Waals interactions), and the protein loses its native structure. T e protein then is in a **denatured state** (see Section 6).

Many pathogenic **mutations** appreciably diminish the stability of a protein by reducing the favorable interactions of functional groups and atoms. Stability can be tested by exposing the protein (typically an enzyme) in vitro to an unphysiologically high temperature.

4. ELEMENTS OF THE THREE-DIMENSIONAL PROTEIN STRUCTURE

T ere are a *f*ew very common con*f*ormations o*f* the peptide backbone. T e most prominent o*f* these are the α-helix and the β-sheet. About two-thirds o*f* the amino acids in globular proteins are a part o*f* an α-helix or a β-sheet. α-Helices are compact structures in which the peptide backbone *f*orms a spiral along a central cylinder, and the side chains project *f*rom the cylinder toward its periphery. β-Sheets consist o*f* peptide segments that line up next to each other in a parallel or antiparallel *f*ashion. Both helices and sheets owe their stability to hydrogen bonds between peptide –N–H and –C=O groups. T e peptide segments that connect helices and sheets are called loop structures. T ey usually have an ordered three-dimensional structure. With the exception o*f* short loops, they di*ff*er *f*rom protein to protein.

4.1. α-Helix

T e peptide backbone makes up the core of an α-helix, while the amino acid side chains point toward the periphery of the helix (Fig. 9.5).

Very generally, approximately one-third of all amino acid residues in proteins are part of α-helices; however, this fraction varies widely between proteins. A typical α-helix in a globular protein contains ~12 amino acids and has ~3 turns (a globular protein has a length less than ~4 times its width).

T e core of the α-helix is packed tightly enough so that van der Waals interactions can take place between atoms. In

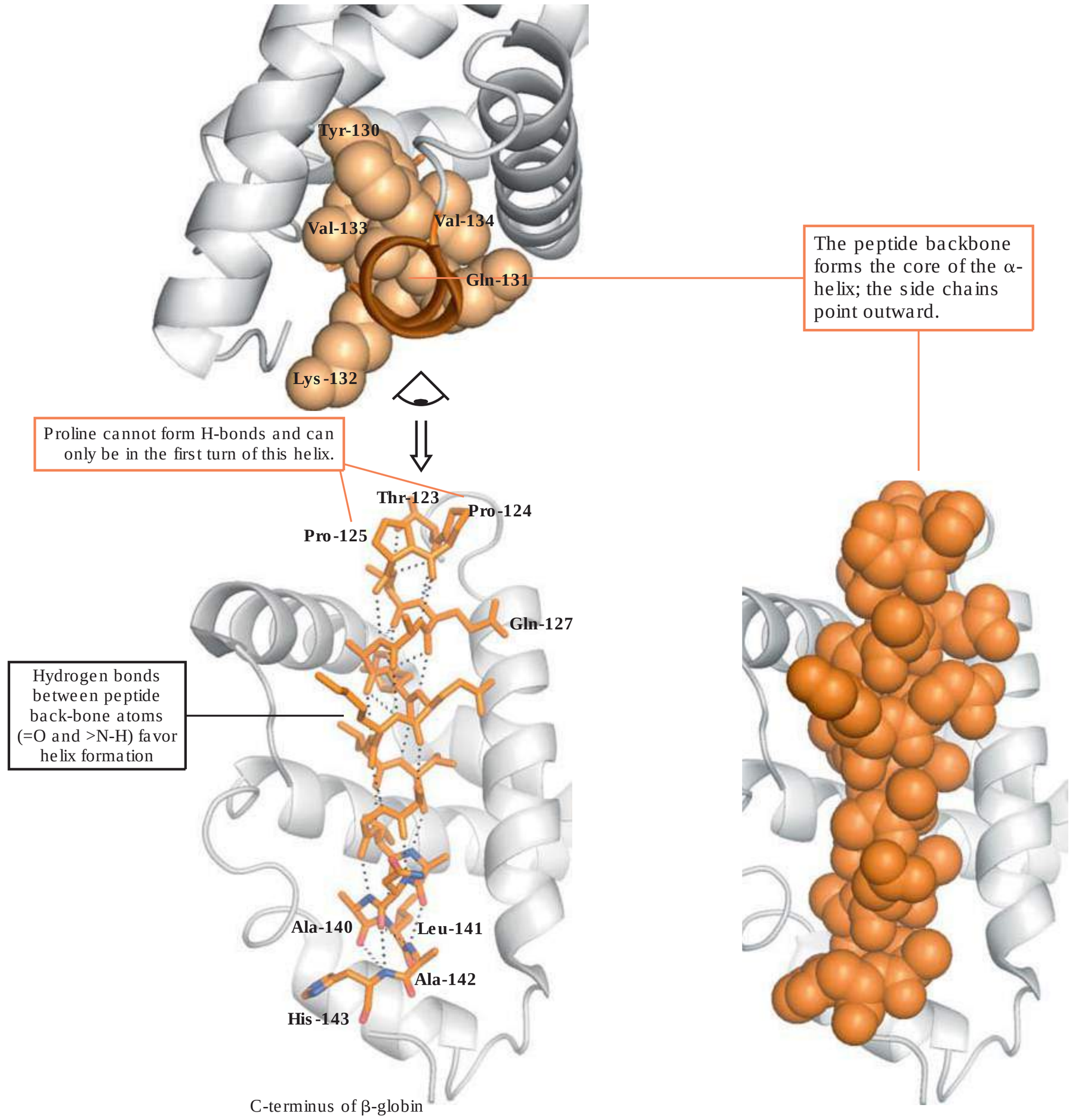


Fig. 9.5 Conformation of an α-helix in human β-globin. β-Globin is a constituent of human hemoglobin A (see Chapter 16). Amino acids in the C-terminal helix of β-globin are shown in orange. At lower left, the orange helix winds its way down from the top. For Ala-140 to His-143 (bottom of the orange helix), N-atoms are shown in blue and O in red. Each β-globin contains several additional α-helices (some are shown in gray). The image on the right shows the same C-terminal helix with space-filled atoms (using van der Waals radii). The image at the top shows a view down the long axis of the same helix. The atoms for amino acid residues 130-134 (midway down the helix) are space filled. Note that there is no empty space in the center of the helix. (Based on Protein Data Bank [www.rcsb.org] file 1HBB from Kavanaugh JS, Rogers PH, Case DA, Arnone A. High-resolution X-ray study of deoxyhemoglobin Rothschild 37 beta Trp—Arg: a mutation that creates an intersubunit chloride-binding site. *Biochemistry*. 1992;31:4111-4121.)

addition, each peptide backbone amide nitrogen [–(CO)–NH–] forms a hydrogen bond with a carbonyl oxygen [>C=O] four residues away from it on the peptide chain. T e α-helix has 3.6 amino acid residues per turn, and it rises by 5.4 Å (54 nm) per turn. T e long axes of the hydrogen bonds have approximately the same direction as the long axis of the helix. Fig. 9.5 illustrates these principles for β-globin.

Amino acids at the end of a helix must find special partners to form hydrogen bonds. Amino acids such as glycine and proline are particularly well suited for capping a helix.

Proline, owing to its structure (lack of an –NH when in a peptide bond), interrupts the hydrogen bonding within an α-helix, except in the first turn of a helix. T e mutation of an amino acid residue inside a helix to proline is usually deleterious because it creates a kink or a break in the helix and thus likely changes the structure of other parts of the protein.

In cartoons of protein structures, helices are commonly shown as screws or cylinders. Fig. 9.6 shows the structure of human serum albumin as an example of such renderings.

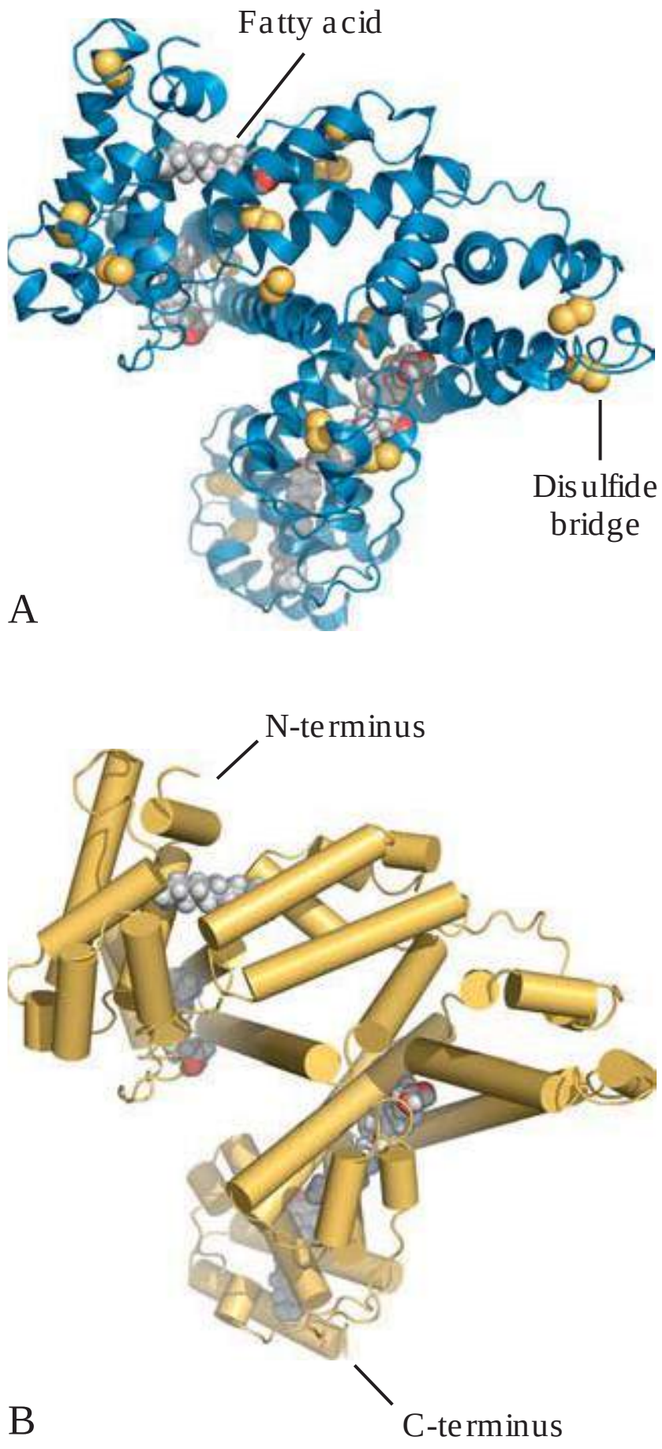


Fig. 9.6 Cartoons of human serum albumin complexed with fatty acids. α-Helices are shown as spirals in **A** and as cylinders in **B**. **A** also shows disulfide bridges (S atoms are space filled). Four bound fatty acid molecules are shown with space-filled atoms. (Based on Protein Data Bank [www.rcsb.org] file 1BJ5 from Curry S, Mandelkow H, Brick P, Franks N. Crystal structure of human serum albumin complexed with fatty acid reveals an asymmetric distribution of binding sites. *Nat Struct Biol.* 1998;5:827-835.)

The sequence of amino acids is often such that one side of the helix is markedly hydrophobic, while the other side is markedly hydrophilic. An example is the **leucine zipper** motif (see Fig. 6.6) that can dimerize due to stabilizing hydrophobic effects.

Membrane-spanning regions of proteins are often α-helical (Fig. 11.8 shows an example). In ion channels, one side of such a helix sometimes contains mostly hydrophobic amino acid residues, while the other contains primarily hydrophilic residues. The hydrophobic side of the helix then faces the hydrophobic core of the membrane, and the hydrophilic side may form part of a hydrophilic transmembrane pore.

Two or more rather long α-helices can form a **coiled coil** (Fig. 9.7). Such structures are found, for example, in some transcription factors that dimerize on DNA promoters (see Chapter 6) and in SNARE complexes, which play a role in docking secretory vesicles to the plasma membrane. These SNARE complexes play a role, for example, in neurotransmission, in peptide hormone secretion (see Chapter 26), and in intracellular trafficking (e.g., inserting GLUT4 glucose transporters into the plasma membrane; see Chapter 18). Coiled coils owe their stability largely to hydrophobic effects.

Many collagens form a **triple helix** (see Fig. 13.1), a structure that is very different from an α-helix.

4.2. β-Sheets

The prefixes α- and β-, for helix and sheet, respectively, have historic roots. Helices were first found in an α-keratin, and sheets were first found in a β-keratin (a great many keratins are now known; they are found in hair, skin, and nails, and also as part of intermediate filaments in virtually every cell). The α-helix and β-sheet structures of proteins were first described in 1951, while the DNA double helix was discovered in 1953.

For a β-sheet to form, two or more strands (called **β-strands**) of one or more proteins must interact. In each strand, the carbonyl-O atoms of consecutive amino acids alternatingly point in opposite directions (like odd zippers with teeth that point into opposite directions; Fig. 9.8).

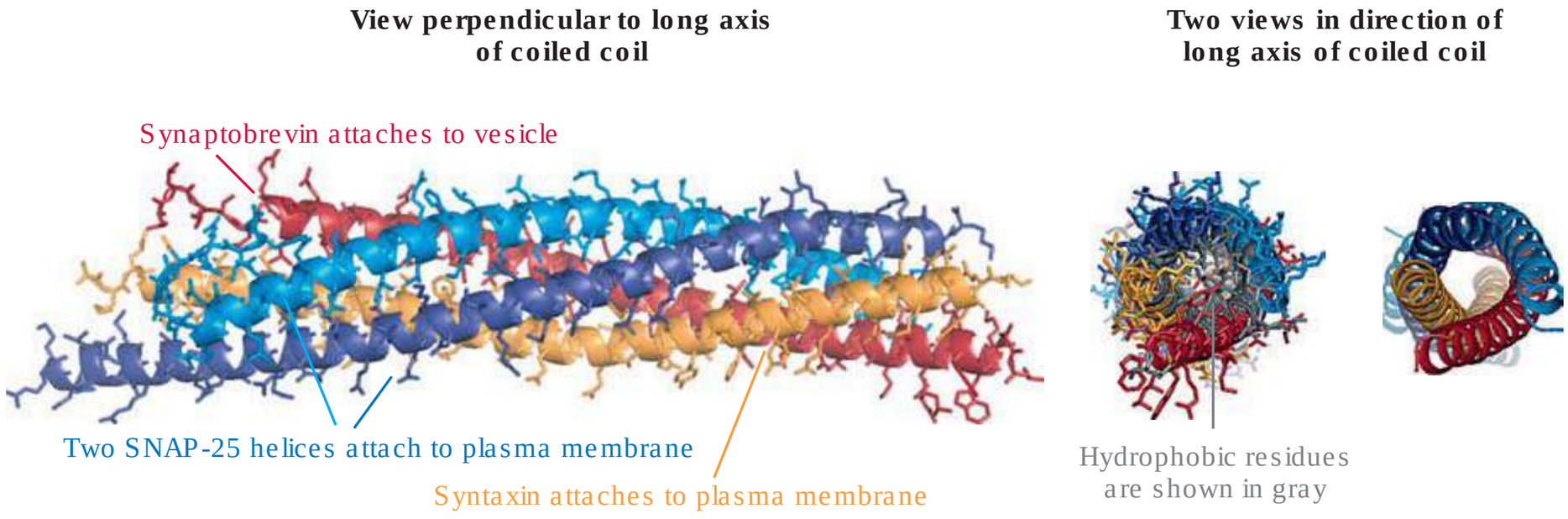


Fig. 9.7 SNARE proteins can form a coiled coil. (Based on Protein Data Bank [www.rcsb.org] file 1SFC from Sutton RB, Fasshauer D, Jahn R, Brunger AT. Crystal structure of a SNARE complex involved in synaptic exocytosis at 2.4 A resolution. *Nature*. 1998;395:347-353.)

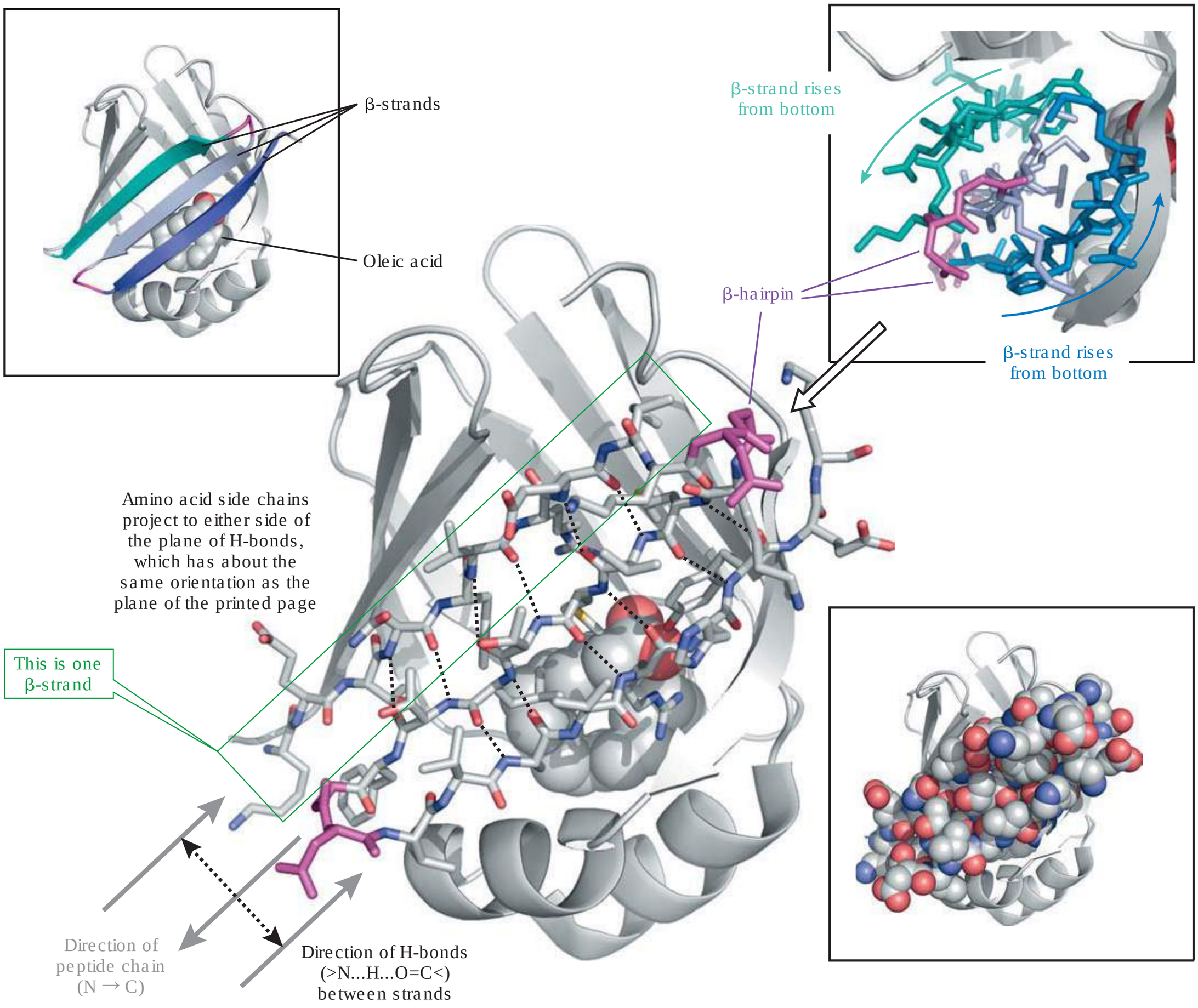


Fig. 9.8 **Model of the crystal structure of human brain fatty acid binding protein in complex with oleic acid.** This protein contains 10 β-strands (insert at top left; shown as ribbon arrows) that participate in β-sheet formation. The three strands at the top left are shown in the central panel as stick models with C-, N- and O-atoms in gray, blue, and red, respectively. H-atoms are not shown. The backbone O-atoms are carbonyl oxygens (>C=O), and the backbone N-atoms are linked to an H-atom (not shown); these H-atoms participate in hydrogen bonds (black dotted lines). The hydrogen bonds are approximately in a plane that is perpendicular to the line of sight. By contrast, the amino acid side chains project above and below the plane that is perpendicular to the line of sight. The solid gray arrows at the lower left indicate the direction of the strands (from N- to C-terminus); the three highlighted strands show an antiparallel organization. The highlighted strands are 10, 8, and 9 amino acid residues long. Two amino acid residues that connect adjacent antiparallel strands are hairpins and are shown in magenta. Oleic acid is shown with space-filled atoms (H-atoms are omitted). Oleic acid is in a folded conformation owing to its double bond and the influence of the protein environment. The inset on the top right shows a view down the long axis of the middle strand (light purplish blue). The strands on either side (cyan and dark blue) are not perfectly parallel to the center strand. Hence, their peptide backbones follow slightly different directions, creating a curvature in the β-sheet (this is common). The side chains point to opposite sides of the β-sheet. The inset on the lower right shows all atoms of the three selected β-strands in a space-filled cartoon (H-atoms are not shown, and the view of oleic acid is almost completely obstructed). Every other side chain of the three β-strands points in the direction of the viewer. (Based on Protein Data Bank [www.rcsb.org] file 1FE3 from Balendiran GK, Schnutgen F, Scapin G, et al. Crystal structure and thermodynamic analysis of human brain fatty acid-binding protein. *J Biol Chem*. 2000;275:27045-27054.)

Carbonyl-O atoms and amide-NH atoms on each strand then form interstrand hydrogen bonds. Two β-strands thus form one set of hydrogen bonds, three strands form two sets, and so forth. Hence, an increase in the number of interacting strands increases the number of hydrogen bonds per strand, which tends to favor β-sheets of unlimited size (see also below).

In some β-sheets the strands run **parallel**, in others they run **antiparallel** with regard to the N→C-terminal direction of the peptide backbone. If the strands of a single polypeptide run in antiparallel fashion, just two extra amino acids suffice to accomplish a turn (called a **β-hairpin**; see Fig. 9.8 and Section 4.3). However, if such strands run in parallel fashion, a much larger intervening sequence must cover the distance from the end of one β-strand to the beginning of the next β-strand.

In cartoons of protein structures, the strands of a β-sheet are commonly shown as flat ribbons, and an arrow at the end sometimes indicates the N→C direction. Fig. 9.8 shows the structure of the β-sheet in the **fatty acid binding protein** of brain. The β-sheet consists of antiparallel strands, and it coils into a cylindrical structure. This protein increases the effective solubility of fatty acids in the cytosol, and it also protects the cell from the detergent effects of the fatty acids (see Chapter 27).

When a drawing shows two ribbons (i.e., β-strands) next to each other, hydrogen bonds link the ribbons and lie in the approximate plane of the two ribbons (see Fig. 9.8). The side chains point at a right angle to the plane of the ribbon, and they alternate between the two sides of the ribbon.

Globular proteins often have parallel and antiparallel β-sheet structures. Fibrous proteins (proteins that have a length >10 times their width) sometimes have antiparallel but usually not parallel β-sheet structures.

β-Sheets are normally curved. Several β-strands can form a single, curved, almost cylindrical β-sheet that is called a **β-barrel**. Alternatively, two or more β-sheets plus connecting loops can form the sides of a polygonal body; this arrangement of β-sheets is called a **β-helix**. Yet another type of structure is called a **β-propeller**; each blade is made up of a β-sheet, whereby the β-strands closest to the center of the propeller are the shortest and those farthest away from the center the longest.

4.3. Loops

The term loop refers to the portion of a polypeptide chain that connects elements of secondary structure (i.e., α-helices and β-sheets). Loops or coils vary tremendously in size and may either move around fairly freely or else have a fixed structure that is neither helical nor sheetlike. In renderings of protein structure, loops are typically shown as thin lines. In globular proteins, approximately one-third of all amino acid residues are a part of loops.

A short **β-turn** typically consists of four amino acid residues and can accomplish a ~180-degree change of direction of the peptide backbone. In a β-sheet with antiparallel β-strands, the first and last residue of a β-turn are often part of the β-sheet; the second and third residues then form a β-hairpin (see Section 4.2 and Fig. 9.8). The second residue (i.e., the residue right after one β-strand) is often Gly, Asp, Asn, or Pro.

4.4. Motifs and Domains

A **motif** is a protein sequence pattern that is preserved through evolution and conveys a predictable property to a variety of proteins. For instance, Gly*Gly**Gly (* = any single amino acid residue) is a motif that helps a protein bind to a nucleotide. Obviously, these motifs are embedded in (and structurally dependent on) neighboring amino acid residues.

A **domain** consists of a contiguous stretch of amino acid residues that can function independently of other portions of the polypeptide chain and that is also physically distinct from them. For instance, among nuclear receptors (see Fig. 6-5) a ligand-binding domain binds a ligand, a dimerization domain mediates dimerization of the receptor when a ligand is bound to it, and a DNA-binding domain allows the protein to hold on to DNA. Another example is glucokinase, which contains two domains that move toward each other when glucose binds (see Fig. 10.2; the large domain is blue, the small domain is gold with a helix highlighted in red).

Sometimes, domains are connected by **hinges**, which are short and flexible segments that allow movement of one domain relative to another; such movements are often a prerequisite for enzyme catalysis.

4.5. Primary, Secondary, Tertiary, and Quaternary Protein Structure

The term **primary structure** refers to the sequence of amino acids in a protein (e.g., Met–Leu–Ser–Ala–).

The term **secondary structure** refers to elements repeatedly seen in the three-dimensional structure of proteins. This term includes helices and sheets. Some authors also consider common structures (e.g., β-turns) a part of the secondary structure, while others count them as a tertiary structure (see below).

The term **supersecondary structure** is sometimes used and refers to the structure of just a few (often three) α-helices and β-sheets.

The term **tertiary structure** refers to a description of the relative location of all atoms of a protein in space. Some of these atoms are part of α-helices, β-sheets, β-turns, other loops, motifs, or domains.

The term **quaternary structure** is used for the description of the composition and three-dimensional structure of a protein complex. Hemoglobin is an example of such a protein complex. As detailed in Chapter 16, hemoglobin consists of a heterotetramer of globin subunits, (e.g., two molecules of α-globin and two molecules of β-globin, each with a heme group bound to it; see Fig. 16.7). The conformation of any one globin subunit affects the conformation of all other globin subunits.

5. UNSTRUCTURED PROTEINS

Some proteins or regions of proteins lack a recognizable secondary structure, which may provide advantages to protein modification and binding to other proteins.

Compared with structured, globular proteins, intrinsically disordered proteins or disordered regions of proteins contain fewer hydrophobic amino acids and more polar amino acids. They also contain more charged amino acids. Therefore the hydrophobic effect, which contributes much to the stability of a structured protein, has significantly less effect on the structure of intrinsically disordered regions. Furthermore, intrinsically disordered proteins contain more glycine and more proline amino acids than well-structured proteins. Glycine adds flexibility (and entropy). Proline attenuates the formation of α-helices and β-sheets.

The lack of structure in intrinsically disordered regions of proteins may facilitate **posttranslational modification** by enzymes and may help **binding** to other molecules. Phosphorylation, sulfation, or acetylation may affect the charge distribution significantly and thus alter dramatically the behavior and conformation of intrinsically disordered proteins. Intrinsically disordered regions generally bind to other molecules with high specificity. They often also show a relatively low binding affinity, and they unbind relatively rapidly. Many intrinsically disordered regions have enough flexibility to bind to several different molecules.

An example of an intrinsically disordered region of a protein is the 51-residue sequence at the C-terminus of **HIF-1α**. Some of the amino acids in this region take on α-helical structure only when bound to transcription coactivators in the nucleus (the function of HIF-1α is described in Section 1.4 and in Fig. 16.5).

Amylin, produced by pancreatic β-cells, is another example of a peptide that has an intrinsically disordered region (see Section 8.4).

6. PROTEIN FOLDING

Proteins fold soon after synthesis, sometimes with the help of chaperones. Disulfide bonds form with the help of an enzyme.

The backbone of a protein can, in theory, assume a huge number of different conformations, even though peptide bonds can adopt only a limited number of torsion angles (see Section 2.1). During and after synthesis, a protein tends to assume a conformation of low energy. This is usually also a conformation with a low amount of internal motion (i.e., a situation of tight packing). However, some native proteins are not in their lowest state of energy; the lowest state may be an aggregated state, to which the proteins do not normally have kinetic access.

It seems likely that crude elements of a secondary structure form right after protein synthesis, and that a few key amino acid residues then form contacts with other residues so that the polypeptide chain quickly adopts a three-dimensional structure that is roughly similar to its native state. Then the protein may settle into its final, compact, native structure; sheets and helices, as well as interactions between amino acid residues that are distant in the primary sequence, become tighter. At this point, electrostatic and van der Waals interactions, which strongly depend on the three-dimensional conformation of a protein, convey specificity to a particular protein conformation. In large proteins, domains are thought to fold first and interact afterward.

The native structure of proteins is only marginally stable. In fact, it is commonly observed that **mutant** proteins have a shorter lifetime than normal proteins; part of this reduction in lifetime can be attributed to decreased stability.

Some proteins do not fold into the correct shape all by themselves but instead depend on **chaperone** proteins that use energy from ATP hydrolysis to guide proteins into the correct folding pattern. Some chaperones detect unfolded or incorrectly folded proteins by exposed patches of hydrophobic amino acid side chains. As described in Chapter 7, chaperone proteins either refold misfolded proteins or lead them to degradation in proteasomes.

When small proteins that do not normally need chaperones for folding are artificially unfolded in vitro, they often refold in less than 1 second.

Members of the protein disulfide isomerase family catalyze **disulfide bond** formation between cysteine side chains.

7. DENATURATION OF PROTEINS

Proteins are intentionally denatured as part of disinfection.

Denaturation of a protein is defined as a loss of its native (physiological) three-dimensional structure. However, a denatured protein may still have some secondary structure, such as α-helices or β-sheets. Once a protein has been denatured in vitro, it often cannot refold to its original native structure. Possible reasons for this are that the protein originally started folding already during biosynthesis so that N-terminal residues could fold before C-terminal residues could interfere, that the protein folded with the assistance of chaperones, and that the protein underwent posttranslational proteolytic or other processing after the normal folding process.

For most proteins, the difference in free energy between the normal physiological and the denatured state is on the order of only ~10 kcal/mol. This amount of energy is roughly equivalent to forming two hydrogen bonds de novo or burying two phenylalanine side chains in the protein interior instead of completely exposing them to water.

Chaperones can unfold proteins inside cells for **transport** from one compartment to another (e.g., from the cytosol to the nucleus, or from the cytosol to mitochondria via specialized pores in the membranes of these organelles).

Denaturation of proteins is often used as a means of **disinfection**. An unphysiologically high **temperature**, as is used in pasteurization, cooking, and sterilization, leads to the loss of the normal protein conformation. This is in large part due to a temperature-induced increase in entropy that destabilizes hydrogen bonds, hydrophobic interactions, electrostatic interactions, and van der Waals forces.

A change in **pH** may also cause denaturation. T is happens physiologically in lysosomes and endosomes in all cells, as well as in the lumen of the stomach. Pathologically, for example, it happens when the eyes are exposed to lye (concentrated NaOH).

Iodine-containing disinfectants (e.g., betadine and isodine; so-called iodophors) iodinate proteins.

Alcohols (e.g., 70% to 90% ethanol or isopropanol), by replacing water as a solvent, lead to diminished hydrogen bonding between the protein and solvent, reduced hydrophobic ef ects, and decreased shielding of electrical charges by the solvent.

Bleach and similar disinfectants release hypochlorous acid and hypochlorite (HOCl and ClO^-, respectively), which chlorinate the α-carbon of amino acids. "**Bleach alternatives**" are made to release hypochlorous acid and hypochlorite at a somewhat acidic pH instead of the strongly alkaline pH of regular bleach (i.e., sodium hypochlorite).

Detergents can solubilize hydrophobic side chains and thereby alter the conformation of a protein.

8. DISEASES THAT ARE ACCOMPANIED BY EXCESSIVE PROTEIN AGGREGATION

Pathologic aggregation o*f* proteins into f brillar structures is a hallmark o*f* degenerative diseases such as Alzheimer and Parkinson diseases. Increased *f*ormation o*f* hydrogen bonds and sequestration o*f* hydrophobic residues *f*rom water both drive aggregation o*f* proteins into f brils.

8.1. Formation of Extracellular Amyloid Fibrils

Amyloid fibrils are fibrillar aggregates of proteins that stain with **Congo Red**. T e term amyloid is partially a misnomer. In the nineteenth century, Virchow found that he could stain these fibers with iodine, like starch, and he named them amyloid fibers (in Greek, amylin means starch). Shortly after that, it became clear that amyloid fibers not only contain carbohydrates, but also proteins. Amyloid fibrils are roughly 100 Å (10 nm) in diameter and have a length up to greater than 100 times their diameter (i.e., a length up to ≥1 μm; for comparison, a red blood cell has a diameter of 7–8 μm). Fibrils may contain thousands of protein molecules.

Amyloid fibrils form as a result of a pathologically increased concentration of one or more normal proteins, the presence of a mutant protein, the presence of a seed structure, or the absence of an inhibitor of aggregation.

All amyloid fibrils contain **β-strands** and **β-sheets** in a **cross-β motif**. T e β-sheets and the cross-β motif extend over the length of the fibril. T e direction of the β-strands of individual molecules is perpendicular to the length of the fibril. Fig. 9.9 shows an example of an amyloid fibril. Amyloid fibrils can serve as seeds to which additional molecules are added such that the entire fibril has the same longitudinal architecture. A single type of peptide can often give rise to several dif erent amyloid fibril structures.

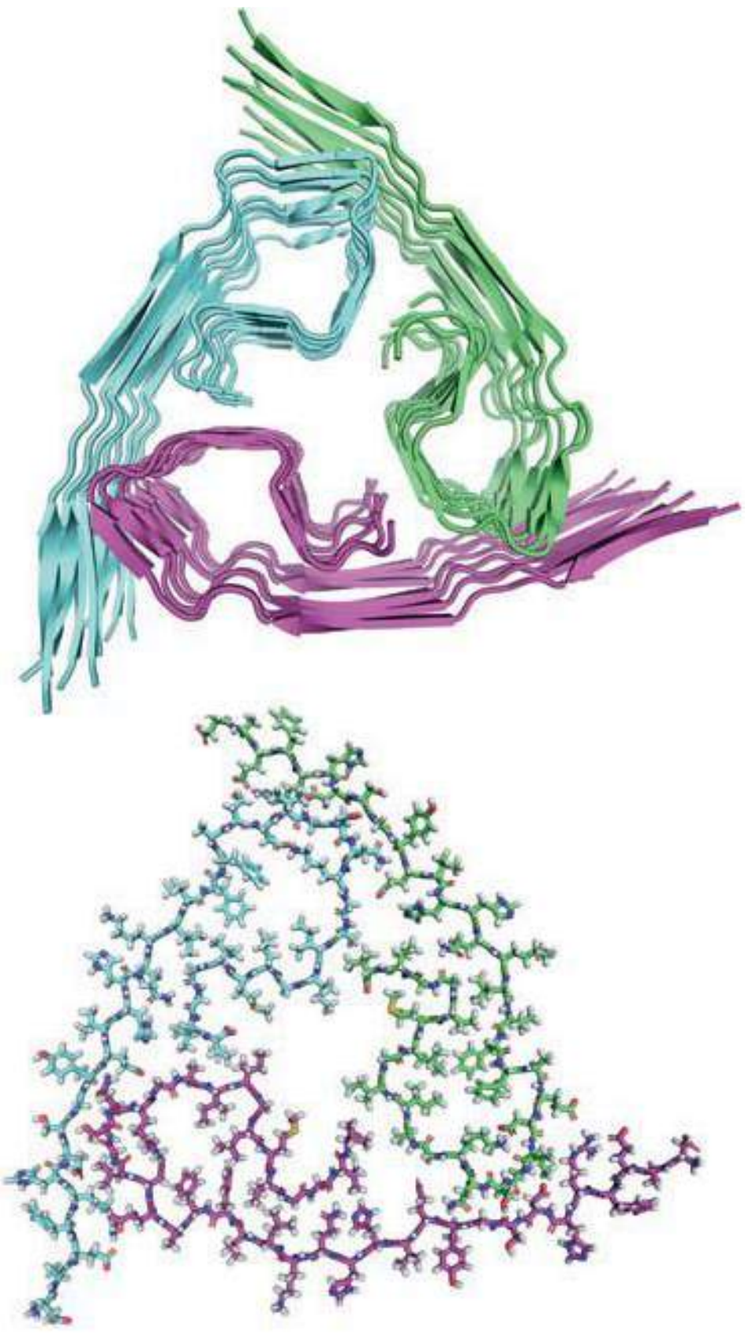


Fig. 9.9 Model of Aβ40 fibrils grown in vitro using seeds from the brain of an Alzheimer patient. The long axis of the fibril is parallel to the line of sight. The fibril resembles a stack of pancakes for which each pancake consists of three Aβ40 molecules (cyan, green, or magenta) stabilized mostly by hydrophobic effects due to nonpolar amino acid side chains. The stack, in turn, is stabilized mostly by hydrogen bonds between backbone atoms of successive molecules. (From Tycko R. Amyloid polymorphism: structural basis and neurobiological relevance. *Neuron*. 2015;6(8):632-645.)

T e formation of amyloid fibrils is energetically favorable for many proteins, but other low-energy states, as well as kinetic barriers, can prevent proteins from becoming part of amyloid.

About 25 dif erent proteins are known to give rise to amyloid fibrils in vivo, and the peptide cores of all of these fibrils are decorated by a number of other molecules (e.g., heparan sulfate proteoglycan, apolipoprotein E, and serum amyloid P). **Heparan sulfate proteoglycan** is a normal part of the extracellular matrix (see Chapter 13); the negatively charged sulfate and carboxyl groups of its carbohydrate chains bind to positively charged amino acid side chains (e.g., Arg, Lys, or His) of several protein molecules in the fibril. **Apolipoprotein E** is a constituent of many lipoprotein particles (see Chapters 28 and 29). **Serum amyloid P protein** is a glycoprotein that is synthesized in the liver, secreted into the blood, and eventually eliminated by the liver. Its half-life in serum is ~1 day. During inflammation, the concentration of amyloid P in the serum increases up to 1,000-fold. T e normal physiological function of serum amyloid P is incompletely

understood. Serum amyloid P belongs to the pentraxin family. T is family of proteins plays a role in opsonizing and neutralizing pathogens as part of the innate immune system that then informs the adaptive immune system. Serum amyloid P protein can stabilize fibrils by binding simultaneously to the monosaccharides of several molecules of the glycosylated, amyloidogenic protein.

Amyloid fibrils are insoluble in water, and extracellular proteases and phagocytic cells do not appreciably degrade amyloid fibrils.

T e formation of amyloid fibrils is associated with impaired function and the death of nearby cells, but the exact pathogenesis is only poorly understood. Amyloid fibrils themselves or much smaller oligomers of their constituents impair cellular function, perhaps by changing the permeability of the cell membrane. In addition, an ever-increasing amount of extracellular amyloid can displace the cells and eventually reduce the supply of nutrients. Common sites of amyloid deposition and cell death are the brain, heart, kidney, and joints.

When patients are suspected of having an amyloid disease (see below), the ultimate **diagnosis** often rests on demonstrating plaques of long, unbranched amyloid fibrils using stains and a light microscope, as well as an electron microscope. To this end, biopsy material may come from gum tissue, abdominal subcutaneous fat, or a kidney. If an amyloid disease involves the brain exclusively, such an analysis is commonly performed postmortem. Currently, amyloid fibrils are identified on the basis of appearance (**plaques** under the light microscope, fibrils of ~10 nm diameter in the **electron microscope** and a length on the order of μm regardless of core protein), staining with **Congo red** ("Congophilia"; if so stained, appearing either red or green in the polarized light microscope when the plane of polarized light is parallel or perpendicular to the long axis of the fibril, respectively, which is due to the orientation of the repeating β-sheets in the fibril). Antisera can be used to determine the protein that gives rise to the amyloid fibrils. Laser microdissection of plaques in a biopsy, followed by mass spectrometry, is another useful option.

8.2. Formation of Intracellular Aggregates

Intracellular aggregates are known to be formed through several mechanisms that range from hydrophobic ef ects to cross–β-sheet formation. In patients who have sickle cell disease, fibers inside the red blood cells form through an aggregation of hemoglobin S (for an extensive discussion, see Section 3.2 in Chapter 17 and Figs. 17.5 and 17.6). T rough the formation of a cross-β structure, normal proteins can give rise to **neurofibrillary tangles**, amyloid fibrils, or **intranuclear inclusions**. T e aggregation is driven by the same basic forces as in extracellular aggregation (see Section 8.1).

T e neurofibrillary tangles of patients with Alzheimer disease, familial frontotemporal dementia with parkinsonism, Down syndrome, or amyotrophic lateral sclerosis contain aggregated, abnormally phosphorylated, and acetylated **tau protein**. Tau is a microtubule-associated protein that stabilizes microtubules. Microtubules are cablelike structures inside cells on which motor proteins "run." T e motor proteins move intracellular organelles as well as chromosomes or chromatids during meiosis and mitosis. T e aggregation of tau is thought to cause a deficiency of free tau, which renders the microtubules less stable, and thus impairs transport along axons so that the neurons no longer work properly. **Tauopathy** is a term used for a disease that is accompanied by the formation of fibrils containing tau.

8.3. Alzheimer Disease

T e extracellular amyloid fibrils seen in Alzheimer disease and in Down syndrome consist of amino acid residues 1-40 and 1-42 of the **Aβ-protein**. T ese polypeptides are derived from the **amyloid precursor protein** (**APP**) through the action of β- and γ-**secretase** (these secretases are proteases). Aβ protein is found throughout the body, but fibrils of Aβ are restricted to the synapses and the basement membranes of the blood vessels in the brain.

T e major **hereditary** forms of Alzheimer disease are caused by mutations in the genes for **APP**, **presenilin 1**, and **presenilin 2**. T e mutations in the APP gene typically af ect amino acids outside the Aβ(1-42)-protein but adjacent to the secretase cleavage sites. Mutations in the presenilins give rise to altered γ-secretase activity.

T e gene for APP is on chromosome 21, and patients with **Down syndrome** have three copies of chromosome 21. Patients with Down syndrome show amyloid deposits by 40 years of age.

For research studies, the amount of Aβ-amyloid in brain can be assessed by **proton emission tomography** (**PET**) using a radioactive (^{18}F-labeled) dye.

Patients with the **apolipoprotein E4** isoform are more likely to have **sporadic Alzheimer disease**. Compared with those who are homozygous for the E3 allele (~60% of the population), each E4 allele approximately doubles the risk and causes onset roughly 5 years earlier. T e apolipoprotein E4 allele is the most important factor in a person's risk for sporadic Alzheimer disease. In the United States, ~2% of the population is homozygous for the E4 allele, and ~20% is heterozygous.

Besides extracellular fibrils made of Aβ (Fig. 9.10), most patients with Alzheimer disease also have intracellular fibrils made of **tau**, and yet other intracellular fibrils made of **α-synuclein** (**Lewy bodies**; see Parkinson disease in Section 8.5). T e tau protein in the intracellular fibrils is aberrantly phosphorylated and acetylated, and it cannot fulfill its normal role in stabilizing microtubules.

8.4. Type 2 Diabetes

In many patients who have **type 2 diabetes** or an insulinoma, pathologic fibrils of **amylin** form in the extracellular space predominantly of the islets of Langerhans in the pancreas. Amylin is a 37-amino acid peptide hormone that is an intrinsically partially disordered peptide (see Section 5). It is also called **islet amyloid polypeptide** (**IAPP**). Amylin plays a role

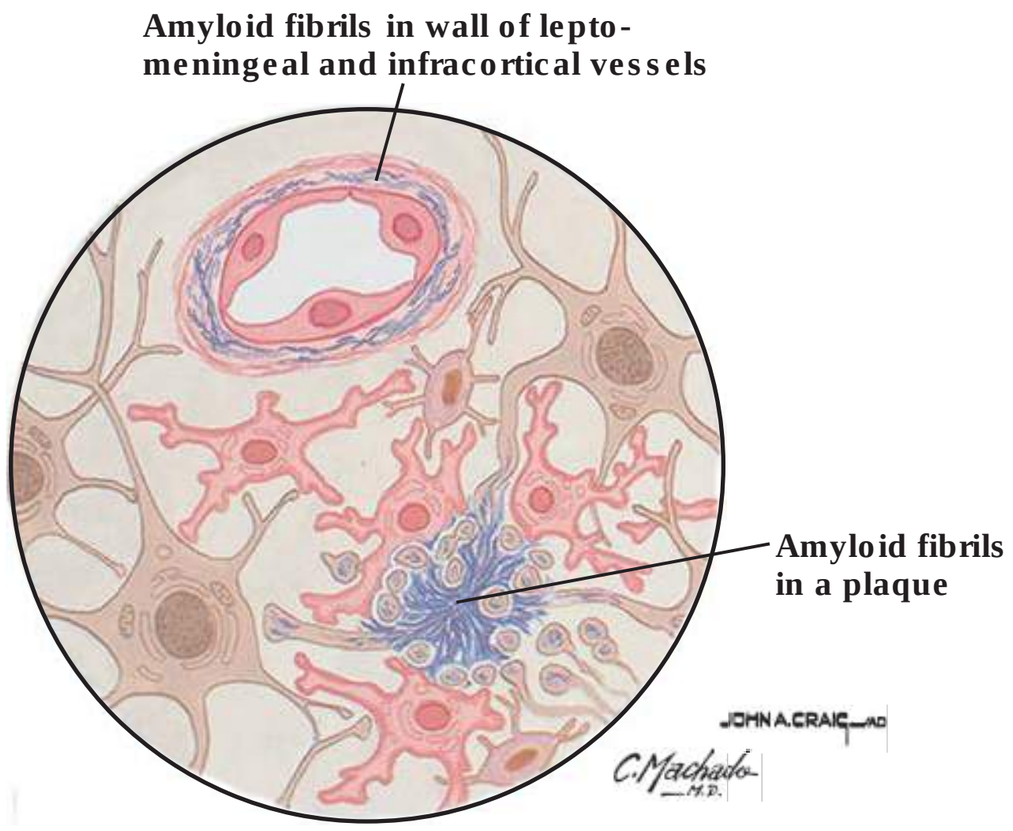


Fig. 9.10 **Extracellular plaques containing β-amyloid in the brain of a patient with Alzheimer disease.**

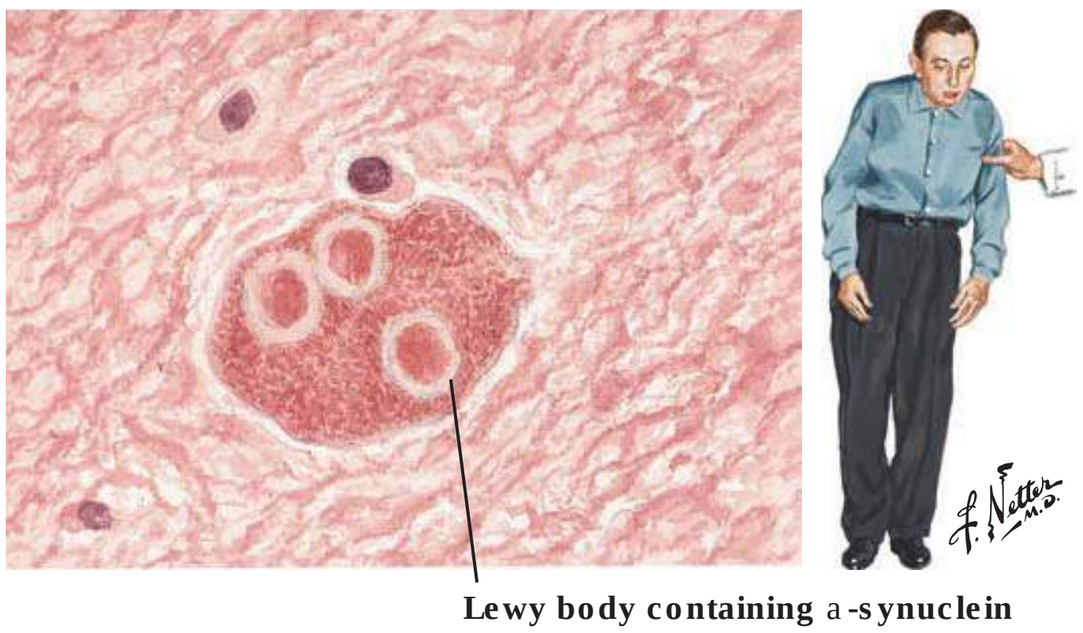


Fig. 9.11 **Pathologic aggregation of α-synuclein in Parkinson disease.**

in regulating glucose metabolism (see Chapters 26 and 39). Together with insulin, amylin is stored inside β-cell secretory vesicles; it is therefore also secreted together with insulin. Inside β-cell secretory vesicles, insulin (but not proinsulin) binds to amylin and thereby keeps amylin from aggregating into fibrils. Inside secretory vesicles, there are ~100 molecules of insulin per molecule of amylin. In the bloodstream, the concentration of amylin is too low to permit appreciable aggregation into fibrils. Hence, aggregation is confined to the extracellular space near β-cells. It appears likely that oligomers of amylin are pathogenic, interact with the plasma membrane, and perhaps alter membrane permeability, resulting in β-cell death. Whether such a mechanism is responsible for some of the deterioration of β-cell function that is characteristic of type 2 diabetes remains to be seen. Like other amyloid fibrils, fibrils of amylin contain a cross-β structure.

Pramlintide is a synthetic, especially soluble analog of amylin that is used to treat patients with **type 1** or **type 2 diabetes** (see Chapter 39). Pramlintide greatly reduces the excursion of the blood glucose concentration after a meal; in fact, when patients take pramlintide they require less insulin to control blood glucose.

8.5. Parkinson Disease

Parkinson disease can arise as a result of various genetic mutations. Through pathways that remain largely unknown, many patients accumulate fibrils (also called **Lewy bodies**; Fig. 9.11) inside cells of the substantia nigra. The fibrils contain mostly **α-synuclein**, a protein that is made in the synapse. The disease is accompanied by a loss of neurons in the substantia nigra. The substantia nigra normally sends messages to the basal ganglia via dopaminergic synapses.

Patients with Parkinson disease show a tremor at rest but not during voluntary movements. They also have rigid muscles, move slowly, and have little expression on their face. Parkinson disease usually becomes clinically apparent in the sixth to eighth decades of life.

In the United States, more than 2% of the population has Parkinson disease. Most of this disease is sporadic. Some of the **hereditary** forms of Parkinson disease are due to missense mutations in α-synuclein.

SUMMARY

- Proteins are synthesized from 21 different amino acids, which are linked by peptide bonds (–CO–NH–). The arrangement in space of these amino acids determines the properties of a protein.
- On average, in the native, folded state, approximately two-thirds of the amino acid residues of a protein are part of α-helices or β-sheets. The amino acids that are not part of helices and sheets are generally part of turns and loops. Tight turns have a common structure, but the longer loop regions usually differ appreciably in structure from protein to protein.
- The native, three-dimensional structure of proteins is a result of H-bonding, hydrophobic effects, electrostatic interactions, and van der Waals interactions between the atoms of amino acids.
- Thermodynamics favor the aggregation of β-strands into infinitely large β-sheets. In nature, such aggregation is usually prevented, often by steric means. However, there are many diseases, collectively called amyloid diseases, that are associated with the aggregation of proteins into ~10 nm diameter, very long fibrils, called amyloid fibrils. Patients with Alzheimer disease form extracellular amyloid fibrils that contain the Aβ-protein; these fibrils, in turn, give rise to plaques. Neurons of patients with Alzheimer disease also contain intracellular fibrils of tau protein, which in turn form neurofibrillary tangles. Extracellular fibrils containing amylin (also called IAPP) are found in patients with type 2 diabetes. In patients with Parkinson disease, α-synuclein gives rise to intracellular fibrils in cells of the substantia nigra; the fibrils aggregate into Lewy bodies.

FURTHER READING

- Finkelstein AV, Ptitsyn OB. Protein Physics: A Course of Lectures, ed 2. London: Academic Press; 2016:528. T ese authors take a rigorous chemical approach that is suitable for those who want to learn more about protein structure.
- Protein Data Bank: <www.rcsb.org>. T is databank provides free access to data for thousands of protein structures.
- Tycko R. Amyloid polymorphism: structural basis and neurobiological relevance. Neuron. 2015;86:632-645.

Review Questions

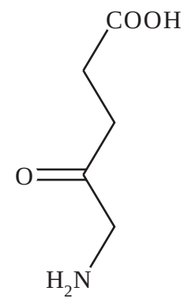
1. T e termini of proteins are usually found near the surface of the protein. T e most likely reason for this is which of the following?

   A. Neither the first nor the last residue of a polypeptide chain can be part of a hydrogen-bonded α-helix or β-sheet.
   B. Proteins fold a*fte*r synthesis by ribosomes has started but b*efo*re it has ended.
   C. T e higher mobility of side chains near the termini prevents their participation in van der Waals interactions.
   D. T e termini are charged, and there would be a large energy penalty for burying these charges in the protein interior.

2. T e codons GGA, GGC, GGG, and GGU all encode the amino acid glycine. In turn, the codons GUA, GUC, GUG, and GUU all encode the amino acid valine. Mutation of the first nucleotide (G) of a codon for Gly is usually much more damaging to the function of a protein than mutation of the first nucleotide (G) of a codon for Val. Which of the following is the best explanation for this finding?

   A. Compared with glycine, valine poses greater constraints on the conformation of the peptide backbone.
   B. Glycine is more likely to be exposed on the surface of a protein than valine.
   C. Glycine residues give rise to greater hydrophobic ef ects than valine residues.
   D. T e side chain of valine is much smaller than that of glycine.

3. Aminolevulinic acid (ALA), which derives from glycine, has the following structure:

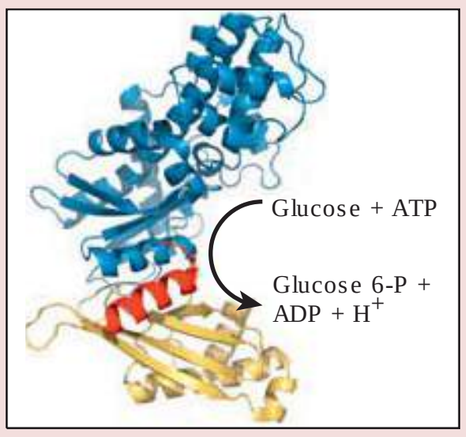


   In dermatology, ALA is sometimes applied to a skin area as an acidic solution or cream. Application of an electric current then moves ALA through the stratum corneum into the underlying epidermis. What is the charge of ALA that moves under these conditions?

   A. −2
   B. −1
   C. 0
   D. +1
   E. +2

4. T e figure above shows the structure of a defensin. T e antiparallel β-sheet (indicated by two long flat ribbons) is most likely primarily stabilized by which of the following?

   A. Electrostatic interactions among oppositely charged atoms of the two β-strands
   B. Hydrogen bonds between amino acid side chains of the two β-strands
   C. Hydrogen bonds between O and N in the peptide backbone of the two β-strands
   D. Hydrophobic ef ects due to the presence of aliphatic and aromatic amino acid side chains among the two β-strands
   E. Van der Waals interactions between neighboring atoms of amino acid side chains



Chapter

10 Enzymes and Consequences of Enzyme Deficiencies

SYNOPSIS

- Enzymes catalyze chemical reactions by lowering the activation energies of these reactions.
- Most enzymes are proteins, but some consist of RNA or a mixture of RNA and protein. Some enzymes need coenzymes (organic nonprotein molecules) for catalysis.
- Thermodynamics determine whether a reaction from reactants to products is energetically possible.
- Physiological or pathological changes in the concentrations of reactants and products allow many reactions in the human body to change direction; such reactions are said to be reversible. Other reactions are favorable in only one direction under conditions that are compatible with life; these reactions are termed physiologically irreversible.
- Regulatory circuits control the activities of many of the enzymes that catalyze physiologically irreversible reactions.
- The three-dimensional arrangement of amino acids in enzymes provides specificity for the binding of substrates.
- The Michaelis-Menten equation describes how enzymatic activity depends on the substrate concentration. This equation is valid for some enzymes.
- Some enzymes show cooperativity and thus are described by a modified Michaelis-Menten equation. Such enzymes become significantly active only above a threshold concentration of the substrate.
- The activity of certain enzymes is regulated by activators, by inhibitors, and/or by covalent modification, such as phosphorylation.
- In all pathways, the enzyme with the lowest rate of reaction limits flux through the pathway. An enzyme deficiency tends to lead to an increased concentration of all upstream reactants and a decreased concentration of all downstream products.

LEARNING OBJECTIVES

For mastery of this topic, you should be able to do the following:

- Examine available resources to determine whether two enzymes catalyze the same reaction or different reactions.
- Define the terms zymogen, proenzyme, ribozyme, coenzyme, cofactor, prosthetic group, apoenzyme, holoenzyme, isozyme, catalytic cycle, and enzyme unit.
- Describe the relationship between thermodynamics and the feasibility of enzyme catalysis of a chemical reaction.
- Explain how an enzyme can increase the rate of a reaction.
- Compare and contrast physiologically reversible and physiologically irreversible reactions in the human body.
- Explain the effect of temperature and pH on an enzyme-catalyzed reaction.
- Compare and contrast the key-lock theory and the induced fit theory of enzymatic action.
- Use the Michaelis-Menten equation to relate K_m, enzyme activity, and substrate concentration to each other.
- Compare and contrast the properties of enzymes that show simple Michaelis-Menten type kinetics and enzymes that show cooperativity.
- Define allostery and provide an example.
- Compare and contrast how competitive and noncompetitive inhibitors affect the rate of enzyme catalysis, taking into account the concentration of substrate.
- List an example each of feedback and feed-forward regulation.
- Predict the effects of a partial or a complete enzyme deficiency on the concentrations of the intermediates of a metabolic pathway.
- Explain why the activity of certain enzymes in the blood is of interest in the diagnosis of disease.

1. NOMENCLATURE OF ENZYMES

The names of enzymes end in -ase. Different naming systems exist, of which the "recommended names" are used here. Enzymes are most often proteins and sometimes require inorganic or organic, nonpeptidic cofactors for catalysis. A few enzymes consist of RNA or a mixture of RNA and protein.

Enzymes are characterized by the suffix **–ase**. Each enzyme can be identified in three different ways: (1) the **recommended name**, which is generally the historically grown name (e.g., hexokinase; Table 10.1); (2) the "**systematic name**," which describes the chemical function of an enzyme (e.g., ATP:D-hexose 6-phosphotransferase; Table 10.2); and (3) the **enzyme classification number** (e.g., EC 2.7.1.1), which is based on the systematic naming system. It is text generally uses only the recommended names, although such a name is occasionally at odds with the physiological function of an enzyme. With the recommended names, some enzymes are named after the compound they degrade (e.g., glucose 6-phosphatase, which degrades glucose 6-phosphate to glucose and phosphate; see Chapters 24 and 25). Others are named more descriptively after the reaction they catalyze (e.g., alcohol dehydrogenase, which removes hydrogen from alcohol [see Chapter 30], or aminotransferase, which transfers an amino group from an amino acid to a keto acid [see Chapter 35]). Some enzymes are named after the reactions they catalyze in test tubes, but not in the human body.

Virtually all enzymes are **proteins**. However, a small number of RNAs can also catalyze chemical reactions. These RNAs are collectively called **ribozymes**, RNA enzymes, or catalytic RNAs. Physiologically important ribozymes typically catalyze reactions that involve RNA as a substrate (e.g., RNA processing by spliceosomes [see Chapter 6] or protein

Table 10.1 Classification of Enzymes by Recommended Name

Recommended Name	Systematic Name	Reaction Catalyzed*	Examples
Kinase	ATP (substrate): phosphotransferase	$A–OH + ATP \leftrightarrow A–P_i + ADP$	Hexokinase, cAMP-dependent protein kinase = protein kinase A
Phosphatase	Phosphohydrolase	$A–P_i + H_2O \leftrightarrow A–OH + H–P_i$	Glucose 6-phosphatase, phosphoprotein phosphatase
Synthase	Oxidoreductase, transferase, ligase (depending on the reaction)	$A + B \leftrightarrow C + D$	Methionine synthase, thymidylate synthase
Dehydrogenase	Oxidoreductase	$AH_2 + B \leftrightarrow A + BH_2$	Glucose 6-phosphate dehydrogenase, malate dehydrogenase, alcohol dehydrogenase
Reductase	Oxidoreductase	$AH_2 + B \leftrightarrow A + BH_2$	Hydroxymethylglutaryl coenzyme A reductase (HMG-CoA reductase), dihydrofolate reductase
Peptidase	Most often: hydrolase	$A + H_2O \leftrightarrow B + C$	Trypsin, elastase, carboxypeptidase

*The reactions are written in the direction that corresponds to the recommended name. The reactions are shown as being reversible (↔) because enzymes work in both directions. The concentrations of reactants and products affect the direction of the reaction.
ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A.

Table 10.2 Classification of Enzymes by Systematic Name

Systematic Name	EC Number	Reactions Catalyzed (Sample)	Examples (Recommended Names)
Oxidoreductase	EC 1.x.x.x.	$AH_2 + B \leftrightarrow A + BH_2$	Glycerol-3-phosphate dehydrogenase, lactate dehydrogenase, malate dehydrogenase
Transferase	EC 2.x.x.x.	$AB + C \leftrightarrow A + BC$	Hexokinase, various amino acid transaminases
Hydrolase	EC 3.x.x.x.	$A + H_2O \leftrightarrow B$	3′,5′-Cyclic-nucleotide phosphodiesterase, methenyltetrahydrofolate cyclohydrolase
Lyase	EC 4.x.x.x.	X ↔ A + B (a bond must be broken by hydrolysis or oxidation; or: a new double bond or a new ring must be formed)	Fructose-bisphosphate aldolase, fumarate hydratase, argininosuccinate lyase
Isomerase	EC 5.x.x.x.	X–A–B–Y ↔Y–A–B–X	Triose-phosphate isomerase, UDP-glucose 4-epimerase, prostaglandin D synthase, phosphoglycerate mutase
Ligase	EC 6.x.x.x.	$A + B + C \leftrightarrow A–B + D + E$	Various amino acid tRNA ligases, DNA ligase, ubiquitin-protein ligase, argininosuccinate synthase, pyruvate carboxylase

EC, enzyme classification number; UDP, uridine diphosphate.

synthesis by ribosomes [see Section 3 in Chapter 7]). Unless specifically stated otherwise, in this chapter the term "enzyme" refers to a protein.

A **zymogen** or **proenzyme** is a precursor protein to an active enzyme that has little or no enzymatic activity; however, once it is processed posttranslationally, often by limited proteolysis, it becomes competent for catalysis. For instance, the pancreas secretes enzymatically inactive trypsinogen, chymotrypsinogen, and proelastase, which become active trypsin, chymotrypsin, and elastase, respectively, in the lumen of the intestine (see Chapter 34).

A **coenzyme** is an organic molecule that binds to an enzyme (transiently or permanently) and participates in catalysis. Coenzymes are typically needed for oxidation-reduction reactions and as temporary acceptors in the transfer of chemical groups. An example is lipoic acid, which participates in the oxidative decarboxylation of pyruvate by pyruvate dehydrogenase (see Chapter 22). Lipoic acid is covalently bound to the

side chain amino group of a lysine residue of pyruvate dehydrogenase. Lipoic acid transiently accepts a CH_3–CH(OH)– group, presents it for oxidation to CH_3–CO–, and transfers CH_3–CO– to yet another coenzyme, coenzyme A. Many enzymes contain tightly bound **metal ions** in their active sites; these metal ions are not usually called coenzymes.

Some terms in enzymology are poorly defined and consequently used in dif erent ways. T us, coenzymes are often also called **cofactors**. Sometimes, the distinction between **substrate** and coenzyme becomes blurry. For instance, NAD^+ and NADH are substrates of many dif erent dehydrogenases, but some scientists also refer to them as coenzymes or cofactors. Some coenzymes are called **prosthetic groups**. In general, the term prosthetic group implies that the coenzyme is tightly or covalently bound to the enzyme and does not dissociate from the enzyme during catalysis. An example of such a prosthetic group is biotin, which is linked covalently to a lysine residue in all human carboxylases (see Section 3 in Chapter 22 and Section 2 in Chapter 27). Other examples are lipoic acid (mentioned in the preceding paragraph) and heme in cytochromes (see Chapters 14 and 23).

T e term **apoenzyme** refers to a coenzyme-dependent enzyme that does not have a coenzyme bound to it, while the term **holoenzyme** refers to such an enzyme that has its coenzyme bound to it, usually tightly. Alternatively, a holoenzyme may be an enzymatically functional assembly of two or more molecules.

Isozymes (**isoenzymes**) are enzymes that catalyze the same reaction yet dif er in their amino acid sequences. Often, isozymes dif er in their maximal velocity of catalysis (i.e., V_{max}, see Section 3) and in the concentration of substrate that is needed for half-maximal activity (i.e., K_m or $S_{0.5}$; see Section 3). An example of this are the hexokinases, which phosphorylate glucose; hexokinases I, II, III, and IV are half-maximally active at glucose concentrations of about 0.03, 0.3, 0.03, and 5 mM, respectively. Some isoenzymes are derived from dif erent genes; others originate from a single gene through alternative use of transcription start sites, alternative RNA splicing, or dif erent posttranslational processing (see Chapters 6 and 7).

2. ENZYME CATALYSIS OF CHEMICAL REACTIONS

T e observed high specif city o*f* the binding o*f* substrates to enzymes is due to steric e*ff*ects and numerous *f*unctional group interactions between enzymes and substrates. An enzyme-catalyzed reaction is *f*aster than an uncatalyzed reaction because it has a lower activation energy. *T*emperature and pH pro*f*oundly in*fl*uence the activity o*f* enzymes.

Chemical thermodynamics allow one to determine whether a chemical reaction can take place, based on an analysis of the temperature, concentration, and standard Gibbs free energy (G^0) of the reactants and products. Only reactions that lead to an overall lowering of Gibbs free energy (those with a **negative ΔG**) occur spontaneously. T e **change in Gibbs free energy** during a reaction (ΔG) depends on the energy state of the atoms and the bonds between them, the movement of atoms and molecules in space (e.g., rotation, tumbling, and oscillation), and the concentrations of reactants and products. Only those reactions can occur spontaneously that result in a decrease in Gibbs free energy; enzymes do not change this fact.

When a chemical reaction is at **equilibrium**, there is no net force to drive it toward either reactants or products. At equilibrium, the thermodynamic driving force ΔG is therefore zero. In cells, there is usually some flux in all metabolic pathways; hence, virtually no reaction is exactly at equilibrium. However, a large number of reactions are close to equilibrium. Examples of such reactions are the isomerization of glucose 6-phosphate and fructose 6-phosphate in glycolysis (see Chapter 19) and gluconeogenesis (see Chapter 25), as well as the redox reaction between malate and oxaloacetate in the citric acid cycle (see Chapter 22).

An enzyme accelerates the rate of a thermodynamically feasible reaction by lowering the **activation energy** of the reaction. Many thermodynamically feasible reactions do not happen because their activation energy is too high. For instance, glucose and ATP in water at pH 7 do not appreciably react with each other. However, the enzyme hexokinase changes the reaction path so that the activation energy is low enough for the reaction to occur with high frequency at normal body temperature.

T e activation energy of a reaction is set largely by the energy level of the transition state that has the highest Gibbs free energy. Hence, the activation energy is also called the **transition state barrier** (Fig. 10.1). Since enzymes only af ect the reaction path but not the nature of the reactants or products, enzymes do not af ect the position of the equilibrium between the reactants and products.

Temperature af ects enzymes in two opposing manners. An increase in temperature facilitates a reaction by increasing the number of molecules that have sufficient energy to cross the transition state barrier. On the other hand, most human enzymes lose their structure as the temperature rises. T e net result is that most enzymes have their highest activity near the physiological core body temperature.

pH af ects enzymatic activity via the degree of protonation of amino acid side chains and peptide termini; these, in turn, af ect the formation of hydrogen bonds and electrostatic interactions. Often, it is the protonation of residues near the catalytic site that is most important in determining the pH optimum of an enzyme. T ese residues can serve as proton donors or acceptors. T e pH may also af ect the protonation of a substrate. Enzymes tend to have the highest activity at the pH at which they normally should be active. For many enzymes that normally reside in the lumen of the intestine, the blood, extracellular space, cytosol, endoplasmic reticulum, secretory vesicles, mitochondria, or nucleus, the optimal pH is in the range of 7 to 8. For many enzymes that must be active in the lumen of the stomach or inside lysosomes, the optimal pH is in the range of 4 to 5. Some of these enzymes become inactive when they are exposed to a near-neutral pH. However, many

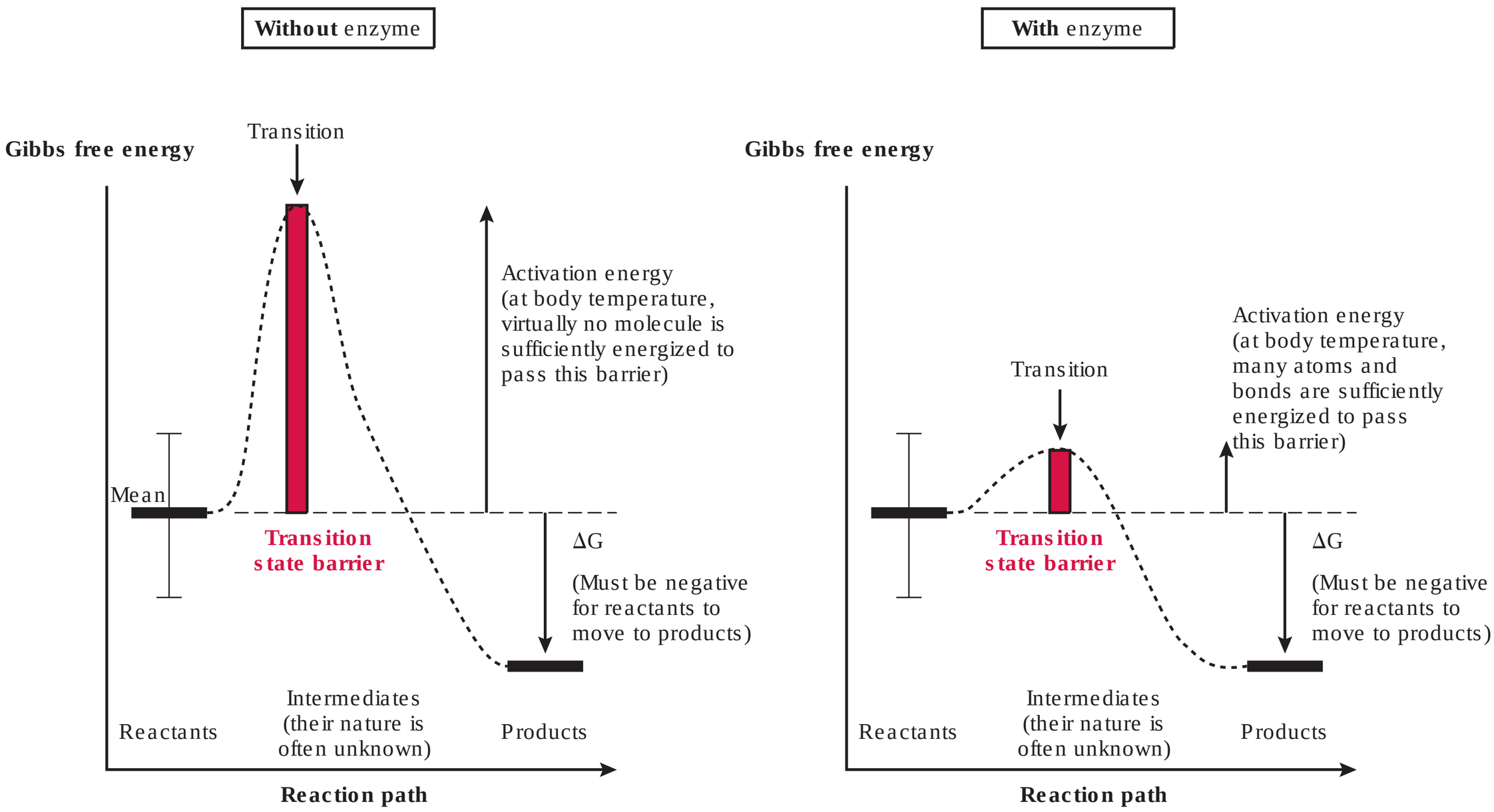


Fig. 10.1 **The effect of an enzyme on the energy diagram of a chemical reaction.** The means of the Gibbs free energy of intermediates on the reaction path are plotted (the thermal energy of individual molecules varies a bit around the mean).

other enzymes are highly active even far from their normal physiological pH.

Most enzymes accept only a narrow range of substrates. Specificity stems from the substrate-binding site, as well as the catalytic mechanism. Substrates are bound with high specificity through a three-dimensional network of electrostatic effects, hydrophobic effects, hydrogen bonds, and van der Waals interactions, as already described for the protein-protein interactions in Section 3 in Chapter 9. Since enzymes consist of L-amino acids, which are chiral, enzymes themselves are chiral (i.e., they have a handedness). Indeed, most enzymes can distinguish the **enantiomers** of substrates if the chiral center of the substrate is near the substrate binding site.

Based on the observed specificity of enzymes for substrates, the **key-lock theory** of the late nineteenth century postulates that the catalytic sites of enzymes fit substrates precisely like a negative fits its corresponding positive. This theory can indeed explain many interactions among enzymes, substrates, and inhibitors, although it presents enzymes and substrates as static, inflexible structures. Based on a more thorough understanding of chemical structures and the interactions of substrates and enzymes, the **induced fit theory** of the mid-twentieth century improved on the key-lock theory. This theory expressly takes into account that the three-dimensional structure of enzymes and substrates is not rigid but pliable. The induced fit theory postulates that the binding of a substrate may induce an appreciable change in the orientation of the reactive groups of the active site of an enzyme and that catalysis often occurs only when these reactive groups have a specific orientation. Fig. 10.2 shows the structure of an enzyme that uses an induced fit mechanism.

Enzymes **catalyze** chemical reactions by providing a reaction path that has a lower transition state barrier than the uncatalyzed reaction and therefore occurs more readily. This can be accomplished by the following means. Some enzymes induce a conformational strain in a substrate as they bind it. A change in enzyme conformation, often induced by the binding of a substrate, sometimes brings the substrate and cofactor into a particularly favorable alignment. Other enzymes preferentially bind substrate molecules that have assumed an optimal orientation and configuration that favors a reaction and thus lowers the transition state barrier. Enzymes facilitate catalysis in a three-dimensional structure that provides well-positioned chemical groups (including those on cofactors), reaction-dependent movement of these chemical groups, or a space that may be free of water or have only one or two strategically placed water molecules. Regarding the latter, binding of a substrate sometimes induces a movement of an enzyme domain that encloses the substrate in a chamber made up of amino acids that no longer have a connection to bulk water. In many enzymes, substrates, cofactors, and intermediates are bound in such a way as to stabilize the transition state.

Enzymes are sometimes organized into **complexes** with closely spaced active sites (e.g., carbamoyl-phosphate synthase II/aspartate carbamoyltransferase/dihydroorotase in pyrimidine nucleotide de novo synthesis; see Chapter 37), or with prosthetic groups that pass intermediates from active site to

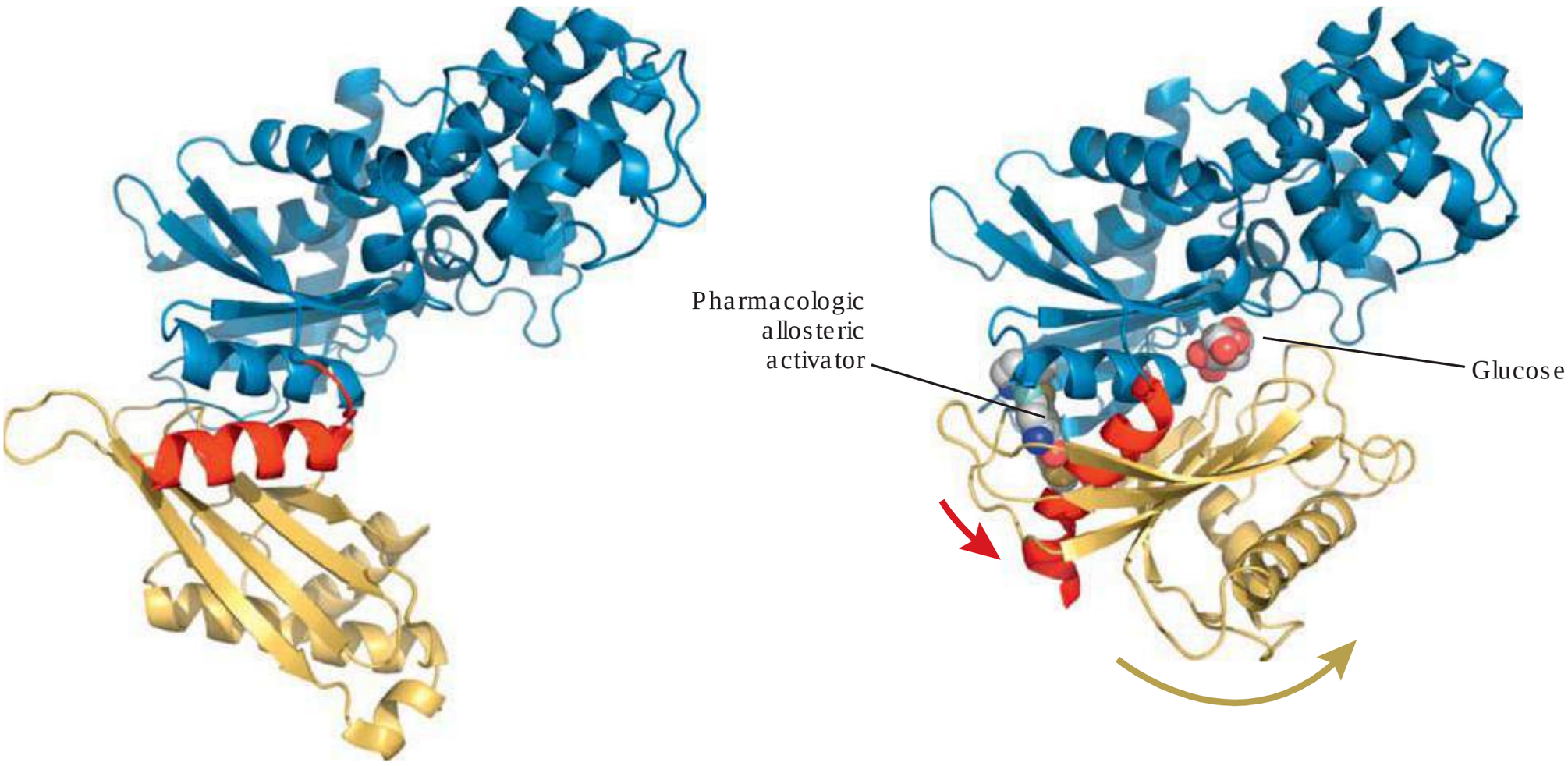


Fig. 10.2 Induced fit in glucokinase. In the insulin-secreting pancreatic β-cells, glucokinase serves as a glucose sensor (see Chapter 26). In the liver, glucokinase produces glucose 6-phosphate mostly in the fed state (see Chapter 19). *Left,* Pure glucokinase. *Right,* Glucokinase with glucose and a pharmacological allosteric activator. The larger domain (blue) is shown in the same position. Upon binding glucose, the smaller domain (gold) rotates by ~100 degrees relative to the larger domain, and the end of the C-terminal helix (red) moves away from the larger domain, with glucokinase thereby enclosing glucose in a chamber. (Based on Protein Data Bank [www.rcsb.org] files 1V4T and 1V4S from Kamata K, Mitsuya M, Nishimura T, Eiki J, Nagata Y. Structural basis for allosteric regulation of the monomeric allosteric enzyme human glucokinase. *Structure*. 2004;12:429-438.)

active site (e.g., fatty acid synthase; see Chapter 27). Such arrangements are favorable due to reduced dif usion, degradation, or competition of substrates, as well as reduced interaction of substrates with the surrounding fluid.

Amino acid side chains of enzymes can participate in catalysis. For instance, Cys can form covalent bonds via the sulfur atom of its sulfhydryl group and Ser via the oxygen atom of its hydroxyl group. Similarly, reversible protonation and deprotonation can occur on a nitrogen atom of the 5-membered ring of His, on an oxygen atom of the carboxyl group of Asp or Glu, or on the oxygen atom of the hydroxyl group of Ser (for structures of amino acids, see Chapter 9).

Enzymes dif er vastly in the time it takes to complete a **catalytic cycle**. A catalytic cycle starts, for example, with the binding of substrate and ends with the release of the product and formation of the enzyme in its initial state. G-proteins by themselves may take up to about 7000 seconds (~2 hours) to complete a catalytic cycle (hydrolysis of GTP to GDP; see Chapter 33). Glucokinase needs about 0.02 seconds to phosphorylate glucose to glucose 6-phosphate (see Chapter 19), while fumarase needs only about 0.001 seconds to convert fumarate + H_2O to malate (see Chapter 22), and carbonic anhydrase needs just about 0.000002 seconds to convert CO_2 + H_2O to HCO_3^- + H^+ (see Chapter 16).

T e **turnover number** of an enzyme (often called $\mathbf{k_{cat}}$) is the maximal number of catalytic cycles an enzyme performs in 1 second. For instance, if a catalytic cycle takes 0.001 seconds, the turnover number is 1/0.001 s = 1000 s^{-1}.

3. ENZYME ACTIVITY AS A FUNCTION OF THE CONCENTRATION OF SUBSTRATES

T e Michaelis-Menten equation describes the activity o*f* simple enzymes as a *f*unction o*f* substrate concentration. A modif ed *f*orm o*f* this equation describes the behavior o*f* enzymes that show cooperativity toward a substrate.

T e **Michaelis-Menten** equation describes enzyme activity as a function of the concentration of a single substrate. T is equation is valid only when the concentration of the product is negligible, and the concentration of the enzyme-substrate complex stays constant.

$$\frac{v}{V_{max}} = \frac{s}{s + K_m}$$

In the above equation, **v** is the rate at which the enzyme gives rise to the product (moles/time). $\mathbf{V_{max}}$ is the maximal rate at which the enzyme can make a product (this is assumed to happen at an infinitely high substrate concentration, but in practice there are limits to the substrate concentration, such as substrate solubility and ef ects of the solution on enzyme structure). T e concentration of substrate is denoted by s. $\mathbf{K_m}$ is the concentration of substrate at which the enzyme gives rise to the product at **half** its **maximal** rate (i.e., $v/V_{max} = 0.5$). A graph of values that fit this equation is shown in Fig. 10.3. V_{max} and K_m are the characteristic properties of individual enzymes.

If an enzyme has **two substrates**, the Michaelis-Menten equation still applies if the concentration of one substrate is

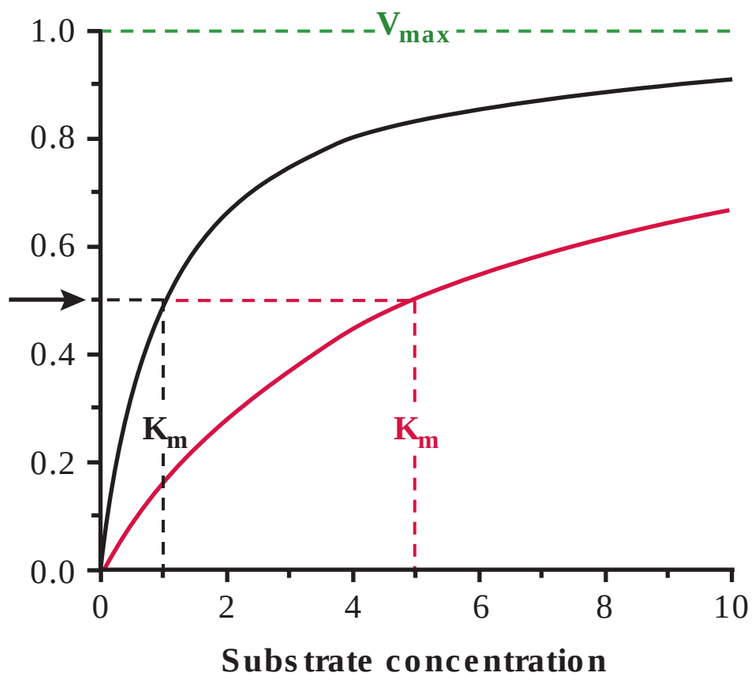


Fig. 10.3 **Activity of two different enzymes as a function of the substrate concentration and as predicted by the Michaelis-Menten equation.**

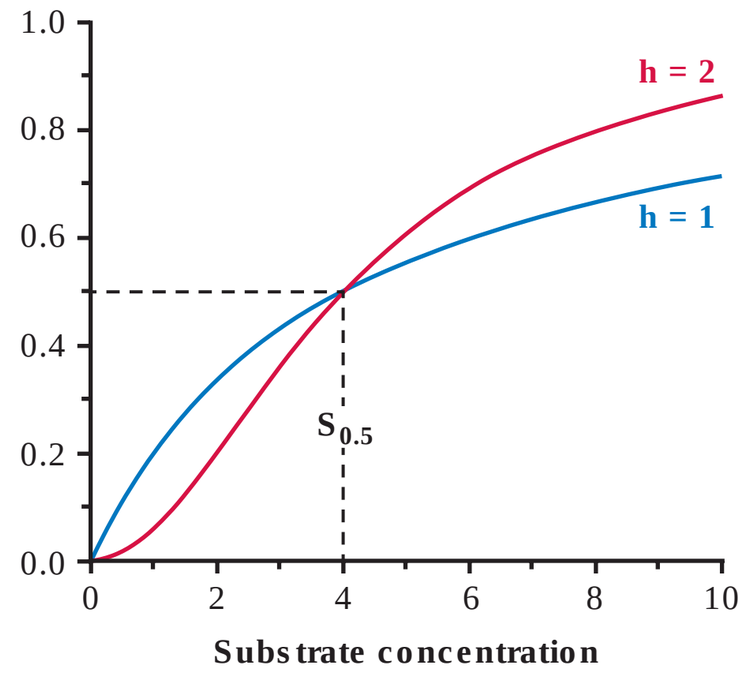


Fig. 10.4 **Enzyme activity as predicted by a modified Michaelis-Menten equation for enzymes that show cooperativity toward their substrate.** No cooperativity: blue. With cooperativity: red.

Table 10.3 **Substrate Concentration and Enzyme Activity as Predicted by the Michaelis-Menten Equation**

Substrate Concentration	Relative Enzyme Activity (v/V_{max})
$0.1 \times K_m$	0.09
$1 \times K_m$	0.50
$10 \times K_m$	0.91
$100 \times K_m$	0.99

held constant and that of the other varied. Furthermore, the product of v/V_{max} for substrate 1 and v/V_{max} for substrate 2 is often a useful approximation of the rate of a reaction that has two substrates.

An equation of the same mathematical form as the Michaelis-Menten equation also applies to the binding of molecules to proteins, such as the binding of insulin to the insulin receptor, or of cobalamin to intrinsic factor. Thereby, actual binding replaces v, and the maximum amount of binding replaces V_{max}. K_s, the concentration at which binding to the protein is half-maximal, replaces K_m, the substrate concentration at which an enzyme is half-maximally active.

According to the Michaelis-Menten equation, enzymatic activity is **not directly proportional** to the substrate concentration. A few key numbers are listed in Table 10.3. (Note that if the substrate concentration is far below the K_m, and less than $0.01 \times K_m$, the reaction rate v is nearly proportional to the substrate concentration s.)

Under physiological circumstances, the substrate concentration is often below the K_m of an enzyme. Hence, most enzymes normally work at only a fraction of their maximal catalytic capacity, and they therefore have a lot of reserve capacity.

The higher the K_m of an enzyme for a substrate, the higher the concentration of the substrate must be to achieve a certain v/V_{max}.

The Michaelis-Menten equation is valid whether the rate-limiting step in the enzyme catalysis is the association between the enzyme and the substrate or the reaction from enzyme-substrate complex to the product. In the latter case, the K_m is a measure of the **substrate affinity** of an enzyme.

Enzyme activity is usually stated in **enzyme units** (**U**). One U is the amount of an enzyme that produces 1 µmol of product per minute. The conditions for this enzyme activity are not standardized across the board, but must be stated in each report. In general, a unit refers to a measured V_{max}.

Some enzymes consist of **several interacting subunits** with mutually dependent conformations: binding of substrate to one subunit induces a conformational change in another subunit, thereby changing its catalytic properties. This is phenomenon is called **cooperativity**. The activity of enzymes that show cooperativity toward a substrate can be described by a **modified Michaelis-Menten equation** as follows:

$$\frac{v}{V_{max}} = \frac{(s)^h}{(s)^h + (S_{0.5})^h}$$

In this equation, $\mathbf{S_{0.5}}$ is the concentration of substrate at which the enzyme shows half-maximal activity; the term $S_{0.5}$ is used in place of K_m because it denotes not one substrate molecule binding to the enzyme and reacting, but a mean of several substrate molecules binding and reacting. h is also called the **Hill coefficient** or **cooperativity coefficient**. It does not have to be an integer. If h is larger than 1, the enzyme is said to display **positive cooperativity**. A linear plot of the activity of such an enzyme versus substrate concentration shows an S-shaped relationship (Fig. 10.4). (Negative cooperativity is not discussed here.)

Examples of enzymes that have cooperativity are **glucokinase**, which has a Hill coefficient of about 1.5 (see Chapters 19 and 26), and phosphofructokinase 1 (see Chapter 19) and

amidophosphoribosyltransferase (see Chapter 38), which have Hill coefficients of about 2.

A formula similar to that for enzyme cooperativity is found for the cooperative binding of ligands to proteins under equilibrium conditions, such as oxygen to hemoglobin (hemoglobin saturation with O_2 = hemoglobin-bound O_2/maximal amount of hemoglobin-bound O_2 = $O_2^h/[O_2^h + P_{50}^h]$; see Chapter 16).

A physiologically important dif erence between an enzyme that follows Michaelis-Menten kinetics and one that shows cooperativity is that the latter may be turned of more fully at substrate concentrations below $S_{0.5}$ (see Fig. 10.4).

4. ACTIVATORS AND INHIBITORS OF ENZYMES

Inhibitors and activators *of* enzymes play major roles in the physiological and pharmacological regulation *of* enzymes. Such modif ers bind in the active site *of* an enzyme, a regulatory site that is remote *f*rom the active site, or any other place on the enzyme. Among the inhibitors, competitive inhibitors compete with the substrate *f*or binding to the active site, while noncompetitive inhibitors do not show such competition.

Many enzymes are controlled by reversibly binding inhibitors and activators. A substrate binds to a site that is variably referred to as the **substrate binding site**, **active site**, or **catalytic site**. Activators typically bind to a site at some distance from the substrate binding site; in this case, they are allosteric **activators** (see Fig. 10.2 and Table 10.4). Inhibitors that compete with the substrate for a binding site are called competitive **inhibitors**. Inhibitors that bind to a site at some distance from the substrate binding site(s) are allosteric inhibitors. Some of these inhibitors are called noncompetitive **inhibitors** (there are also mixed and uncompetitive inhibitors, which are not discussed here). Physiological noncompetitive inhibitors often bind to specific regulatory sites on enzymes.

If an enzyme is exposed to a competitive inhibitor, the substrate competes with the inhibitor. A high concentration of the substrate can, therefore, make the presence of inhibitor irrelevant to enzyme activity. A competitive inhibitor increases the apparent K_m, but it has no ef ect on the V_{max} of an enzyme. In contrast, a noncompetitive inhibitor decreases the V_{max}, but it has no ef ect on the K_m.

In enzymology, the adjective "allosteric" is often misused as a substitute for "cooperative." In a strict sense, an allosteric activator or inhibitor (also called allosteric ef ector) by definition binds to an enzyme at some distance from the catalytic site. Most allosterically regulated enzymes are also multimeric complexes of several identical subunits, and they do show cooperativity (see Section 3). However, this does not mean that all allosterically regulated enzymes must show cooperativity (cooperativity usually means that the conformation of one subunit af ects the conformation of other subunits in a multimeric enzyme complex).

If an enzyme accepts **dif erent substrates into the same site**, each substrate acts as a competitive inhibitor of the other substrate. For instance, alcohol dehydrogenase accepts ethanol, ethylene glycol ("antifreeze"), and methanol as substrates. In methanol- or ethylene glycol-poisoned patients, ethanol can therefore be used to diminish the formation of a toxic product from either methanol or ethylene glycol (see Chapter 36).

While practically all physiological and most pharmacologically used inhibitors act reversibly, others act irreversibly. Among these **irreversible inhibitors**, some simply dissociate from the enzyme extremely slowly, and they are thus irreversible only for practical purposes. Truly irreversible inhibitors react with an enzyme by forming a covalent bond that cannot be split readily. T ese inhibitors can modify any portion of the enzyme. In terms of enzyme kinetic analysis, they never exhibit purely competitive inhibition. Some of these irreversible inhibitors resemble substrates but then trap the enzyme irreversibly in one phase of its catalytic cycle. T ese latter inhibitors are also called **suicide inhibitors**; Table 10.4 provides three examples. An enzyme that has reacted with a suicide inhibitor cannot regain its catalytic activity and must be degraded and replaced.

T e commonly used terms feedback inhibition, product inhibition, and feed-forward activation are based on knowledge of metabolic pathways. **Feedback inhibition** refers to the inhibition of the activity of an enzyme by a downstream product. **Product inhibition** refers to the inhibition of an enzyme by its product. Some scientists view product inhibition as a form of feedback inhibition. Others reserve the term feedback inhibition for inhibitors that are not the immediate product of the reaction in question, and they therefore do not consider product inhibition to be a form of feedback inhibition. **Feed-forward activation** refers to the activation of an enzyme by an upstream metabolite. T e following are examples of feedback inhibition, product inhibition, and feed-forward activation in glycolysis (see Chapter 19): glucose 6-phosphate product inhibits hexokinase, ATP feedback inhibits phosphofructokinase 1, and fructose 1,6-bisphosphate feed-forward activates pyruvate kinase.

Some enzymes change activity in response to **covalent modification**. T e best-known examples are enzymes that are influenced by **phosphorylation** (the addition of a phosphate group) at one or more key residues. Kinases add phosphate groups, and phosphatases remove them. Examples are glycogen synthase and glycogen phosphorylase (see Chapter 24).

5. ENZYME ACTIVITY AND FLUX IN METABOLIC PATHWAYS

For convenience, enzyme-catalyzed reactions are categorized into physiologically reversible and irreversible reactions. T e irreversible reactions occur physiologically in only one direction, regardless *of* the concentrations *of* the reactants and products. T ese reactions commit molecules to pathways, and the activity *of* enzymes that catalyze them is *oft*en regulated by the concentration *of* a product *of* the pathway. A partial def ciency *of* an enzyme *of* a metabolic pathway may limit *fl*ux through every step *of* the pathway.

Table 10.4 Examples of Commonly Encountered Activators and Inhibitors of Enzymes

Type and Subtype	Pathway	Example
Allosteric activator	Glycolysis	Fructose-2,6-bisphosphate activates phosphofructokinase 1 by increasing its affinity for the substrate fructose-6-phosphate (see Chapter 19).
	Glycogen synthesis	Glucose 6-phosphate activates glycogen synthase in part by increasing its affinity for the substrate UDP-glucose (see Chapter 24).
	Signaling	cAMP activates cAMP-dependent protein kinase by causing the dissociation of regulatory subunits that inhibit catalytic subunits (see Chapter 33).
	Synthesis of urea	N-acetylglutamate induces an active conformation of carbamoylphosphate synthase I, which catalyzes an early step in the elimination of nitrogen as urea (see Chapter 35).
REVERSIBLE INHIBITORS		
Competitive inhibitor	Cholesterol synthesis	The widely used **statin** drugs inhibit HMG-CoA reductase by binding to the active site in place of HMG-CoA (see Chapter 29).
	Synthesis of dTMP	The antineoplastic drug **methotrexate** inhibits dihydrofolate reductase by binding to the same site as the substrate dihydrofolate (see Chapter 37).
Noncompetitive inhibitor	Glycolysis	ATP allosterically inhibits phosphofructokinase 1 by decreasing its affinity for the substrate fructose-6-phosphate (see Chapter 19).
IRREVERSIBLE INHIBITORS		
Suicide inhibitor	Synthesis of dTMP	The body converts the antineoplastic/cancer chemotherapeutic drug **5-fluorouracil** to 5F-dUMP, which reacts in place of dUMP (i.e., 5H-dUMP) with thymidylate synthase and N^5, N^{10}-methylene tetrahydrofolate, leading to an intermediate that cannot go forward or backward in the reaction sequence (see Chapter 37).
	Formation of urate	Xanthine oxidase normally converts hypoxanthine to xanthine and then to urate. **Allopurinol** gets converted to oxypurinol, which inhibits xanthine oxidase irreversibly (see Chapter 38).
	Acidification of stomach	An H^+, K^+-ATPase normally pumps H^+ out of parietal cells and into the lumen of the stomach (see Chapter 34). **Omeprazole** and related proton pump inhibitors form a covalent bond with a cysteine residue of the H^+, K^+-ATPase that is very long lived.

ATPase, adenosine triphosphatase; cAMP, cyclic adenosine monophosphate; dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; UDP, uridine diphosphate.

In clinical biochemistry, it is useful to divide enzyme-catalyzed reactions into groups of physiologically reversible and irreversible reactions.

All chemical and enzyme-catalyzed reactions can only proceed in the direction of liberating Gibbs free energy (see Section 2). In theory, reactants and products of any chemical reaction can be chosen such that the reaction proceeds forward or backward (i.e., every chemical reaction is reversible). Enzymes also catalyze reactions in both directions. However, inside an organism, only a relatively narrow range of concentrations of the reactants and products is compatible with life.

T is text uses the terms reversible and irreversible as follows. Physiologically **reversible reactions** are often close to equilibrium and they can proceed forward or backward, depending on the prevailing concentrations of the reactants and products (these reactions generally have a change in Gibbs free energy, ΔG, between 0 and about −20 kJ/mol). **Physiologically irreversible** reactions are far from equilibrium and proceed only in one direction because the concentration of the products relative to that of the reactants is never high enough to reverse the reaction (these reactions generally have a change in Gibbs free energy, ΔG, between about −20 and −130 kJ/mol). In the liver, for example, the reversible reactions in glycolysis proceed in one direction in the fed state (see Chapter 19), and in the opposite direction in the fasting state (see Chapter 25); in the fasting state, enzymes that catalyze the irreversible reactions of glycolysis are made quite inactive and enzymes that catalyze dif erent irreversible reactions for gluconeogenesis are made active.

Irreversible reactions commit chemicals to pathways, and the rate of these reactions does not depend on the concentration of the products. In the pathway shown in Fig. 10.5, reaction 1 irreversibly feeds A into the pathway. Such a reaction is typically regulated, often via inhibition from a downstream product (i.e., feedback inhibition; see Section 4). Furthermore, such a reaction often also limits flux through the pathway (flux is the number of molecules that pass through per unit of time). In glycolysis, for example (see Chapter 19), A is fructose 6-phosphate, and B is fructose 1,6-bisphosphate; reaction 1 is

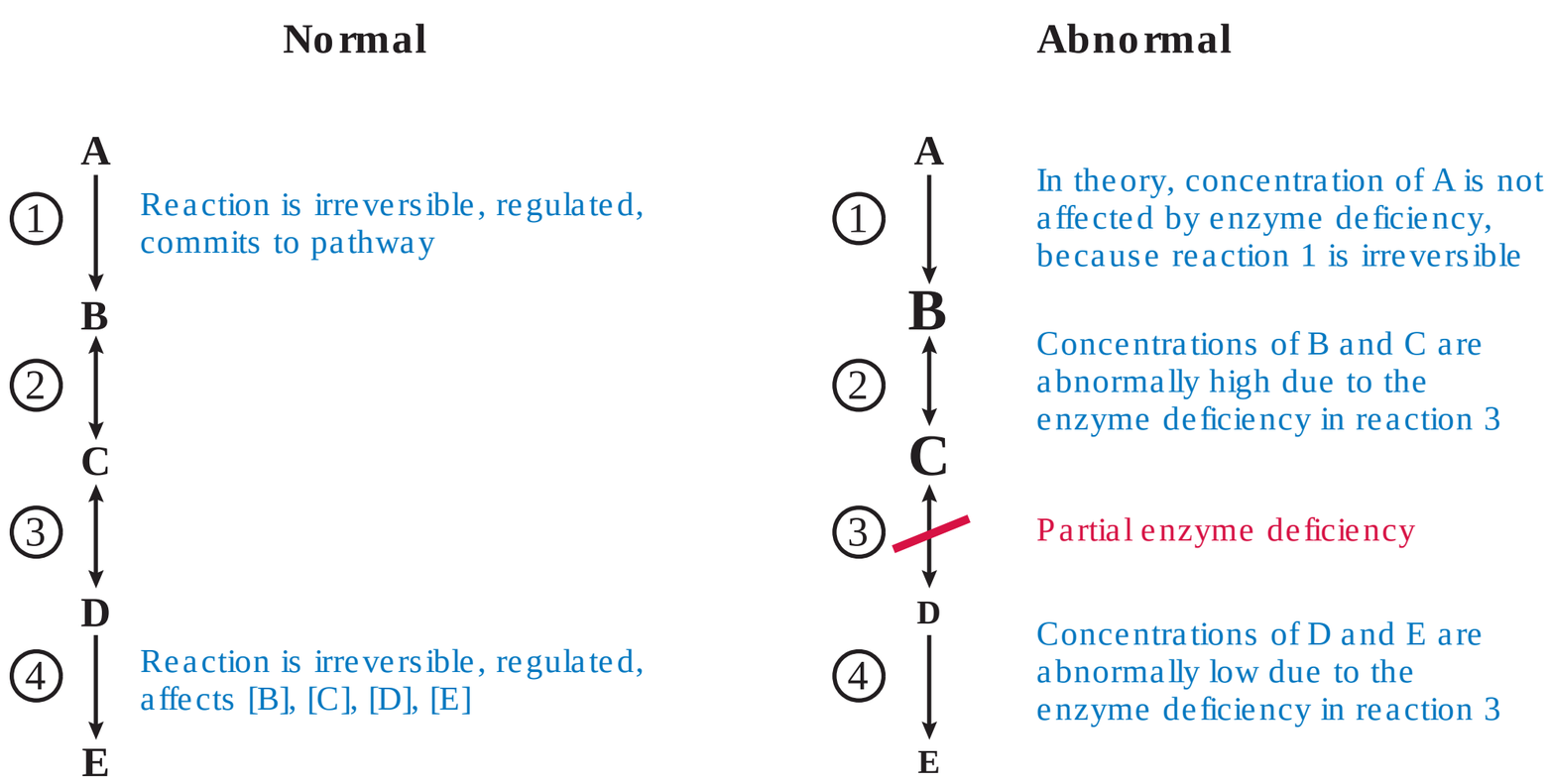


Fig. 10.5 Normal and abnormal regulation of flux through a metabolic pathway. Single-headed arrows indicate irreversible reactions; double-headed arrows indicate reversible reactions.

catalyzed by phosphofructokinase 1, and this enzyme is regulated by the concentrations of AMP and ATP. T ese nucleotides can be viewed as feedback regulators since glycolysis lowers the concentration of AMP and maintains or increases the concentration of ATP.

In the pathway discussed above (see Fig. 10.5), B is converted to C and then to D by the reversible reactions 2 and 3. Under normal circumstances, there is usually an excess of enzyme activity to catalyze such reactions. Additionally, the activity of enzymes that catalyze these reactions is not regulated. T e relative concentrations of B, C, and D depend largely on chemical thermodynamics, and to a lesser degree on the rate of flux through the pathway (at a low rate of flux, the concentrations of B, C, and D are closer to equilibrium than at a high rate of flux). Examples of reversible reactions in glycolysis are those shown with bidirectional arrows in Fig. 19.2 in Chapter 19.

In the pathway shown in Fig. 10.5, reaction 4, which is irreversible, determines the concentrations of intermediates B, C, and D. T e rate of the irreversible reaction 4 depends only on the concentration of D. If the rate of reaction 4 is less than that of reaction 1, the concentration of intermediates B, C, and D increases, which also enhances the rate of reaction 4. T us, the concentrations of B, C, and D depend on the amount of enzyme that catalyzes reaction 4, as well as on the kinetic properties of this enzyme. An example of reaction 4 in glycolysis is the conversion of phosphoenolpyruvate to pyruvate, which is catalyzed by pyruvate kinase (see Chapter 19). Some intermediates of glycolysis are needed for other pathways so that a control of the concentrations of these intermediates becomes important. Indeed, the activity of pyruvate kinase is controlled not just by the amount of enzyme that is present and active, but also by an intermediate of the pathway, fructose 1,6-bisphosphate (e.g., B in the pathway of Fig. 10.5).

Steady state is defined as a state in which the concentrations of reactants and products do not change. Many metabolic reactions can be considered to be very close to steady state. If a linear pathway of metabolism is in steady state, every reaction also shows the same flux. If the pathway shown in Fig. 10.5 is in a steady state, flux through the reaction A → B is therefore the same as through reaction B → C, and so forth. In cells, steady state is typically achieved within seconds. Samples taken from patients (e.g., blood) are expected to reflect steady-state conditions.

If there is a partial or complete **deficiency** of the enzyme that catalyzes reaction 3 in the pathway shown in Fig. 10.5, there is an increase in the concentrations of B and C, and a decrease in the concentrations of D and E. T e concentration of A is not af ected because it is followed by an irreversible reaction, the rate of which is not influenced by the concentration of product B. Again, steady state is generally reached within seconds. If a patient has a deficiency in enzyme 3, the build-up of B and C, or the depletion of D and E, can have clinical significance.

6. ENZYMES IN THE BLOOD THAT HAVE DIAGNOSTIC SIGNIFICANCE

Data on the activity o*f* certain enzymes in the blood provide the clinician with in*f*ormation about tissue damage.

Tissues normally lose a small fraction of their enzymes into the bloodstream. Tissue damage enhances this loss. Measurements of the activities in the blood of some of these enzymes provide the clinician with useful data (Table 10.5). In blood, these enzymes play no physiological role.

7. ENZYME-LINKED IMMUNOSORBENT ASSAY

Enzyme-linked immunosorbent assays (ELISAs) allow the measurement o*f* a very small amount o*f* an antigen or antibody due to the high sensitivity with which enzyme activity o*f* an enzyme-antibody or the enzyme-antigen conjugate can be measured.

Table 10.5 Enzymes in the Blood That Are Useful for Diagnosis

Enzyme	Interpretation of Abnormal Activity
γ-Glutamyl transpeptidase	Elevation may indicate liver damage.
Alanine aminotransferase	Elevation may indicate liver damage.
Aspartate aminotransferase	Elevation may indicate damage to liver or heart.
Alkaline phosphatase	Elevation may indicate obstructive liver disease or a bone disorder.*
Creatine kinase	Elevation may indicate damage to skeletal or cardiac muscle. Isozymes exist, and isozyme composition can be determined.
Lactate dehydrogenase	Elevated total activity may indicate damage to one of many tissues. Isozymes exist.
Amylase	Elevation may indicate an obstruction of the pancreatic duct or damage to the pancreas.
Lipase	Elevation may indicate damage to the pancreas.

*Normally elevated in pregnant women due to loss from the placenta.

ELISAs are carried out with an enzyme that is covalently linked to an antigen or an antibody. In the simplest form of the assay (Fig. 10.6), an antigen is immobilized on the surface of a small container, an enzyme-antibody conjugate is added, and the activity of the bound enzyme is then measured (often by measuring a light-absorbing or a fluorescent product). One such application is the measurement of ferritin in serum (see Section 7 in Chapter 15). Alternatively, antibodies in a patient's serum are adsorbed, an antigen-enzyme conjugate is added, and the activity of the bound enzyme is measured. T is method is currently used to measure antibodies against the hepatitis C virus or the human immunodeficiency virus.

SUMMARY

- Enzymes can be identified by systematic name as oxidoreductases, transferases, hydrolases, lyases, isomerases, or ligases; they can also be identified by an enzyme classification (EC) number that is based on these categories. However, clinically relevant enzymes are for the most part still identified by their recommended names, which often have historical roots.
- Enzymes often contain nonpeptidic molecules as coenzymes. When such coenzymes are tightly or covalently bound, they are often called prosthetic groups; examples are biotin, heme, and lipoic acid.
- Isoenzymes (isozymes) are enzymes that catalyze the same reaction but dif er in their amino acid sequence. Often, isoenzymes also dif er in their kinetic properties; the hexokinases are an example.
- Enzymes act as catalysts. An enzyme speeds up a thermodynamically feasible reaction (one with a negative ΔG) by decreasing its activation energy. Enzymes do not change the position of the chemical equilibrium.

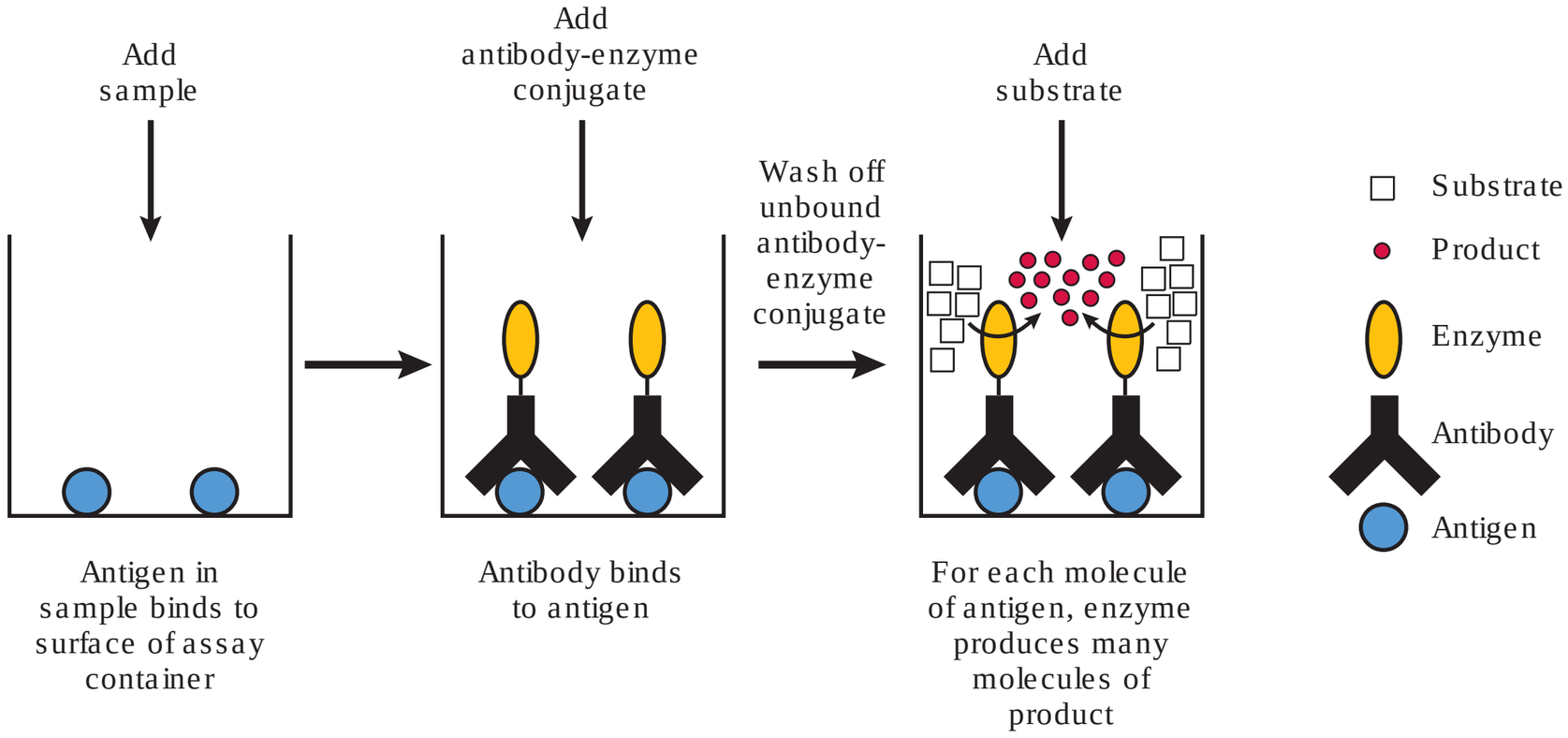


Fig. 10.6 Basic principle of the enzyme-linked immunosorbent assay. The antigen may bind directly to the surface of the assay container, or to antibodies with which the surface has been precoated. The product is usually measured by the absorbance of light, fluorescence upon excitation with light, or emission of light (luminescence).

- Enzymes have a pH and temperature optimum.
- The lock-and-key theory is an older and the induced-fit theory a more recent attempt to model enzyme-substrate interactions.
- The Michaelis-Menten equation is $v/V_{max} = s/(s + K_m)$. The K_m is the concentration of substrate s, at which the enzyme works at half its maximal velocity (i.e., s, at which $v/V_{max} = 0.5$). A doubling of substrate concentration does not usually lead to a doubling of enzyme activity because the v (or v/V_{max}) versus s plots are not linear.
- For enzymes that show cooperativity for a substrate, the Michaelis-Menten equation can be modified and written as $v/V_{max} = s^h/(s^h + S_{0.5}{}^h)$. $S_{0.5}$ is the substrate concentration at which the enzyme works at half its maximal velocity. h is the Hill coefficient.
- An enzyme unit (U) is usually the amount of enzyme that produces product at the rate of 1 µmol/min (based on the calculated V_{max}).
- Allosteric regulatory sites are by definition located at a site of the enzyme that is removed from the catalytic (or active) site. Competitive inhibitors compete with the substrate for binding to the catalytic site, while noncompetitive inhibitors bind to another site. Some drugs are suicide inhibitors (i.e., they typically bind to the catalytic site and then irreversibly inhibit the enzyme).
- The flux in metabolic pathways is often regulated by feed-forward activation, feedback inhibition, or product inhibition.
- In a linear metabolic pathway, a partial enzyme deficiency leads to an increase in the concentrations of metabolites that precede the impaired reaction (except metabolites that are upstream of an irreversible reaction) and a decrease in the concentrations of metabolites that follow the impaired reaction.
- The activities of some enzymes in the bloodstream are indicative of damage to a specific tissue. These activities are reported as U or IU per volume of the blood, plasma, or serum.
- Enzyme-linked immunosorbent assays (ELISAs) are used to measure low concentrations of proteins of clinical interest. These assays derive their specificity from antigen-antibody interactions, and their sensitivity from an antigen- or antibody-linked enzyme that produces many molecules of product for each protein molecule of interest.

FURTHER READING

- Biomolecular Movies and Images for Teaching. Induced fit and hexokinase. <http://www.chem.ucsb.edu/~molvisual/ABLE/induced_fit/index.html>.
- BRENDA: The Comprehensive Enzyme Information System. <http://www.brenda-enzymes.org>. This site provides a list of enzymes along with names, reactions, kinetic parameters, and a set of links to literature data.
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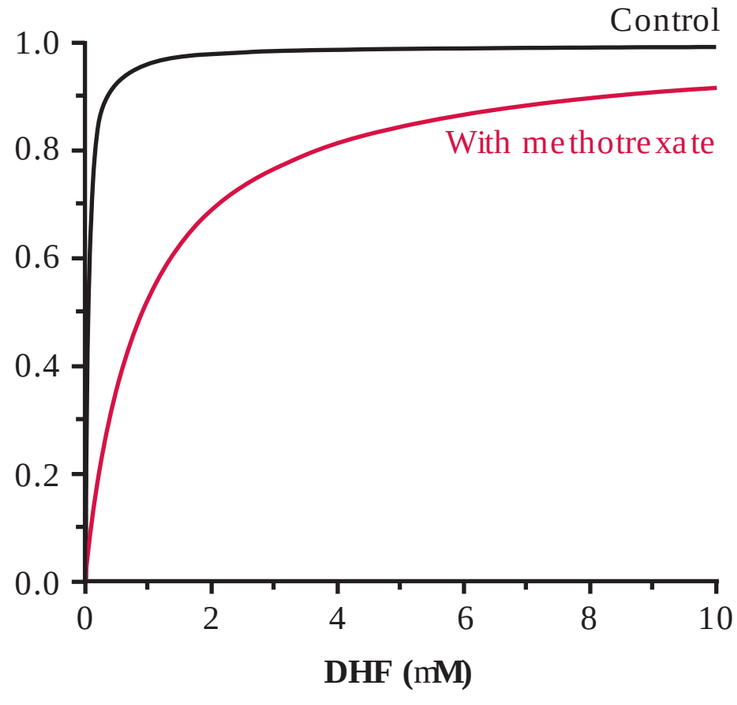
Review Questions

1. An enzyme that is normally active in the cytoplasm is tested both at pH 7 and at pH 5. Then the enzyme activities at these two conditions are compared. None of the catalytically active amino acid side chains has ionizable groups. At pH 5, protons most likely behave like which one of the following?

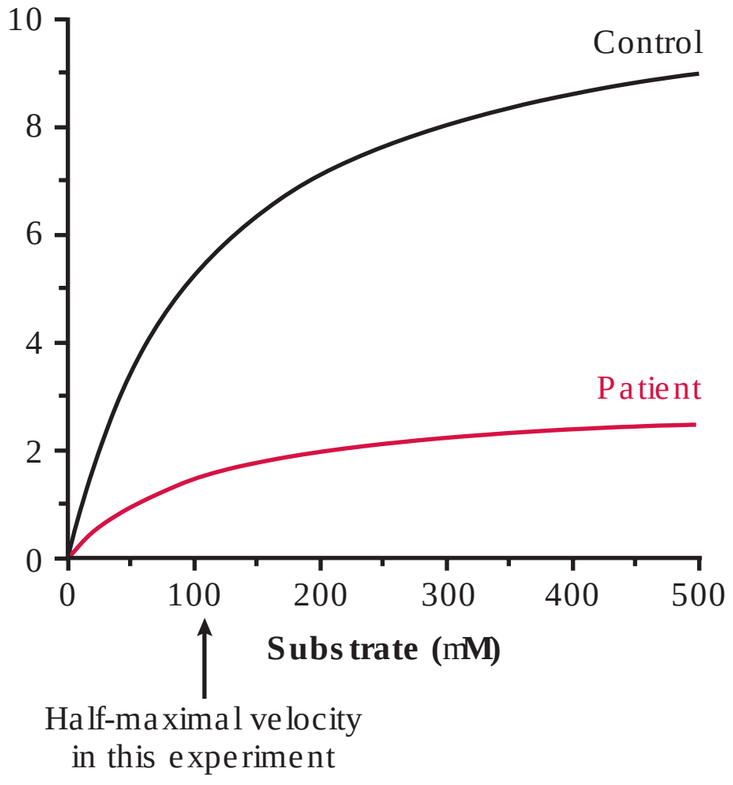
   A. Allosteric activator
   B. Competitive inhibitor
   C. Irreversible inhibitor
   D. Noncompetitive inhibitor
   E. Suicide inhibitor

2. Dihydrofolate reductase catalyzes the reaction DHF + $NADPH + H^+ \rightarrow THF + NADP^+$. The inhibitor methotrexate binds to the catalytic site of dihydrofolate reductase in place of DHF. The graph below shows the activity of dihydrofolate reductase with and without methotrexate. Which of the following is the major effect of methotrexate?

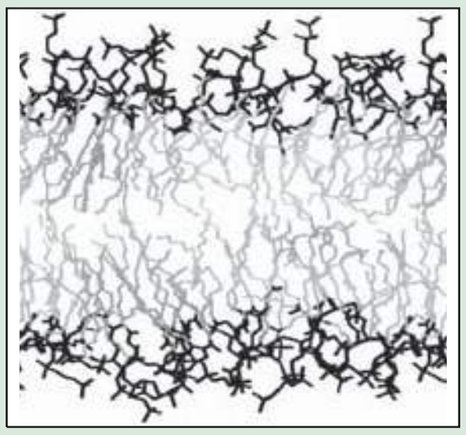


   A. Decrease of the K_m of dihydrofolate reductase for DHF
   B. Decrease of the V_{max} of dihydrofolate reductase
   C. Increase of the K_m of dihydrofolate reductase for DHF
   D. Increase of the V_{max} of dihydrofolate reductase

3. An enzyme was purified from fibroblasts of a healthy patient. In addition, this enzyme was also purified from the fibroblasts of a patient who has a disorder of metabolism. As judged by electrophoresis and staining of the protein, the purity of both enzymes was 97%. Results of the kinetic analyses of equal concentrations of purified protein are shown in the graph below. Based on these data, the mutant enzyme causes disease chiefly by having which of the following?



A. A decreased K_m
B. A decreased V_{max}
C. An increased K_m
D. An increased V_{max}



Chapter

11

Biological Membranes

SYNOPSIS

- The membranes that enclose cells and their subcellular compartments consist of phospholipids, glycosphingolipids, cholesterol, and proteins that are lipid embedded or bound to the membrane surface. The extracellular side of plasma membranes contains many sugar residues, which are covalently linked to phospholipids and membrane proteins.
- Plasma membranes and the membranes that delimit most organelles contain two layers. Lipids and proteins in each layer diffuse laterally.
- Substances that are sufficiently hydrophobic (including oxygen and many currently used orally effective drugs) can cross biological membranes. However, charged or highly hydrophilic compounds do not diffuse through membranes; rather, such compounds cross membranes only with the aid of transport proteins.
- Some transport proteins facilitate passive transport of substances across membranes; others use the energy from nucleotide hydrolysis or from an electrochemical gradient to transport compounds actively across a membrane.

LEARNING OBJECTIVES

For mastery *of* this topic, you should be able to do the *following*:

- Describe the structure and content of a biological bilayer membrane, list at least five types of lipids in such membranes, describe the membrane distribution of these lipids, and characterize the processes that maintain or destroy membrane lipid asymmetry, paying special attention to phosphatidylserine.
- List the means by which proteins can be anchored in a membrane.
- Explain how ethanol, O_2, CO_2, NH_3, K^+, Na^+, and glucose are transported across membrane bilayers.
- Compare and contrast the transport mechanism and transport rate of channels or pores, transmembrane carrier proteins, and pumps; then, provide an example of each.

1. STRUCTURE AND COMPOSITION OF MEMBRANES

Membranes *f*orm barriers to hydrophilic substances and thus, compartmentalize metabolic reactions, signaling, electrical charges, and so *f*orth. Membranes consist o*f* a bilayer o*f* lipids, into which proteins are embedded. T e major membrane lipids are diacylglycerophospholipids, cardiolipin, plasmalogens, sphingomyelin, gangliosides, and cholesterol. Proteins o*ft*en use α-helices to span membranes. Other proteins are anchored to the membrane via a phospholipid, a *f*atty acid, or a prenyl group.

1.1. Physiological Roles of Membranes

T e term **membrane** is usually reserved for lipid **bilayers**, such as the plasma membrane, the inner and outer nuclear and mitochondrial membranes, and the membranes that delimit lysosomes, the smooth and rough endoplasmic reticulum, peroxisomes, secretory vesicles, and synaptic vesicles. In contrast, lipid storage droplets inside cells and lipoprotein particles in the blood are delimited by a **monolayer** of phospholipids (as well as proteins) (see Chapters 28 and 29).

Membranes delimit cells and subcellular compartments. Membranes can separate synthesis and degradation (e.g., in the liver, fatty acid synthesis in the cytosol from fatty acid degradation in the mitochondria; see Chapter 27). T ey can delimit zones for regulation or signaling (e.g., Ca^{2+} signaling in the cytosol, whereby the extracellular space, mitochondria, and the endoplasmic reticulum play accessory roles; see Chapter 33). T ey can serve as a scaf old for forming enzyme complexes (e.g., adenylate cyclase and protein kinase A; see Chapter 24) and serve as a reservoir for phospholipids that are needed in signaling (e.g., by phospholipases; see Chapter 33) or for biosyntheses (e.g., of prostaglandins; see Chapter 32). T ey can also act as electrical insulators between ions and thus participate in energy production (see Chapter 23) or electrical signaling (e.g., in pancreatic endocrine cells; see Chapter 26).

1.2. Structure of Lipids

Lipid bilayer membranes contain phospholipids, sphingolipids, cholesterol, and proteins. A classification of phospholipids and sphingolipids is shown in Fig. 11.1. Phospholipids and sphingolipids can contain various fatty acids. Section 1 in Chapter 27 provides an introduction to saturated, cis-unsaturated, and trans-unsaturated fatty acids.

T e **phospholipids** always contain a phosphomonoester or a phosphodiester (see Fig. 11.1). Among them, the **diacylglycerophospholipids** are **esters** of glycerol and two fatty acids. T e most abundant diacylglycerophospholipids in membranes are **phosphatidylcholine** (popularly called **lecithin**), **phosphatidylserine**, **phosphatidylethanolamine**, and **phosphatidylinositol**. Phosphatidylinositol can be phosphorylated and then hydrolyzed as part of signal transmission (see Chapter 33).

T e **plasmalogens** are also phospholipids, but they contain a **vinyl ether** and an ester (see Fig. 11.1). T e plasmalogens carry the prefix plasmenyl (e.g., in plasmenylethanolamine). Plasmalogens probably serve as **antioxidants**. T eir vinyl

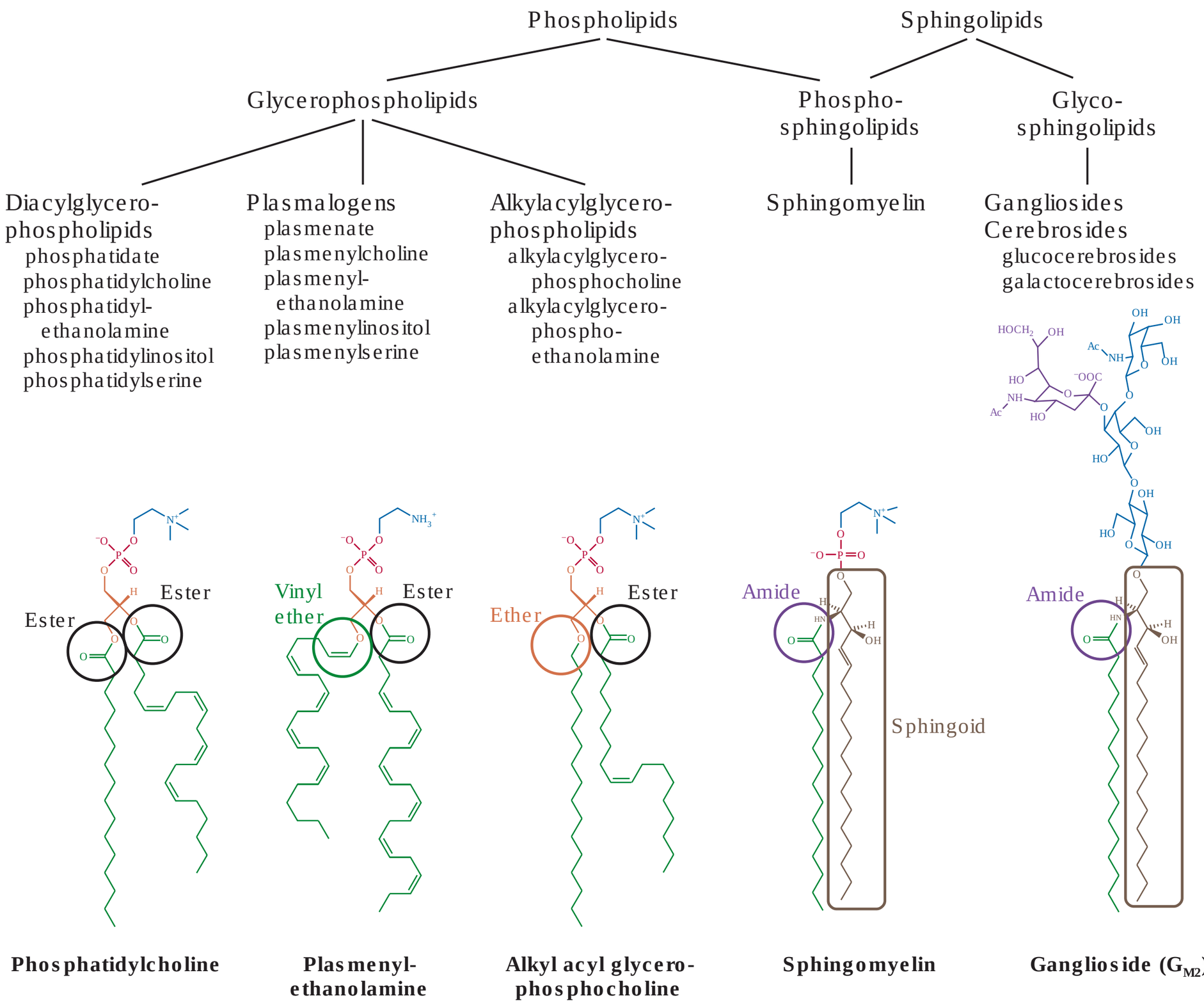


Fig. 11.1 Classification of phospholipids and sphingolipids. For each molecule shown, the fatty acid content may vary significantly.

ether more readily reacts with reactive oxygen radicals than does an ester in a diacylglycerophospholipid. As a result, plasmalogens protect polyunsaturated fatty acids (e.g., arachidonic acid and docosahexaenoic acid) from a radical attack. Of all the phospholipids in the brain and cardiac and skeletal muscle, 20% or more are plasmalogens. In **myelin**, most of the phospholipids are plasmalogens, and without plasmalogens, nerve conduction is profoundly impaired. Many **peroxisome disorders** impair plasmalogen biosynthesis and thus the function of the nervous system.

The diacylglycerophospholipids and plasmalogens have similar **head groups** (i.e., choline, serine, ethanolamine, or inositol) (Fig. 11.2).

Cardiolipin in the inner membrane of mitochondria is a unique glycerophospholipid in that it contains four fatty acids (see Fig. 11.3). When the function of mitochondria is compromised, cardiolipin moves to the outer mitochondrial membrane and becomes a signal for **apoptosis** of the cell. Antibodies to cardiolipin are found in the autoimmune disorder **antiphospholipid syndrome**, which has a prevalence of about 0.5% and leads to vascular thrombosis (blood clot forming inside a blood vessel).

In **alkylacylglycerophospholipids**, one substituent of glycerol is an alkyl group, and the other substituent is an acyl group. Hence, alkylacylglycerophospholipids contain a plain ether (not a vinyl ether) and an ester.

The **sphingolipids** (see Fig. 11.1) contain a **sphingoid**, which is synthesized from serine and a fatty acid. The sphingolipids are divided into two categories, the **phosphosphingolipids** and the **glycosphingolipids**. The phosphosphingolipids are also phospholipids because they contain a phosphate group. The most common phosphosphingolipid is **sphingomyelin**. Glycosphingolipids are found primarily in the outer leaflet of the plasma membranes. The most commonly encountered glycosphingolipids are the **gangliosides**, which contain one or more sialic acid residues (usually N-acetylneuraminic acid). Gangliosides are particularly abundant in the plasma membranes of neurons, and their degradation is impaired in **Tay-Sachs disease**.

Ceramides consist of a sphingoid that is acylated on its amino-nitrogen. Ceramides, in contrast to sphingomyelin, contain no phosphate or head group. Ceramides play a role in signaling. Ceramides can be synthesized from serine and palmitate; they can be produced directly from sphingomyelin, or

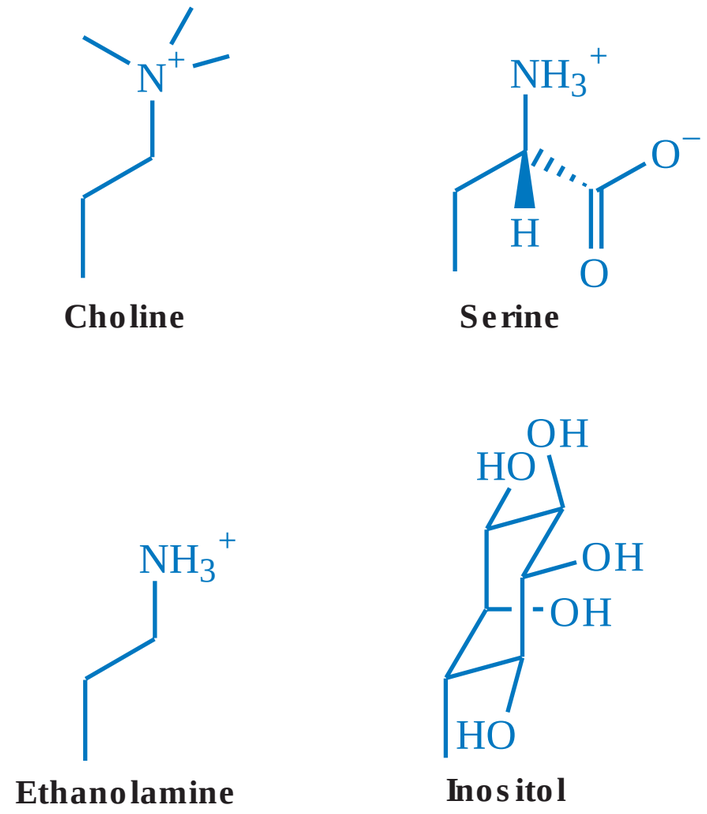


Fig. 11.2 Structure of head groups in diacylglycerophospholipids and plasmalogens.

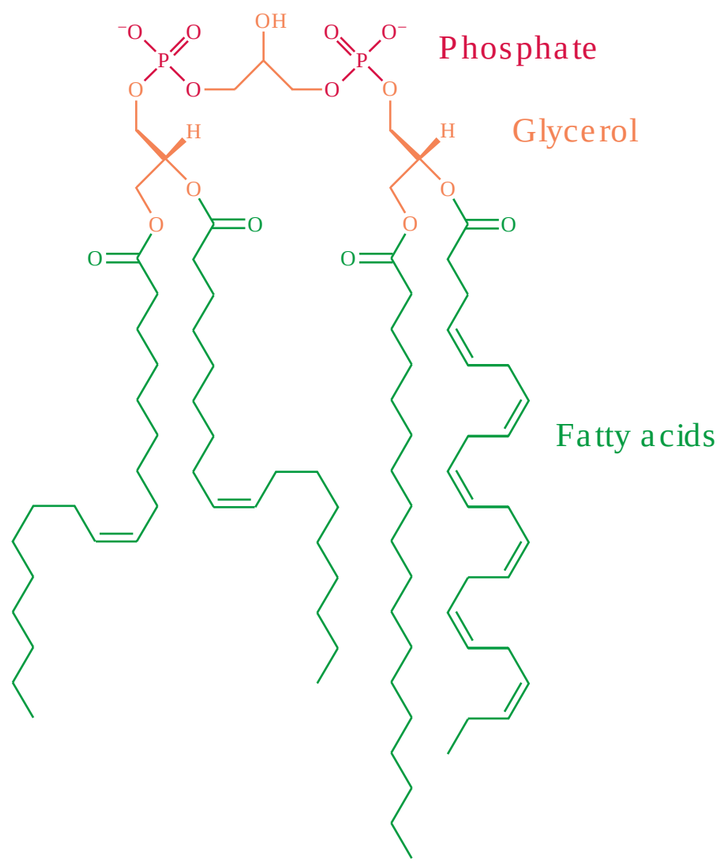


Fig. 11.3 Structure of a cardiolipin. Various fatty acids can be part of cardiolipin.

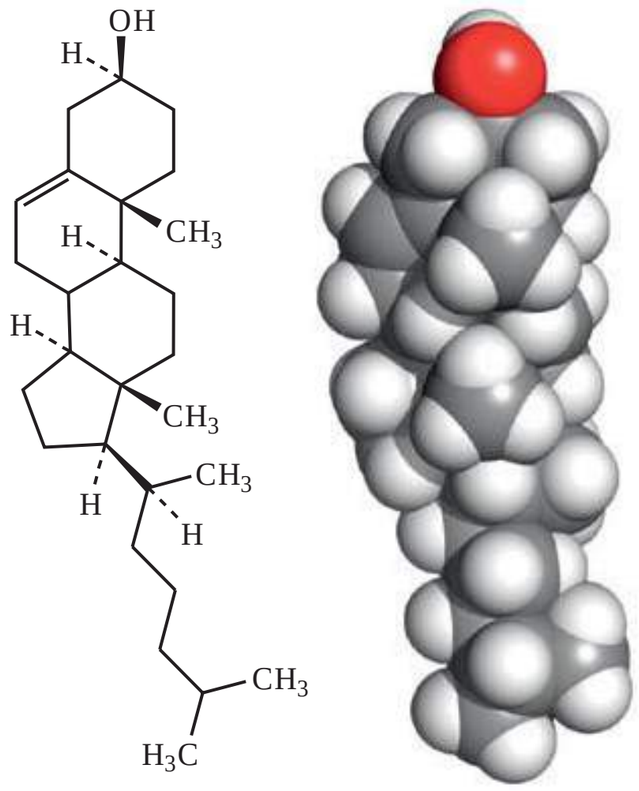


Fig. 11.4 Structure of cholesterol. The hydroxyl group is hydrophilic and is primarily located at the water interface of membranes, while the remaining portion of cholesterol is hydrophobic and preferentially associates with sphingomyelin. In this space-filled model, C is gray, H white, and O red. (Based on svn.cgl.ucsf.edu/chimera/trunk/devel/RasMol/RasMol_Scripts/BILAYR1P/CHOLESTE.pdb.)

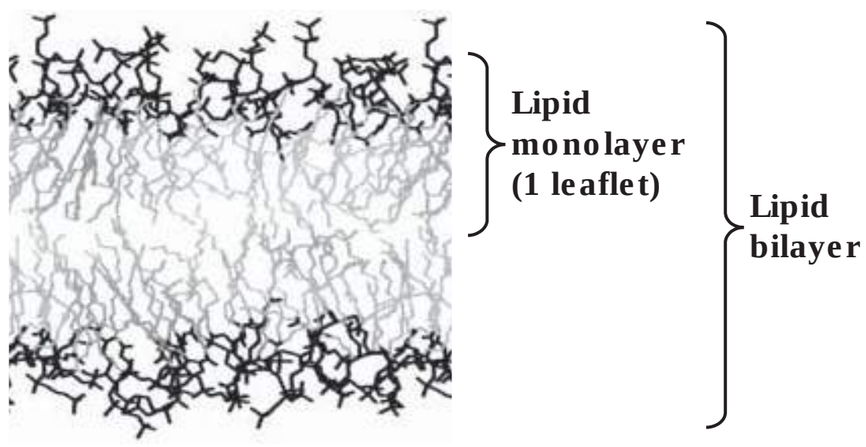


Fig. 11.5 Basic structure of a lipid bilayer membrane. A computer simulation of a bilayer that consists of phosphatidylcholine that contains two 14-carbon saturated fatty acids. The head groups are shown in black, and the rest of each molecule is shown in gray. (Modified from Robinson AJ, Richards WG, Thomas PJ, Hann MM. Head group and chain behavior in biological membranes: a molecular dynamics computer simulation. *Biophys J*. 1994;67:2345–2354.)

they can be generated from a sphingoid that is released from lysosomes, which degrade sphingolipids (this is called the salvage pathway).

Lysophospholipids are phospholipids (glycerophospholipids or sphingolipids) that have lost one of their constituent fatty acids. This is commonly due to the action of various phospholipases, such as in the digestive tract (see Chapter 28) or in signaling (see Chapter 33). When present in the plasma membrane at a sufficiently high concentration, lysophospholipids lead to cell lysis (hence their name).

Membranes also contain **cholesterol** (Fig. 11.4). Membranes of different organelles differ in their lipid composition. Cholesterol is commonly found in plasma membranes, as well as in the membranes of lysosomes and the trans-Golgi, but it is scarce in the endoplasmic reticulum membrane.

1.3. Composition and Structure of Bilayer Membranes

The lipid **bilayer** of membranes (Fig. 11.5) contains phospholipids, sphingolipids, and cholesterol. The lipid bilayer consists of an inner and an outer **leaflet**, each of which is a monolayer of lipids. The lipids in each monolayer are **amphipathic**; their hydrophobic portions aggregate to form the hydrophobic core of the membrane; their hydrophilic portions (phosphate- and head groups; see Fig. 11.1) face the water phase on either side of the membrane. Such bilayer membranes also form spontaneously in vitro from lipid mixtures in water. (Triglycerides are not sufficiently amphipathic to form bilayer membranes.)

Lipid bilayer membranes form a **barrier** against hydrophilic substances, while they permit the passage of many uncharged, lipophilic substances (see Section 2).

Membrane **proteins** are embedded into one or both lipid layers (see Section 1.5). Some of these proteins help transport hydrophilic substances across membranes (see Section 2.2).

Detergents can destroy membranes by solubilizing proteins and lipids into mixed micelles. T is is the basis of the antimicrobial ef ect of soap.

Liposomes (lipid vesicles) are used as vehicles for drug delivery. Liposomes consist of a spherical lipid bilayer membrane that encloses a small amount of liquid. Liposomes are made in the laboratory. T ey are much smaller than cells. Drug-loaded liposomes have a tendency to accumulate in the extracellular space of tumor tissues, owing to leaky capillaries; there, liposomes act as slow-release vesicles, either in the extracellular space or after uptake into cells. Macrophages tend to take up most of the liposomes. Coating liposomes with polyethylene glycol (PEG) dramatically prolongs the time they are circulating.

In biological membranes, at physiological temperature, phospholipids and sphingolipids are free to dif use within their leaflet, but they cannot readily flip from one leaflet to the other. Proteins that are embedded in the membrane (see Section 1.5) likewise can dif use within the plane of the bilayer membrane, but they cannot flip from one side of the membrane to the other. T is state in which the long axis of the phospholipids always points in the same direction, yet molecules can move laterally, is also called a liquid crystalline state. In contrast, at a considerably lower temperature, the lipids enter a gel state in which lipids and membrane proteins have greatly reduced lateral mobility.

Unsaturated fatty acids increase membrane **fluidity** (i.e., they favor a liquid crystalline state). In contrast, **saturated fatty acids** lower membrane fluidity.

Under physiological conditions, **cholesterol** increases the mechanical strength of the membranes and reduces their permeability to water and small molecules. Cholesterol binds most strongly to phospholipids with saturated acyl chains, which are predominantly found in sphingomyelin and to a lesser extent in phosphatidylcholine). Under physiological circumstances, cholesterol has the ef ect of ordering lipids and condensing the bilayer, hence the increased strength and decreased permeability.

Commonly found **fatty acids** in phospholipids include palmitic (16:0; for nomenclature, see Section 1 in Chapter 27), stearic (18:0), oleic (18:1), linoleic (18:2), arachidonic (20:4), and docosahexaenoic acid (22:4). Within the same membrane, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, and sphingomyelin often dif er appreciably in their fatty acid composition.

T e two leaflets of plasma membranes have a dif erent **lipid composition**. Among phospholipids, sphingomyelin and phosphatidylcholine predominate in the **outer leaflet**, while phosphatidylserine and phosphatidylethanolamine are found mostly in the **inner leaflet** (Table 11.1 provides an example).

T e distribution of **cholesterol** between the membrane leaflets is approximately equal or skewed in favor of the sphingomyelin-rich outer leaflet. However, cholesterol also spontaneously flips between the membrane leaflets. Plasma membranes contain approximately equal numbers of cholesterol and phospholipid molecules.

Table 11.1 Asymmetrical Distribution of Phospholipids in Human Red Blood Cell Membranes

	% of Total	
Phospholipid	**Inner Leaflet**	**Outer Leaflet**
Sphingomyelin	2	20
Phosphatidylcholine	7	20
Phosphatidylserine	13	2
Phosphatidylethanolamine	23	7
Others	3	3

Data from Zachowski A. Phospholipids in animal eukaryotic membranes: transverse asymmetry and movement. Biochem J. 1993;294:1-14.

When **phosphatidylserine** moves from the inner to the outer leaflet of the plasma membrane, it acts as a signal. Macrophages in the reticuloendothelial system of the spleen, for example, recognize phosphatidylserine on the surface of **senescent red blood cells** and then remove these cells. Elsewhere in the body, macrophages similarly recognize an increased concentration of phosphatidylserine in the outer leaflet of the membrane of cells that undergo **apoptosis** (programmed cell death; see Chapter 8). In **platelets**, an increased concentration of phosphatidylserine in the outer leaflet of the membrane plays a role in activating other platelets, a prerequisite for blood clotting.

Biological membranes contain **membrane rafts**. T ere are two types of rafts: (1) short-lived planar rafts and (2) long-lived caveolae. T e **planar rafts** are thought to consist mainly of saturated phospholipids and sphingolipids, cholesterol, and membrane proteins that either have a glycophosphatidylinositol anchor (and face the extracellular space) or a fatty acid anchor (and face the cytosol; see Section 1.5). T e planar rafts are most likely very small (a diameter of <1% of the diameter of a red blood cell) and short lived (a half-life in the millisecond range). **Caveolae** are flask-like invaginations of the plasma membrane that are particularly abundant in adipocytes, vascular endothelial cells, and smooth muscle cells. Caveolae play a role in signaling, membrane maintenance, and endocytosis. T e invaginations are created and stabilized by integral and peripheral membrane proteins (e.g., the caveolins and cavins; see Section 1.5).

1.4. Transport of Lipids Inside Membranes

Several enzymes move phospholipids between membrane leaflets (Fig. 11.6). T e transport of phospholipids from the outer leaflet to the inner leaflet is also called flipping, and the transporters responsible for this are called **flippases**.

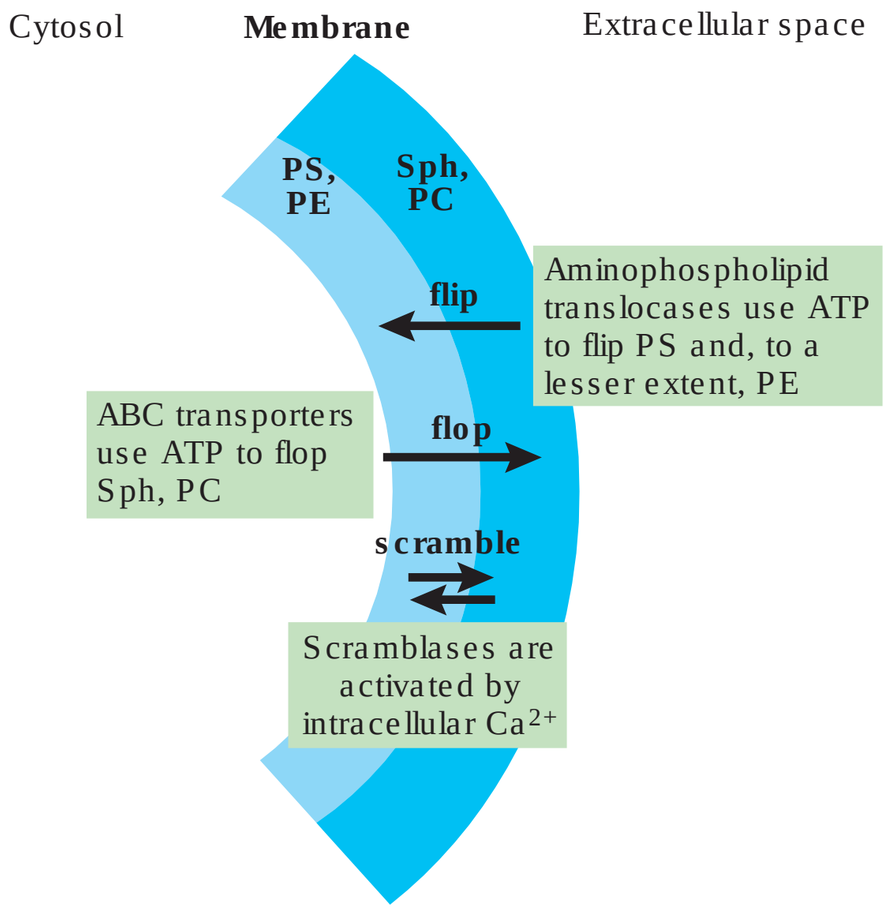


Fig. 11.6 Enzymes that maintain or abolish the asymmetric distribution of phospholipids in bilayer membranes. *ABC*, ATP binding cassette; *ATP*, adenosine triphosphate; *PC*, phosphatidylcholine; *PE*, phosphatidylethanolamine; *PS*, phosphatidylserine; *Sph*, sphingomyelin.

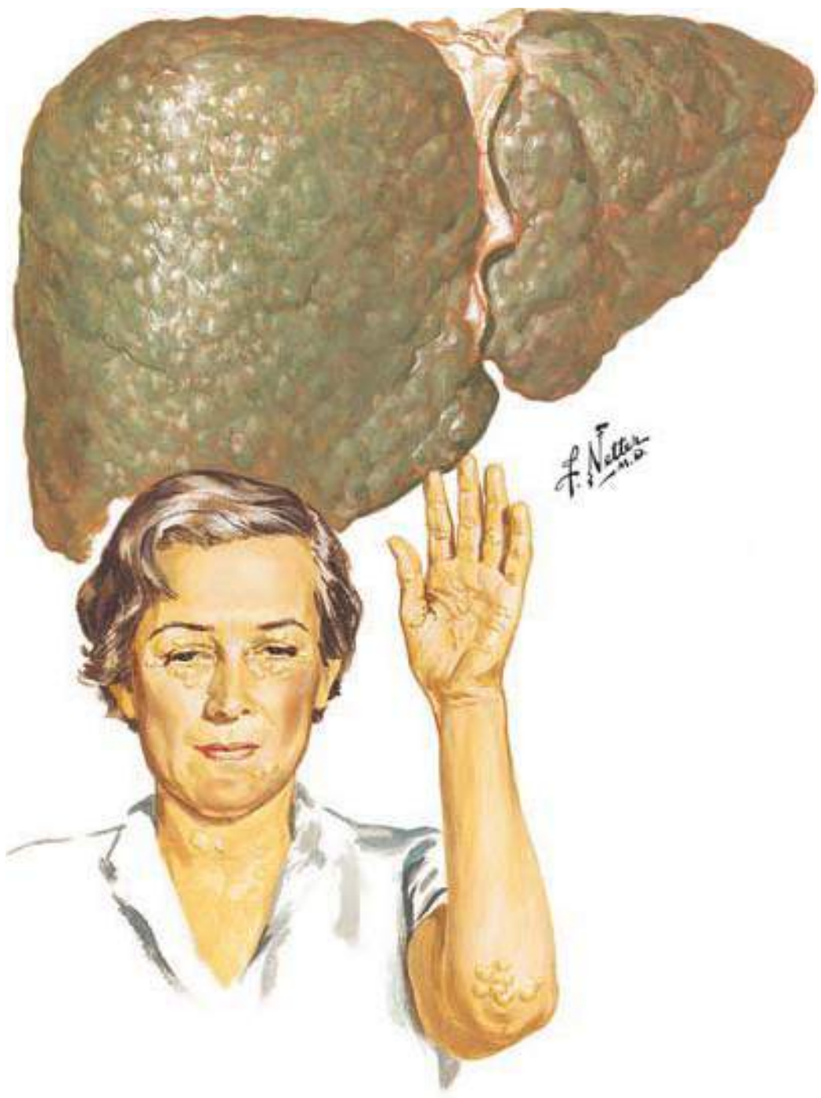


Fig. 11.7 Intrahepatic cholestasis. The disorder leads to jaundice and xanthomas of the face, neck, palms, and elbows.

Conversely, outward transport is called flopping and is accomplished by **floppases**. **Scramblases** move phospholipids between leaflets in either direction.

Mutant **multidrug resistance protein 3** (**MDR3**) is a floppase encoded by the ABCA4 gene and gives rise to a form of **intrahepatic cholestasis** (Fig. 11.7). MDR3 transports phosphatidylcholine from the inner to the outer leaflet of the plasma membrane. Secretion of phospholipids from the liver into bile is important to attenuate the toxic effects of bile salts and to prevent the formation of cholesterol stones. Severely affected patients develop cirrhosis and liver failure, and they are at risk for a cholangiocarcinoma.

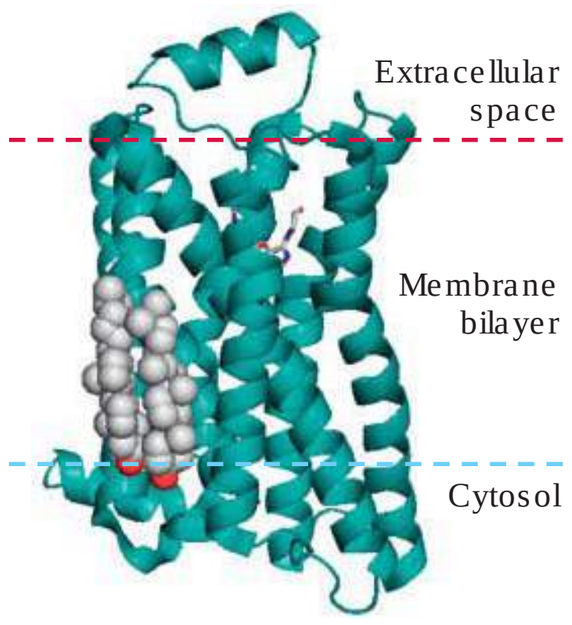


Fig. 11.8 Part of the human β_2-adrenergic receptor. Two molecules of cholesterol are shown as spheres, with the O of the hydroxyl group shown in red. To obtain a crystal for analyses, one amino acid residue was mutated, and a 26-residue cytosolic loop was replaced by another protein (not shown). (Based on Protein Data Bank file 3D4S. From Hanson MA, Cherezov V, Griffith MT, et al. A specific cholesterol binding site is established by the 2.8 A structure of the human beta2-adrenergic receptor. *Structure*. 2008;16:897-905.)

1.5. Membrane Proteins

Proteins can be part of, or tied to, a membrane in many different ways. Some proteins contain one or more **helices** with hydrophobic amino acids that **span** the membrane (e.g., the human β-adrenergic receptor) (Fig. 11.8). Less commonly, proteins span a membrane with **β-sheets** that form a **β-barrel** (aquaporin is an example). Other proteins anchor themselves with a loop or helix of hydrophobic amino acids in only one membrane leaflet. Proteins with amino acid membrane anchors are called **integral** (or **intrinsic**) **membrane proteins**. In the lab, detergents can free such integral membrane proteins; physiologically, proteases can cut off a domain that protrudes from the membrane. For example, in the brain, the extracellular domain of the amyloid precursor protein is freed by a secretase (see Chapter 9). Yet, other proteins bind to lipids or proteins in the plasma membrane largely via electrostatic interactions, so that in the lab they can be removed from the membrane with a concentrated salt solution. These latter proteins are called **peripheral membrane proteins**. An example of such a protein is cubilin (see Fig. 36.9), which binds to the integral membrane protein amnionless and plays a role in cobalamin absorption. Other such peripheral membrane proteins play a role in signaling (see Chapter 33) by binding to specific lipids, such as phosphatidylinositol 4,5-bisphosphate (PIP2), phosphatidylinositol (3,4,5)-trisphosphate (PIP3), phosphatidylinositol 4-phosphate (PI4P), or diacylglycerol (DAG). Finally, there are proteins that are anchored in the membrane by a posttranslationally added

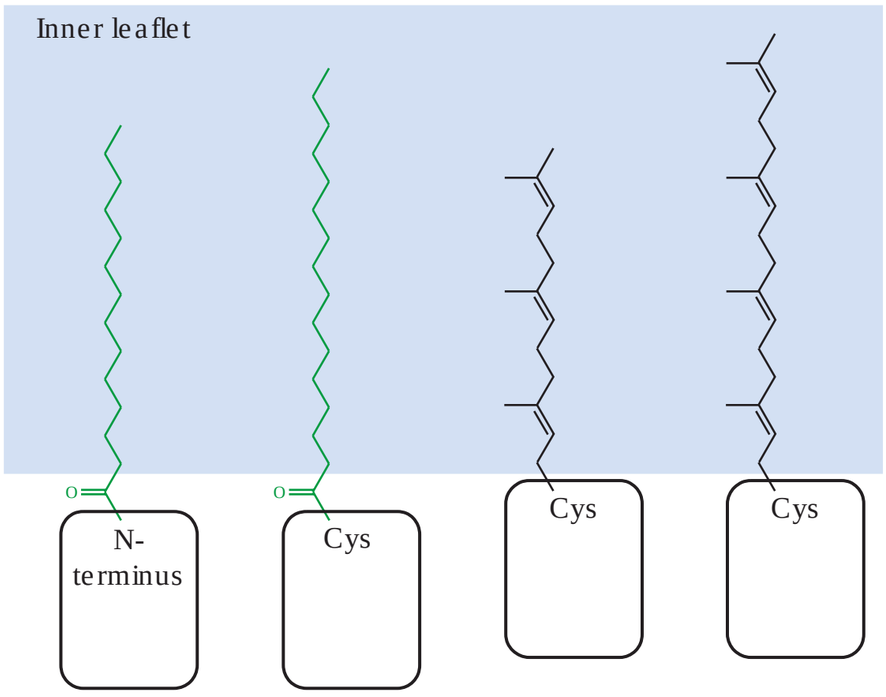


Fig. 11.9 **Proteins tethered to the inner leaflet of the plasma membrane.**

lipid, (e.g., a fatty acid, an isoprene, or a glycosylphosphatidylinositol). Some of these proteins can be cut loose from their anchor, and some can even be reanchored.

Posttranslationally acquired inner-leaflet lipid anchors for proteins (Fig. 11.9) may consist of a **myristoyl** group (a 14-carbon fatty acid), a **palmitoyl** group (a 16-carbon fatty acid), a **farnesyl** group (a 15-carbon isoprene), or one to two **geranylgeranyl** groups (a 20-carbon isoprene). Proteins that contain a farnesyl or geranylgeranyl group are called **prenylated**. Some prenylated proteins also carry a palmitoyl anchor. The synthesis of isoprenes has several steps in common with the synthesis of cholesterol, and isoprene synthesis, like cholesterol synthesis, is inhibited by the **statin** drugs (see Fig. 29.4 and Section 2.1 in Chapter 29). The products of several proto-oncogenes and oncogenes (e.g., Ras proteins) have isoprene anchors. Statin use is associated with a lower risk of certain cancers. Whether statins also have a place in cancer treatment remains unknown. Prenylation is irreversible, but certain proteins can extract prenylated proteins from a membrane or deliver them to a membrane. Palmitoylated proteins can be released from their membrane anchor.

On the cytosolic side of the plasma membrane, the **cytoskeleton**, a network of cytosolic and transmembrane proteins, shapes and stabilizes the membrane.

Glycosylphosphatidylinositol (**GPI**) can anchor proteins in the outer leaflet of the plasma membrane (Fig. 11.10). Proteins with GPI anchors are on the extracellular face of the plasma membrane, and GPIases can cut the anchor so as to release the protein into the extracellular space.

Paroxysmal nocturnal hemoglobinuria is caused by an attack of the innate immune system on circulating red blood cells that lack GPI-anchored proteins that normally suppress this immune reaction. In most patients, this is an acquired mutation of hematopoietic stem cells. The product of the mutated gene normally catalyzes the first step in the synthesis of the GPI anchor. Formation of blood clots is the main problem in this disease.

The luminal side of intestinal enterocytes and blood vessel endothelial cells contains proteins in the plasma membrane that are glycosylated so extensively that the glycosyl residues

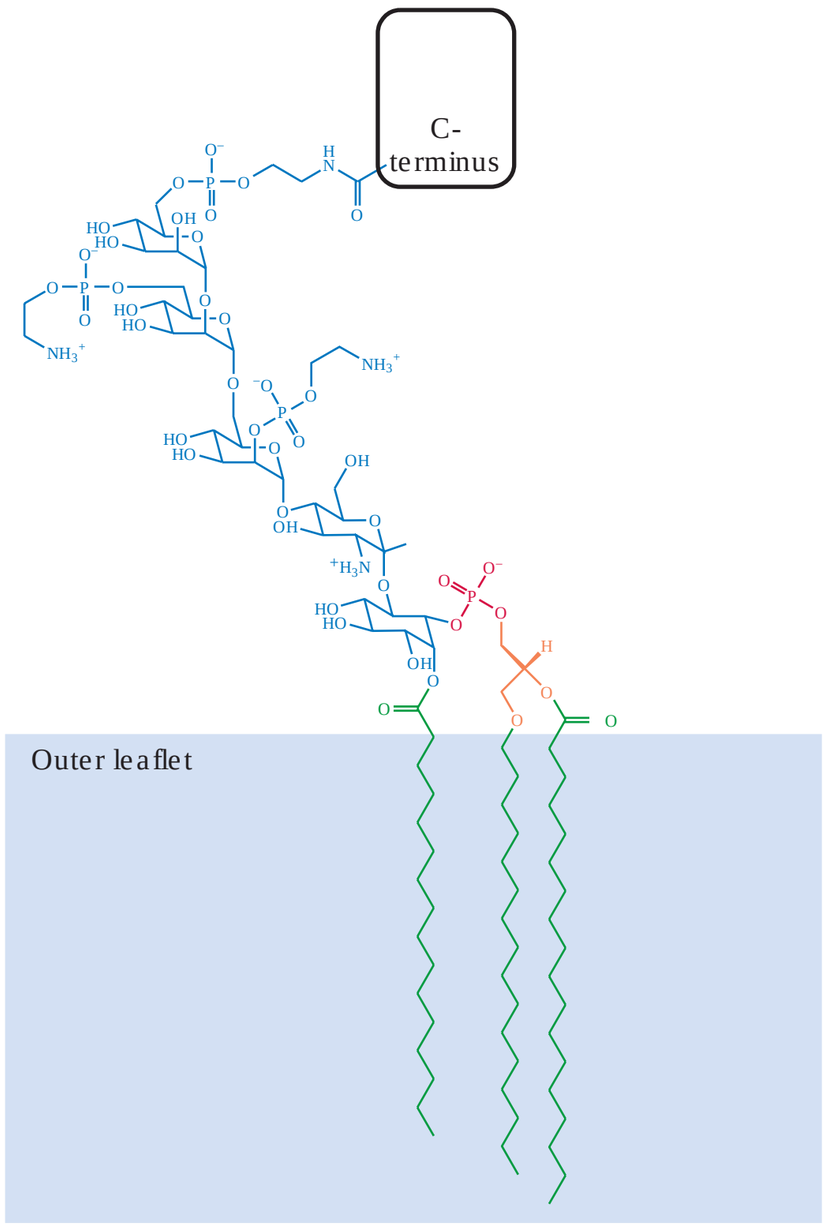


Fig. 11.10 **Glycosylphosphatidylinositol-anchored proteins are tethered to the outer leaflet of the plasma membrane.**

form part of a **glycocalyx**, the thickness of which may be many times that of the membrane itself.

2. MOVEMENT OF MOLECULES ACROSS MEMBRANES

Membranes are permeable to moderately hydrophobic substances, but they are a barrier to hydrophilic substances, particularly charged species. Some transport proteins *form* pores through which molecules can move, while many other transport proteins bind a molecule, undergo a conformational change, and then release the molecule on the other side of the membrane. Some of these latter proteins transport a second, different molecule in the same or opposite direction, and some are powered by ATP hydrolysis.

2.1. Simple Diffusion of Molecules Through the Hydrophobic Core of the Lipid Bilayer

Some substances can cross a lipid bilayer by **simple diffusion**. Such substances must be soluble in the water phase on either side of the membrane, and they must also be sufficiently lipophilic to shed water and dissolve in the hydrophobic core of the lipid bilayer membrane. Substances that have a net electrical charge are usually too hydrophilic to dissolve in the hydrophobic membrane core. Indeed, as a rule in metabolism, charged molecules do not cross membranes by simple diffusion. Examples of molecules that pass through membranes by

simple dif usion are O_2, CO_2, NH_3, ethanol, and many orally taken drugs.

2.2. Transport of Molecules Through Transport Proteins in Membranes

Charged, appreciably hydrophilic, or large molecules may cross a lipid bilayer only with the help of a transport protein. T e transport protein may simply provide a means for the molecule to move down an electrochemical gradient (a combination of an electrical charge gradient and a chemical concentration gradient). If this is the case, such movement is called **facilitated dif usion**.

Some transport proteins provide (at least temporarily) a **pore** (also called a **channel**), through which specific molecules can move. Examples of channels are Na^+ channels and the water-transporting aquaporins. Na^+, K^+, and Cl^- can move through ion channels at rates up to more than 1 million ions per second per channel. Pores provide the fastest mode of transport of molecules across a membrane.

Carrier transport proteins bind a molecule on one side of a membrane, undergo a conformational change, and then release the molecule on the other side of the membrane. T is type of transport is called **carrier-mediated transport** and is a form of facilitated dif usion. In general, carriers operate at a much lower transport rate than pores. GLUT-type glucose transporters are an example of a carrier (see Chapter 18).

Some carrier transport proteins move molecules against their electrochemical gradient by using energy either from nucleotide hydrolysis or the electrochemical gradient of another molecule; this is called **active transport** and is a subcategory of carrier-mediated transport. Some of the proteins that catalyze active transport are also called **pumps**. Examples of active transport are the Na^+-coupled amino acid transporters (see Chapter 34) and the Na^+:K^+-ATPase, a pump that uses ATP to move Na^+ and K^+ ions in opposite directions (against their respective electrochemical gradients).

Carrier transport proteins that facilitate the simultaneous transport of two dif erent molecules in the same direction are also called **cotransporters** or **symporters** (or symport); those that transport in opposite directions are called **antiporters** (or antiport) or **exchangers**. An example of a cotransporter is the Na^+-coupled glucose transporter (SGLT1) in the epithelium of the small intestine (see Chapter 18). An example of an exchange transporter is the antiporter for aspartate and glutamate in the inner mitochondrial membrane that is part of the malate-aspartate shuttle (see Chapters 19, 23, and 25).

SUMMARY

- Bilayer membranes enclose cells, subcellular organelles, and intracellular vesicles. T ese membranes may contain cholesterol, glycerophospholipids, and sphingolipids.
- Phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol, plasmenylethanolamine, plasmenylcholine, and sphingomyelin are all phospholipids that are commonly found in biological bilayer membranes. Gangliosides are found in plasma membranes, and cardiolipin is in the inner membrane of mitochondria.
- Impaired synthesis of plasmalogens in peroxisomes impairs nerve conduction.
- Patients who have antiphospholipid syndrome have antibodies to cardiolipin. T is syndrome is common, and it is associated with vascular thrombosis.
- Common sphingolipids are sphingomyelin and gangliosides.
- Cholesterol is a major constituent of the plasma membrane. It increases the mechanical strength of the membrane and decreases its permeability to water and small molecules.
- T e leaflets of bilayer membranes often dif er in phospholipid composition (e.g., phosphatidylserine is predominantly in the inner leaflet, while gangliosides and sphingomyelin are in the outer leaflet of plasma membranes). In part, such asymmetry is a result of leaflet-specific synthesis in the endoplasmic reticulum or mitochondria. However, lipid transporters also actively maintain phospholipid asymmetry. Aminophospholipid transporters are flippases that move phosphatidylserine and phosphatidylethanolamine from the outer leaflet of the plasma membrane to the inner leaflet. Conversely, certain ABC transporters are floppases that move sphingomyelin and phosphatidylcholine from the cytosolic leaflet to the outer leaflet of the plasma membrane.
- Impaired flopping of phosphatidylcholine from the inner to the outer plasma membrane leaflet leads to a form of intrahepatic cholestasis.
- Scramblases can move phosphatidylserine to the outer membrane leaflet, where it is a signal for platelet activation or apoptosis.
- Membranes contain short-lived planar rafts and comparatively long-lived invaginated rafts in the form of caveolae that play a role in signaling and membrane traffic.
- Membrane proteins can contain sequences that span the membrane several times, or they can contain a peptide anchor that penetrates only one membrane leaflet. Proteins can also acquire posttranslationally a membrane anchor of myristic acid, palmitic acid, farnesol, or geranylgeranol for anchoring in the inner leaflet of the plasma membrane. Yet other proteins acquire a glycosylphosphatidylinositol (GPI) anchor for anchoring in the outer leaflet of the plasma membrane.
- O_2, CO_2, and ethanol cross membranes without the aid of a transporter. Channels or pores rapidly transport small molecules, such as Na^+, K^+, Cl^-, and water. Carriers more slowly transport a variety of molecules, sometimes with the help of an electrochemical gradient or the input of energy from ATP hydrolysis. Certain carrier transport proteins are called antiports, exchangers, symports, or cotransporters.

FURTHER READING

- Kovtun O, Tillu VA, Ariotti N, Parton RG, Collins BM. Cavin family proteins and the assembly of caveolae. J Cell Sci. 2015;128:1269-1278.

Review Questions

1. Which of the following best describes the reason that phosphatidylserine by itself fails to cross the plasma membrane?

   A. Phosphatidylserine contains an ether-linked alkenyl chain.
   B. Phosphatidylserine contains two hydrophobic isoprenes.
   C. Phosphatidylserine is a glycolipid.
   D. Phosphatidylserine is electrically charged.

2. An antiporter that transports amino acids with a positively charged side chain between the extracellular space and the cytosol in exchange for the efflux of amino acids with an uncharged side chain is most likely also which of the following?

   A. A flippase
   B. A glycolipid
   C. A membrane-spanning protein
   D. An aminophospholipid translocase

Chapter

12 Collagen, Collagenopathies, and Diseases of Mineralization

SYNOPSIS

- Various types of collagens provide tensile strength in bone, tendons, and ligaments or filter fluids in the kidney glomeruli.
- Collagen α-chains are synthesized inside cells as precursors of mature collagen molecules. The precursors aggregate into trimers that form a collagen triple helix. The trimers are exported into the extracellular space. In some types of collagen, after proteolysis of flanking prosequences, the triple helices assemble to form fibrils or networks. Covalent cross linking further improves the stability of these complexes.
- The deposition of calcium phosphate within collagen fibrils, a process called mineralization, stiffens bone.
- Achondroplasia is caused by mutations that activate a fibroblast growth factor receptor, which in turn leads to decreased production of collagen in bone, causing short stature.
- The more severe forms of osteogenesis imperfecta are most often due to mutations in type I collagen that impair the formation of a triple helix, which greatly weakens bones and leads to numerous fractures.
- Most patients who have the classical Ehlers-Danlos syndrome have a mutant type V collagen that leads to hyperextensible and fragile skin.
- Insufficient mineralization of bone causes rickets in children and osteomalacia in adults.
- Paget disease of bone is associated with certain foci of excessive growth and other foci of excessive destruction in one or multiple bones.
- In persons with osteoporosis, loss of bone mineral density increases fracture risk.
- Mutations in some of the network-forming type IV collagen chains cause thin basement membrane nephropathy (which causes hematuria) or Alport syndrome (a disorder that leads to early kidney failure).
- Mutations in an anchoring fibril-forming type VII collagen α-chain cause dystrophic epidermolysis bullosa, a severe blistering disease.

LEARNING OBJECTIVES

For mastery of this topic, you should be able to do the following:

- Explain how fibrillar collagens are synthesized inside the cell and how they form fibrils in the extracellular space. Describe the role of ascorbate in this process.
- Compare and contrast the structure of a collagen triple helix with the structure of an α-helix (e.g., an α-helix in hemoglobin).
- Name the cause and signs of achondroplasia.
- List signs of vitamin C deficiency and explain why this deficiency is associated with poor wound healing.
- Describe the mineralization of collagen fibers and explain how mineralization is abnormal in rickets, osteomalacia, and osteoporosis.
- Explain why mutation of a Gly residue to another amino acid in a collagen triple helix is usually inherited in an autosomal dominant fashion.
- Describe the signs of classical Ehlers-Danlos syndrome, define the cause of this disorder, and explain the pattern of inheritance.
- Compare and contrast signs and symptoms of rickets and osteomalacia, and describe the main causes of these disorders.
- Describe the signs, symptoms, and broad cause of Paget disease of bone and list the most common treatment option.
- Describe the signs of osteogenesis imperfecta, define the most common cause of this disorder, list treatment options, and explain the most common pattern of inheritance.
- Compare and contrast the signs, causes, and treatment options of thin basement membrane nephropathy, Alport syndrome, and Goodpasture syndrome. Explain the pattern of inheritance of thin basement membrane nephropathy and Alport syndrome.
- Compare and contrast the causes of dystrophic epidermolysis bullosa and of epidermolysis bullosa acquisita.

1. BIOSYNTHESIS AND DEGRADATION OF FIBRILLAR COLLAGENS

Protein synthesis produces fibrillar procollagens inside the endoplasmic reticulum. In the endoplasmic reticulum, many proline and some lysine residues are hydroxylated in vitamin C–dependent reactions. Three procollagen chains associate near their C-termini, and a triple helix forms. After secretion into the extracellular space, peptidases cleave the N- and C-terminal propeptides. A dioxygenase oxidizes some lysyl and hydroxylysyl residues to aldehydes. Triple helices aggregate and become cross-linked, forming microfibrils. The microfibrils in turn aggregate into fibrils and fibers. Collagens are resistant to most proteases. Matrix metalloproteinase-1 cleaves fibrillar collagens, which causes the helix to unwind and thereby become susceptible to degradation by other proteases.

1.1. Overview of Types of Collagen

Collagens are the major component of the extracellular matrix and amount to about 30% of the protein mass in a human.

Many different collagens are known; they are numbered in the order of discovery. The collagens are commonly grouped into fibrillar and nonfibrillar collagens.

Fibrillar collagens provide mechanical strength, for instance, in bone, tendons, ligaments, and cartilage. This group encompasses collagens I, II, III, V, and XI. Mutations in

some of these collagens cause diseases such as osteogenesis imperfecta (brittle bones; see Section 2.4) or Ehlers-Danlos syndrome (hyperelastic skin, hyperextensible joints; see Section 2.5).

Nonfibrillar collagens serve a variety of purposes. In this chapter, they are grouped into network-forming collagens and collagens of anchoring fibrils. **Network-forming collagens** are present in the sheet-like basement membranes, including the kidney glomeruli, where they help filter fluids. T e main representative of this group is collagen type IV. Mutations in some of the type IV collagen chains cause thin basement membrane nephropathy or Alport syndrome (a disorder that leads to early kidney failure). T e collagens of **anchoring fibrils**, such as type VII collagen, anchor the basement membrane to collagen fibrils that are present in the underlying connective tissue. Mutations in this collagen give rise to dystrophic epidermolysis bullosa (a severe skin-blistering disorder).

All collagens contain a **triple-helix domain,** which is formed from three collagen **α-chains** (β is only used in reference to a pair of cross-linked α-chains). Some collagen triple helices contain three identical α-chains (e.g., type II collagen). Other collagens contain two to three dif erent α-chains (e.g., type IV collagen); in this case, the dif erent α-chains are distinguished as α1, α2, and so on. Humans have close to 50 dif erent genes that encode collagen α-chains.

According to the common **terminology** for **collagen protein, α-chains** are described in the form "αn(#)," such as α2(IV), where "n" is a number for the type of α-chain and "#" is a Roman number for the type of collagen that results from the assembly of several α-chains. Human **collagen-coding genes** are named in the form "COL#An," where "#" is an Arabic number for the type of collagen (e.g., 4 for collagen type IV), "A" stands for α-chain, and "n" is still the number that follows the character α in the naming of collagen protein α-chains. For example, the collagen α-chain α2(IV) is the product of the gene COL4A2.

1.2. Biosynthesis and Posttranslational Modification of Fibrillar Collagens

Collagen is synthesized from **preprocollagen**. Fig. 12.1 provides an overview of the posttranslational processing of preprocollagen. Further details are provided below.

T e pre- or **signal** sequence ensures transfer of the growing peptide chain into the endoplasmic reticulum. Inside the endoplasmic reticulum, a signal peptidase cleaves the signal sequence (see Fig. 7.6 and Section 3 in Chapter 7).

T e fibrillar procollagens contain a central domain that eventually participates in the formation of a triple helix; this domain contains many repeats of Gly-X-Y, where X is most often proline and Y is most often hydroxyproline. Another common amino acid in the Y-position is lysine. Inside the endoplasmic reticulum, **procollagen-proline 4-dioxygenases** (**proline 4-hydroxylases**) and **procollagen-lysine 5-dioxygenases (lysine 5-hydroxylases)** hydroxylate

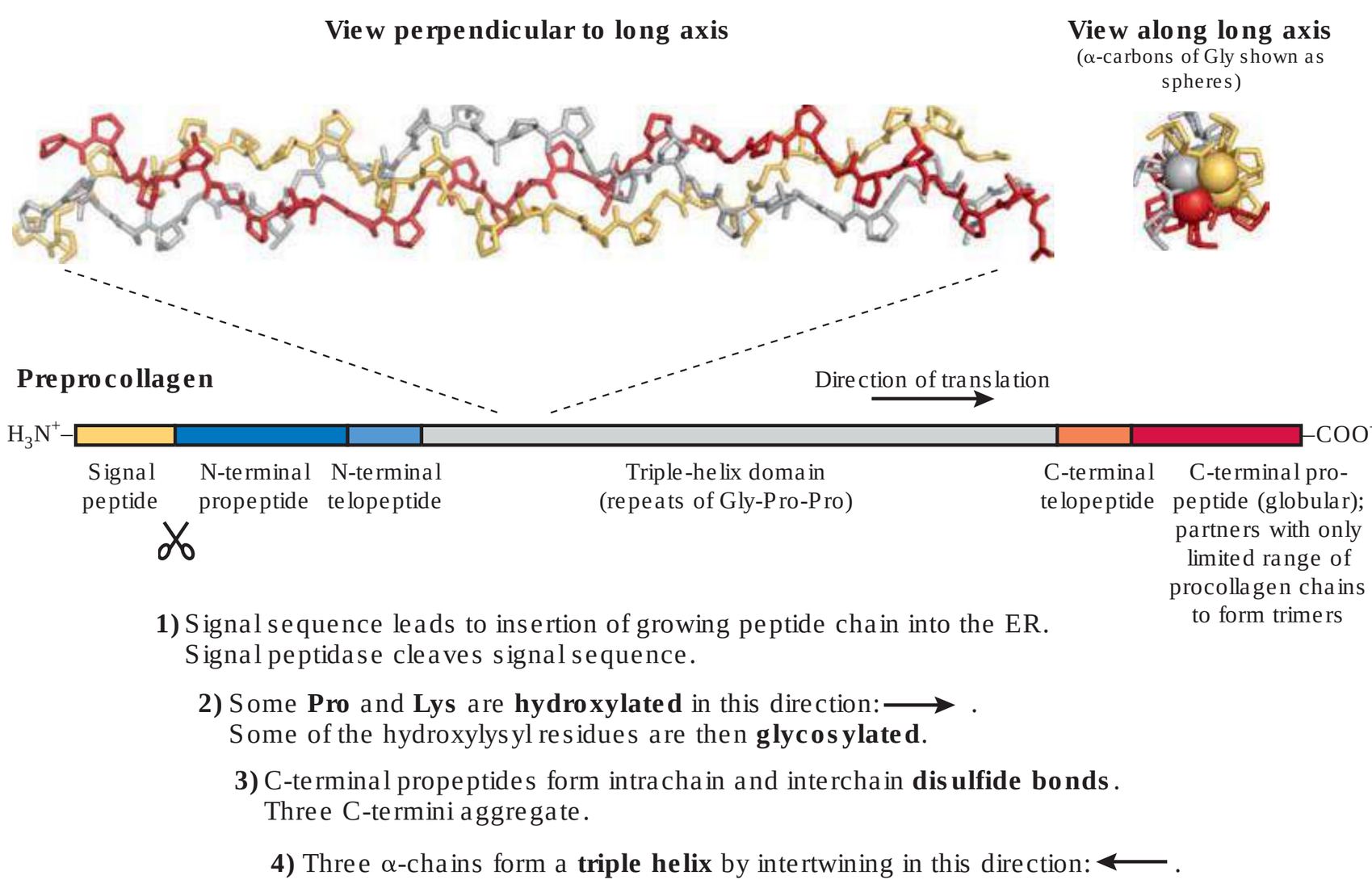


Fig. 12.1 Overview of the processing of a fibrillar preprocollagen in the endoplasmic reticulum (ER). The domains are not drawn to scale. As an example of the structure of a collagen triple helix, a trimer of the artificial peptide $(Pro\text{-}Hyp\text{-}Gly)_4\text{-}(Pro\text{-}Hyp\text{-}Ala)\text{-}(Pro\text{-}Hyp\text{-}Gly)_5$ is shown (colors identify three different molecules). The N-termini of the three molecules are on the left and toward the viewer. (Based on Protein Data Bank file 1CGD of Bella J, Brodsky B, Berman HM. Hydration structure of a collagen peptide. *Structure*. 1995;3:893-906.)

many Y-position prolines and also some lysine side chains (Fig. 12.2). Hydroxylation proceeds from the N-terminus (which is synthesized first) toward the C-terminus. The extent to which lysine residues are hydroxylated varies greatly among collagens and tissues. The proline- and lysine-hydroxylating enzymes require **vitamin C** (**ascorbate, ascorbic acid**; see Section C below).

Some of the **hydroxylysyl** residues are **O-glycosylated** in the endoplasmic reticulum and in the Golgi.

Once the proline and lysine dioxygenases have reached the C-terminus of the procollagen α-chains, a **protein disulfide isomerase** forms disulfide bridges in the C-terminal propeptide.

Once the folded C-termini of three collagen α-chains aggregate, the **triple helix** forms from the C-terminal side (Fig. 12.2 and Section 2.3), assisted by peptidyl-prolyl cis-trans isomerase. The isomerase can produce the required trans configuration of the peptide bond. The triple helix is stabilized by hydrogen bonds, water bridges, hydrophobic interactions, and van der Waals interactions.

Replacement of glycine residues in a collagen triple helix is strongly pathogenic, whereas replacement of some amino acids in positions X and Y is less damaging. In the triple helix, the glycine residues of one chain are always opposite the X or Y positions of the other chains. Nonglycine residues impair triple-helix formation because there is no room for a side chain larger than –H (as in Gly). Charged or hydrophobic amino acids in positions X and Y influence the lateral aggregation of collagen triple helices in the extracellular space (see below and Fig. 12.3). Compared to helix formation, lateral aggregation is less dependent on the amino acid side chain structure.

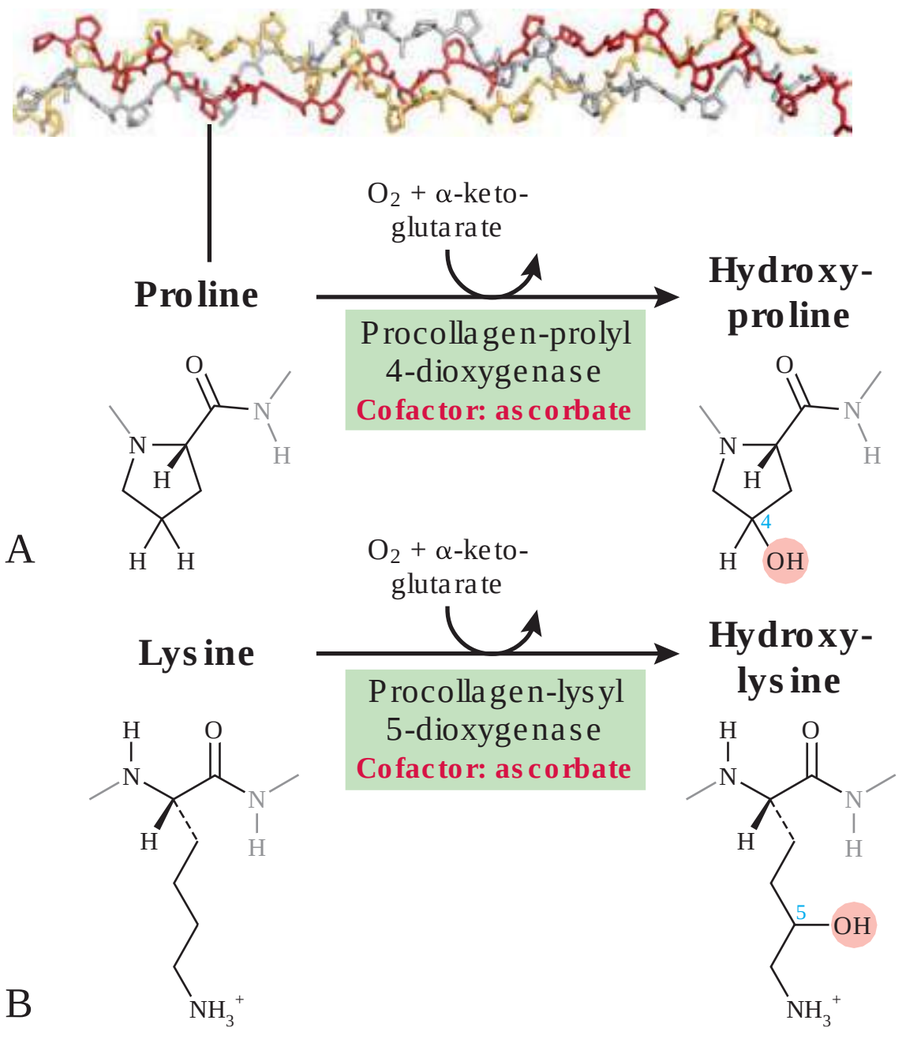


Fig. 12.2 Posttranslational formation of hydroxyproline and hydroxylysine in procollagen. Numbers in enzyme names refer to amino acid carbons (1 is at the –CO– end).

The procollagen trimers are sorted through the Golgi apparatus and emerge from the Golgi inside secretory vesicles. Collagen molecules that are not part of a triple helix (i.e., surplus chains, mutant chains) are degraded. The procollagen trimers are then secreted into the extracellular space.

In the extracellular space, **procollagen N-endopeptidases** and **procollagen C-endopeptidases** cleave the N- and C-terminal propeptides of procollagens (see Figs. 12.1 and 12.3). The remaining triple-helix domains, framed by N- and C-terminal **telopeptides**, spontaneously aggregate into **microfibrils**, helped by telopeptides and by patches of charged or hydrophobic amino acid side chains in X and Y positions within the triple-helix domains. It is a quirk of the collagen research field that a trimeric complex of collagen molecules that is formed in the extracellular space is called a collagen monomer in a context of multimonomer fibrils.

Collagen microfibrils (diameter ~0.003 µm) aggregate into **collagen fibrils** of various diameters, which in turn aggregate into **collagen fibers** that may reach a diameter of ~10 µm (by comparison, the diameter of a red blood cell is ~7 to 8 µm). The dermatan sulfate-containing proteoglycan (see Fig. 13.4 and Section 2.1 in Chapter 13) **decorin** binds to collagen microfibrils in regular intervals and thereby determines spacing between microfibrils, as well as fibril shape. Furthermore, **glycosylation** of hydroxylysyl residues may alter the lateral aggregation of collagen triple helices. The fibers can be as long as a tendon.

Lysyl oxidases in the extracellular space can oxidize a lysyl or hydroxylysyl side chain in each one of the telopeptides of collagens I, II, or III. (These lysyl oxidases are not to be confused with the lysine hydroxylases in the endoplasmic reticulum as mentioned above.) Oxidation of lysine residues yields **allysine** residues, and oxidation of hydroxylysine residues yields **hydroxyallysine** residues. These residues spontaneously participate in intramolecular and intermolecular cross-linking reactions with lysine residues in telopeptides and triple-helix regions of neighboring molecules (Fig. 12.3). When there is a pulling force on collagen fibers, the collagen monomers normally slide past each other. Cross-links decrease such sliding and therefore increase the tensile strength but decrease the elasticity of collagen fibers. Furthermore, if there are too many cross-links, the capacity of the fibrils to absorb energy decreases. (These lysyl oxidases described here are also involved in oxidizing lysyl side chains in elastin; see Section 1.1 in Chapter 13.)

1.3. Mineralization of Fibrillar Collagens in Bone

Mineralization of collagen refers to the deposition of **calcium phosphate** in and around collagen fibers.

In bone, collagen provides the overall structure, flexibility, and tensile strength, whereas mineralization increases stiffness and reduces compressibility.

Collagen fibrils contain holes, which serve as nucleation sites for the deposition of calcium phosphate. As shown in Fig. 12.3, there is a gap between consecutive collagen triple helices in a fibril. These gaps are connected into channels so that

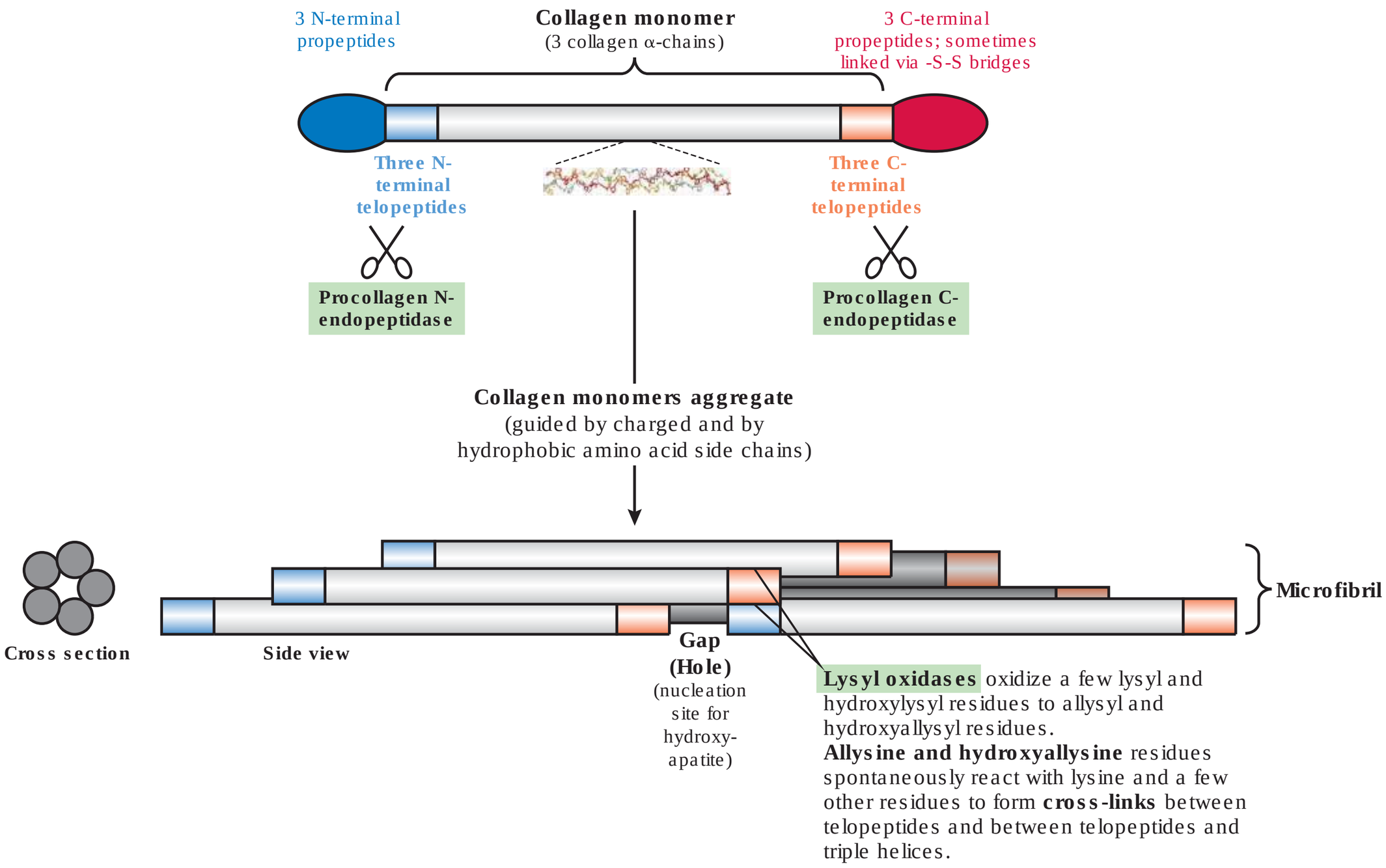


Fig. 12.3 In the extracellular space, processing of procollagen trimers and the aggregation of collagen triple helices yields collagen microfibrils. Relative to their length, the collagen trimers are shown at 10 times their actual width. Furthermore, for simplicity, the trimers are shown as cylinders, when in reality the trimers are twisted around each other.

the collagen fiber can be mineralized after assembly. Electrically charged amino acid side chains pointing into the holes facilitate the binding of the first calcium cations and phosphate anions; that is, they facilitate nucleation of a **hydroxyapatite** crystal. The hydroxyapatite crystals are only approximately two-thirds the thickness and in their longest dimension one-third the length of a collagen triple helix.

Bone-forming osteoblasts contain **bone alkaline phosphatase** on their surface and release some of it into the blood. The enzyme contributes to alkaline phosphatase activity commonly measured in serum for diagnostic purposes. Children have higher serum alkaline phosphatase activity than adults. Adults who have excessive osteoblast activity (e.g., in Paget disease of bone or in osteomalacia; see Sections 2.5 and 2.6) also have elevated serum alkaline phosphatase activity.

Bone is the main **reservoir** of calcium phosphate and serves as a reservoir for these ions; however, it can cover only a portion of daily needs for phosphate (the remainder has to be absorbed from food).

1.4. Degradation of Fibrillar Collagens

Degradation of fibrillar collagens occurs during growth, bone remodeling, wound healing, and arthritis. Fibrillar collagens normally have half-lives of many weeks or months. Most proteases cannot degrade an intact collagen triple helix. Proteolysis of collagen triple helices is catalyzed by the **matrix metalloproteinases** MMP-1, MMP-2, MMP-8, MMP-13, and MMP-14 (the prefix metallo- indicates that these enzymes contain a metal, such as Zn^{2+}). These enzymes are secreted into the extracellular space as procollagenases; there, other proteases cleave the N-terminus, yielding active collagenases. The collagenases cleave the α1 or α2 chain of a type I collagen monomer at a bond approximately three-fourths of the length of a collagen molecule. The resulting fragments then unfold, and the chains are degraded further by other proteases (e.g., gelatinases).

The extracellular matrix contains a reservoir of **tissue inhibitors of metalloproteinases**, as well as other protease inhibitors. Tissue inhibitors of metalloproteinases inhibit matrix metalloproteinases (such as collagenases) by binding into their active site.

2. DISEASES OF BONE THAT ARE ASSOCIATED WITH FIBRILLAR COLLAGENS

Various mutations in the type I collagen genes cause osteogenesis imperfecta, which is characterized by inadequate production of the extracellular matrix in bone. Severe forms of the disease cause death in utero or shortly after birth; less

severe forms can result in lifelong disability. Mutations in type V collagen cause the classic Ehlers-Danlos syndrome manifesting as hyperelastic, poorly healing skin. Patients who have a vitamin C deficiency have decreased hydroxylation of extracellular matrix proteins. These patients bruise easily and show impaired wound healing. Paget disease of bone is caused by increased turnover of bone and the deposition of abnormal new bone, causing fissures and bone pain. Osteomalacia is a syndrome of "weak" bones due to deficient mineralization, which in turn commonly has its origins in a deficiency of circulating vitamin D or phosphate. Patients who have osteoporosis have an abnormally low bone mineral content.

2.1. Overview and General Comments

The majority of mutations in **collagen** genes cause disease when present in a heterozygous state, either by haploinsufficiency of the remaining normal allele, or by a dominant-negative effect of the mutant allele (see Table 5.1 and Section 2 in Chapter 5). Dominant-negative effects can be explained with the following example. If a heterozygous patient synthesizes a mutant α1(I)-procollagen chain at a normal rate, the chance that three normal type I collagen α-chains [i.e., two α1(I)-chains and one α2(I)-chain] come together to form a normal triple helix is only 25%. [On the other hand, if the mutation were in the α2(I)-chain, the chance would be 50%.) If triple helices that contain one or more mutant α-chains are exported into the extracellular space, most collagen microfibrils and practically all collagen fibers contain one or more mutant α-chains.

Most mutations in collagen **processing enzymes** cause disease only when present in a homozygous or compound heterozygous state.

Fig. 12.4 provides an overview of the location of the molecular defect in collagen synthesis, mineralization, and degradation that is observed in the diseases of bone that are discussed in this section.

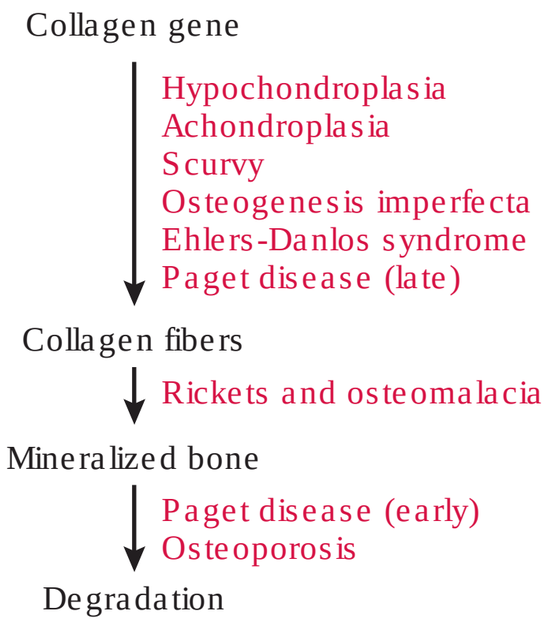


Fig. 12.4 Diseases that involve collagen.

2.2. Hypochondroplasia and Achondroplasia

Hypochondroplasia and achondroplasia are caused by activating mutations in the fibroblast growth factor receptor-3 (FGFR3). When active, this receptor stops growth of bones. About 80% of affected patients have a de novo mutation, and the others inherited the mutation in autosomal dominant fashion. Each of these dysplasias occurs in ~1:15,000 births. Persons who have hypochondroplasia or achondroplasia make up most of the people who have markedly short stature.

Persons who have achondroplasia have short arms and legs, a large head, and altered facial features (Fig. 12.5). Those with hypochondroplasia have milder symptoms than those who have achondroplasia. In an affected fetus, short limbs are noticeable by ultrasound late in pregnancy.

FGFR3 signals through the RAS-RAF-MEK-ERK pathway (see Fig. 8.3), as well as other pathways. After binding one of the fibroblast growth factors, FGFR3 forms a dimer that has tyrosine kinase activity. While ligand-activated FGFR3 receptors can stimulate the growth of many types of cells (e.g., fibroblasts), it inhibits the growth of matrix-producing chondrocytes in the growth plates via cell cycle inhibition (the mechanism is not well understood).

The most common mutations that give rise to hypochondroplasia and achondroplasia are a glutamine to lysine mutation (N540K) and a glycine to arginine mutation (G380R) in FGFR3, respectively. The mutant amino acid side chains lead to a modest increase in the fraction of dimerized (and thus active) receptors, even in the absence of a fibroblast growth factor.

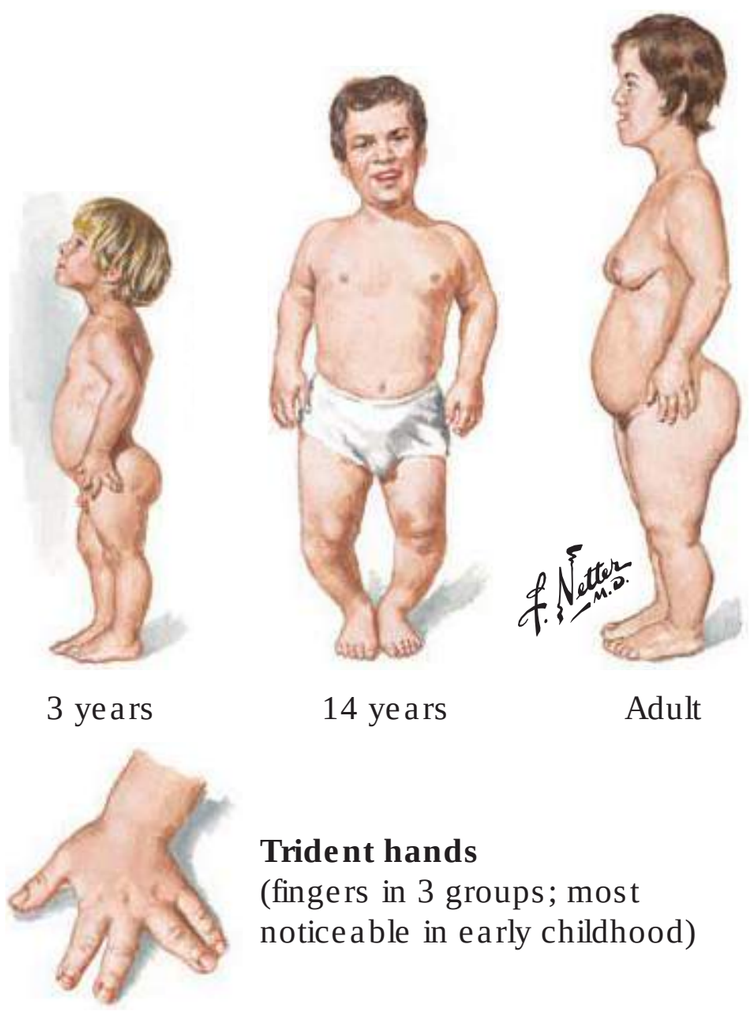


Fig. 12.5 Achondroplasia.

2.3. Vitamin C (Ascorbate) Deficiency

We obtain ascorbate mostly from **plants** (see Table 12.1). Plants in turn synthesize relatively large amounts of ascorbate via a pathway that starts with glucose 6-phosphate. The structure of ascorbate (vitamin C) is shown in Fig. 21.7. The concentration of ascorbate inside cells is in the submillimolar to millimolar range. Ascorbate is needed for the hydroxylation of proline and lysine residues (see Fig. 12.2).

In the United States, the **recommended daily dietary allowance** for vitamin C for people who are at least 19 years old is 90 mg for men, 75 mg for nonpregnant women, 85 mg for pregnant women, and 120 mg for lactating women. Smokers should take in an extra 35 mg per day.

Ascorbate crosses human cell membranes via one of the **sodium-dependent vitamin C transporters** (SVCT-1 or SVCT-2; genes are named SLC23A1 and SLC23A2, respectively), whereas dehydroascorbic acid crosses it via one of the **glucose transporters** (GLUT-1, GLUT-3, or GLUT-4). Inside cells, dehydroascorbic acid is reduced to ascorbate. Normally, SVCTs provide cells with most of their ascorbate; however, during oxidative stress or inflammation, uptake of dehydroascorbate via GLUTs becomes more important. The intracellular concentration of ascorbate in the brain is ~3 mM and that of muscle is about ~0.5 mM.

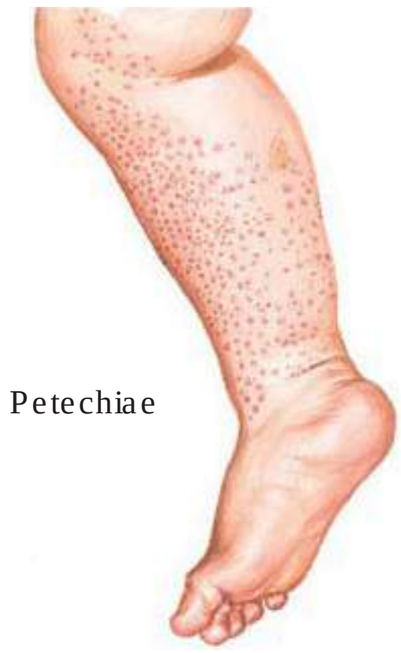
Vitamin C deficiency, characterized by a concentration of ascorbate in serum of less than 11 µM, is common worldwide. Vitamin C deficiency is most common among refugee populations, among people who consume few vitamin C–rich fruits and vegetables, and among smokers (oxidants in smoke react with ascorbate). Patients who had gastric bypass surgery often become deficient in vitamin C unless they take a vitamin C supplement. A marked deficiency also often occurs in patients who receive renal dialysis. These patients are asked to exclude potassium-rich foods from their diet; however, some of these foods also happen to be important sources of vitamin C. To make matters worse, these patients lose a large amount of ascorbic acid through dialysis.

In patients with vitamin C deficiency, the rate of hydroxylation of prolyl and lysyl side chains is diminished; **collagen** therefore forms at a decreased rate. With a decrease in the rate of hydroxylation, triple-helix formation is delayed and slowed. Furthermore, collagens produced by ascorbate-deficient cells have decreased thermal stability and tensile strength. Indeed, patients severely deficient in vitamin C bruise easily, their gums may bleed, and their wounds heal poorly (Fig. 12.6).

Table 12.1 Sources of Vitamin C (Ascorbate)

Food	Vitamin C Content (mg/100 g Edible)
Pepper, sweet, red, raw	128
Kiwi, green	75
Cauliflower, boiled	73
Cabbage, raw	72
Cabbage, boiled	38
Strawberries	57
Pineapple	36
Orange juice	33
Tomato	20
Potato, boiled in skin	13
Apple	6
Walnuts	1
Pasta (dry)	0
Milk (whole, from cow)	0

Bleeding gums



Petechiae

Multiple spots of subcutaneous bleeding

Fig. 12.6 Deficiency of vitamin C (ascorbate).

Petechiae occur, especially around hair follicles. Patients report joint pain and often have swollen joints. Vitamin C–deficient patients are also at an increased risk for cardiovascular disease.

2.4. Osteogenesis Imperfecta

In ~90% of patients, osteogenesis imperfecta (**brittle bone disease**) is caused by an **autosomal dominantly** inherited mutation in one allele of the COL1A1 or COL1A2 gene; these genes encode the type I collagen α-chains. More than 1,500 pathogenic mutations are known. The remaining patients have mutations in genes, the products of which interact with type I collagen, and these latter mutations are generally inherited in autosomal recessive fashion. Osteogenesis imperfecta occurs in ~1 in 15,000 newborns.

Haploinsufficiency due to a mutation in the COL1A1 gene usually leads to the mildest form of osteogenesis imperfecta (see Fig. 12.7). A single functional COL1A1 allele is not sufficient to give rise to normally structured bone, although all of the collagen is normal. The frequency of fractures varies widely. In affected children, fractures in the arms and legs may occur when they start to walk.

In general, **dominant-negative mutations** in the COL1A1 or COL1A2 gene lead to a more severe phenotype than a mutation that causes haploinsufficiency (Fig. 12.7). In dominant-negative mutations, an amino acid substitution impairs the formation of a collagen triple helix and therefore also the formation of all higher-order structures, such as bone. The substitution is often in a glycine codon. As shown in Fig. 12.1, at glycine positions, there is no room for an amino acid side chain in the collagen triple helix. Some of these mutations also lead to an accumulation of misfolded proteins in the endoplasmic reticulum, a decrease in the rate of translation (except mRNAs that encode chaperones), and eventually apoptosis. Dominant-negative mutations result in disease that ranges from thin bones and fractures in utero (which can be seen by ultrasound), followed either by death on the first day of life or lifelong deformities, to normal stature at birth but fragile teeth and osteoporosis in adulthood. About half of these patients also develop hearing loss at an unusually early age.

Clinically, a finding of **bluish sclerae**, which is especially noticeable at birth and during the early years of life, should raise suspicion that the patient has osteogenesis imperfecta.

Bisphosphonates are widely used in the treatment of osteogenesis imperfecta. These drugs (e.g., pamidronate, zoledronate) bind to hydroxyapatite crystals and then cause apoptosis of osteoclasts, which degrade bone. Bisphosphonate therapy increases bone mineral density; in some patients, it also reduces the rate of fractures. Bisphosphonates are not used in patients who have a type of osteogenesis imperfecta that is caused by defective mineralization.

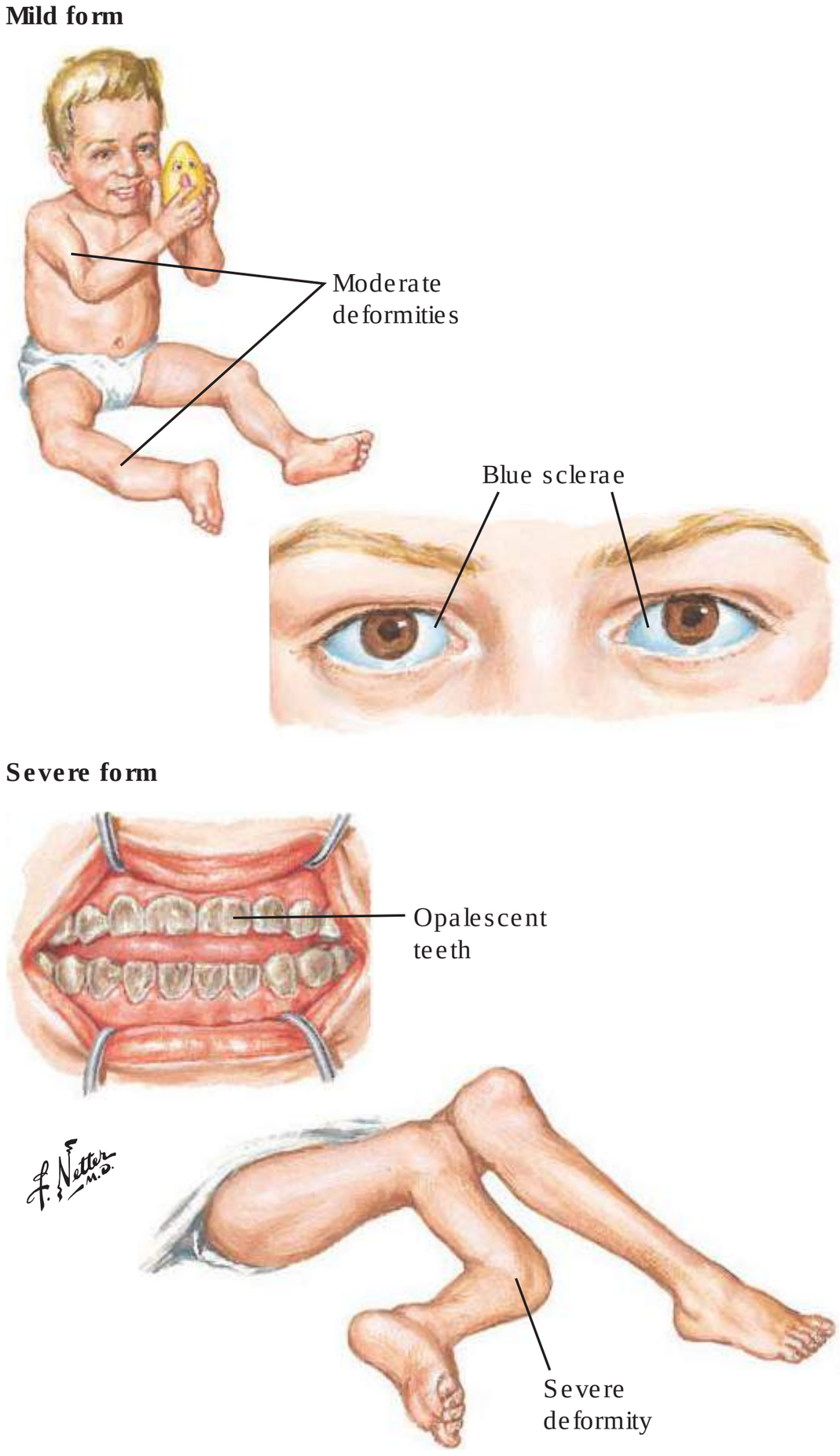


Fig. 12.7 Osteogenesis imperfecta. *Top*, Haploinsufficiency of the α1(I)-chains leads to a mild form of the disease. The sclerae are typically blue at birth. Most fractures occur as the child starts to walk; however, these fractures heal rapidly. *Bottom*, Dominant-negative mutations lead to broken bones in utero and after birth. Bone deformities and fragility typically require multiple surgeries and may prevent patients from walking.

2.5. Ehlers-Danlos Syndrome

Ehlers-Danlos syndrome (EDS) is a collection of diseases with various causes. EDS is classified based mostly on clinical findings. Common signs include altered skin, joint hypermobility, and ready bruising (Fig. 12.8). The prevalence of EDS in all its varieties is ~1 in 7,000. The most common forms of EDS are classic EDS and hypermobility EDS, which show autosomal dominant inheritance.

About 90% of patients who have **classic EDS** have a mutation in one allele of the COL5A1 or COL5A2 gene. Type V collagen consists of two different α-chains. Most pathogenic alleles result in haploinsufficiency. In women who are pregnant with an affected fetus, the membranes, which are of fetal origin, commonly rupture prematurely. Walking is delayed in children. The skin of affected patients is fragile. Scars from

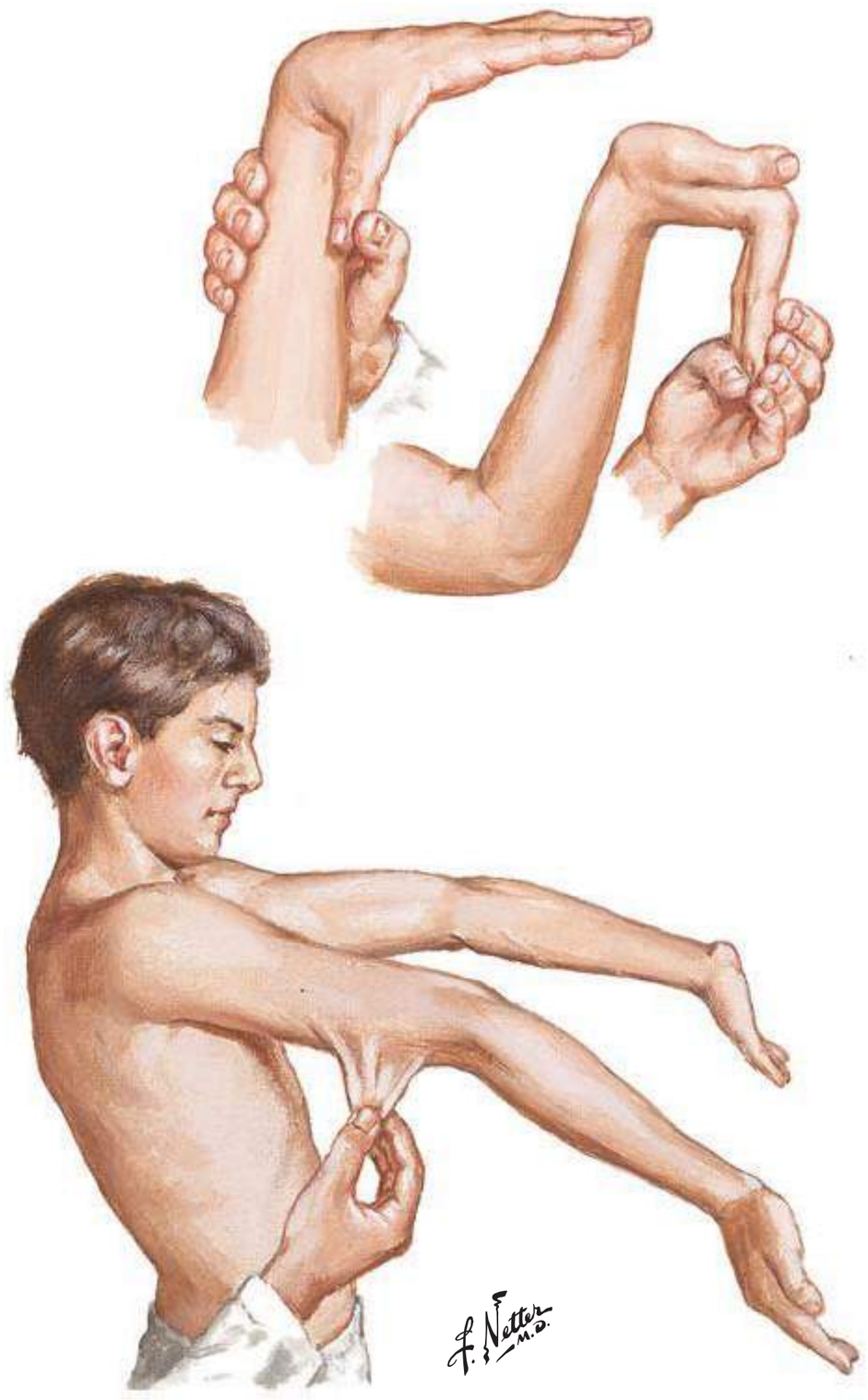


Fig. 12.8 **Joint hypermobility and skin hyperelasticity in Ehlers-Danlos syndrome.**

minor falls start in early childhood and have the appearance of cigarette paper. After surgery, sutures must be left in place about twice the normal length of time.

T e cause of most forms of **hypermobility EDS** is still unknown. Patients typically have musculoskeletal pain, recurrent joint dislocation, and mitral valve prolapse.

2.6. Rickets and Osteomalacia

Rickets and **osteomalacia** are mainly due to decreased **mineralization** of matrix proteins; in turn, this is generally due to a vitamin D deficiency, a calcium deficiency, or phosphate deficiency (Fig. 12.9). T e term rickets is usually reserved for the syndrome seen in children; the term osteomalacia is reserved for adults. In af ected patients, bones develop painful fissures even after minor trauma, and the skeleton may deform (more so in children).

Worldwide, **vitamin D deficiency** is often due to malnutrition. However, in developed countries there are many other prevalent causes, such as low exposure to sunlight, disease-induced decreased absorption in the intestine, or chronic kidney disease (see also Section 5 in Chapter 31). In the absence of adequate calcitriol (the active form of vitamin D

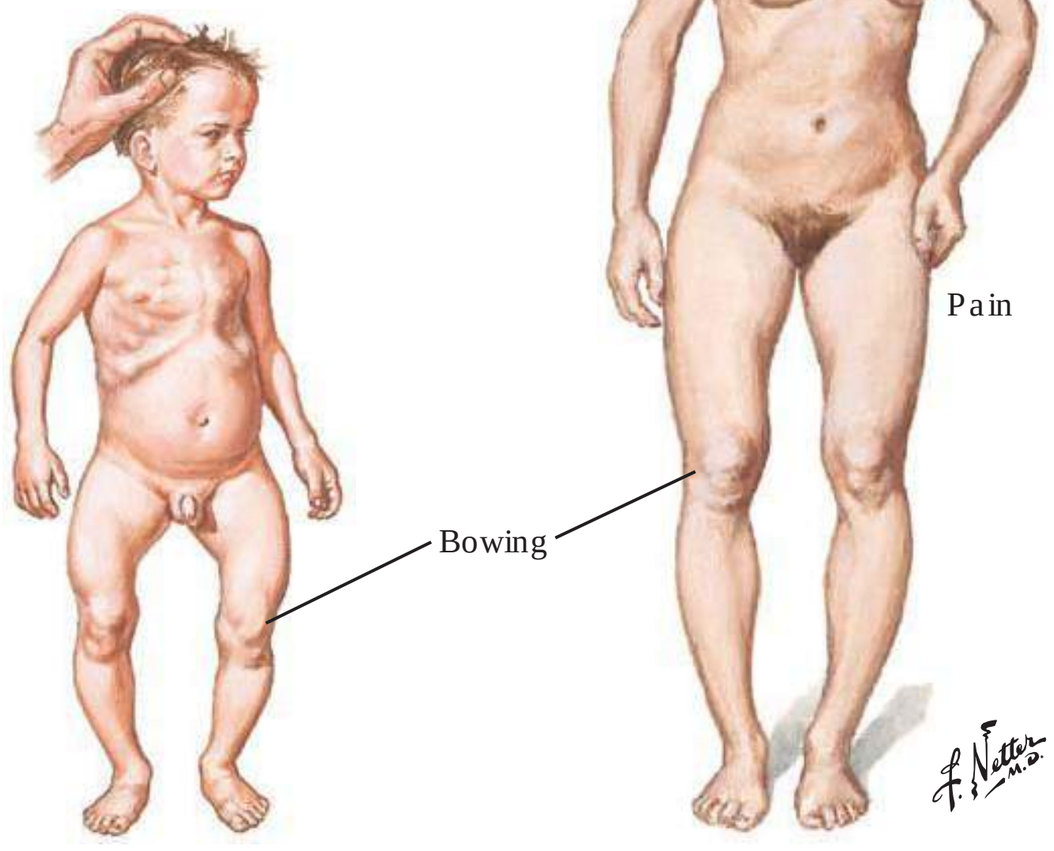


Fig. 12.9 **Rickets and osteomalacia.**

that acts as a signal), there is decreased absorption of calcium in the intestine and kidneys as well as decreased release from bones.

A **dietary calcium deficiency** that causes rickets occurs mostly among children in developing countries. In adults, a calcium deficiency is predominantly associated with osteoporosis (see Section 2.8) rather than osteomalacia.

A **phosphate deficiency** can develop in patients who absorb too little phosphate due to gastrointestinal disease or who have excessively high concentrations of the phosphaturic hormone **fibroblast growth factor 23 (FGF23)**. T e concentration of circulating FGF23 is elevated in patients who have a (usually benign) **tumor** that secretes FGF23 and in patients who have **X-linked hypophosphatemia** (~1 in 20,000 births) due to a mutation in the PHEX gene. Patients can be treated with phosphate and calcitriol (the activated form of vitamin D), unless they have hypercalcemia.

Basic laboratory evaluation of rickets includes measurements of serum calcium, phosphate, alkaline phosphatase, and calcidiol (the form of vitamin D that is stored in blood; see Section 5 in Chapter 31).

2.7. Paget Disease of Bone (Osteitis Deformans)

Paget disease of bone (Fig. 12.10) is accompanied by increased bone remodeling and an abnormal structure of bone. T ere are foci of excessive degradation and other foci of excessive formation of new bone that has an abnormal structure. Degradation predominates early in the disease, whereas bone formation prevails later on.

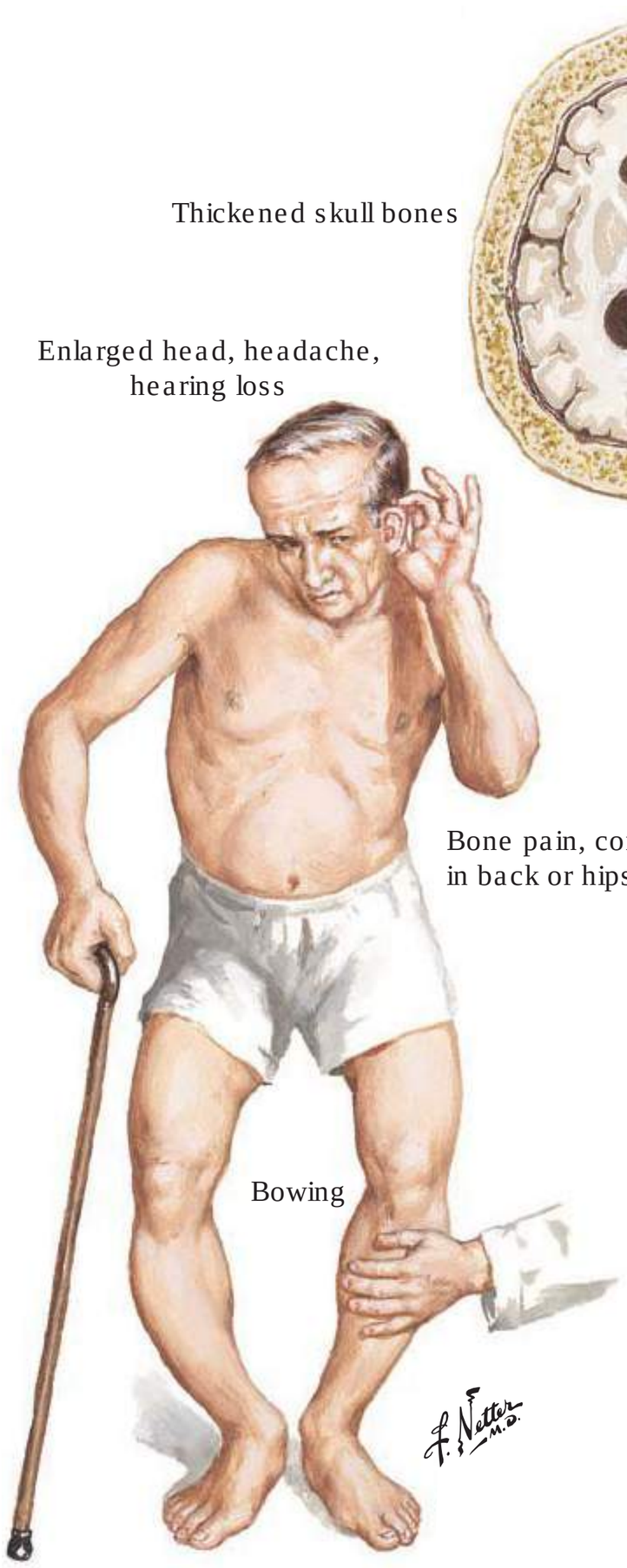


Fig. 12.10 Advanced Paget disease of bone.

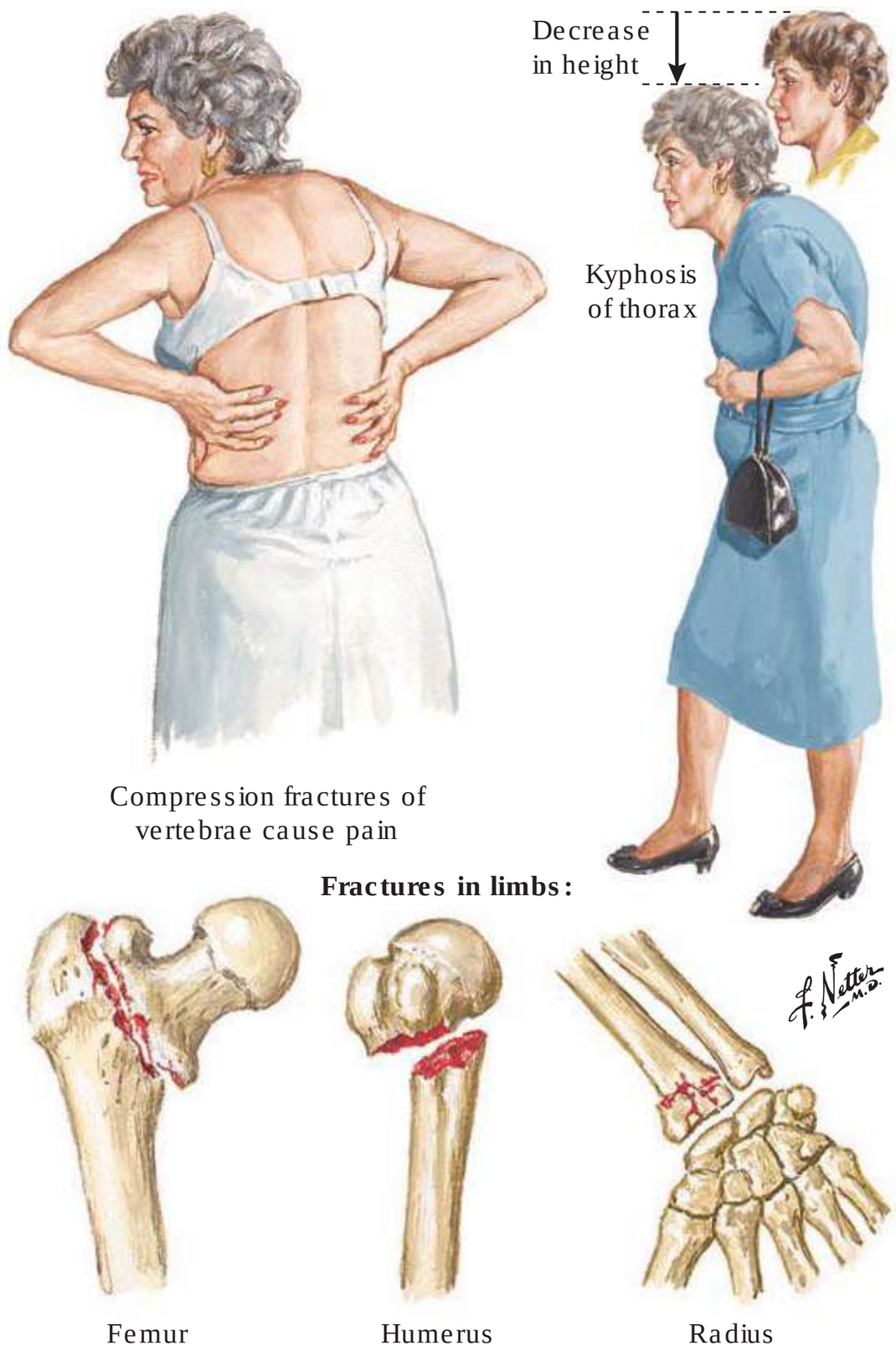


Fig. 12.11 Common fracture sites in osteoporosis. Osteoporosis is also accompanied by a decrease in body height.

Paget disease of bone is found in ~2% of persons aged 60 years and older. Most commonly, a diagnosis is incidentally made based on an x-ray image. The disease is almost twice as prevalent among men as among women. At diagnosis, almost all patients are older than 55 years.

About half of the patients who have familial Paget disease have a mutation in the **sequestosome-1** (*SQSTM1*) gene. The mechanism of pathogenesis is unclear.

Patients who have Paget disease of bone may have pain, deformities, deafness, decreased bone strength, and neurologic deficits. The disease may affect only one bone, or it may be systemic and affect multiple bones. In the long term, patients are at an increased risk of developing a **sarcoma** in bone.

Most patients who have Paget disease are treated with **bisphosphonates**, which inhibit osteoclast activity (for details on these drugs, see Section 2.8). Disease activity can be monitored with measurements of serum **alkaline phosphatase** activity, which reflects osteoblast activity.

2.8. Osteoporosis

Osteoporosis is associated with changes in the structure of bone that reduce bone strength and increase fracture risk (Figs. 12.11 and 12.12). This is the most common disorder of bone.

Bone mass peaks in a person's early twenties and gradually decreases afterward, thus increasing the risk of fracture. Women experience an additional loss of bone mass when the concentration of estrogens drops because of menopause. In persons with osteoporosis, common fracture sites are the wrist, hip, and vertebrae.

Bone mineral density is commonly assessed at the neck of the femur and at the lumbar vertebrae levels using **dual x-ray absorptiometry** (**DXA scan**). Low bone mineral density is associated with increased fracture risk.

Bone undergoes ongoing resorption by **osteoclasts** and formation by **osteoblasts** to remove microdamage and maintain

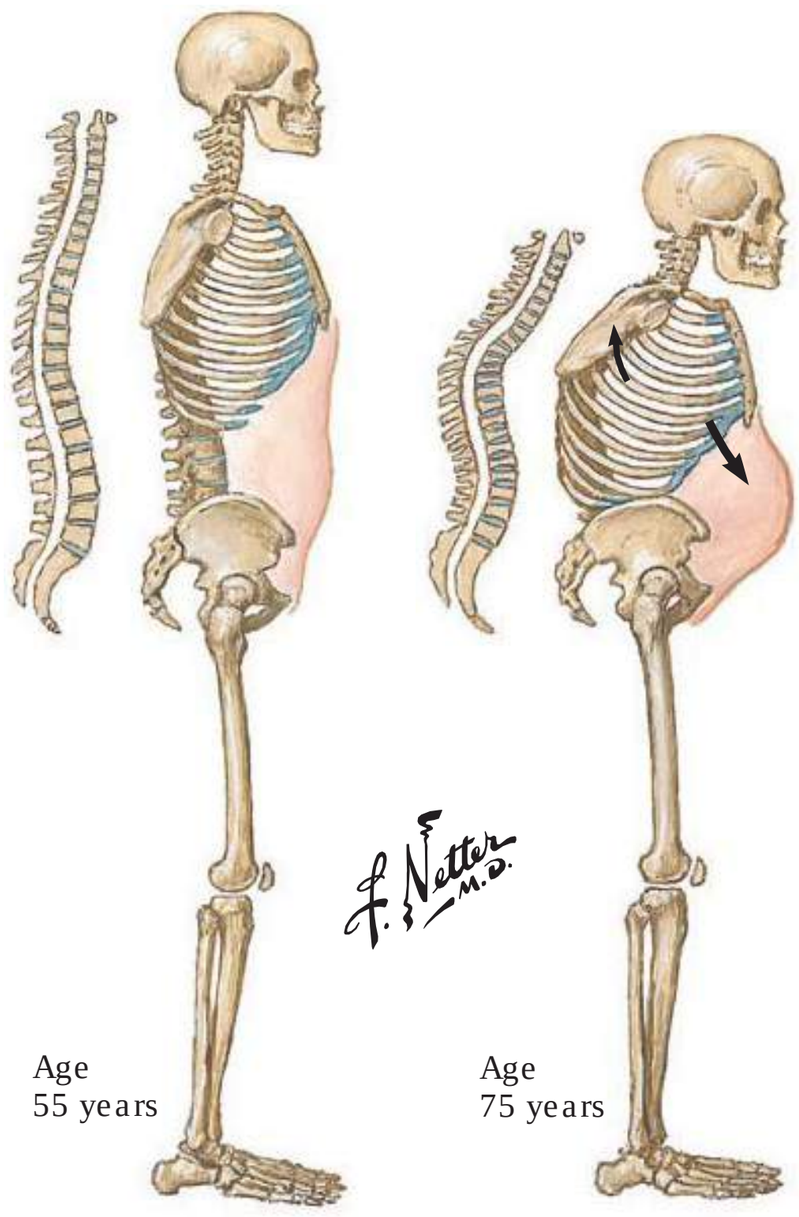


Fig. 12.12 Skeletal changes in osteoporosis.

strength. An imbalance between resorption and formation leads to bone with abnormal mechanical properties. Furthermore, an increased rate of remodeling leads to decreased secondary mineralization, which decreases bone strength.

The formation of osteoclasts from precursor cells depends in large part on the protein **RANKL** on the surface of osteoblasts; RANKL activates the receptor **RANK** on the surface of osteoclasts. **Osteoprotegerin** is a glycoprotein that binds to RANKL and thereby prevents RANKL from activating RANK.

In women before menopause, the high concentration of estrogens inhibits the formation of RANKL while promoting the formation of osteoprotegerin. Thus the net effect of estrogens is to reduce the formation and activation of osteoclasts.

Apart from aging, bone mass also decreases in response to **vitamin D deficiency** (see Section 2.6), **hyperparathyroidism**, and prolonged use of systemic **glucocorticoids** (e.g., **prednisone**) at a high concentration. Parathyroid hormone stimulates the release of calcium from bone as part of the regulation of the concentration of calcium in the blood. Glucocorticoids inhibit osteoblast activity and increase osteoclast activity via increased synthesis of RANKL and RANK.

The nonpharmacological treatment of osteoporosis involves weight-bearing **exercise**, adequate intake of **calcium** and **vitamin D**, **smoking** cessation, abstention from excessive **alcohol** use, and limitation of the risk of **falls**.

The options for pharmacological treatment of osteoporosis include inhibition of osteoclast activity with a bisphosphonate, an antibody to RANKL, or the estrogen agonist/antagonist raloxifene, as well as activation of osteoblast activity through intermittent exposure to a parathyroid hormone analog.

The nitrogen-containing **bisphosphonates** (**zoledronate**, **risedronate**, **alendronate**) induce **apoptosis** of osteoclasts. Alendronate and risedronate are taken orally on a weekly basis, whereas zoledronate is injected intravenously (IV) once a year, often for 3 years. These bisphosphonates tightly and selectively bind to hydroxyapatite in bone, where the longest acting among them stay for many years. Once these bisphosphonates are taken up into an osteoclast, they inhibit **farnesyl pyrophosphate synthase** in the cholesterol synthesis pathway (see Fig. 29.4). The reduction in farnesyl pyrophosphate production reduces the conjugation of signaling proteins with farnesylpyrophosphate and geranylgeranyl pyrophosphate (see also Section 1.5 in Chapter 11), leading to apoptosis.

Bisphosphonates are also used to prevent the increased destruction of bone that accompanies Paget disease of bone (see Section 2.7) or osteomalacia (see Section 2.6); they are effective in the treatment of osteogenesis imperfecta (see Section 2.4), and they can be coupled to a radioactive element (e.g., a γ-emitting isotope of technetium) for detection of bone metastases.

The estrogen agonist/antagonist (formerly selective estrogen receptor modulator [SERM]) **raloxifene** is an option for the prevention and treatment of osteoporosis in women. Raloxifene also reduces the incidence of invasive **breast cancer**; however, it can cause hot flashes and thromboembolisms.

Denosumab is an antibody that binds to **RANKL** and thus prevents the formation and activation of osteoclasts. Denosumab is injected subcutaneously every 6 months.

Teriparatide is a recombinant fragment of **parathyroid hormone**. Teriparatide is injected subcutaneously every day. This generates a pattern of exposing bone to waves of teriparatide, which activates osteoblasts more than osteoclasts (this is contrary to chronic exposure to a high concentration of parathyroid hormone, which activates osteoclasts more than osteoblasts).

3. TYPE IV COLLAGEN: A NETWORK-FORMING COLLAGEN

Like the fibrillar collagens described previously, the network-*f*orming type IV collagens also contain a long triple helix; however, instead o*f* fibrils, they *f*orm a meshwork. Mutations in some o*f* the type IV collagen genes cause thin basement membrane disease or Alport syndrome. An autoimmune reaction to the α3(IV) chain causes Goodpasture syndrome.

Like the fibrillar collagens, type IV collagen contains a long triple-helix region, as well as N- and C-terminal propeptides. However, the triple-helix trimers (i.e., collagen monomers; see Fig. 12.3) form mesh-like networks. Type IV collagen is the primary collagen of all **basement membranes** (**basal lamina**). A basement membrane is a sheet of extracellular matrix that affects tissue permeability, serves as a base for the attachment of epithelial cells, and allows passage of leukocytes. For instance, in the skin, the basement membrane separates the

epidermis from the underlying dermis (Fig. 12.15). In the small intestine, the basolateral portion of the epithelial cells that absorbs nutrients is attached to the basement membrane. Besides collagen, basement membranes also contain laminins; these are proteins and are not discussed here.

There are six different α-chains for type IV collagen, which are matched up to form three different networks.

The **α1/α2 network** contains collagen that consists of two α1- and one α2-chain. It is found in most basement membranes.

The **α3/α4/α5 network** contains collagen that consists of one α3-, one α4-, and one α5-chain. It is found especially in filtration barriers, such as in glomeruli (see Fig. 12.13). It is also found in membranes in the cochlea (i.e., in the inner ear).

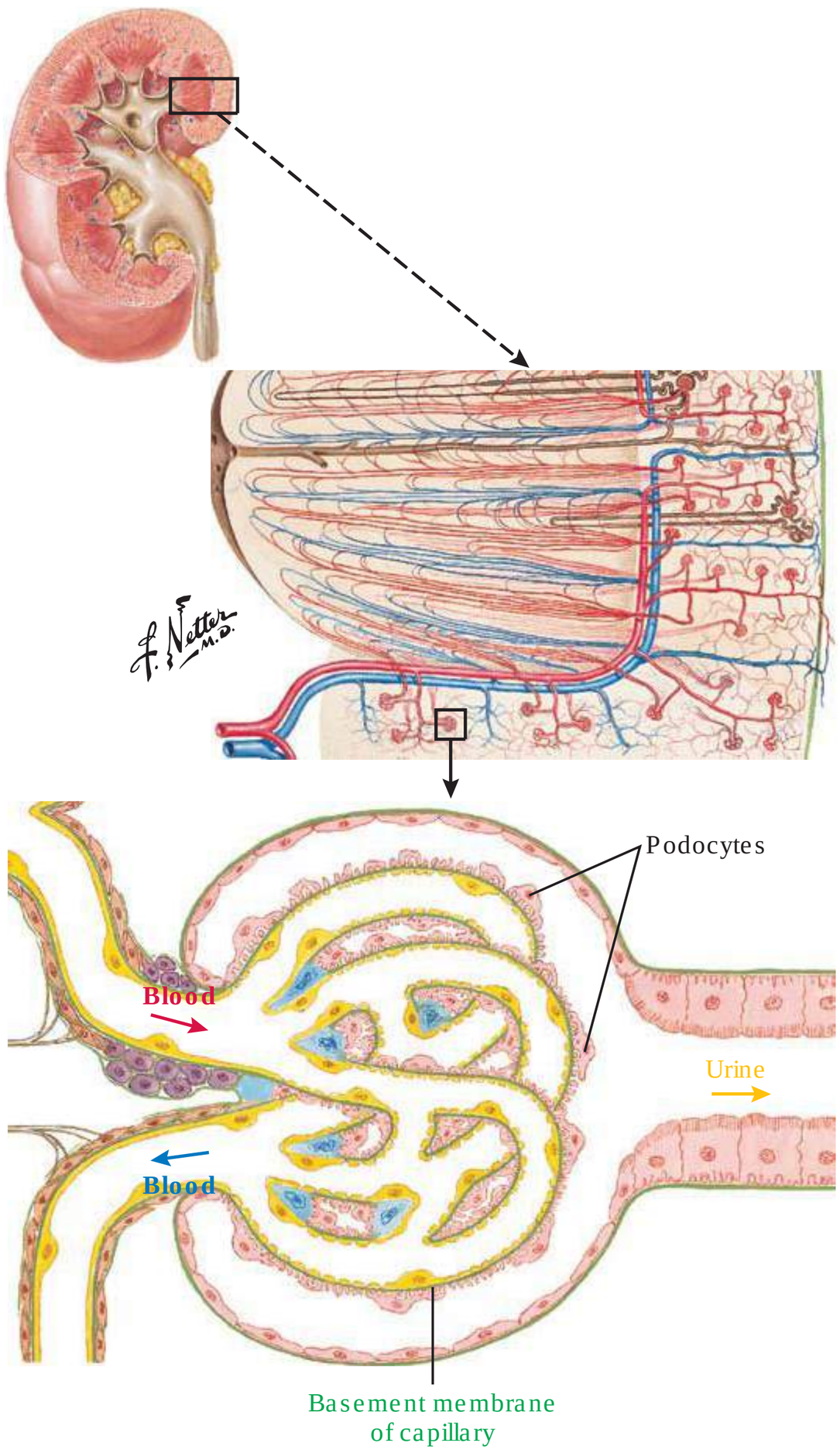


Fig. 12.13 The basement membrane of the capillaries in the glomeruli of the kidneys contains type IV collagen. *Top,* Numerous glomeruli in the parenchyma of a kidney. *Bottom,* A single glomerulus.

The C-termini of triple helices aggregate end to end, and the N-termini aggregate into tetramers, thus forming a mesh.

The **α1/α2/α5/α6 network** contains collagen that consists of two α1-chains and one α2-chain, which in turn is linked to another collagen that consists of two α5-chains and one α6-chain. This collagen network is found in basement membranes that are frequently stretched, such as in vessels, viscera, and the epidermis.

Mutations in the collagen type IV genes cause **Alport syndrome** (**hereditary nephritis**), which has a prevalence of ~1 in 8,000. About 85% of affected patients have **X-linked** Alport syndrome, which is caused by a mutation in the COL4A5 gene on the X chromosome. The remaining ~15% of patients have **autosomal recessively** inherited Alport syndrome and are homozygous or compound heterozygous for mutations in either the COL4A3 or the COL4A4 gene. In total, hundreds of pathogenic mutations in collagen type IV genes are known. The rate of de novo mutations is ~15%.

Starting in childhood, patients with Alport syndrome have **hematuria** and **progressive proteinuria**. Without treatment, they typically develop **end-stage renal disease** in their early twenties. These patients often also have **hearing loss**.

The **basement membrane of the glomeruli** not only contains a type IV collagen α3/α4/α5 network but also a type IV collagen α1/α2 network. As glomeruli develop, the α1/α2 network of type IV collagen is laid down first. Later, podocytes replace this network with a α3/α4/α5 network, which provides greater long-term stability.

In patients with Alport syndrome, the α3/α4/α5 network of type IV collagen is typically absent from the glomerular basement membrane. With time, the original α1/α2 network undergoes proteolysis and increasingly fails to retain large molecules. Accompanying fibrosis eventually impairs kidney function.

Women with autosomal recessively inherited Alport syndrome and men with X-linked or autosomal recessively inherited Alport syndrome have a fairly similar, severe set of symptoms. Because of random and patchwise inactivation of one X chromosome, women who are heterozygous for X-linked Alport syndrome have a clinical course that is anywhere from mild to severe.

The **treatment** of Alport syndrome involves a reduction in **blood pressure** via the renin-angiotensin-aldosterone system (see Fig. 31.17 and Section 4.1 in Chapter 31) to reduce fibrosis and delay the onset of kidney failure. First-line treatment with an **angiotensin-converting enzyme inhibitor**, such as ramipril, and second-line addition of an **angiotensin receptor blocker**, such as losartan, is common.

Thin basement membrane nephropathy (**TBMN**, **familial hematuria**) is due to heterozygosity for a pathogenic mutation in the COL4A3 or COL4A4 gene; that is, affected patients are carriers for autosomal recessive Alport syndrome. TBMN is a much milder disease than Alport syndrome. Affected patients have intermittent or persistent hematuria (based on examination with a microscope) and minimal proteinuria; otherwise, kidney function is nearly normal. About 1% of the population has TBMN due to a mutant type IV collagen.

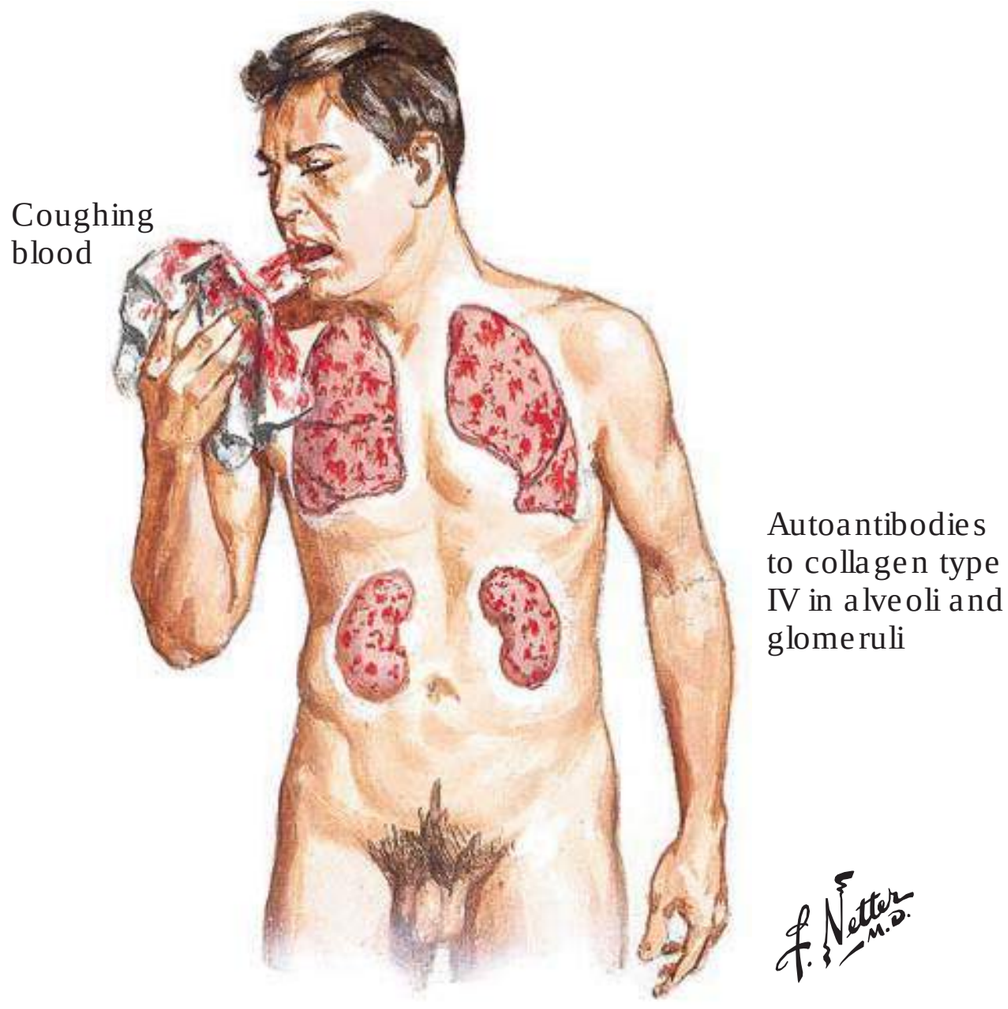


Fig. 12.14 Goodpasture syndrome.

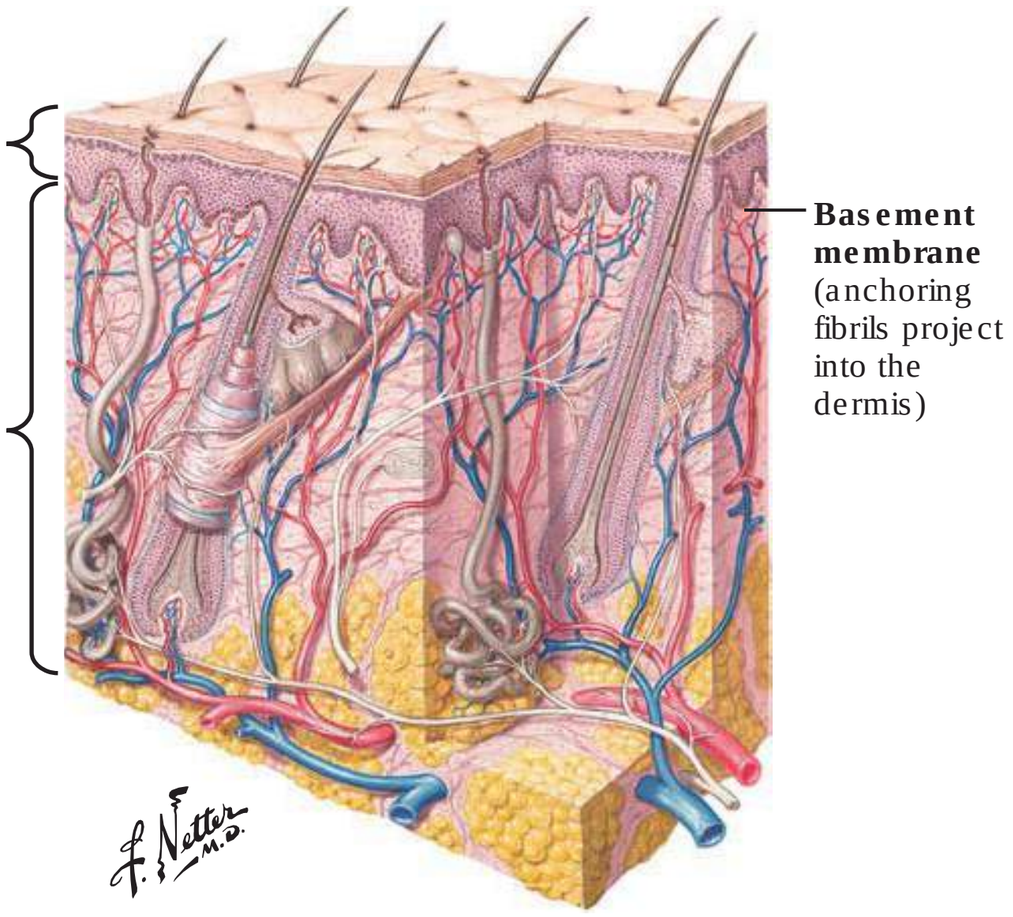


Fig. 12.15 Cross section of skin. Collagen type VII forms anchoring fibrils that anchor the basement membrane in the underlying dermis.

Goodpasture syndrome (also called **antiglomerular basement membrane disease**) is most often the consequence of an autoimmune reaction against the C-terminal domain of the α3-chain of type IV collagen. About 1 in 15,000 people eventually develop Goodpasture syndrome. Smokers and persons exposed to breathing hydrocarbons (e.g., gasoline fumes) are most likely to be af ected. In the alveoli and glomeruli, autoantibodies to type IV collagen bind to basement membranes, and proteases from macrophages and neutrophils destroy the α3/α4/α5 collagen network. Af ected patients often have cough, shortness of breath, proteinuria, and hematuria; smokers may even cough up blood (Fig. 12.14). Af ected patients are treated with **plasmapheresis** (to remove autoantibodies) and **immune suppression** (to inhibit formation of autoantibodies). T ese patients commonly have a shortened life expectancy.

4. TYPE VII COLLAGEN: THE COLLAGEN OF ANCHORING FIBRILS

Anchoring f brils connect the epidermis to the underlying dermis. T ese anchoring f brils consist o*f* type VII collagen. An altered structure o*f* anchoring f brils is the cause o*f* various types o*f* epidermolysis bullosa, which is characterized by blistering o*f* the skin and mucous membranes *f*ollowing mild mechanical trauma.

Type VII collagen consists of a trimer of α1(VII)-chains. Collagen type VII forms the **anchoring fibrils** that connect the basement membrane of the epidermis to the underlying papillary dermis (Fig. 12.15).

Dystrophic epidermolysis bullosa (**DEB**) is caused by a mutation in the COL7A1 gene, which encodes the α-chain that forms a homotrimer that is **type VII collagen**. More than 300 pathogenic mutations are known.

In patients who are af ected with DEB, the skin and mucous membranes are fragile and blister after minor trauma. Upon healing, the blisters form scars. Electron microscopy reveals that the tissue separates below the lamina densa (the lamina densa is a part of the basement membrane). T e anchoring fibrils may be altered, reduced in number, or completely absent.

Dominantly inherited forms of DEB are due to mutations in glycine codons of type VII collagen.

Autosomal recessively inherited forms of DEB have varied causes. T e **Hallopeau-Siemens** type, the most severe of all forms of DEB, is due to mutations that truncate the collagen type VII α-chain. T e resulting mRNA is degraded by nonsense-mediated RNA decay (see Section 3 in Chapter 7). On analysis, there is no immunoreactive type VII collagen.

Besides DEB, there are several other forms of epidermolysis bullosa (e.g., simplex, junctional). T ose diseases are due to mutations in other proteins of the epidermal-dermal junction, which are not discussed in this book.

Epidermolysis bullosa acquisita is an autoimmune disease that is caused by **antibodies** against **type VII collagen** (usually the N-terminal propeptide). Disease onset is typically during adulthood.

SUMMARY

- Fibrillar collagens are synthesized as procollagens. In the endoplasmic reticulum, using ascorbate (vitamin C) as a cofactor, proline and lysine dioxygenases hydroxylate some

proline and lysine residues in the triple-helix domain and in the telopeptides. Three procollagen molecules then aggregate. These trimers are secreted into the extracellular space. There, procollagen endopeptidases remove the propeptides, and the remaining trimeric complexes, now called collagen monomers, aggregate into collagen microfibrils. Lysyl oxidases oxidize some lysyl and hydroxylysyl residues, producing allysine and hydroxyallysine residues, which spontaneously form cross-links with lysyl and other residues in neighboring telopeptides and triple-helix regions. The cross-links increase the tensile strength of collagen microfibrils but decrease their elasticity.

- In holes (gaps) between collagen monomers, collagen fibers in bone are mineralized with hydroxyapatite, which increases stiffness and reduces compressibility. Bone serves as a reservoir of calcium and phosphate.
- The triple-helix region of collagens is resistant to most proteases. However, collagenases, a subclass of matrix metalloproteinases, do cleave a collagen α-chain in a triple helix about three-fourths of its length from the N-terminal end. Subsequently, the triple helix unravels, and the α-chains are digested by other enzymes. Tissue inhibitors of metalloproteinases inhibit collagenases and other matrix metalloproteinases.
- Achondroplasia and hypochondroplasia are caused by an activating mutation in the fibroblast growth factor receptor-3 (FGFR3) that leads to cessation of osteoblast activity, diminished collagen production, and short stature. About 80% of mutations occur de novo. Inheritance is autosomal dominant.
- Vitamin C (ascorbate) deficiency causes a decreased rate of collagen Pro and Lys hydroxylation, which in turn leads to decreased secretion of collagen and decreased cross-linking of collagen monomers.
- Osteogenesis imperfecta is due to a mutation in collagen α1(I)- or α2(I)-chains. Owing to the formation of structurally impaired trimers, microfibrils, and so forth, most of these mutations show dominant-negative effects. Affected patients have an increased rate of bone fractures, in severe cases already in utero.
- Most forms of classic Ehlers-Danlos syndrome (EDS) are caused by mutations in collagen type V that cause abnormally elastic skin, easy bruising, and delayed healing of sutured skin.
- Rickets and osteomalacia are due to demineralization of collagen in bone, often due to a vitamin D deficiency or an excessive need for calcium and phosphate from the bone reservoir.
- Early in the disease, Paget disease of bone shows mostly foci of increased bone degradation; later in the disease, foci of bone formation predominate. The disease is associated with bone pain, an enlarged head, and impaired hearing. It is more common among men and inherited in autosomal dominant manner.
- Osteoporosis is associated with reduced bone mineral density (measurable with a DXA scan) and an increased risk for bone fractures. Patients should be advised about exercise, supplementary calcium and vitamin D, smoking cessation, and moderation of alcohol use. Options for pharmacological treatment include bisphosphonates, the estrogen agonist/antagonist raloxifene, the RANKL antibody denosumab, and the parathyroid hormone fragment and analog teriparatide.
- Type IV collagen is a typical network-forming collagen. It is found in basement membranes.
- Mutations in type IV collagen can cause thin basement membrane nephropathy (TBMN, familial hematuria), a disorder accompanied by persistent microscopic hematuria. Patients of either sex who are homozygous or compound heterozygous for such mutations, and patients who are hemizygous for mutations in an X-linked type V collagen gene can have Alport syndrome. Alport syndrome typically leads to kidney failure in adults as well as hearing loss.
- Autoantibodies to type IV collagen, often induced by smoking and exposure to hydrocarbon vapors (e.g., from gasoline), are the cause of Goodpasture syndrome. The syndrome primarily affects lung function and, in severe cases, kidney function as well.
- Type VII collagen is part of the anchoring fibrils that link the epidermis of the skin to the underlying dermis. Mutations in type VII collagen can cause dystrophic epidermolysis bullosa (DEB, a severe blistering disease).

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Review Questions

1. A woman and her male partner both have hematuria (determined by microscopy). Biopsies of their kidneys showed thinning of the basement membrane. Compared with the general population, these patients are at a much increased risk of having of spring with which of the following?

   A. Alport syndrome
   B. Ehlers-Danlos syndrome
   C. Marfan syndrome
   D. Osteoarthritis
   E. Osteoporosis

2. A 20-year-old male patient has persistent hematuria. His mother also has hematuria, but his father does not. T is patient most likely has which one of the following disorders?

   A. Autosomal recessive Alport syndrome
   B. Ehlers-Danlos syndrome
   C. Goodpasture syndrome
   D. X-linked Alport syndrome

Chapter

13 Pathologic Alterations of the Extracellular Matrix That Involve Fibrillin, Elastin, or Proteoglycans

SYNOPSIS

- Elastic fibers, consisting mainly of elastin and fibrillin, can be stretched and will recoil. Abnormal elastic fibers are found in patients with emphysema, α-1-antitrypsin deficiency, Marfan syndrome, or Williams syndrome.
- Proteoglycans are proteins that are heavily glycosylated with glycosaminoglycans (polysaccharides containing aminated sugars), such as heparan sulfate, keratan sulfate, chondroitin, and dermatan sulfate. Proteoglycans are synthesized inside the cell and then secreted. In the extracellular space, various proteins bind to specific portions of the glycan chains. Proteoglycans take up compressive forces (e.g., in knee joints and intervertebral disks).
- Hyaluronic acid, another glycosaminoglycan, is a long polysaccharide that is synthesized inside the cell and extruded into the extracellular space. Hyaluronic acid serves as a lubricant and as a scaffold for binding proteoglycans.
- Osteoarthritis is associated with degradation of joint cartilage, which contains proteoglycans.
- The main degradation of glycans of proteoglycans occurs inside lysosomes. Such degradation is impaired in patients who have a heritable mucopolysaccharidosis. In these patients, glycosaminoglycans accumulate in lysosomes, thereby damaging the central nervous system, liver, heart, lungs, or various other tissues.
- Remodeling of the extracellular matrix takes place in wound healing, in fibrosis, and during pregnancy-induced changes of the cervix.

LEARNING OBJECTIVES

For mastery of this topic, you should be able to do the following:

- Describe the signs, major pathology, and treatment of Marfan syndrome.
- Describe the consequence of haploinsufficiency for elastin.
- Describe the pathogenesis of emphysema as it relates to elastic fibers.
- Describe the signs, diagnosis, and treatment of hereditary α-1-antitrypsin deficiency.
- Outline the synthesis and structure of proteoglycans.
- Outline the synthesis, structure, and roles of hyaluronate.
- Describe the fate of cartilage in patients who have osteoarthritis.
- Describe the general cause of the mucopolysaccharidoses, and list successful approaches to treatment of type I mucopolysaccharidosis (Hurler syndrome).
- Define fibrosis and explain how it can impair organ function.

1. ELASTIN AND FIBRILLINS

Elastic fibers allow the extracellular matrix to stretch and recoil. Elastic fibers consist mostly of elastin and to a lesser extent fibrillin that forms microfibrils. The microfibrils may serve as guides for the deposition of elastin. Like the collagens, a precursor of elastin is intracellularly synthesized. After export, extracellular processing, and attachment to integrin, elastin becomes cross-linked. In patients who have emphysema, excessive degradation of elastin impairs lung function. In patients with Marfan syndrome, a mutant fibrillin alters production and maintenance of elastic fibers. This can lead to life-threatening rupture of a major artery.

1.1. Synthesis of Elastic Fibers

Elastin is predominantly found in large arteries, lung, ligaments, tendons, skin, and elastic cartilage (e.g., in the front of the rib cage; Fig. 13.1). In large arteries, elastic fibers form several cylindrical layers (called elastic lamellae) along the long axis of the vessel. These lamellae buffer pressure changes during the pumping cycle of the heart (large arteries also contain collagen fibers, which limit the stretching). In lung and in the cartilage of the auricles, elastic fibers form a lattice. In ligaments and tendons, the elastic fibers lie next to each other with the major axis parallel to the major physiological force.

Elastic fibers consist of **elastin** and **microfibrils**. Elastin makes up the core of an elastic fiber and constitutes about 90% of the weight of such a fiber. The microfibrils appear to serve as guides for the deposition of elastin. Some of these microfibrils are interspersed in the core of the elastic fiber, whereas others surround the mature elastic fiber like a sheath. The microfibrils have a diameter of about 0.01 µm (about three times the diameter of collagen microfibrils). Microfibrils contain many different proteins, including fibrillin-1 (see below).

Elastin is the product of cross-linked monomers of **tropoelastin**. Elastin is mainly synthesized by fibroblasts, smooth muscle cells, and some chondrocytes during development of the fetus and shortly after birth. Normally, elastin turns over slowly enough so that most of it lasts for a person's lifetime. There is only one gene for tropoelastin; however, tropoelastin RNA can be spliced in different ways, giving rise to several isoforms. During and after translation, **elastin-binding protein** binds tropoelastin, keeps it from aggregating, and thus chaperones it through the Golgi and into secretory vesicles. Tropoelastin has a globular shape. In the extracellular space, **lysyl oxidase** and **lysyl oxidase-like proteins** oxidize about 40 lysine side chains of tropoelastin to **allysine** side chains These side chains then form di-, tri- or tetravalent crosslinks to yield elastin. Lysyl oxidase also converts lysyl residues in collagen to allysyl residues (see Section 1.2 in

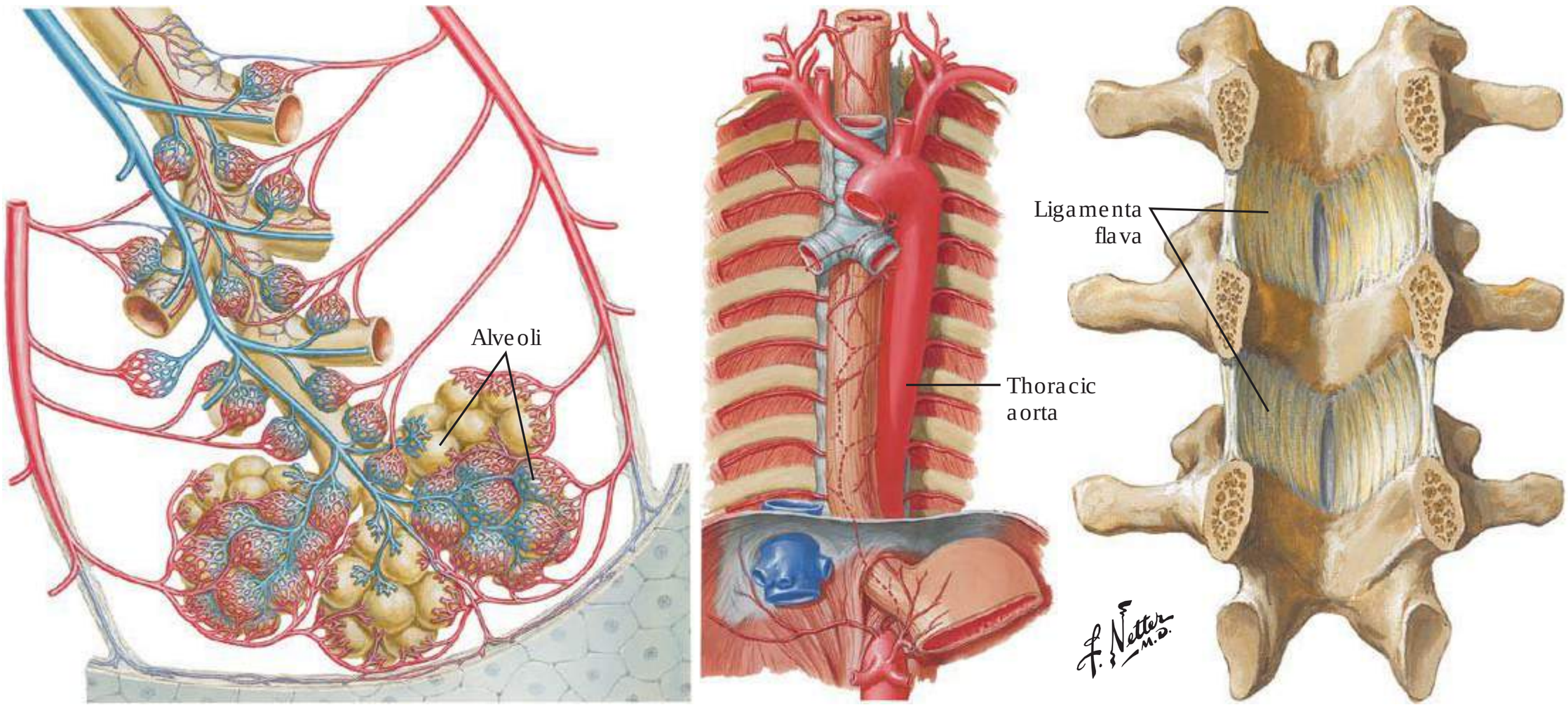


Fig. 13.1 **Elastic fibers are prevalent in the lungs, large blood vessels, and ligaments.**

Chapter 12), which form crosslinks with collagen as well as elastin.

The **elasticity** of elastin is thought to be due to a stretching-induced decrease in entropy and an **entropy**-driven spontaneous return to the unstretched state. Current models assume that when elastin is stretched, hydrophobic amino acid side chains become exposed to water, or unordered sequences or β-turns become ordered (i.e., they lose some of their freedom to assume different conformations). In all of these cases, entropy decreases. When the stretching force vanishes, the natural tendency of molecules toward disorder (i.e., an increase in entropy) is thought to drive recoil.

Fibrillin-1 on the outside of elastic fibers binds **latent transforming growth factor β binding proteins**, which in turn bind the inactive precursor of the protein **transforming growth factor β** (**TGF-β**). Matrix-bound inactive TGF-β serves as a reservoir for the production of active TGF-β. TGF-β affects various processes, including production and maintenance of the extracellular matrix.

1.2. Marfan Syndrome

Mutations in **fibrillin-1**, the most abundant protein of microfibrils in elastic fibers, cause the classic **Marfan syndrome**. Marfan syndrome is characterized by abnormalities of the skeleton, cardiovascular system, and the eyes (Fig. 13.2). There are more than 1,000 known pathogenic mutations of the fibrillin-1 gene (FBN1). In addition, within a family, the phenotype of the same mutation varies greatly. About 1 in 5,000 persons has Marfan syndrome.

The pathogenesis of Marfan syndrome is only partially understood and seems to be due to excessive **TGF-β** signaling (cause unclear), as well as structural deficiencies of microfibrils that contain mutant fibrillin-1 and may induce counterproductive repair mechanisms. The related Loeys-Dietz syndrome, which shows some of the same pathology, is most often caused by mutations in TGF-β receptors and unexpectedly also leads to excessive TGF-β signaling.

Patients with Marfan syndrome typically show increased **joint flexibility**, **skeletal abnormalities**, and, with increasing age, **lens dislocation** as well as **dilation of the aorta**. The ascending aorta and the root of the aorta dilate, leading to **dissection of the aorta** (a life-threatening emergency), **prolapse of the mitral valve,** and **regurgitation**. Dissection of the aorta is also a key feature of Loeys-Dietz syndrome.

The rate of dilation of the aorta is slowed by reducing the pulse pressure with the **β_1-adrenergic receptor antagonist atenolol** or the **angiotensin II receptor blocker losartan**; losartan also reduces excessive TGF-β signaling (and is an effective treatment for Loeys-Dietz syndrome).

Marfan syndrome is usually inherited in **autosomal dominant** fashion. About two-thirds of all patients inherit the mutation from an affected parent. About one-third of all patients have a de novo mutation, which occurred in the germline of an unaffected parent. Either way, offspring of an affected patient have a 50% chance of inheriting the faulty fibrillin-1 (FBN1) allele.

1.3. Supravalvular Aortic Stenosis

Patients with supravalvular aortic stenosis have an abnormally narrow ascending aorta as well as abnormally narrow coronary arteries, carotid arteries, renal arteries, and pulmonary arteries due to hemizygous loss of function of an allele of the **elastin** gene. Haploinsufficiency for elastin leads to narrowing of arteries via decreased production of tropoelastin,

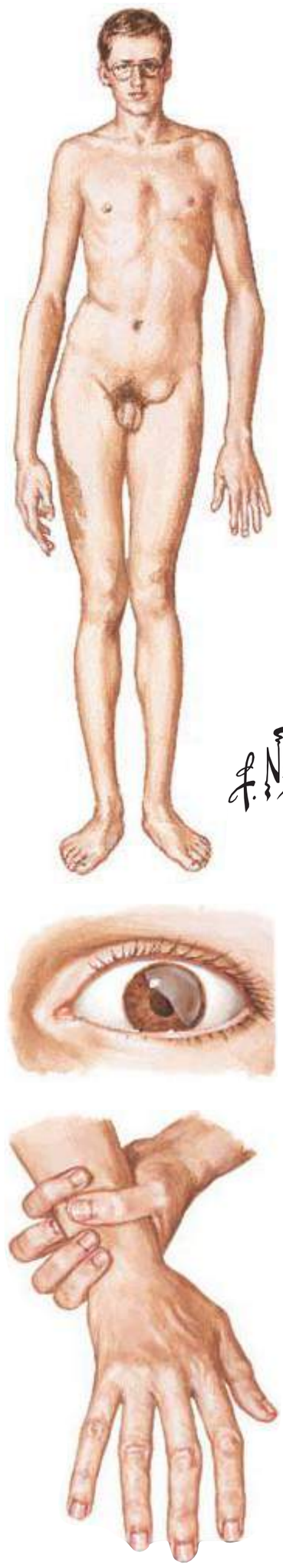


Fig. 13.2 Signs of classic Marfan syndrome. Affected patients frequently have scoliosis (pathologic sideways curvature of the spine). Compared with the length of the upper body, the arms and legs are relatively long. Many patients have a dislocated lens. When wrapping their hand around their wrist, the thumb and little finger overlap.

hyperproliferation of smooth muscle cells, and increased deposition of collagen.

Most often, haploinsufficiency for elastin is a consequence of **Williams syndrome**, a microdeletion on chromosome 7, which includes the elastin gene and occurs in ~1 in 10,000 births. Williams syndrome can be detected by prenatal screening using fluorescence in situ hybridization (see Section 1 in Chapter 4). The syndrome is associated with a low IQ and particularly outgoing behavior.

1.4. Degradation of Elastic Fibers, Emphysema, and α-1-Antitrypsin Deficiency

While turnover of elastic fibers is normally minimal, degradation of elastic fibers is enhanced in wounds. In wounds, neutrophils release the protease **elastase**. In healthy tissue, **α-1-antitrypsin** inhibits elastase as well as other proteases, including trypsin. α-1-Antitrypsin in the serum stems mostly from the liver.

Emphysema is a part of **chronic obstructive pulmonary disease** (**COPD**), which is most often associated with **smoking**. COPD is characterized by persistent, pathologically reduced airflow. In the lungs of a patient with emphysema, the walls between alveoli are progressively and irreversibly destroyed, creating large spaces with a much-reduced surface for gas exchange (Fig. 13.3). Oxygen supplementation helps by increasing the net transfer of oxygen to blood.

Emphysema is accompanied by a marked loss of elastin in the lungs. Smoking attracts an increased number of **neutrophils** and **macrophages** into the lungs, where these cells release **elastases** that degrade elastin and other proteins of the extracellular matrix. In addition, smoking leads to the inhibition of the synthesis of several components of the extracellular matrix.

A **deficiency of α-1-antitrypsin** can lead to emphysema and sometimes liver disease. Worldwide, ~1 in 2,000 individuals is homozygous or compound heterozygous for a pathogenic allele of α-1-antitrypsin. In the United States, ~1 in 3,000 persons is homozygous for the Z allele (Glu342Lys, E342K) of α-1-antitrypsin. The α-1-antitrypsin of these patients has less than 10% of the normal inhibitory effect of α-1-antitrypsin; this is considered a severe deficiency. In the absence of an insult from the environment, antitrypsin deficiency may not damage the lungs. However, smokers develop symptoms of emphysema at ~40 years of age.

The **diagnosis** of α-1-antitrypsin deficiency is based on measurements of α-1-antitrypsin in serum, analysis of α-1-antitrypsin by isoelectric focusing, and genotyping by polymerase chain reaction–based methods. It is recommended that patients who have COPD (see above) be tested for α-1-antitrypsin deficiency, although only ~1% of all these patients have α-1-antitrypsin deficiency and even though antitrypsin deficiency is most common among the youngest patients. If a patient tests positive for antitrypsin deficiency, the patient's relatives should be informed about testing options.

Patients who can blow out only a reduced volume of air in 1 second and who have serum antitrypsin activity below a certain threshold are given **augmentation therapy** with weekly **intravenous antitrypsin**. The antitrypsin is made from pools of human plasma. Augmentation therapy delays the progression of emphysema.

A minority of patients with severe α-1-antitrypsin deficiency develop clinically apparent liver disease at 1 to 2 months of age; affected infants typically have persistent jaundice and an elevated concentration of liver enzymes in serum. Such liver disease is seen only with certain mutations, including the Z allele (i.e., E342K). The pathogenic mutant α-1-antitrypsin accumulates inside hepatocytes. Some of the patients with the

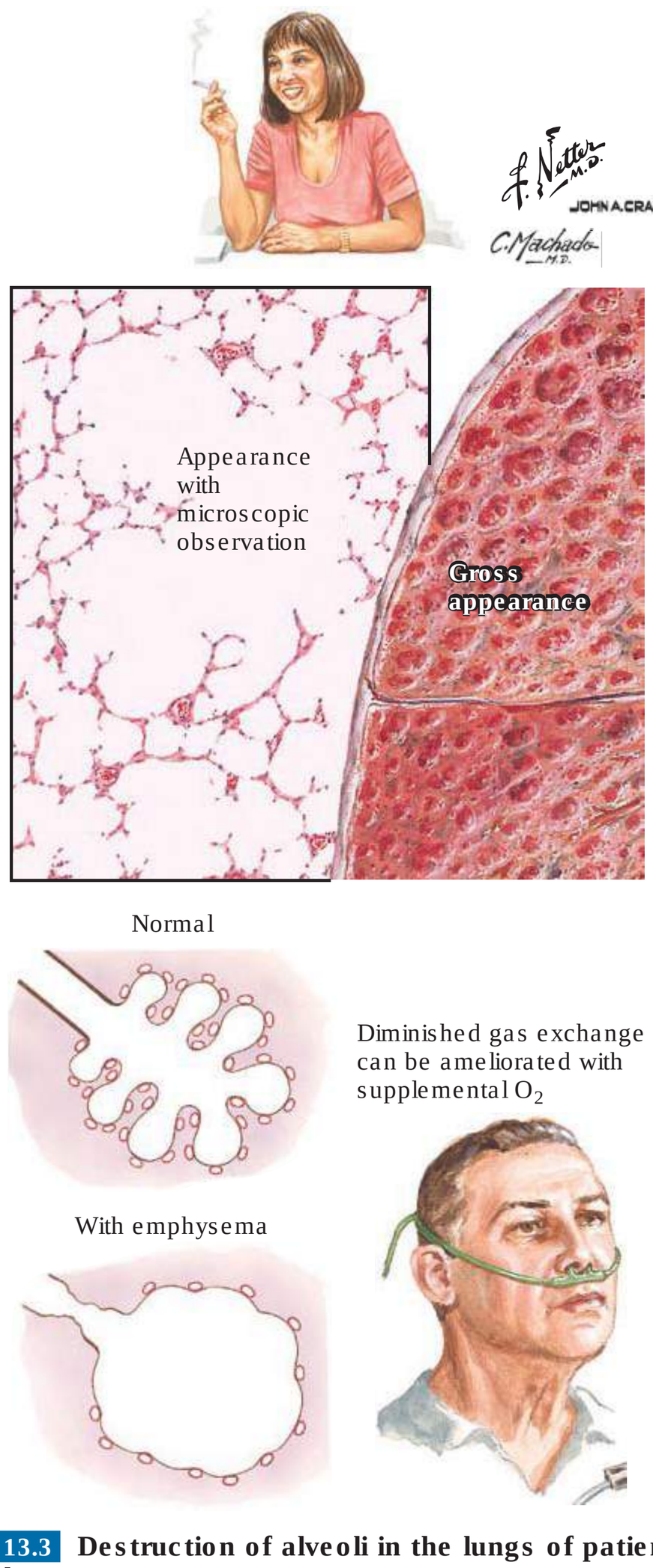


Fig. 13.3 **Destruction of alveoli in the lungs of patients with emphysema.**

Z allele eventually develop liver cirrhosis or hepatocellular carcinoma.

2. PROTEOGLYCANS AND GLYCOSAMINOGLYCANS

Proteoglycans consist of a core protein that is linked to numerous long polysaccharide chains. These polysaccharides are glycosaminoglycans, which are subdivided into heparan sulfates, keratan sulfates, chondroitin sulfates, and dermatan sulfates. Modification of sugar residues in these polysaccharides creates specific binding sites for proteins, such as growth

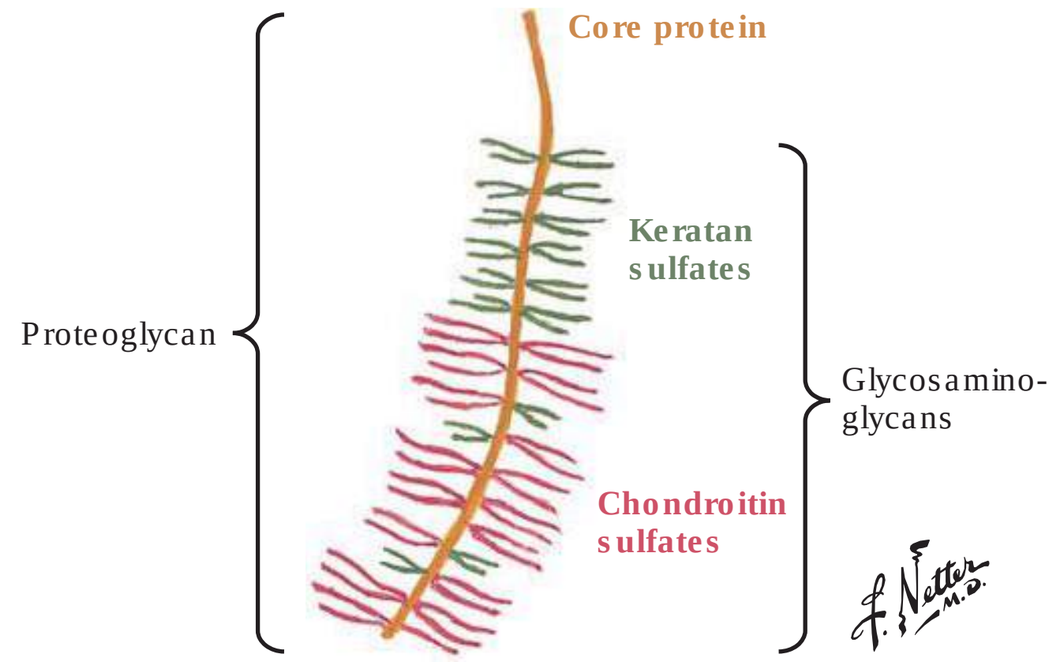


Fig. 13.4 **Structure of a proteoglycan.**

factors and coagulation factors. Chondrocytes can synthesize the polysaccharide hyaluronate and extrude it into the extracellular space. In the extracellular space, hyaluronate binds proteoglycans and other proteins. Degradation of glycosaminoglycans requires several sulfatases and hydrolases in lysosomes. A deficiency of any one of these enzymes leads to a mucopolysaccharidosis, whereby glycosaminoglycans accumulate in lysosomes and cause progressive damage, generally to the nervous system.

2.1. Synthesis and Degradation of Proteoglycans Containing Heparan, Keratan, Chondroitin, or Dermatan Sulfate

Proteoglycans consist of a core **protein** to which **glycosaminoglycans** (**mucopolysaccharides**) are covalently linked (Fig. 13.4). Examples of core proteins are aggrecan, syndecan, perlecan, decorin, glypican, agrin, and collagen XVIII. Glycosaminoglycans are **polysaccharides** that contain aminated sugars (see below).

The glycosaminoglycans are subdivided into **heparan sulfates, keratan sulfates**, **chondroitin sulfates**, and **dermatan sulfates**, all of which contain dozens to hundreds of disaccharide repeats (Fig. 13.5). Each of these disaccharide repeats contains an aminated sugar: glucosamine or galactosamine. Sulfation of these glycosaminoglycans is highly variable and can change within a molecule from one disaccharide repeat to the next. A few sulfated sugar residues suffice to provide a specific binding site for a protein.

Heparan sulfates are ubiquitous, and keratan sulfates are found in the cornea and in cartilage. Chondroitin sulfates are the predominant glycosaminoglycan in cartilage, and dermatan sulfates predominate in skin (Fig. 13.6).

The **core protein** of a proteoglycan is synthesized by ribosomes that are bound to the endoplasmic reticulum. During synthesis, the nascent protein chain is translocated into the lumen of the endoplasmic reticulum. From the endoplasmic reticulum, the protein is transported into the Golgi apparatus. Starting in the endoplasmic reticulum and ending in the Golgi apparatus, a **tetrasaccharide** (first xylose, then galactose, galactose, and finally glucosamine) is added to hydroxyl

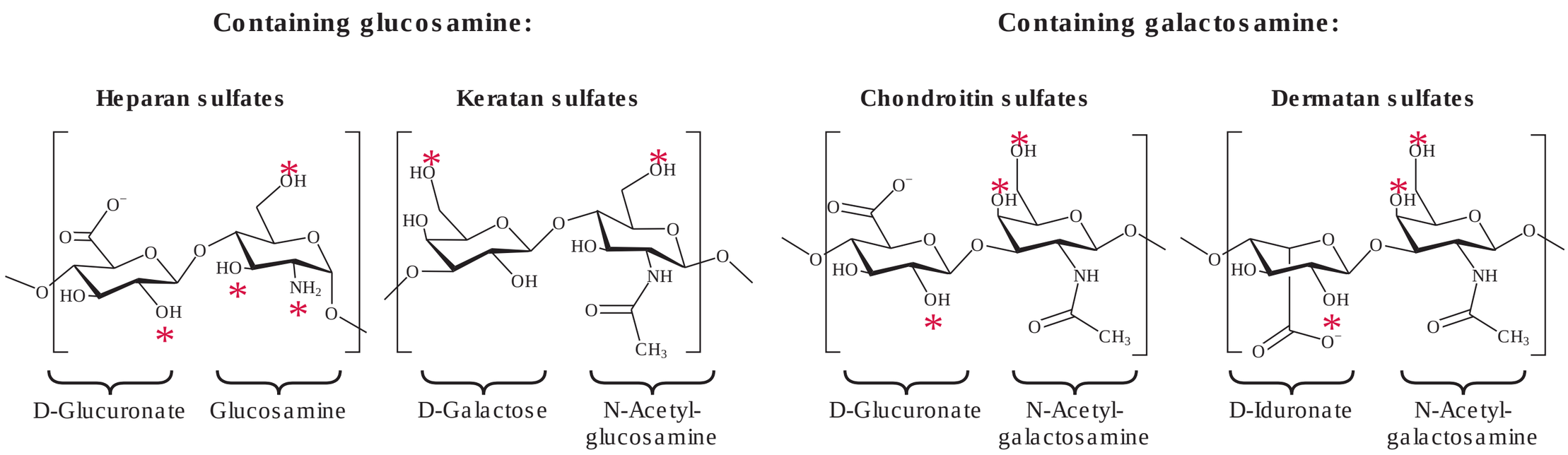


Fig. 13.5 Basic structure of glycosaminoglycans. Asterisks indicate possible sites of sulfation. Brackets enclose the most common disaccharide-repeating unit of different types of glycosaminoglycans.

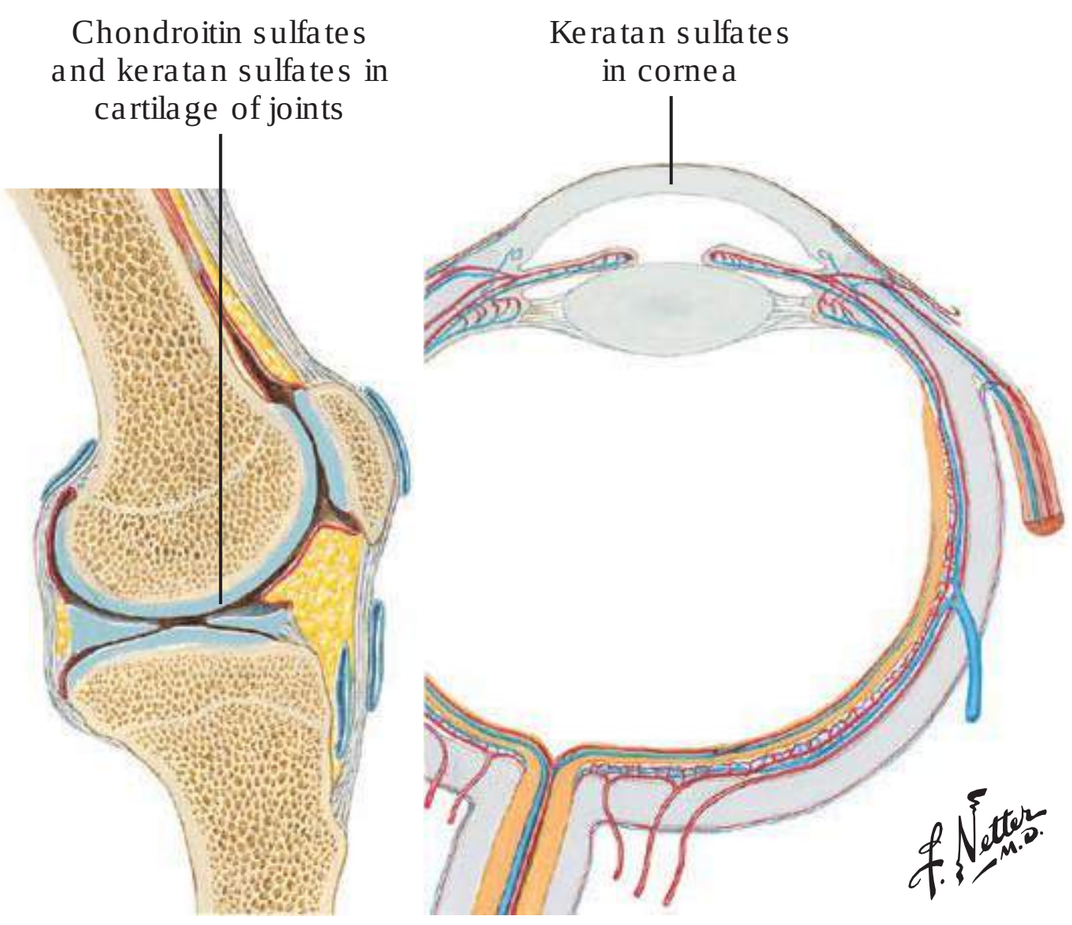


Fig. 13.6 Sites of proteoglycan deposition: the knee joint and the eye.

groups of certain serine or threonine side chains of the core protein. In the Golgi apparatus, the tetrasaccharide is extended by the addition of monosaccharides to generate the repeating disaccharide units shown in Fig. 13.5. Uridine diphosphate sugars serve as substrates for all of these glycosylation reactions. The iduronate residues in heparan sulfate and dermatan sulfate are the result of C5 epimerization of glucuronate residues by an epimerase.

Several **sulfotransferases** sulfate some of the sugar residues; the sulfate derives from **3′-phosphoadenosine 5′-phosphosulfate** (PAPS; see Fig. 36.16 and Section 9 in Chapter 36). Sulfated sugar residues typically occur in clusters.

Proteoglycans emerge from the Golgi in secretory vesicles, which are released into the extracellular space. Proteoglycans can have an anchor in the plasma membrane, or they can at first be free in the extracellular space and then attach to the extracellular matrix. In the extracellular space, **6-O-endosulfatases** can remove 6-O-sulfate groups.

The aforementioned epimerization, sulfation, and desulfation yield polysaccharides with very specific sequence and sulfation patterns, which can be recognized by proteins. However, how these patterns are determined is still unclear. Heparan sulfates, for example, can contain binding sites for antithrombin, coagulation factor X_a, and thrombin, all of which influence **coagulation** (blood clotting). Throughout the extracellular matrix, glycosaminoglycans bind (and serve as reservoirs for) cytokines and growth factors depending on sulfation patterns. Many of these cytokines and growth factors are released during **matrix remodeling** or **wound healing**.

Proteoglycans are particularly abundant in **cartilage**, where they are produced by **chondrocytes** inside the cartilage. Cartilage in the human body includes morphological cartilage (e.g., in the nose), elastic cartilage (e.g., in front of the rib cage), fibrocartilage (e.g., in intervertebral disks), and hyaline cartilage (e.g., at the growth plate and in movable joints on top of bones, see Fig. 13.6).

Proteoglycans help cartilage, such as in knee joints and intervertebral disks (see Figs. 13.6 and 13.10), absorb mechanical energy and deform without fracturing. The proteoglycans are enclosed by a semipermeable capsule of other matrix proteins (mostly collagens; see Fig. 13.10). Within this capsule, the glycosaminoglycans maintain a sizable swelling pressure due to Donnan osmotic pressure. This pressure has its origins in osmotically active cations that neutralize sulfate and carboxylate groups on the glycosaminoglycans.

Heparin is an analog of heparan sulfate and is used as an anticoagulant. Heparin is isolated from the intestines of pigs. It contains more iduronate and sulfate groups than most heparan sulfates. Heparin and some heparan sulfates both have anticoagulant activity. The anticoagulant activity of the negatively charged heparin can be inhibited by **protamine**, a protein from salmon sperm that contains many positively charged arginine residues (protamine is also used to complex insulin; see Section 4.3 in Chapter 39).

In the extracellular space, glycosaminoglycans are hydrolyzed by both exoglycosidases and endoglycosidases. **Exoglycosidases** degrade glycosaminoglycans from one end, one residue at a time; **endoglycosidases** hydrolyze internal

glycosidic bonds, thereby producing fragments that are ~40 sugar residues long. **Sulfatases** remove sulfate groups.

2.2. Hyaluronate, a Glycosaminoglycan That Binds to Link Proteins

Hyaluronate (**hyaluronic acid**, **hyaluronan**) is a long, unbranched polysaccharide that consists of hundreds to thousands of repeating units of glucuronyl N-acetylgalactosamine (Fig. 13.7) and serves as both a lubricant and a platform for the binding of certain proteins. Hyaluronic acid is a glycosaminoglycan like heparan sulfate, keratan sulfate, chondroitin sulfate, and dermatan sulfate. However, hyaluronate is not sulfated, and it is synthesized very differently from these other glycosaminoglycans; **hyaluronan synthases** are embedded in the plasma membrane and directly extrude hyaluronate into the extracellular space.

Free hyaluronate binds a large amount of water, is an excellent **lubricant**, and is found in synovial fluid, covering cartilage.

In the extracellular space, **link proteins** connect hyaluronate to extracellular matrix proteins (mostly proteoglycans; Fig. 13.8). These interactions generate huge complexes. Hyaluronic acid–protein complexes are found in skin and cartilage.

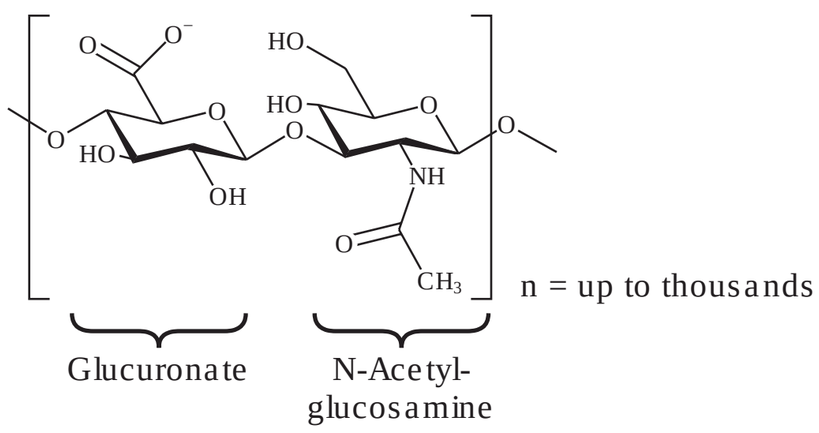


Fig. 13.7 Basic building block of the polysaccharide and glycosaminoglycan hyaluronate.

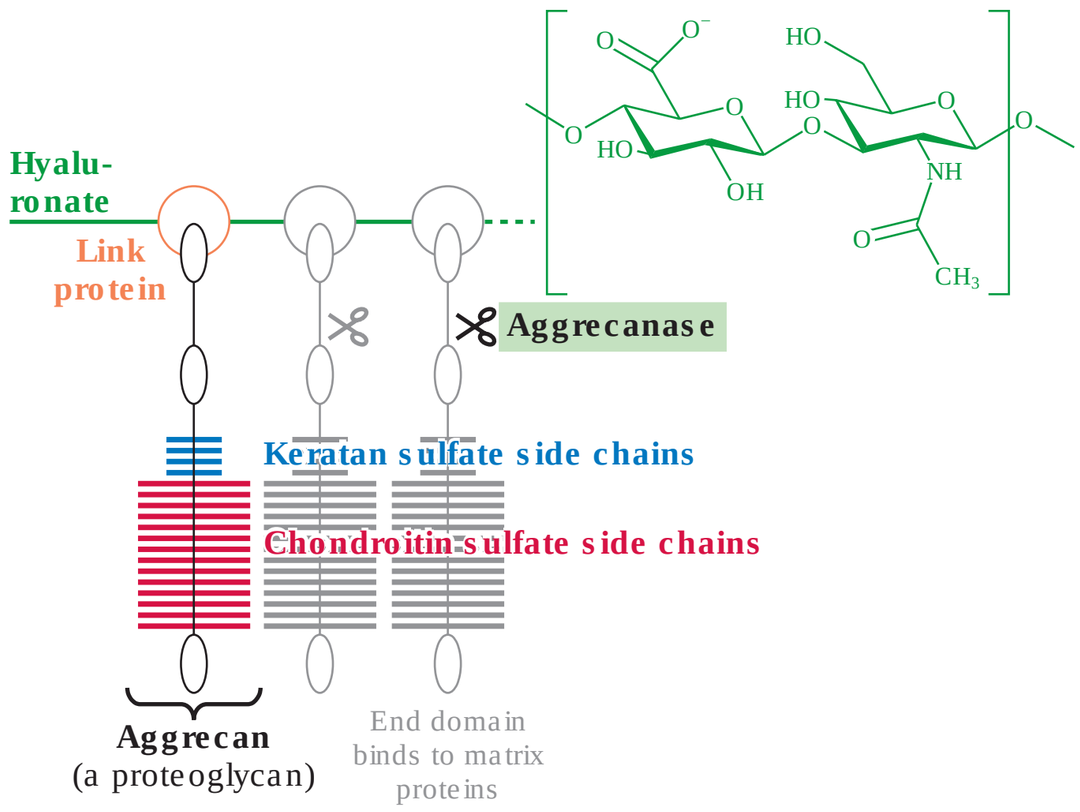


Fig. 13.8 Structure of a hyaluronate-proteoglycan complex. Each molecule of hyaluronate can bind up to ~100 molecules of link protein. Ovals in aggrecan indicate globular domains in the protein.

Aggrecan is one of the proteoglycans that is bound to hyaluronate via a link protein. Aggrecan contains glycosaminoglycans that consist of keratan sulfate or chondroitin sulfate.

In **hyaline cartilage** (e.g., in the knee joint; see Fig. 13.6), hyaluronate-proteoglycan complexes are trapped inside a matrix of collagen. Normal function of hyaline cartilage depends on hyaluronate and aggrecans of adequate length, adequate glycosylation of aggrecan, and adequate sulfation of keratan and chondroitin. An abnormality of any one of these parameters impairs the function of hyaline cartilage and can cause **arthritis**.

Hyaluronidases degrade hyaluronate (the glycosaminoglycan to which link proteins attach; see Fig. 13.8). Hyaluronic acid in the skin has a half-life of about 1 day; in cartilage, it has a half-life of 1 to 3 weeks. One type of hyaluronidase is anchored to the outside of plasma membranes; it cuts hyaluronate to fragments of about 100 sugar residues.

In the extracellular matrix, the **aggrecanases** ADAMTS4 and ADAMTS5 continuously degrade aggrecan, but remnants of aggrecan can remain on hyaluronate until the hyaluronate is degraded (see Fig. 13.8).

Chondrocytes, macrophages, keratinocytes, and other cells express plasma membrane receptors for fragments of hyaluronate through which the fragments are endocytosed. Receptors for hyaluronate fragments that reach the circulation are removed chiefly by receptors in the lymph nodes. Once the hyaluronate fragments reach the lysosomes, a hyaluronidase degrades them to tetrasaccharides. The tetrasaccharides are then hydrolyzed to monosaccharides, which are released into the cytosol.

2.3. Osteoarthritis

Osteoarthritis is characterized by a loss of joint **cartilage** that causes pain with movement (Fig. 13.9). It is the most common form of arthritis. Osteoarthritis affects mostly the hands, feet, knees, hips, and spine, and it is the leading indication for implantation of an artificial joint.

Early in the disease process, the osteoarthritic cartilage contains inordinately few proteoglycans, in part because the **aggrecanases** ADAMTS4 and ADAMTS5 degrade aggrecan.

There are many different causes of osteoarthritis; among them are inflammation of the joint, improper alignment of bones, joint laxity, injury, excess body weight, and, less commonly, mutations in the COL2A1 gene (type II collagen is the most abundant protein in cartilage).

In osteoarthritis, inflammation causes granulocytes and chondrocytes to produce cytokines that induce chondrocytes to synthesize and release more matrix metalloproteinases and aggrecanases. These enzymes, in turn, gradually destroy joint cartilage. With the chronic nature of osteoarthritis, there is insufficient compensatory synthesis of the extracellular matrix.

Treatment aims to strengthen the joint, lessen the load on it, and decrease both pain and inflammation. These improvements may happen with orthoses, weight loss, exercise, intermittent heat treatment, acetaminophen, oral or topical nonsteroidal antiinflammatory agents, topical capsaicin, or

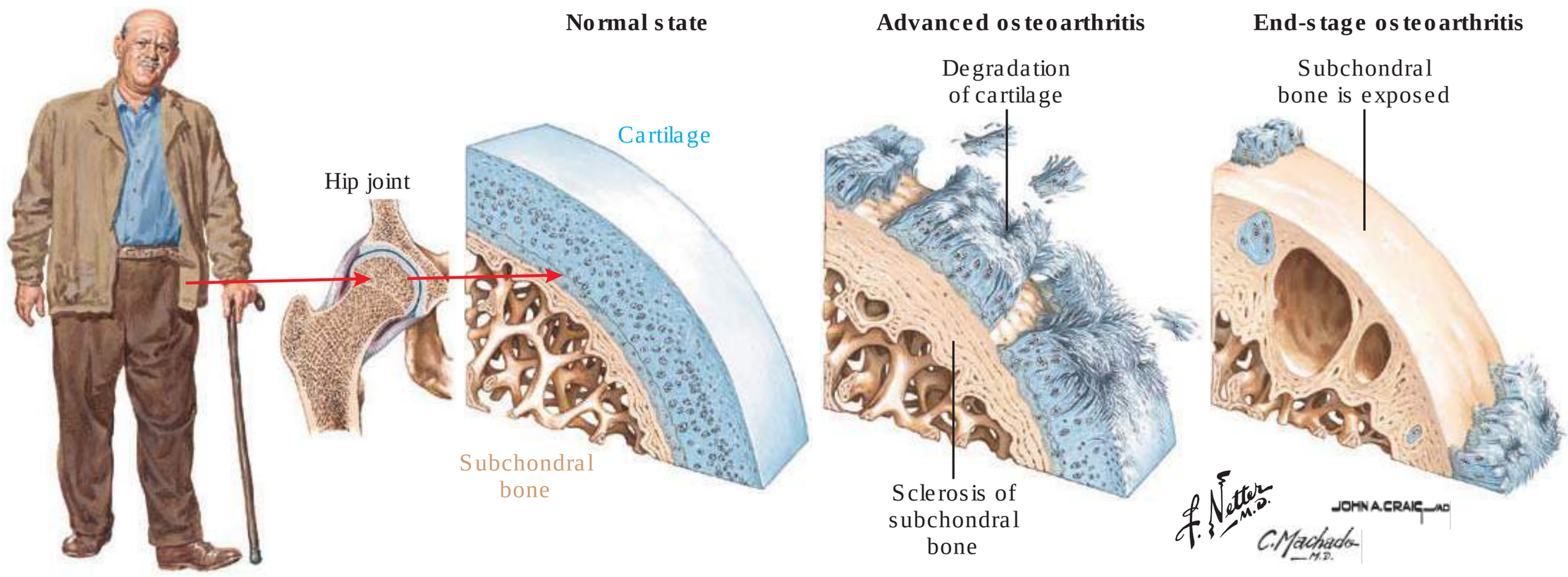
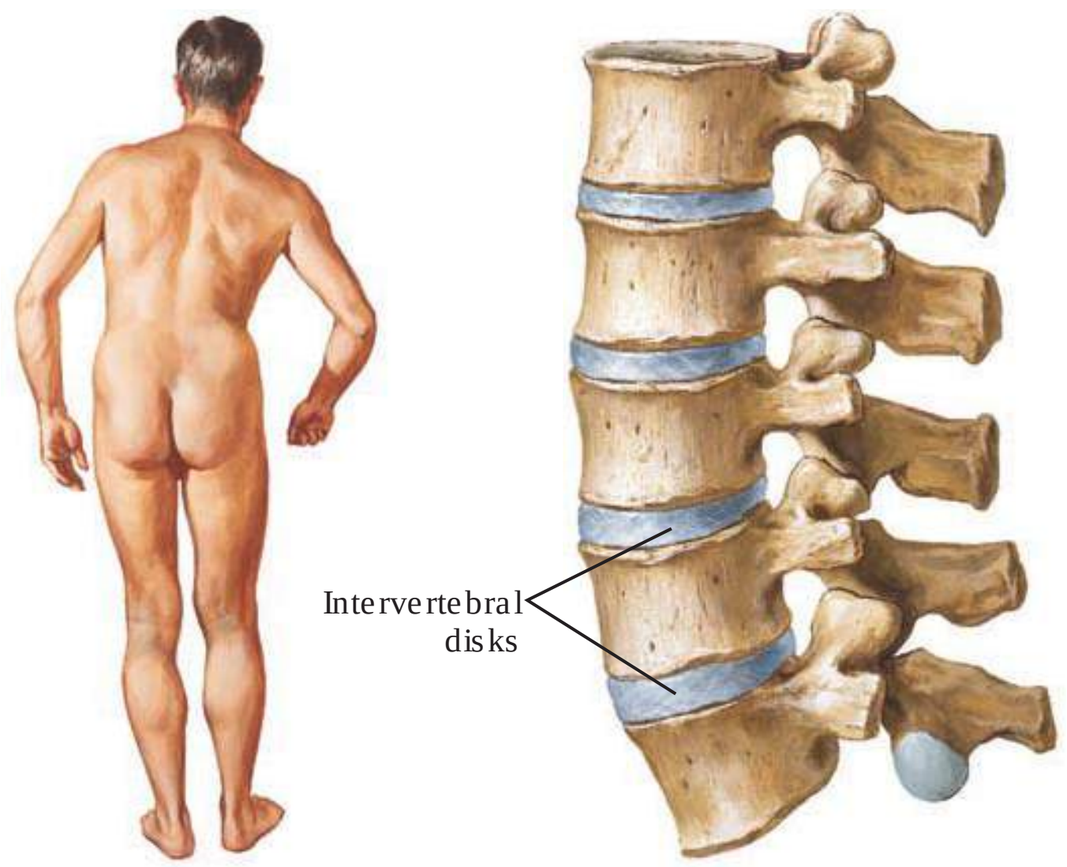


Fig. 13.9 **Destruction of joint cartilage, calcification, and abnormal growth of bone in osteoarthritis.**

glucocorticoids injected into the joint. Total joint replacement (arthroplasty) is used as a last resort.

Degeneration of intervertebral disks typically leads to low-back pain and is often considered a form of osteoarthritis (Fig. 13.10). Intervertebral disks allow motion of the spine in different directions while bearing a load. A disk consists of an annulus fibrosus and a nucleus pulposus. The annulus fibrosus contains mostly collagens (types I and II), which provide form to the disk. The nucleus pulposus is rich in proteoglycans, which bind water and provide resistance to compression; the nucleus also contains some collagen. Among the proteoglycans, the glycosaminoglycan side chains contain mostly chondroitin sulfate. With degeneration of the disk, the matrix of the nucleus pulposus loses proteoglycans and gains collagen.

Glucosamine and **chondroitin**, taken by mouth, are often used in the treatment of osteoarthritis. However, these saccharides are of uncertain benefit.



2.4. Mucopolysaccharidoses

The lysosomes of patients with a mucopolysaccharidosis are missing one of the many enzyme activities that are needed to degrade glycosaminoglycans (previously called **mucopolysaccharides**). The prevalence of mucopolysaccharidosis is ~1 in 30,000. Disease becomes apparent only if enzyme activity is less than ~2% of the normal. Because of inadequate degradation, glycosaminoglycans accumulate in lysosomes of most cells and eventually cause progressive organ damage. Affected patients may develop a dysmorphic face, hepatomegaly, splenomegaly, hernias, deafness, cardiomyopathy, lesions of the heart valves, airway obstruction, caries, or dysfunction of the central nervous system. Diagnosis relies heavily on measurements of enzyme activities in various cells or body fluids.

The most common forms of mucopolysaccharidosis are **Hurler syndrome** (a severe form and subtype of mucopolysaccharidosis type I) and **Sanfilippo syndrome** (mucopolysaccharidosis type III, which has four subtypes denoted A, B,

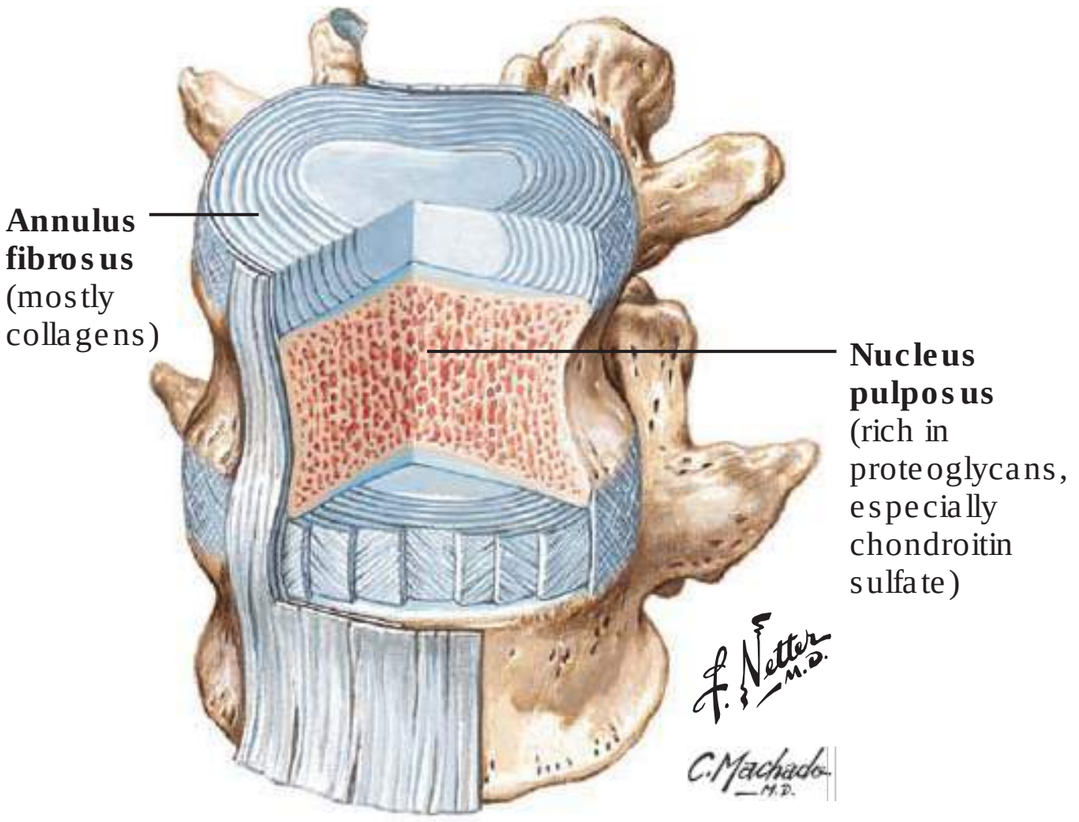


Fig. 13.10 **Structure of intervertebral disks.**

C, and D). Both disorders are inherited in an autosomal recessive manner. Hurler syndrome is due to a deficiency of L-iduronidase, which hydrolyzes the terminal iduronate residue from dermatan sulfate and heparan sulfate. As a result, dermatan sulfate and heparan sulfate accumulate in lysosomes. Most severely affected patients have facial dysmorphism from birth and develop symptoms during the second year of life. Sanfilippo syndrome is caused by a deficiency in one of the four enzymes that play a role in degrading heparan sulfate; as a result, heparan sulfate accumulates in lysosomes. Besides the signs and symptoms common to all mucopolysaccharidoses (see above), most patients develop severe hyperactivity and aggression, seizures, sleep disturbance, and retinopathy.

Intravenous enzyme replacement therapy is available for mucopolysaccharidosis type I. The exogenous enzyme contains covalently linked mannose 6-phosphate (the localization signal for lysosomes), binds to mannose 6-phosphate receptors on the plasma membrane, is endocytosed, and ends up in lysosomes. However, infused enzyme does not readily cross the blood-brain barrier.

Transplantation of hematopoietic stem cells from bone marrow or cord blood is feasible for the treatment of patients with Hurler syndrome. Descendants of transplanted stem cells are thought to leak enzymes into their environment (including the enzyme missing from the host cells, L-iduronidase). Enzymes that contain mannose 6-phosphate then end up in lysosomes. Although transplantation reduces hepatomegaly, splenomegaly, and deterioration of the central nervous system, it does not prevent skeletal problems or retinopathy.

3. REMODELING OF THE EXTRACELLULAR MATRIX

Alterations of the extracellular matrix that involve collagens, elastic fibers, and proteoglycans are involved in a number of physiologic and pathologic processes. During and after pregnancy, the cervix normally undergoes substantial remodeling of its extracellular matrix. Wound healing is accompanied by removal of extracellular matrix followed by resynthesis of matrix. Osteoporosis manifests itself in progressive weakening of bone. Fibrosis is characterized by excessive deposition of extracellular matrix to the detriment of organ function.

3.1. Wound Healing

A few days after injury, a transient extracellular matrix forms that is gradually replaced by a stronger matrix (Fig. 13.11). Immediately after skin is wounded, **clotting** prevents further blood loss. Peaking about 1 day after injury, **neutrophils** move into the wound. Neutrophils remove bacteria and cellular debris, including proteins of the extracellular matrix. Unwounded tissue has a sufficient amount of extracellular protease inhibitors so that its extracellular matrix is not degraded. Within about a day, the neutrophils die by apoptosis. One to three days after the initial injury, **monocytes** from the blood and from healthy tissue near the wound move into the wound and transform into macrophages. These macrophages phagocytose the remains of the neutrophils and also further degrade extracellular matrix in the wound.

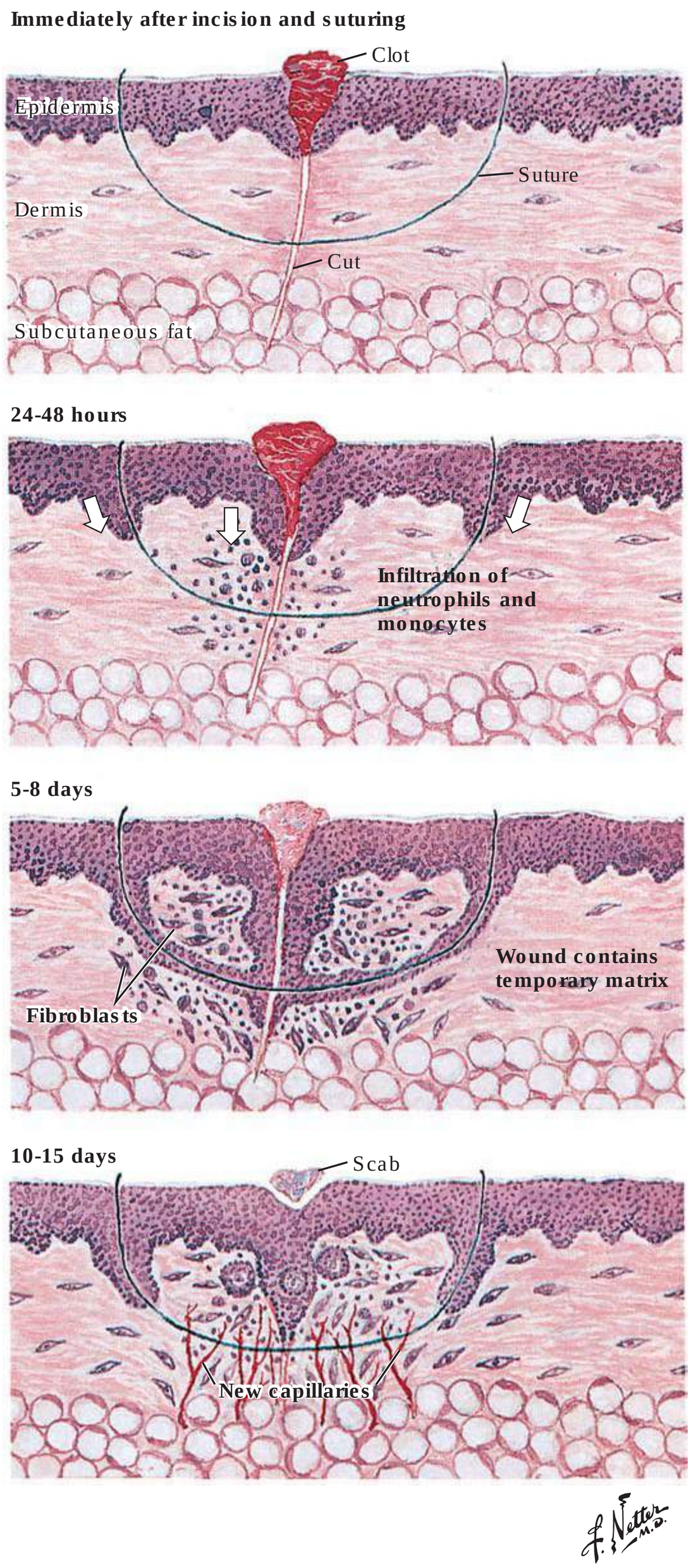


Fig. 13.11 **Healing of sutured skin.**

About 3 to 7 days after injury, epithelial cells at the edge of the wound start to proliferate, and **fibroblasts** from surrounding healthy tissue migrate into the wound. T e fibroblasts start to synthesize collagen. Initially, they produce a matrix of type I and type III collagen that has only very low tensile strength. Starting about 1 week after injury and then lasting months, the early collagen matrix is degraded and replaced by a stronger matrix, which contains about 80% type I collagen and about 20% type III collagen. However, collagen in the scar never attains the same organization as exists in uninjured skin. A few days after injury, responding to signals from fibroblasts, **keratinocytes** move from surrounding tissue into the wound, where they proliferate, dif erentiate, and reform the epidermis.

Within 3 months of injury, wounded skin regains about 80% of its original tensile strength. T ereafter, tensile strength does not increase significantly. Vitamin C deficiency, infection, hyperglycemia, hypoxia, ischemia, malnutrition, an elevated concentration of glucocorticoids, or a hereditary extracellular matrix disorder reduces the rate of healing and decreases the strength of scarred skin. If collagen synthesis is excessive, a hypertrophic scar (i.e., a keloid) forms.

3.2. Remodeling of the Cervix

During pregnancy, parturition, and the postpartum period, the cervix softens, ripens, dilates, and then repairs. Softening starts about 1 month after conception and lasts well into the third trimester. It results in reduced collagen cross-linking and reorganization of type I and type II collagen fibrils, which provide tensile strength. Ripening starts several weeks before labor and involves the addition of proteoglycans and hyaluronate; these compounds are thought to help disperse collagen. During labor, hyaluronidase activity in the cervix increases. Dilation of the cervix is also facilitated by invading leukocytes that release proteases, which degrade the extracellular matrix. During the postpartum repair phase, proteoglycans are degraded, and dense connective tissue is formed once again. Premature ripening and dilation of the cervix are frequent causes of preterm deliveries.

3.3. Fibrosis

Fibrotic diseases are caused by overactive mesenchymal cells that generate an excessive, disorganized extracellular matrix. Fibrosis means replacement of normal tissue with scar tissue that has an abnormal extracellular matrix. For fibrosis to occur, there must be an interaction between the immune system and connective tissue that leads to an excessive wound healing response to injury. Wound healing normally involves activation of mesenchymal cells (i.e., hepatic stellate cells, mesangial cells, fibroblasts in the skin or in the lungs). Fibrosis can af ect a variety of organs, such as the liver, kidneys, intestine, heart, lungs, skin, and eyes.

Liver cirrhosis (Fig. 13.12) can be caused by hepatitis virus B or C infection, excessive alcohol ingestion (see Section 4.4 in Chapter 30), hemochromatosis (see Fig. 15.10 and Section 9.2 in Chapter 15), cholestasis, or α-1-antitrypsin disease (see Section 1.4). Liver cirrhosis is seen in up to 10% of human autopsies; however, the condition often remains clinically silent. Cirrhosis is associated with proliferation of a fibrotic (i.e., poorly organized) extracellular matrix, of which collagen types I and III are the most abundant proteins. T e outcome of liver cirrhosis can be portal hypertension and impaired hepatocellular function. Furthermore, most cases of liver cancer arise from liver cirrhosis. Fibrosis typically starts with injury to hepatocytes, such as by excessive alcohol consumption (with accumulation of acetaldehyde), a hepatotoxic drug (e.g., methotrexate), or excessive iron stores. T e injury activates inflammatory cells that in turn activate matrix-producing hepatic stellate cells (lipocytes, Ito cells).

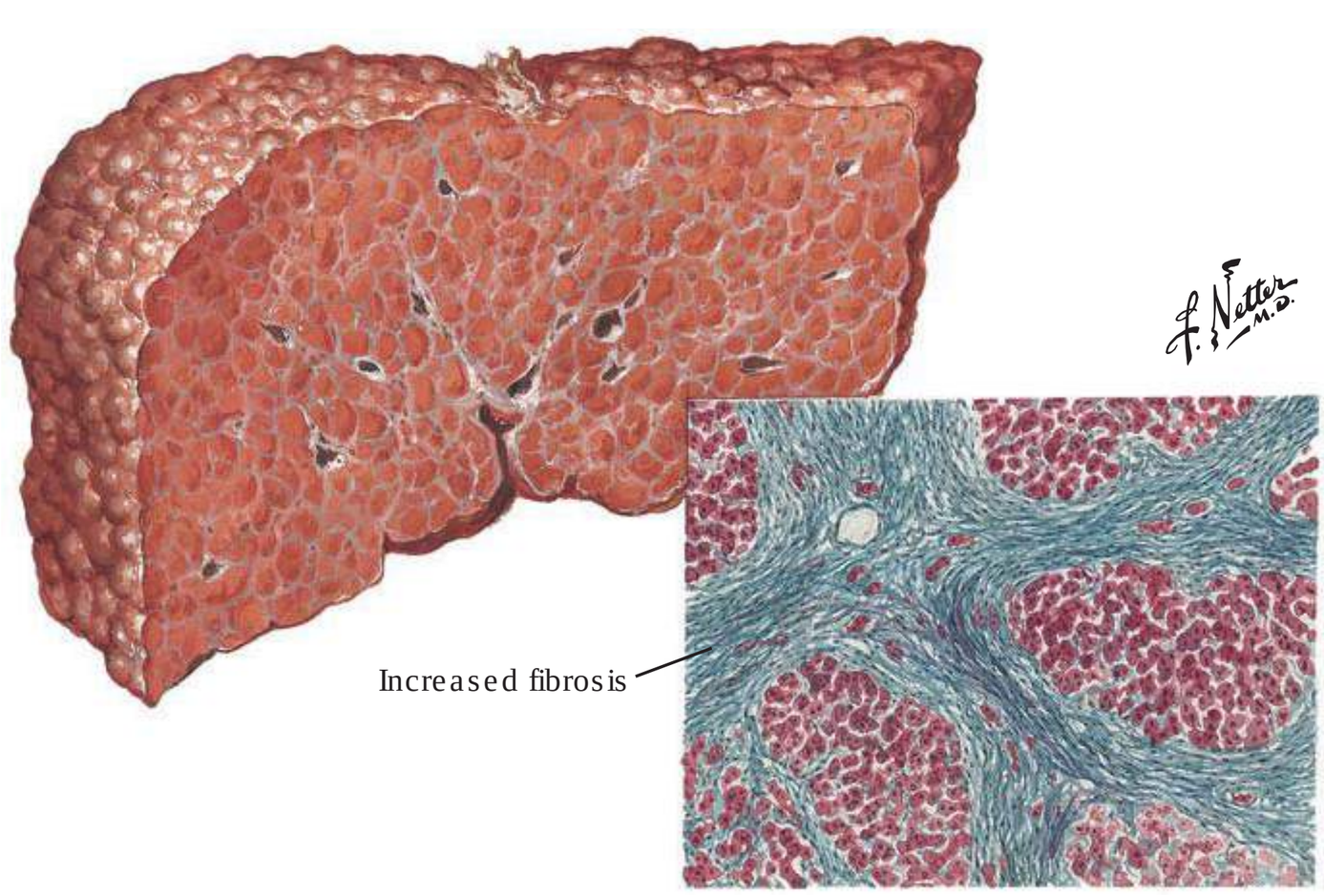


Fig. 13.12 **Liver cirrhosis.**

SUMMARY

- Elastic fibers consist mostly of elastin. The core and periphery of elastic fibers contain microfibrils, a major component of which is fibrillin. Mutations in fibrillin-1 can cause Marfan syndrome, which can be accompanied by dilation and dissection of blood vessels, skeletal abnormalities, and poor eyesight.
- Elastase degrades elastin. In chronic smokers, neutrophils and macrophages destroy elastin in the lungs, causing emphysema. α-1-Antitrypsin is a natural inhibitor of elastase. A heritable deficiency of α-1-antitrypsin affects about 1 in 2,000 individuals. Most patients predominantly have emphysema; some also have liver disease and are at a high risk of liver cirrhosis and hepatocellular carcinoma.
- Proteoglycans consist of a core protein and linked long glycosaminoglycans (carbohydrate chains) that consist of repeats of heparan sulfate, keratan sulfate, chondroitin sulfate, or dermatan sulfate. The sulfation of the saccharides is highly variable. Certain sequences of a few sulfated saccharides serve as binding sites for specific proteins. Proteoglycans are particularly abundant in cartilage, where they play a role in absorbing compressive forces. Heparin is used as an anticoagulant. It is typically isolated from pig intestine.
- Hyaluronic acid is also a glycosaminoglycan; however, it is not covalently linked to a core protein. Hyaluronic acid serves as a scaffold for link proteins to bind to, which in turn bind proteoglycans, such as aggrecan.
- Osteoarthritis is characterized by a gradual loss of cartilage from joints. It is unclear whether oral glucosamine and chondroitin have a beneficial effect.
- Deficiencies of enzymes that degrade glycosaminoglycans in lysosomes can cause one of the mucopolysaccharidoses (e.g., Hurler syndrome, Sanfilippo syndrome). Affected patients typically develop a dysmorphic face, hepatomegaly, splenomegaly, hernias, deafness, cardiomyopathy, lesions of the heart valves, airway obstruction, caries, or dysfunction of the central nervous system.

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- Campos MA, Lascano J. α1 Antitrypsin deficiency: current best practice in testing and augmentation therapy. Ther Adv Respir Dis. 2014;8:150-161.
- Cheng S, Mohammed TL. Diffuse smoking-related lung disease: emphysema and interstitial lung disease. Semin Roentgenol. 2015;50:16-22.
- The Marfan Foundation. <http://www.marfan.org/dx/home>. This site contains helpful information for the diagnosis of Marfan syndrome.

Review Questions

1. A 30-year-old man with Marfan syndrome undergoes genetic testing in anticipation of preimplantation testing of embryos. Testing of the man's DNA is expected to reveal a pathogenic mutation in the gene for which one of the following proteins?

   A. α-1-Antitrypsin
   B. Elastin
   C. Elastase
   D. Fibrillin-1
   E. TGF-β receptor 1

2. A 39-year-old man with a 20-year history of smoking two packs of cigarettes per day is diagnosed with emphysema. His 40-year old brother has a similar history. Further testing for this patient should involve a search for a pathogenic mutation in the gene for which one of the following proteins?

   A. α-1-Antitrypsin
   B. Elastin
   C. Elastase
   D. Fibrillin-1
   E. TGF-β receptor 1

3. Which one of the following drugs is most likely useful in the treatment of Hurler syndrome?

   A. α-1 proteinase inhibitor (antitrypsin)
   B. Chondroitin
   C. Glucosamine
   D. Laronidase (iduronidase)
   E. Losartan

Chapter 14 Heme Metabolism, Porphyrias, and Hyperbilirubinemia

SYNOPSIS

- Heme is an organic molecule that consists of a porphyrin ring that binds to and coordinates an iron ion (Fig. 14.1).
- Heme is a part of hemoglobin, myoglobin, and the cytochromes. Heme iron can bind oxygen or facilitate redox reactions.
- Most of the daily heme synthesis occurs in immature red blood cells and the liver. Other cells synthesize heme only at a low rate.
- Porphyrias are caused by deficiencies in enzymes of heme synthesis that lead to the accumulation of toxic intermediates. Some porphyrias primarily affect the nervous system, while others affect the skin.
- Lead poisoning is associated with decreased heme synthesis and damage to the nervous system.
- When heme-containing proteins are degraded, the amino acids and the heme iron are recycled, while the porphyrin moiety is converted to bilirubin glucuronates, which are secreted into bile.
- A high concentration of bilirubin in the blood indicates the premature death of red blood cells, reduced glucuronidation of bilirubin, or impaired excretion of bilirubin glucuronides.

LEARNING OBJECTIVES

For mastery of this topic, you should be able to do the following:

- Describe the main inciting events, symptoms, pathology, and treatment of acute intermittent porphyria and porphyria cutanea tarda.
- Describe the effect of lead poisoning on the synthesis of heme and hemoglobin.
- Describe the main source and production of bilirubin and conjugated bilirubin, paying attention to the tissues in which this occurs as well as the transport of intermediates between these tissues.
- Interpret lab reports on bilirubin measurements.
- Define the signs and symptoms of hyperbilirubinemia.
- Define cholestasis and explain how it affects lab values for bilirubin.
- Compare and contrast the causes of neonatal jaundice and Gilbert syndrome.
- List the drugs that require special consideration when given to patients who have Gilbert syndrome or severe hyperbilirubinemia.
- List the proteins that remove heme and hemoglobin from blood plasma, and relate laboratory data for these proteins to hemolysis.

1. HEME SYNTHESIS

Most of the body's heme synthesis occurs in two compartments: the bone marrow synthesizes heme while synthesizing hemoglobin, and the liver synthesizes heme as part of the synthesis of cytochrome P450 enzymes. At elevated concentrations, intermediates of heme synthesis are toxic. Cells synthesize heme only on demand.

1.1. Use of Heme in Proteins

Heme is a prosthetic group that reversibly binds oxygen or accepts and donates electrons. Heme consists of a **porphyrin** (an organic molecule) and an **iron** ion (see Fig. 14.1). A **prosthetic group** is an organic, nonpeptidic molecule (e.g., heme) that binds to a protein (see Section 1 in Chapter 10).

Heme is found principally as the prosthetic group of **hemoglobin**, **myoglobin**, and the **cytochromes**, and also of **catalase**. In hemoglobin and myoglobin, heme reversibly binds oxygen (see Chapter 16). The cytochromes encompass cytochrome P450 enzymes, cytochrome b_5, and the cytochromes that are involved in mitochondrial respiration. **Cytochrome P450** enzymes are located in the membrane of the endoplasmic reticulum. These enzymes hydroxylate endogenous and exogenous compounds (e.g., steroids and most drugs). Hydroxylation makes these compounds more water soluble. Drug intake often leads to a marked increase in the synthesis of cytochrome P450 enzymes. **Cytochrome b_5** is involved in electron transfer, often from NADH. The cytochromes that are involved in **mitochondrial respiration** transport electrons from ubiquinol to oxygen. In all of these cytochromes, heme is the prosthetic group that reversibly accepts and donates electrons (see Chapter 23). **Catalase** is an enzyme that degrades hydrogen peroxide (it catalyzes the reaction $2\ H_2O_2 \rightarrow 2\ H_2O + O_2$; see Chapter 21).

1.2. Pathway and Regulation of Heme Synthesis

Most of the heme synthesis occurs in the bone marrow and the liver. A healthy person synthesizes approximately 0.3 g of heme per day. About 80% of the daily heme synthesis occurs in erythropoietically active bone marrow for the production of hemoglobin. About 15% of the daily heme synthesis occurs in the liver as part of the synthesis of cytochrome P450 enzymes and catalase.

Fig. 14.2 shows the **pathway** of heme synthesis. In essence, glycine and the citric acid cycle intermediate succinyl–coenzyme A (succinyl-CoA) give rise to aminolevulinic acid, eight molecules of which eventually give rise to a ring-shaped porphyrinogen. Removal of carboxyl groups and oxidation generates a more hydrophilic porphyrin, into which an iron ion is inserted (iron enters mitochondria via mitoferrin, see Section 5 in Chapter 15). The porphyrins, possessing an extensive system of conjugated double bonds, absorb visible light.

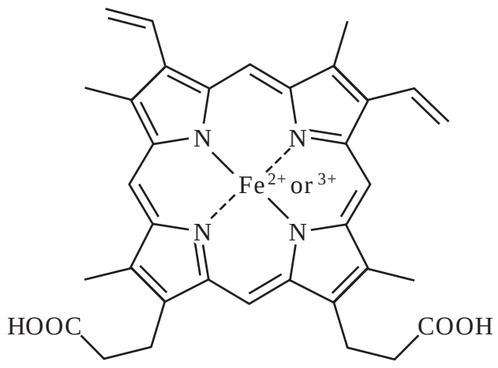

Fig. 14.1 **Heme.**

Hemoglobin, for instance, looks reddish. Parts of heme synthesis take place in the mitochondria; others occur in the cytosol.

The physiological **regulation** of heme synthesis occurs chiefly at the level of **aminolevulinic acid synthase** (ALA synthase; see Fig. 14.2). In the first step in heme synthesis, ALA synthase inside mitochondria catalyzes the condensation of succinyl-CoA and glycine to **ALA** (**5-aminolevulinic acid**, **δ-aminolevulinic acid**). As this is the first committed step (i.e., the first irreversible reaction) in heme synthesis, it is regulated. There are two types of ALA synthase that differ in regulation and are derived from different genes: the erythroid type and the housekeeping type.

The **erythroid-type ALA synthase** (ALA synthase 2) is synthesized only when **iron** is present. This ALA synthase is expressed chiefly in hemoglobin-synthesizing precursor cells of red blood cells (i.e., polychromatophilic erythroblasts and all reticulocytes; see Fig. 16.3). The mRNA for ALA synthase contains an **iron response element** (IRE; see Section 3 in Chapter 15). Only in the presence of iron can this mRNA be translated. In erythropoietic cells, the supply of iron is commonly borderline low. Iron-dependent synthesis of ALA synthase ensures that there is enough iron to insert into the penultimate product of the heme synthesis pathway (see Fig. 14.2). Otherwise, protoporphyrin IX would accumulate and damage cells (see below).

The activity of the **housekeeping-type ALA synthase** (ALA synthase 1) is regulated principally by feedback inhibition from free **heme** (see Fig. 14.2). This enzyme is expressed in most cell types, but it is most abundant in the liver. The liver needs heme mainly as a cofactor for the enzymes of the cytochrome P450 pathway (see above). Free heme is noxious to cells. As the concentration of free heme increases, the housekeeping ALA synthase is progressively more inhibited. Conversely, when a relatively large amount of heme is needed for cytochrome P450 synthesis, the concentration of free heme decreases, and the activity of ALA synthase increases.

Both ALA synthases use **pyridoxal phosphate** as a cofactor, which is derived from **vitamin B_6** (also called **pyridoxine**). Pyridoxal phosphate is also a cofactor for many aminotransferases (see Fig. 35.4). Indeed, the ALA synthases are also a type of aminotransferase. A vitamin B_6 deficiency can impair synthesis of red blood cells (see Section 2.6).

2. DISEASES ASSOCIATED WITH HEME SYNTHESIS

Several diseases (mostly porphyrias) are associated with impaired heme synthesis. All of these diseases are uncommon, except for lead poisoning. The porphyrias can be categorized into those that damage primarily the nervous system (e.g., acute intermittent porphyria), those that damage the skin (e.g., porphyria cutanea tarda), and those that damage both the nervous system and the skin.

2.1. General Considerations

A patient who produces an excessive concentration of any porphyrinogen or porphyrin is susceptible to **photo damage** of the skin. Hydroxymethylbilane can spontaneously cyclize to uroporphyrinogen. All **porphyrinogens** in heme synthesis (i.e., uro-, copro-, and protoporphyrinogen) readily and spontaneously oxidize to the respective **porphyrins** (Fig. 14.3; also see Fig. 14.2). When these porphyrins reach the skin via the bloodstream, they absorb sunlight and give rise to oxygen **radicals** that damage the skin. A system of several conjugated double bonds is needed for a molecule to absorb light from the sun (see Fig. 14.3). For this reason, only porphyrins (e.g., protoporphyrin, heme) appreciably absorb **sunlight**, while their precursors do not.

For diagnostic purposes, plasma, urine, and stool can be tested for intermediates of heme synthesis. If a patient has neurovisceral symptoms (see Section 2.3), urine tests for ALA and PBG are ordered. If the patient is photosensitive (see Section 2.4), plasma is first tested for total porphyrins. If this test is positive, urine and stools are tested for specific porphyrins. The most water-soluble **porphyrins** are excreted in the **urine**, while the less soluble ones are excreted in the **stool** (via the bile). Carboxyl groups (–COOH) increase the water solubility of porphyrinogens and porphyrins. Hence, at the extremes, uroporphyrin (the oxidation product of uroporphyrinogen, eight –COOH) are mostly found in urine, while protoporphyrin (the oxidation product of protoporphyrinogen, two –COOH) is found mostly in stool. Porphyrins with intermediate numbers of carboxyl groups distribute accordingly. Data on individual porphyrins usually help make a diagnosis.

Lead poisoning is common and inhibits heme synthesis; porphyrias (deficiencies of enzymes of the heme synthesis pathway) are uncommon. Acute intermittent porphyria is the most prevalent porphyria with damage predominantly to the nervous system, while porphyria cutanea tarda is the most prevalent porphyria with damage predominantly to the skin.

2.2. Use of Porphyrins for Photodiagnostic Purposes and Photodynamic Therapy

Exogenous aminolevulinic acid (ALA), or an analog, is used for photodiagnosis and photodynamic therapy. Normally, the rate of synthesis of ALA determines flux in the heme synthesis pathway. Exogenous ALA bypasses the control of ALA synthase, and cells convert this ALA to protoporphyrin IX. Protoporphyrin IX tends to accumulate more in tumor cells than in normal cells. ALA can be given orally. ALA or ALA esters can also be applied to the skin. ALA crosses plasma membranes with the help of transporters for dipeptides and amino acids.

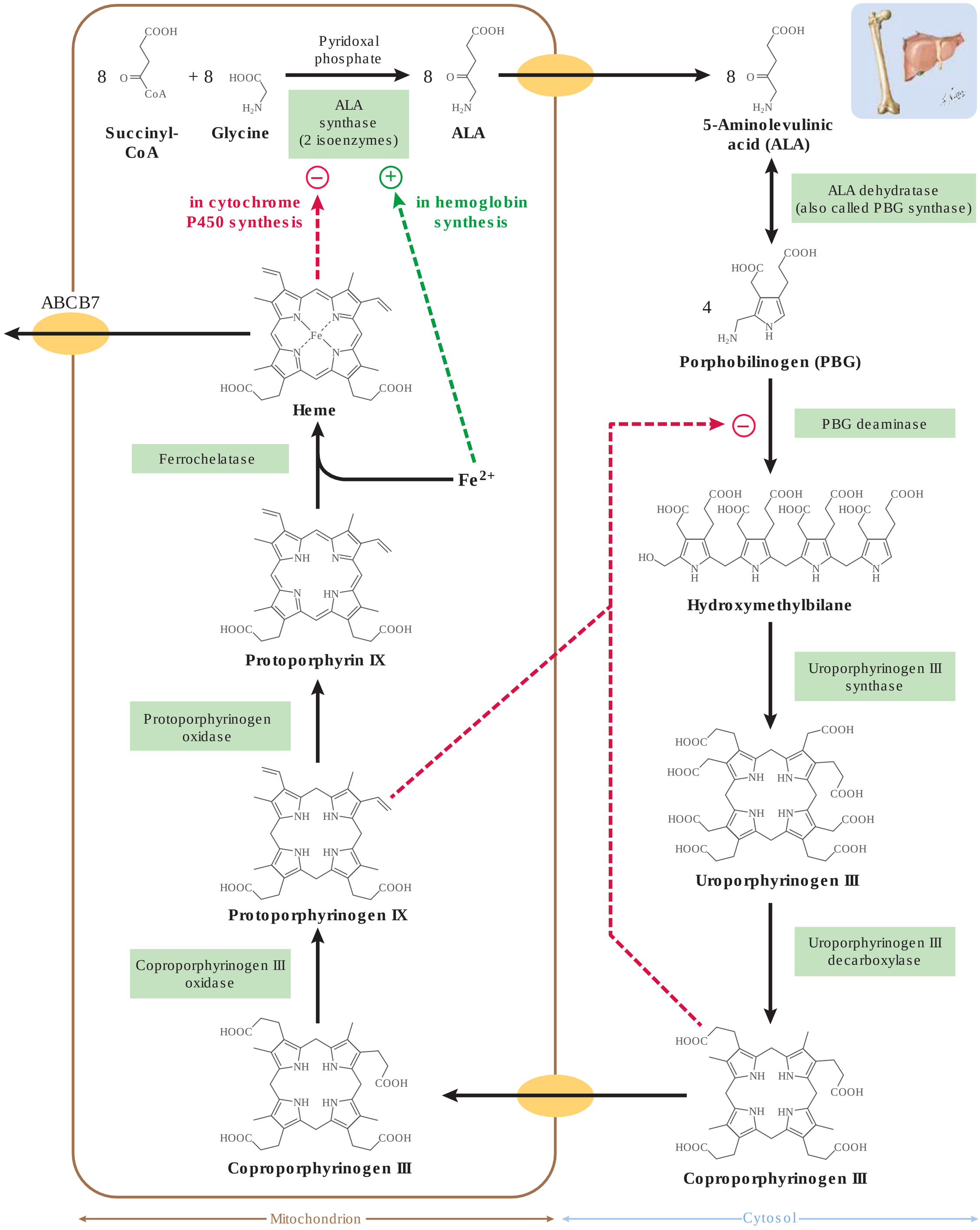

Fig. 14.2 **Biosynthesis of heme.** Most of the heme is synthesized in red blood cell precursors and in the liver.

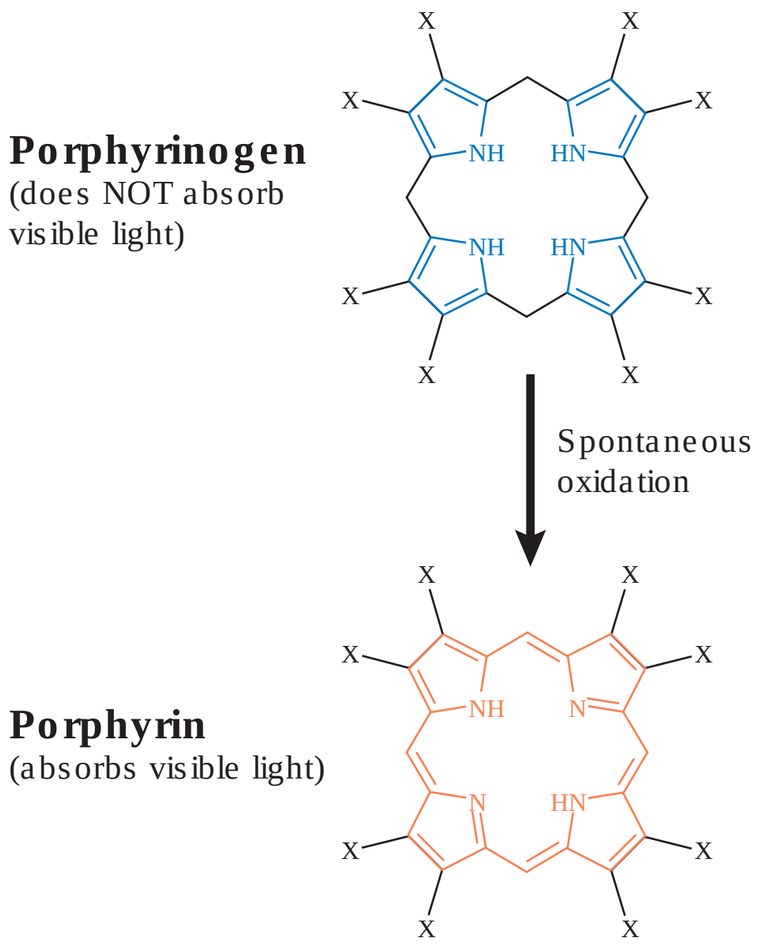

Fig. 14.3 Spontaneous oxidation of porphyrinogens to porphyrins. Porphyrinogens possess only the isolated, relatively small, aromatic pyrrole rings (*blue*), while the porphyrins contain a more extensive system of delocalized electrons (*orange*). As a consequence, porphyrinogens do not absorb sunlight, whereas porphyrins do. X is CH_3, $CH{=}CH_2$, $CH_2{-}COOH$, or $CH_2{-}CH_2{-}COOH$.

Fluorescence detection after excitation with blue light is the most sensitive means of locating **protoporphyrin IX** for **diagnostic** purposes. T is technique is chiefly used to locate **neoplasms**.

For **photodynamic therapy**, protoporphyrin IX formed inside cells is activated with blue or red light, depending on the desired tissue depth of activation (red light penetrates further into tissues). When protoporphyrin IX absorbs light, it rapidly reacts with oxygen (O_2) to produce the highly reactive **singlet oxygen** (an energy-rich form of O_2 that is a toxic "reactive oxygen species" [ROS]; see Chapter 21). Photodynamic treatment is used for the destruction of superficial or internal tumors (e.g., lung cancer and intraductal cholangiocarcinoma).

2.3. Diseases of Heme Synthesis That Primarily Affect the Nervous System

2.3.1. Acute Intermittent Porphyria

Acute intermittent porphyria is caused by reduced activity (<50% of normal) of **porphobilinogen deaminase**. T is disorder is inherited in autosomal dominant fashion. In North America, at least one person in 10,000 has insufficient enzyme activity (approximately half of the normal amount), and most of these people have an attack sometime during their life. T e disease starts in the liver and manifests itself as a disease of the nervous system. Episodic attacks are brought on by an increased demand for heme in the liver, such as by substances that induce the **cytochrome P450** system (e.g., barbiturates, contraceptives, sulfonamides, or alcohol). Under normal circumstances, the patient's liver has enough porphobilinogen deaminase activity to synthesize an adequate amount of heme.

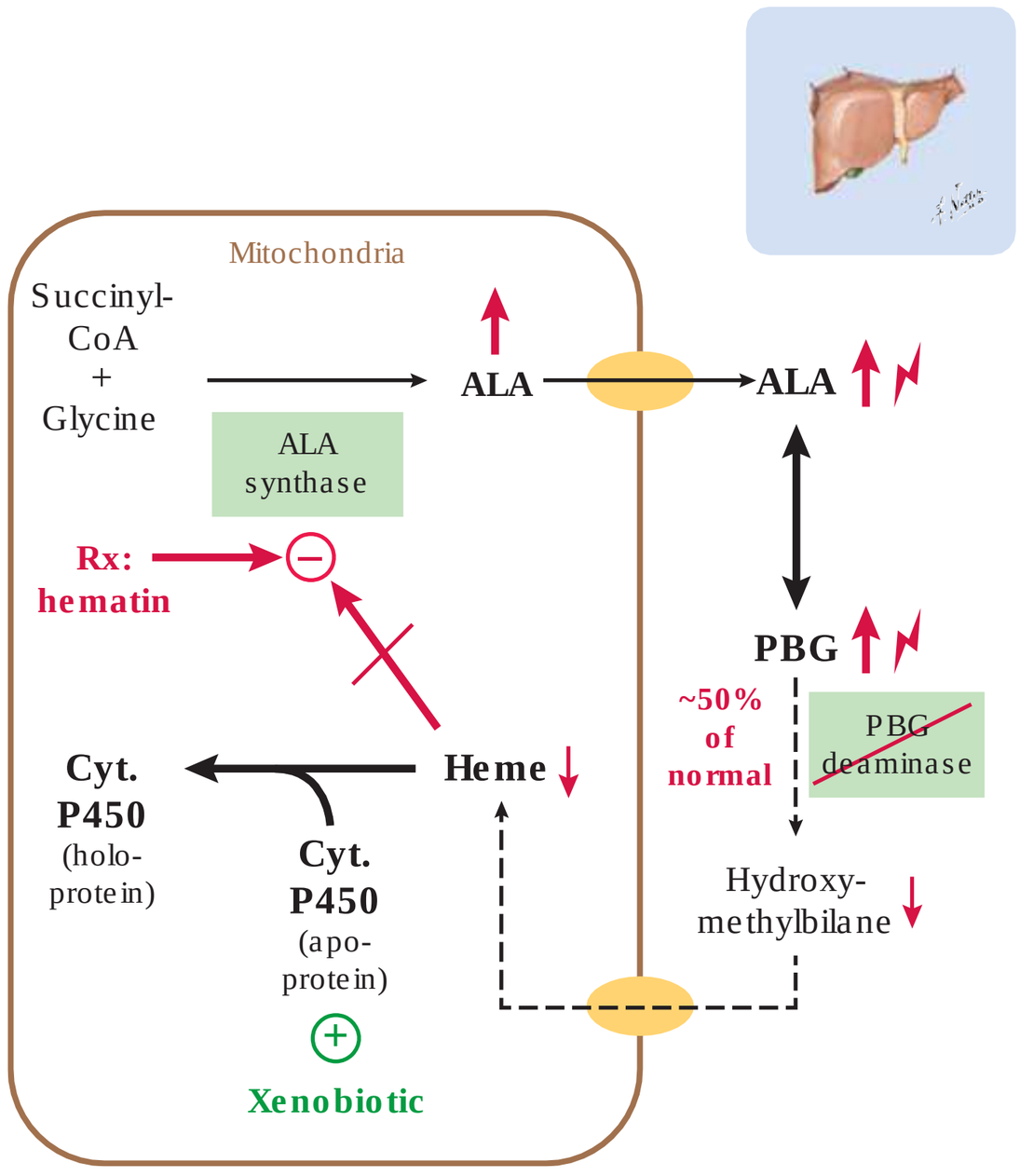

Fig. 14.4 Pathogenesis of an attack in a patient with acute intermittent porphyria.

However, when demand for heme is increased, the liver of these patients does not have enough porphobilinogen deaminase activity. T e resulting low concentration of heme deinhibits ALA-synthase and thus creates a detrimental accumulation of **ALA** (5-aminolevulinic acid) and **PBG** (porphobilinogen); see Figs. 14.2 and 14.4.

During an attack, patients with acute intermittent porphyria present with intense central **abdominal pain**. T e abdominal pain may be due to the accumulation of PBG in the liver, as well as the neurotoxic ef ects of ALA excess and heme deficiency. Patients also present with vomiting, hypertension, and tachycardia. Furthermore, some patients have motor neuropathy (that may lead to respiratory paralysis), anxiety, depression, psychosis, or life-threatening seizures. Patients with acute intermittent porphyria are not photosensitive. During an attack, the urine contains abnormally high amounts of ALA (20–100 mg/day; normal, <7 mg/day) and PBG (50–200 mg/day; normal, <4 mg/day).

Treatment of acute intermittent porphyria includes inhibition of ALA synthase with a high dietary **carbohydrate** intake (oral or intravenous; mechanism of action not understood), intravenous **hematin** (**hydroxyheme,** sometimes called **hemin**) to replenish the heme pool and inhibit ALA synthase, and pain management.

2.3.2. Lead Poisoning and ALA Dehydratase-De ficient Porphyria

An **acquired** decrease in the activity of **ALA dehydratase** is seen in patients with **lead poisoning**. As with patients who

have acute intermittent porphyria, patients who have lead poisoning can have abdominal pain, motor neuropathy, and depression. The concentration of lead in the blood helps the clinician differentiate between acute intermittent porphyria and lead poisoning.

Lead poisoning most often occurs through ingestion or inhalation of lead-containing particles or fumes. Some paints used to be made with lead (white lead carbonate and/or yellow lead chromate). Children sometimes get lead poisoning due to eating chips of old, lead-based paint. Adults get lead poisoning through workplace exposure (batteries, paints). While the blood of nonsymptomatic patients typically contains about 2-µg lead/dL (~0.1 µM), a concentration more than 10 µg/dL is a cause for follow-up and intervention. Children are especially vulnerable because they absorb and retain much more lead than adults, and they are also more sensitive to the neurotoxic effects of lead than adults. Long-term lead exposure increases an adult's risk for cognitive decline. Long-term exposure is best assessed by measuring lead in the tibia with a special radiographic procedure.

In patients who have blood lead levels more than about 45 µg/dL, **chelation therapy** may be indicated to remove lead from the body. This is commonly involves the use of oral **succimer** (dimercaptosuccinic acid), intravenous or intramuscular **Ca^{2+}-EDTA** (ethylenediamine tetraacetic acid complexed with calcium), or, less commonly, intramuscular **dimercaprol** (dimercaptopropanol).

The **neurotoxic** effects of lead are complex and incompletely understood. Clearly, lead (Pb^{2+}) binds to ALA dehydratase in place of zinc (Zn^{2+}) and thereby inhibits ALA dehydratase activity; this inhibition causes a rise in the concentration of ALA. ALA then spills into the blood and from there into the nervous system. Lead may also bind to DNA-binding proteins that contain a certain subtype of Zn-finger, which would alter transcription. Besides these effects on Zn^{2+}-containing proteins, lead also binds into the Ca^{2+}-binding site of Ca^{2+}-activated protein kinase C and of synaptotagmin. Ca^{2+}-activated protein kinase C is involved in intracellular signal transmission, and synaptotagmin is involved in neurotransmitter release. Many other proteins that contain Zn^{2+} or Ca^{2+} are not affected by lead poisoning because their affinity for Pb^{2+} is too low. Lead can also be toxic to the kidneys and cause **gout** (see Chapter 38).

An abnormally high concentration of ALA is also seen in patients with the extremely rare **hereditary** disease **ALA-dehydratase-deficient porphyria**. These patients, too, present with neuropathy.

2.4. Diseases of Heme Synthesis That Affect Only the Skin

2.4.1. Porphyria Cutanea Tarda

In porphyria cutanea tarda, **uroporphyrinogen III decarboxylase** activity is deficient (<20% of normal activity); Fig. 14.5. Porphyria cutanea tarda is the most prevalent porphyria that involves photosensitivity. Porphyria cutanea tarda is usually an acquired disease of the liver but not of the blood-forming tissues. An elevated **iron** content of the liver appears to be a prerequisite. Additionally, iron overload (often due to hereditary hemochromatosis; see Chapter 15), hepatitis C, AIDS, certain drugs (e.g., estrogens), excessive alcohol intake, or smoking can lead to insufficient uroporphyrinogen III decarboxylase activity. It is currently thought that these coexisting conditions lead to the formation of an inhibitor of uroporphyrinogen III decarboxylase. Less frequently, the uroporphyrinogen III decarboxylase deficiency is inherited rather than acquired. In this case, even environmental influences of low potency can render a patient symptomatic.

Porphyria cutanea tarda involves **photosensitivity** but not neurologic symptoms. Patients with porphyria cutanea tarda are photosensitive because uroporphyrinogen III and partially decarboxylated intermediates between uroporphyrinogen III and coproporphyrinogen III are present at an abnormally high concentration. These porphyrinogens leak from the liver into the bloodstream and spontaneously oxidize to porphyrins, which have an extensive system of conjugated double bonds (see Fig. 14.3). In the skin, upon exposure to light, the porphyrins give rise to reactive oxygen radicals, which in turn damage the cells and cause blisters that take up to 1 month to heal, often with scarring (Fig. 14.6). From the blood, porphyrins reach the **urine** and give it a **red** to **purple** color.

Laboratory tests on 24-hour urine usually reveal elevated amounts of **uroporphyrin**, which has eight carboxyl groups, as well as porphyrins with seven, six, and five carboxyl groups (named **hepta-**, **hexa-**, and **penta-carboxyporphyrin,** respectively). These porphyrins are oxidized intermediates of the impaired decarboxylation reactions between uroporphyrinogen (eight carboxyl groups) and coproporphyrinogen (four carboxyl groups). Patients with porphyria cutanea tarda can synthesize heme at a near normal rate because the loss of uroporphyrinogen III is made up by increased synthesis, owing to reduced feedback inhibition from heme as well as protoporphyrinogen and coproporphyrinogen. The excretion of ALA and PBG in the urine is normal.

The treatment of porphyria cutanea tarda has three main objectives: (1) to improve liver function by treatment of underlying disorders, such as hepatitis C or iron overload (excess iron stores can be depleted with **phlebotomy**; see Chapter 15); (2) to reduce formation of porphyrin radicals in the skin by **avoidance of sun exposure** and the use of sunscreen lotion; and (3) to prevent damage to the skin by porphyrin radicals with oral supplements of **β-carotene**, an antioxidant (see Chapter 21).

2.4.2. Other Porphyrias That Affect the Skin but Not the Nervous System

Insufficient activity of **uroporphyrinogen III synthase** causes **congenital erythropoietic porphyria**, and insufficient activity of **ferrochelatase** causes **erythropoietic protoporphyria**. Neither of these porphyrias gives rise to neurovisceral symptoms.

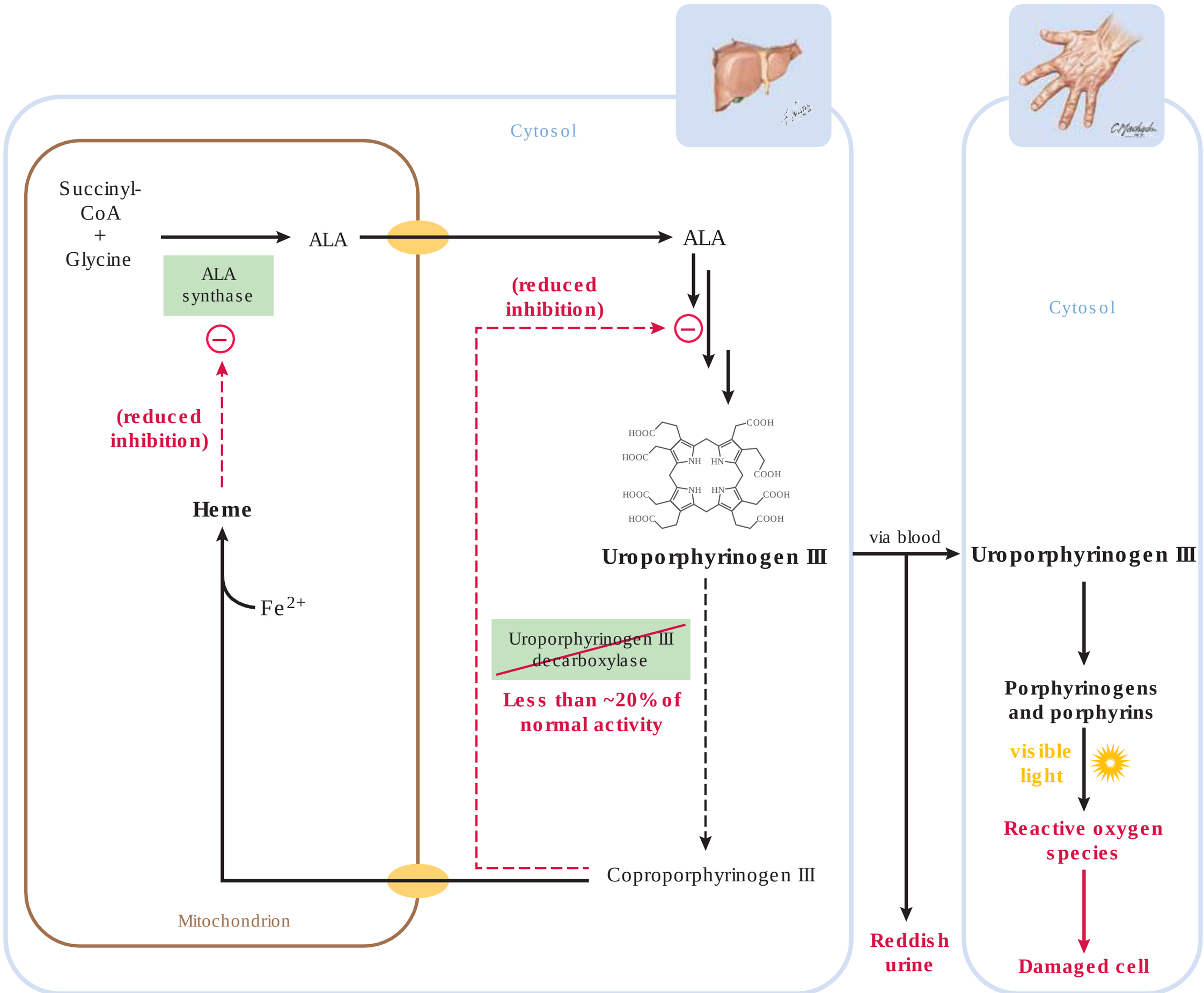

Fig. 14.5 **Pathogenesis of photosensitivity in a patient with porphyria cutanea tarda.**

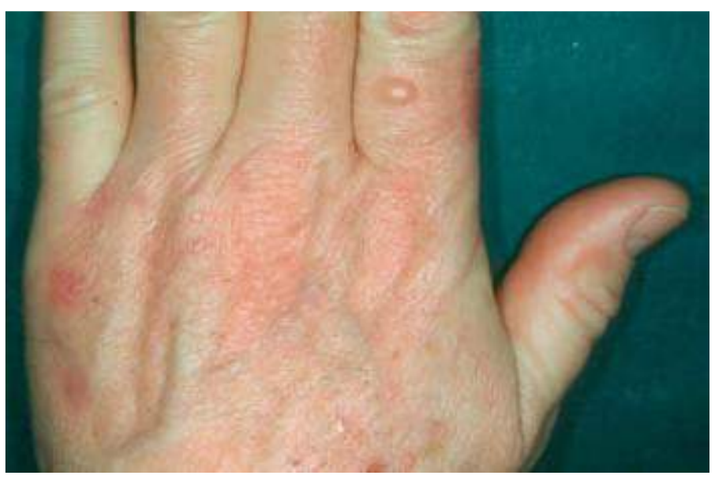

Fig. 14.6 **Porphyria cutanea tarda.** (From Frank J, Poblete-Gutiérrez P. Porphyria cutanea tarda: when skin meets liver. *Best Pract Res Clin Gastroenterol.* 2010;24:735–745.)

2.5. Porphyrias That Affects Both the Nervous System and the Skin

Insufficient activity of **coproporphyrinogen I oxidase** causes **hereditary coproporphyria**, and **protoporphyrinogen oxidase** causes **variegate porphyria**. T ese porphyrias lead to the accumulation of coproporphyrinogen and protoporphyrinogen, respectively, which feedback inhibit PBG deaminase, the enzyme that is deficient in acute intermittent porphyria (see Figs. 14.2 and 14.4). Hence, these porphyrias share symptoms of neurotoxicity. T e accumulating porphyrinogens spontaneously oxidize to porphyrins (see Fig. 14.3), which render patients photosensitive, akin to those who have porphyria cutanea tarda (see above).

2.6. Diseases of Heme Synthesis That Cause Anemia but Not Neurotoxicity or Skin Damage

A severe **deficiency of pyridoxal phosphate** can impair heme synthesis by diminishing the activity of ALA synthase. Pyridoxal phosphate is derived from pyridoxine (vitamin B_6; see Fig. 35.4). Vitamin B_6 deficiency is rare. Women who use oral contraceptives and patients who regularly abuse alcohol are most at risk.

An extremely rare, heritable deficiency of the erythroid form of **ALA synthase** (see Fig. 14.2) causes **X-linked sideroblastic anemia**, which is characterized by life-threatening iron accumulation.

3. DEGRADATION OF HEME TO BILIRUBIN

When red blood cells are degraded, the iron in heme is recycled, while the porphyrin moiety is degraded to bilirubin and eventually excreted. An excessive amount of bilirubin in the blood indicates reduced survival of red blood cells and/or insufficient excretion of bilirubin. Excretion may be inadequate due to impaired conjugation with glucuronic acid in the liver or impaired secretion of conjugated bilirubin from the liver into the intestine.

In a healthy patient, about 80% of heme degradation reflects degradation of **aged red blood cells** (i.e., greater than 120-day-old cells), and about 15% reflects the degradation of heme by enzymes in the liver and the kidneys. Less than 3% of heme degradation results from the degradation of excess heme in the normal erythropoietic bone marrow. Though myoglobin (a heme-containing and oxygen-binding protein in muscle) is abundant, it turns over too slowly to contribute significantly to heme degradation.

In blood plasma, **haptoglobin** and **hemopexin** bind free hemoglobin and free heme, respectively, and deliver these to the liver. Free hemoglobin in blood plasma stems from intravascular hemolysis. Free heme in blood plasma has leaked from cells or has been spontaneously lost from free methemoglobin, which in turn is formed from free hemoglobin in blood plasma. Removal of haptoglobin-hemoglobin complexes can occur at a much greater rate than de novo haptoglobin synthesis. Hence, a low concentration of haptoglobin in the serum indicates the presence of intravascular hemolysis.

The spleen removes aged red blood cells and turns the heme into bilirubin (Fig. 14.7). In an asplenic patient, the liver removes the aged red blood cells. **Macrophages** in the spleen (**Kupffer cells** in the liver) break down hemoglobin into globin and heme. Globin is then degraded into its component amino acids, most of which are eventually reused for protein synthesis (see Chapter 34). Heme oxygenase degrades heme into **iron** and **biliverdin**. The iron is recycled. Biliverdin is reduced to **bilirubin** and released into the bloodstream. Carbon

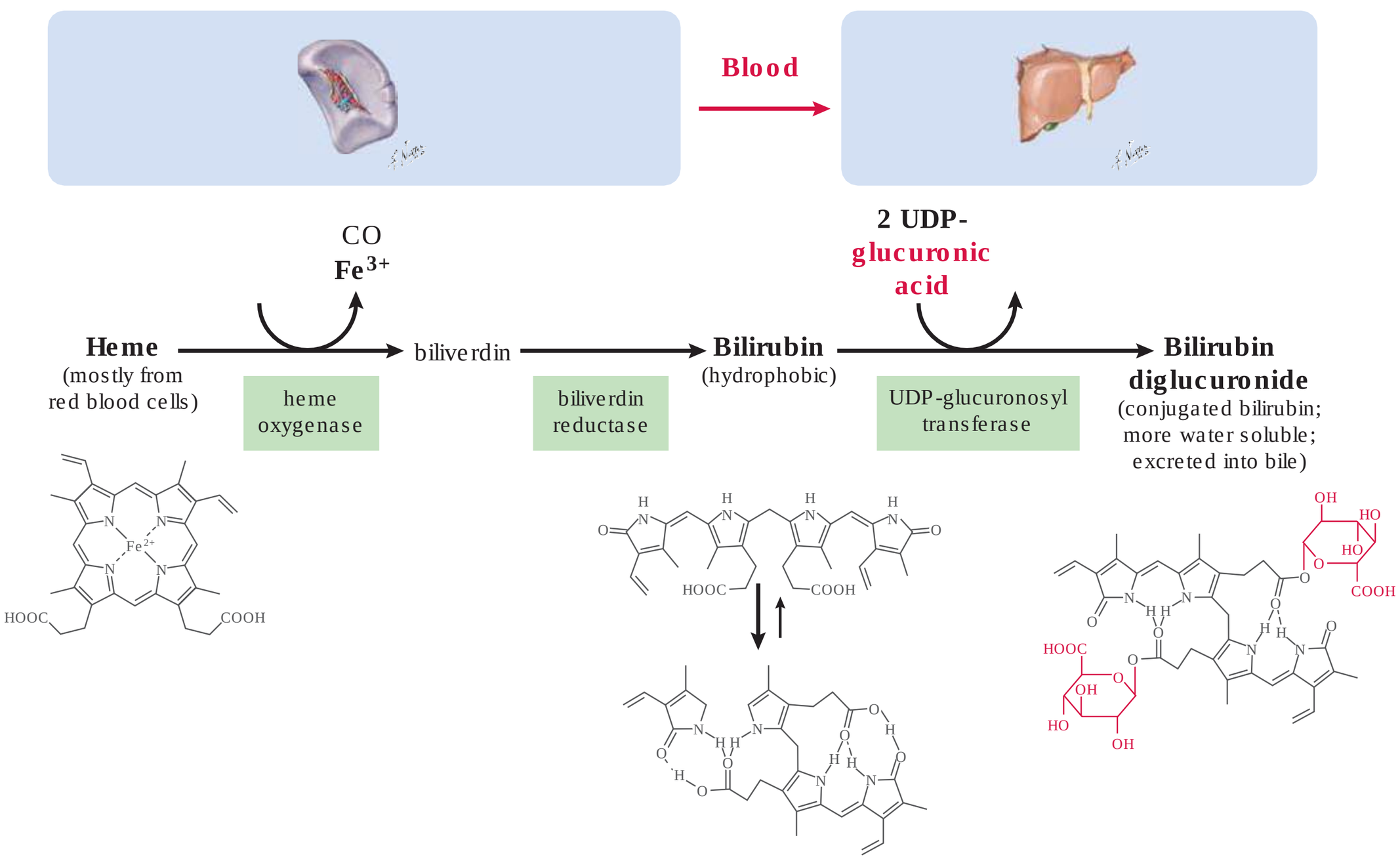

Fig. 14.7 **Degradation of heme to bilirubin and conjugation with glucuronic acid to bilirubin diglucuronide.**

monoxide (CO) from the oxidation of heme is carried away in blood, bound to hemoglobin, and eventually exchanged for oxygen; CO production from heme degradation is normally too small to cause appreciable toxicity.

In water, bilirubin forms internal **hydrogen bonds** that conceal the hydrophilic groups from water (see Fig. 14.7), and thus render it poorly water soluble. Hence, in the bloodstream, bilirubin binds to **albumin** (a protein that is secreted by the liver and has a half-life in the blood of about 3 weeks). T is complex does not pass through the glomerular membrane in the kidneys nor is it excreted into the bile.

Bilirubin is an abundant fat-soluble **antioxidant** (see Chapter 21). It protects **polyunsaturated fatty acids** from **peroxyl radicals** and proteins from **hydroxyl radicals** (see Chapter 21). However, besides this beneficial role, bilirubin at a high concentration is also neurotoxic (see the following discussion on kernicterus and Crigler-Najjar syndrome).

T e liver takes up bilirubin from the blood, conjugates it, and excretes **bilirubin glucuronides** into the bile duct (see Fig. 14.7). **Bilirubin UDP-glucuronosyl transferase** in the membrane of the endoplasmic reticulum conjugates bilirubin with **glucuronic acid** to form conjugated bilirubin (i.e., bilirubin monoglucuronide and bilirubin diglucuronide). **Conjugated bilirubin** is more hydrophilic than bilirubin. (T e UDP-glucuronic acid for the conjugation reaction is produced by the oxidation of UDP-glucose by UDP-glucose dehydrogenase.) Conjugated bilirubin (together with other glucuronides, such as those from drugs conjugated with glucuronic acid) is actively secreted into the **bile**.

Conjugation with glucuronic acid, catalyzed by a number of dif erent UDP-glucuronosyl transferases, is a general and important pathway of **detoxification**. Another important pathway of detoxification is hydroxylation by the cytochrome P450 system (see Section 1.1).

4. LAB ASSAYS: DIRECT, TOTAL, AND INDIRECT BILIRUBIN

T e total and direct-reacting bilirubin in blood are commonly measured. I*f* the total bilirubin is raised and i*f* the direct/total bilirubin ratio is high, bile likely cannot *fl*ow out o*f* the liver into the duodenum.

Normally, 95% or more of the bilirubin in the blood is unconjugated and 5% or less is conjugated (Fig. 14.8). Most of the conjugated bilirubin is bilirubin diglucuronide.

In the laboratory, bilirubin is measured as the colored (green) product of a reaction with a **diazo reagent**. In alcohol, the diazo dye reacts with all of the bilirubin (conjugated and unconjugated). T e result is reported as the **total bilirubin** (see Fig. 14.8). In water, the diazo dye also reacts with both conjugated and unconjugated bilirubin, but it reacts more quickly with conjugated (water-soluble) than unconjugated (fat-soluble) bilirubin. T is reaction is not allowed to proceed to completion and is stopped after a few minutes. T e result is called **direct bilirubin**. T is fraction contains all of the conjugated and some of the unconjugated bilirubin.

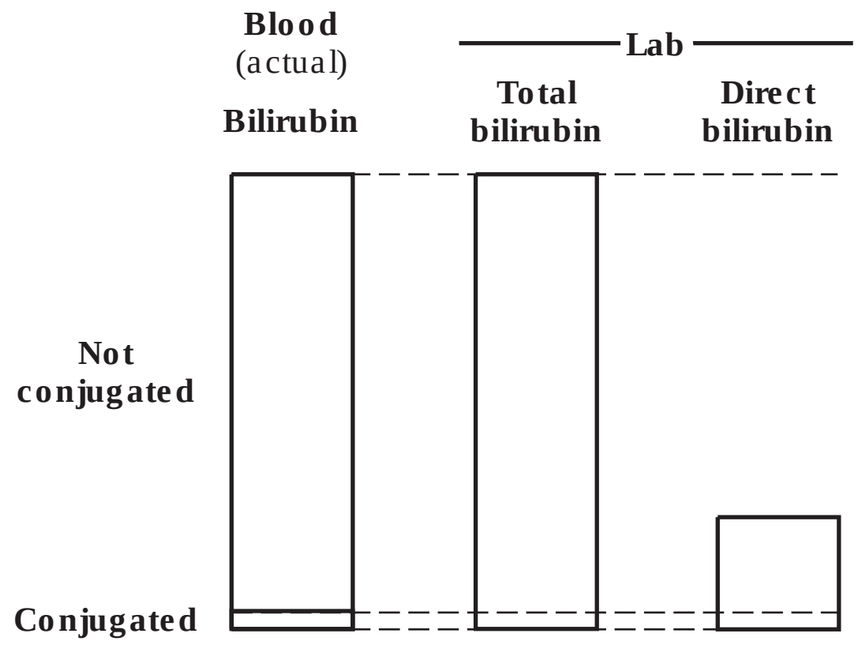

Fig. 14.8 Relationship of bilirubin lab values to the actual concentrations of bilirubin species in the blood of a healthy patient.

A normal value for the total bilirubin is 0.1 to 1.0 mg/dL; for the direct bilirubin, it is 0.3 mg/dL or less. T e direct bilirubin is always smaller than the total bilirubin.

T e mathematical dif erence between the total bilirubin and the direct bilirubin is called the **indirect bilirubin**. Virtually all of the indirect bilirubin represents unconjugated bilirubin.

T e lack of specificity of the direct bilirubin assay for conjugated bilirubin complicates the interpretation of abnormalities. Only a major elevation in conjugated bilirubin generates a lopsided direct bilirubin/total bilirubin ratio.

T ere is a convenient **15% rule of thumb** to interpret bilirubin measurements: If a patient has marked hyperbilirubinemia (e.g., >3 mg/dL), and **if the direct bilirubin is 15% or more of the total bilirubin**, the patient is said to have a **direct hyperbilirubinemia,** which is most likely due to **cholestasis** (i.e., the conjugated bilirubin cannot properly get out of the liver and into the duodenum).

A patient who has marked hyperbilirubinemia (e.g., >3 mg/dL) and a direct bilirubin of 15% or less of the total bilirubin is said to have an **indirect hyperbilirubinemia**. Such a patient either produces too much bilirubin or has a problem conjugating bilirubin.

Note that the 15% rule of thumb does not apply to patients who have a normal or only mildly elevated total bilirubin.

5. PROBLEMS WITH THE DEGRADATION OF HEME

Jaundice is an indication o*f* hyperbilirubinemia. Bilirubin, at a high concentration, is neurotoxic. Hyperbilirubinemia is commonly seen in newborns and in patients who have a hemolytic anemia, Gilbert syndrome, liver disease, or blockage o*f* the bile system.

5.1. General Considerations

Jaundice is due to a deposition of bilirubin in the sclerae and skin, which is visible when the total bilirubin in the blood is greater than ~3 mg/dL (see, for example, Figs. 14.10 and 14.12).

In patients with severe hyperbilirubinemia, **alternative pathways of bilirubin excretion** become appreciable. Unconjugated bilirubin can spontaneously oxidize to less hydrophobic products that can be excreted into the bile. Unconjugated bilirubin can also photoisomerize to a more water-soluble diastereomer, which is excreted in the urine (see neonatal jaundice and "bili-lights" below). Conjugated bilirubin can be filtered in the glomeruli and lost into the urine, thereby markedly darkening the urine.

5.2. Hyperbilirubinemia Due to Impaired Excretion of Conjugated Bilirubin

If conjugated bilirubin is not adequately secreted from the liver into the bile system and from there into the intestine, it may spill into the bloodstream, thereby giving rise to jaundice and a direct bilirubin that is **more than 15%** of the total bilirubin (Fig. 14.9).

5.2.1. Acquired Cholestasis

Cholestasis, a lack of adequate bile flow, causes an accumulation of conjugated bilirubin in the liver. Cholestasis may be due to a problem within the liver or outside of it (see Fig. 14.10).

Intrahepatic cholestasis may be due to **primary biliary cirrhosis**, an autoimmune disease seen in about 0.1% of women over 40 years of age and much less frequently among men. Primary biliary cirrhosis is associated with the destruction of interlobular (or microscopic) bile ducts. Intrahepatic cholestasis may also be due to **primary sclerosing cholangitis** (suspected to be of autoimmune origin) that causes persistent inflammation and stricturing of bile ducts.

Extrahepatic cholestasis is due to the physical obstruction of the common bile duct. T e obstruction may be created by

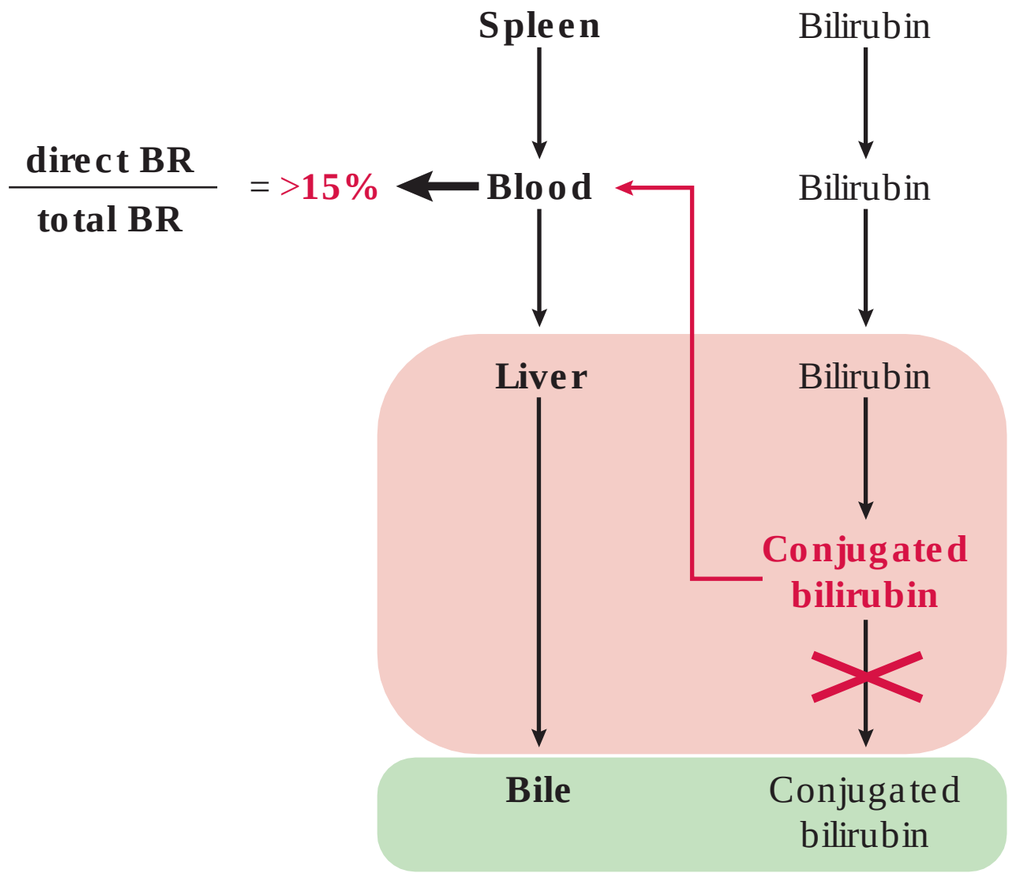

Fig. 14.9 Deficient secretion of conjugated bilirubin (BR) from the liver into the bile and then the duodenum markedly increases the concentration of conjugated bilirubin in the blood, causing a direct hyperbilirubinemia.

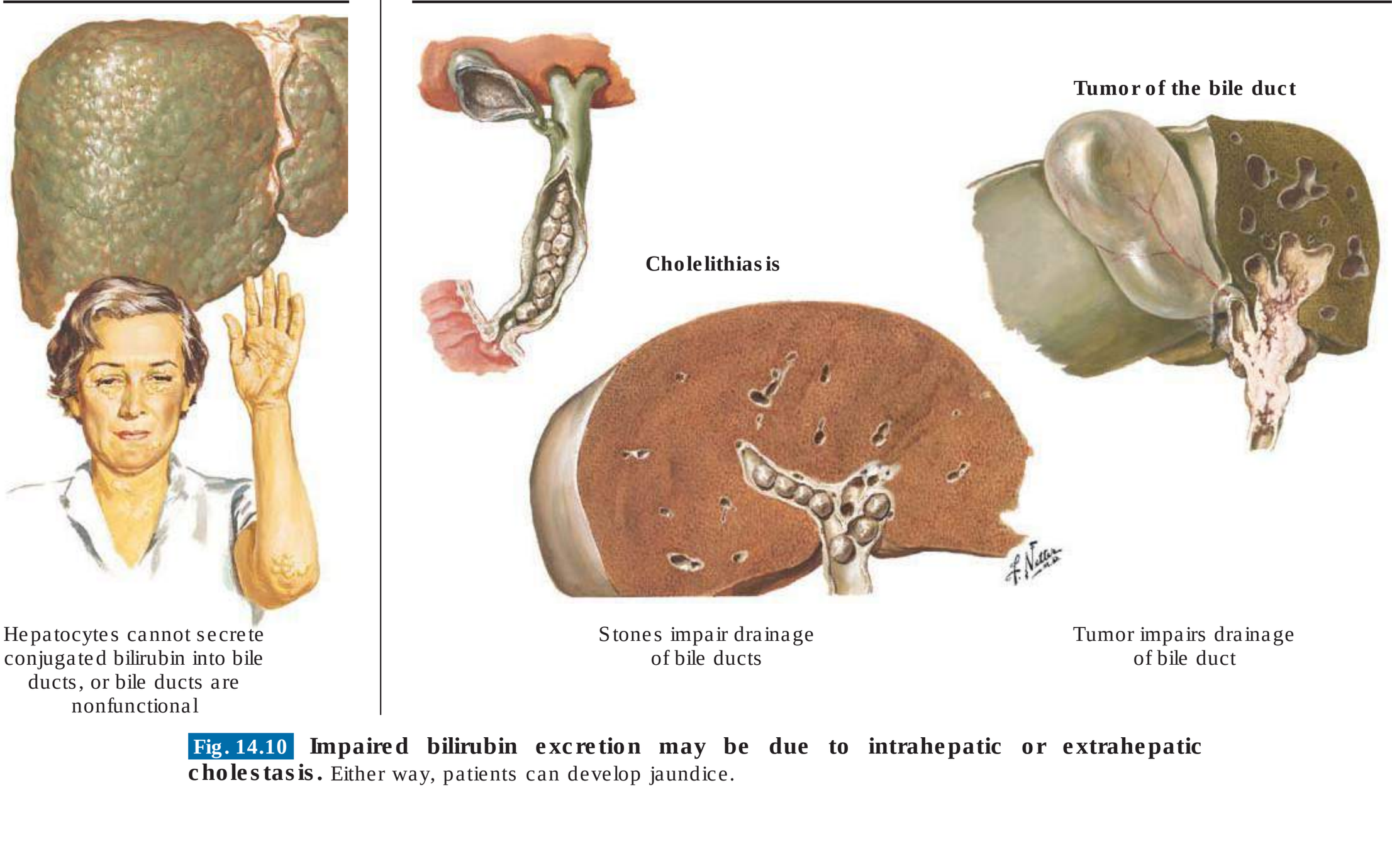

Fig. 14.10 Impaired bilirubin excretion may be due to intrahepatic or extrahepatic cholestasis. Either way, patients can develop jaundice.

gallstones or tumors (e.g., **pancreatic head cancer**, **carcinoma of the ampulla of Vater**) that obstruct the bile ducts.

5.2.2. Congenital Impairment of Hepatic Bilirubin Diglucuronide Secretion: Dubin-Johnson Syndrome

Dubin-Johnson syndrome is due to a hereditary deficiency in the transporter that excretes conjugated bilirubin and other glucuronides into the bile canaliculi. The transporter is variably called the **multidrug resistance-associated protein 2** (MRP2) or the **ATP-binding cassette subfamily C member 2** and is encoded by the ABCC2 gene. Dubin-Johnson syndrome is inherited in an autosomal recessive fashion. Prevalence is unclear but is thought to be much less than 1 in 1,000. It is reasonable to suspect Dubin-Johnson syndrome (or another heritable problem of bilirubin excretion) when laboratory data show direct hyperbilirubinemia but no abnormalities in other parameters that reflect liver function (e.g., enzymes, coagulation factors, or albumin). Patients with Dubin-Johnson syndrome are more sensitive to the antifolate drug methotrexate because their cells eject methotrexate at an abnormally low rate (see Section 5.3 in Chapter 37).

5.3. Hyperbilirubinemia Due to Increased Degradation of Heme

If a hyperbilirubinemia is caused only by increased degradation of heme, a patient has an indirect hyperbilirubinemia (i.e., the total bilirubin is elevated, and the direct bilirubin usually amounts to **less than 15%** of the total bilirubin, provided that the total bilirubin is greater than about 3 mg/dL; Fig. 14.11). Increased degradation of heme and subsequent increased production of bilirubin can be due to ineffective erythropoiesis or hemolytic disease.

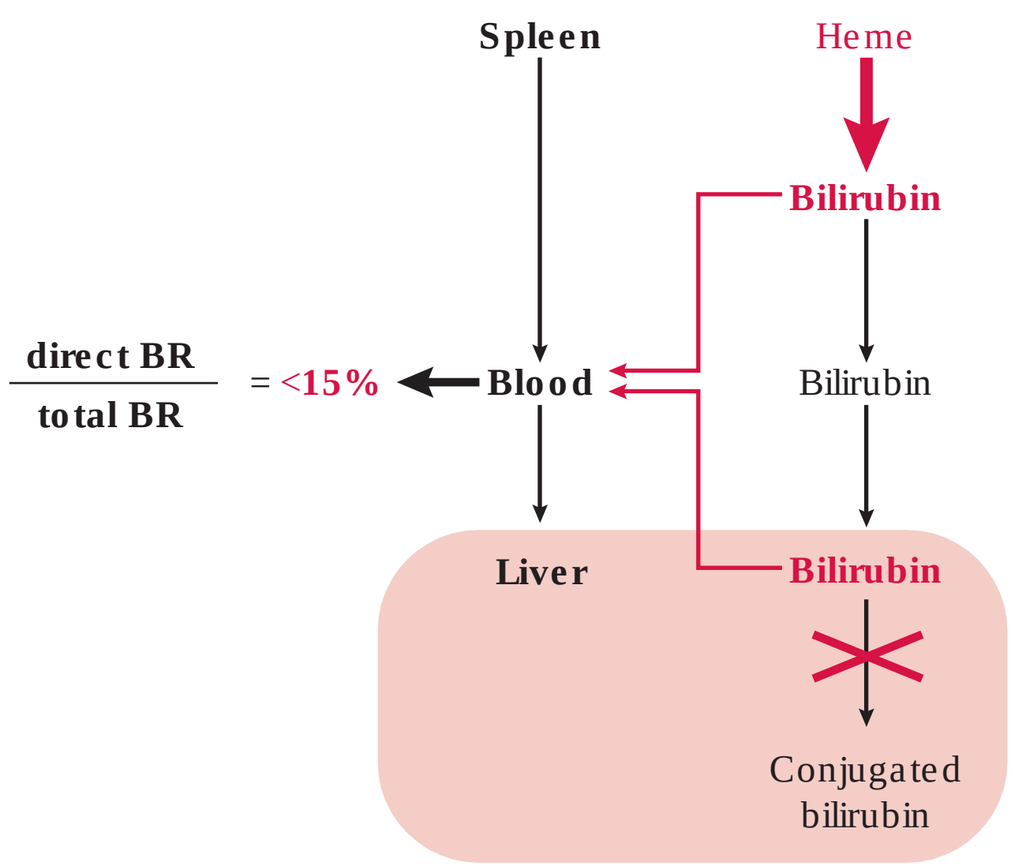

Fig.14.11 **Excess production of bilirubin (BR) and inadequate conjugation of bilirubin can each lead to indirect hyperbilirubinemia in which the direct bilirubin is less than 15% of the total bilirubin.**

Inef ective erythropoiesis is associated with the destruction of heme-containing red blood cell precursors (i.e., polychromatophilic erythroblasts and reticulocytes; see Fig. 16.3). Inef ective erythropoiesis is seen in **lead poisoning** (see Section 2.3.2 above), **congenital erythropoietic porphyria** (see Section 2.4.2), **thalassemia major** (due to a problem with globin synthesis; see Chapter 17), and **megaloblastic anemia** (due to a problem with DNA replication; see Chapter 36).

Hemolytic disease is due to the premature destruction of red blood cells. Examples include **sickle cell anemia** (see Chapter 17), **glucose 6-phosphate dehydrogenase deficiency** (see Chapter 21), and **pyruvate kinase deficiency** (see Chapter 19).

5.4. Hyperbilirubinemia Due to Inadequate Conjugation of Bilirubin

The diseases discussed in this section (i.e., neonatal jaundice, Crigler-Najjar syndrome, Gilbert syndrome, and acquired deficiencies of bilirubin conjugation) are all associated with an indirect hyperbilirubinemia (i.e., with a direct bilirubin of <15% of the total bilirubin).

5.4.1. Neonatal Jaundice

Newborns (especially premature babies) often have insufficient amounts of **bilirubin UDP-glucuronosyl transferase**, since the liver starts synthesizing this enzyme only around the time of birth. As a result, these newborns have an abnormally high concentration of bilirubin in the blood, and they have neonatal jaundice (see Fig. 14.12). In utero, the fetus' enzyme deficiency is not a problem because bilirubin dif uses across the placenta and is conjugated in the mother's liver.

Albumin acts as a buf er for (unconjugated) bilirubin so that the concentration of free bilirubin rises significantly only when albumin is nearly saturated with bilirubin. For this reason, a total bilirubin concentration in blood plasma that does not rise beyond 20 mg/dL is usually safe. For comparison, a healthy 3-day-old baby has a bilirubin concentration of ~6 mg/dL.

Kernicterus (acute bilirubin encephalopathy; see Fig. 14.12) is a severe form of jaundice in which aggregates of (unconjugated) bilirubin and phospholipids form on the membrane surfaces of neurons, predominantly in the basal ganglia, which initiates an encephalopathy that may cause permanent brain damage (especially to the auditory system) or death. Since the aggregates form and dissolve slowly, there may be a lag time between changes in the concentration of bilirubin in the blood and changes in the degree of encephalopathy. With vigilance, kernicterus should be avoidable. Newborns with an unrecognized hemolytic disorder (e.g., glucose 6-phosphate dehydrogenase deficiency; see Chapter 21) are at an especially high risk of developing kernicterus.

In babies with hyperbilirubinemia, the physician must be careful not to introduce drugs (e.g., **sulfonamides**) or **fatty acids** that bind to albumin and thereby displace

Fig. 14.12 **Neonatal jaundice and kernicterus.**

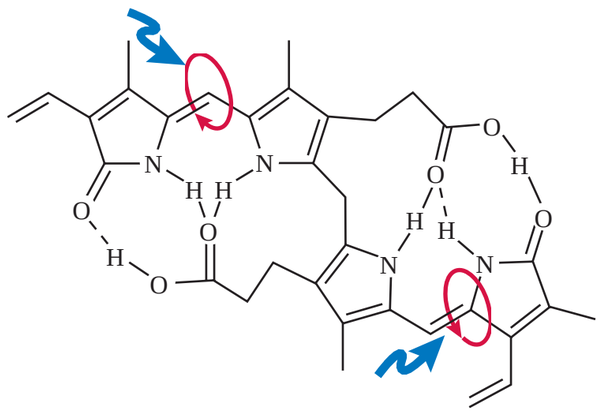

Fig. 14.13 **Light can induce the isomerization of bilirubin to a more water-soluble diastereomer.**

(unconjugated) bilirubin. The physician also must be careful not to introduce drugs (e.g., **steroids**) that are detoxified by UDP-glucuronosyl transferase, the enzyme that conjugates bilirubin.

Patients with very high concentrations of (unconjugated) bilirubin can be treated in three main ways: (1) **exchange transfusion** can be used to remove bilirubin and red blood cells that are prone to hemolysis due to hemolytic disease; (2) an infusion of **albumin** can help bind some bilirubin, but it may also increase blood pressure; and (3) most importantly, **phototherapy** ("**bili-lights**") can be used to isomerize bilirubin in the skin in a water-soluble form (Fig. 14.13). Blue-green light appears to be suited best to this purpose. The physiological isomer of (unconjugated) bilirubin has the Z,Z-configuration. Light can excite bilirubin and allow the rotation of one or two double bonds to produce diastereomers of the E,Z-, Z,E-, or E,E-configuration. These latter diastereomers form fewer intramolecular hydrogen bonds than bilirubin in the Z,Z-configuration, and they expose more hydrophilic groups (–OH, =NH, =O) to water. As a consequence, these diastereomers do not bind to albumin as tightly as does bilirubin in the Z,Z-configuration, and appreciable amounts of them can be filtered through the glomeruli and excreted via the urine.

5.4.2. Crigler-Najjar Syndrome

Crigler-Najjar syndrome is due to a very rare, inherited deficiency of bilirubin **UDP-glucuronosyl transferase**. In **type I** disease, the enzyme deficiency is nearly complete, and in the absence of treatment, patients die from kernicterus within a few years of life. This syndrome is occasionally seen among the **Amish** or **Mennonites**. The lifelong, extreme hyperbilirubinemia requires daily treatment with bili-lights. Bili-lights are less effective in adults than in babies because of less translucent skin and a smaller surface/volume ratio. Replacement of the patient's liver with a transplanted normal liver (i.e., orthotopic liver transplantation) can restore normal bilirubin concentrations but is irreversible and requires immunosuppression. In **type II** disease, the deficiency is only partial, and the affected patients develop kernicterus only episodically during trauma or sepsis. Early identification of these patients helps prevent brain damage because bili-lights can be used promptly. Globally, dozens of different pathogenic mutations have been identified in UDP-glucuronosyl transferase. Most of these mutations affect the amino acid sequence.

5.4.3. Gilbert Syndrome

Gilbert syndrome, seen in about 9% of the population, is due to a hereditary, marked deficiency of **bilirubin UDP-glucuronosyl-transferase**. The deficiency is much less severe than what is seen in Crigler-Najjar syndrome. The disease is usually due to an autosomal recessively inherited mutation in the **promoter** of the gene, which reduces gene expression to approximately one-third of the normal levels. Neonates with Gilbert syndrome have more pronounced and longer-lasting hyperbilirubinemia than neonates normally do. Children and adults with Gilbert syndrome show hyperbilirubinemia in the absence of liver disease (i.e., liver enzymes and coagulation are normal). Hyperbilirubinemia develops predominantly during periods of high rates of heme degradation (e.g., during illness or stress, or after **alcohol** consumption; mostly in males, possibly due to higher turnover of hemoglobin). Ongoing therapy is not necessary. Of clinical importance, however, is the fact that patients with Gilbert syndrome conjugate the topoisomerase inhibitor and anticancer drug **irinotecan** (see Chapter 1) abnormally slowly, which makes the drug more toxic to them.

5.4.4. Acquired Deficiency of Bilirubin Conjugation

Damage to liver cells can impair the conjugation of bilirubin. Causes of such damage may be acquired liver disease, liver ischemia, intrahepatic cholestasis, and extrahepatic cholestasis. In addition to conjugation, these diseases can also affect the excretion of conjugated bilirubin into the bile system, so that the direct bilirubin is 15% or more of the total bilirubin.

Acquired liver disease causing jaundice can be due to **fatty liver** disease (see Fig. 28.11), **viral hepatitis**, **autoimmune hepatitis**, **hemochromatosis** (see Figs. 15.4 and 15.10), **Wilson disease** (leading to copper overload of the liver), liver toxins (including some drugs), excessive **alcohol** ingestion, **carcinoma**, or **α1-antitrypsin deficiency** (possibly due to harmful accumulation of aggregates of mutant α1-antitrypsin in hepatocytes; jaundice then occurs mostly in affected newborns).

SUMMARY

- Heme is synthesized mainly in the bone marrow for the benefit of erythropoiesis. Heme synthesis for red blood cells depends on the availability of iron.
- The second major site of heme synthesis is in the liver, where heme is needed chiefly for the cytochrome P450 system, which plays a role in the detoxification of metabolites and drugs. In the liver, heme synthesis is regulated primarily by the concentration of free heme.
- The two possible major manifestations of various porphyrias are skin damage and neurologic dysfunction with intense abdominal pain. Neurologic effects and abdominal pain are seen in a setting of ALA accumulation. Photodamage to the skin occurs when porphyrinogens or porphyrins accumulate.
- Acute intermittent porphyria is the most common porphyria that affects the nervous system but not the skin. The disease is due to a heritable ~50% decrease in the activity of PBG deaminase. The disease is inherited in autosomal dominant fashion. Acute attacks may be induced by an increased demand for the cytochrome P450 system and may be accompanied by life-threatening neurologic dysfunction.
- Porphyria cutanea tarda is the most common porphyria that affects only the skin. The disease is due to a deficiency of uroporphyrinogen decarboxylase in the liver, most often as a consequence of liver disease in a setting of elevated liver iron stores.
- The initial steps in the diagnosis of a porphyria involve measurements of ALA and porphobilinogen (PBG) in urine and the measurement of porphyrins in blood plasma.
- In the treatment of porphyrias, the synthesis of ALA can be inhibited with intravenous hematin and a high glucose intake, while photodamage by porphyrins can be prevented with light avoidance, sunscreen, and β-carotene. Iron overload can be reduced by phlebotomy.
- Most of the heme that is to be degraded stems from the removal of aged red blood cells in the spleen. The spleen degrades heme into iron and bilirubin. The iron is recycled, and the bilirubin is released into the bloodstream where it binds to albumin. The liver takes up bilirubin and conjugates it with glucuronic acid to form conjugated bilirubin, which it secretes into bile.
- Jaundice is caused by an excessive concentration of bilirubin in the bloodstream (the total bilirubin is more than ~3 mg/dL).
- In a hyperbilirubinemic patient, a direct bilirubin that is greater than 15% of the total indicates a problem with the excretion of conjugated bilirubin. This may be caused by the defective excretion of conjugated bilirubin into the bile ducts or by cholestasis, which in turn may be intrahepatic or extrahepatic. Intrahepatic cholestasis may be due to stricturing or destruction of the bile ducts, which happens with primary biliary cirrhosis and primary sclerosing cholangitis. Extrahepatic cholestasis may be due to obstruction of bile flow by gallstones or tumors.
- In a hyperbilirubinemic patient, a direct bilirubin that is less than 15% of the total indicates excessive production of bilirubin or inadequate conjugation of bilirubin. Excessive production of bilirubin occurs in ineffective erythropoiesis and hemolytic anemia. Inadequate conjugation of bilirubin is common in neonates, due to yet inadequate synthesis of bilirubin UDP-glucuronosyl-transferase. Inadequate conjugation is also observed in patients who have Gilbert syndrome and in patients who have the rare Crigler-Najjar syndrome.
- Liver disease, such as viral hepatitis or liver cancer, and bile duct blockage (by stones or tumor tissue) may affect both the conjugation of bilirubin and the excretion of bilirubin glucuronides.

- Kernicterus, a bilirubin encephalopathy with high mortality and morbidity, can occur at a high concentration of unconjugated bilirubin (generally >20 mg/dL).

FURTHER READING

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Review Questions

1. Which one of the following diseases is a son most likely to inherit from his father when his mother is neither a carrier nor af ected by the disease?

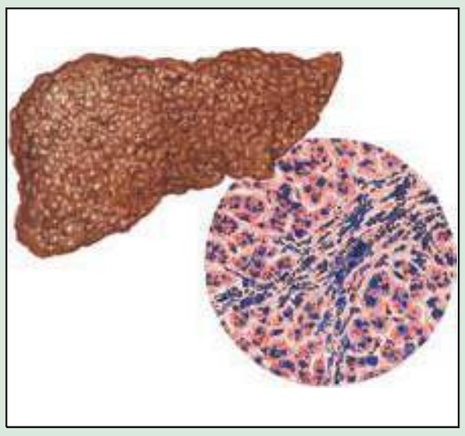
   A. Acute intermittent porphyria
   B. Glucose 6-phosphate dehydrogenase deficiency
   C. Hemoglobin H disease
   D. Porphyria cutanea tarda
   E. Sickle cell anemia

2. A blood sample from an ill 50-year-old woman shows the following values: hematocrit, 39%; MCV, 90 fL; transferrin saturation, 35%; total bilirubin, 9.0 mg/dL; direct bilirubin, 6.3 mg/dL. T ese lab results are most consistent with which one of the following diseases?

   A. Acute intermittent porphyria
   B. Autoimmune destruction of bile ducts
   C. Gilbert syndrome
   D. Lead poisoning
   E. Mild glucose 6-phosphate dehydrogenase deficiency

3. Parenteral nutrition is instituted for a 4-day-old infant who has a total bilirubin of 18 mg/dL and a direct bilirubin of 3.0 mg/dL. Which one of the following macronutrients in the parenteral nutrition should be restricted in this patient?

   A. Carbohydrate
   B. Fat
   C. Protein



Chapter

15 Iron Metabolism: Iron-Deficiency Anemia and Iron Overload

SYNOPSIS

- Iron is needed mostly for the synthesis of heme (see Chapter 14) in hemoglobin. Lesser amounts of iron are used to synthesize heme in myoglobin, the cytochromes P450, as well as the cytochromes and iron-sulfur clusters that participate in oxidative phosphorylation.
- When iron-containing proteins are degraded, the iron is recycled.
- There is no regulated process of iron excretion. Iron is lost mainly through bleeding, pregnancy, and lactation.
- To replace lost iron, healthy children and adults need to take in iron. Epithelial cells in the small intestine absorb a portion of dietary iron. A Western diet usually contains enough iron to meet normal needs.
- The liver secretes the hormone hepcidin, which regulates how much iron the intestinal epithelial cells release into the bloodstream. The amount of iron transferred into the blood is inversely related to the concentration of hepcidin. In addition, hepcidin also regulates the release of iron from other stores in the body.
- Iron homeostasis and body iron content can be assessed by measuring the serum concentration of iron, the total iron-binding capacity as a proxy for transferrin (an iron transport protein), and ferritin (an iron storage protein).
- Chronic blood loss or long-term dietary iron deficiency depletes tissue iron and eventually causes anemia.
- A long-term excess of iron uptake leads to iron overload, which can be associated with dysfunction of the liver, endocrine organs, joints, and the heart.
- Acute poisoning due to oral iron supplements first damages the gastrointestinal tract and then organs throughout the body.

LEARNING OBJECTIVES

For mastery *of* this topic, you should be able to do the *following*:

- Describe the uptake of iron from the diet into the intestine, the release of iron from the intestine into the blood, the movement of iron around the body, storage of iron in tissues, the major use of iron, and loss of iron from the body. Describe the role of hepcidin in regulating iron flux.
- Calculate the transferrin saturation from the serum iron and total iron-binding capacity, and use this value together with other lab tests to assess a patient's iron status, paying special attention to iron deficiency, overload, and poisoning.
- Compare and contrast the daily amount of iron that is absorbed from dietary sources and the daily dose of supplemental iron that is used to prevent or treat iron deficiency, considering the patient's age, gender, and reproductive status.
- Describe the dangers of iron supplements to young children. Explain the biochemical processes that underlie the pathology of acute iron poisoning. Identify potential treatment methods for acute iron poisoning.
- Explain how ineffective erythropoiesis and transfusions can lead to an iron overload.
- Describe the genetic basis and inheritance pattern of the most common form of hereditary hemochromatosis. Describe current genetic testing for this disorder and list the prevalence of pathogenic genotypes.
- Compare and contrast phlebotomy and chelation therapy in the treatment of iron overload or acute iron toxicity. Identify disease conditions for which phlebotomy is usually appropriate. Identify disease conditions for which chelation therapy is usually appropriate.

1. THE BODY'S PRINCIPAL IRON STORES

Most *of* the body's iron is contained in blood as part *of* heme. Free iron is toxic. In cells, nonheme iron is stored inside the storage protein *f*erritin and in its derivative hemosiderin. Most iron leaves the body as a result *of* bleeding, the delivery *of* a *f*etus, or lactation.

Iron is principally found in **heme**, and heme, in turn, is principally found in red blood cells (Fig. 15.1). Heme is a small organic molecule that contains iron (see Fig. 14.1). Heme is found mostly in hemoglobin, myoglobin, in cytochrome P450, and in the cytochromes that participate in oxidative phosphorylation (see Chapter 23). Red blood cells and their **hemoglobin**-synthesizing precursors contain most of the body's heme; therefore, they normally also contain most of the body's iron, about 2.4 g in a healthy adult. As a rule, 1 mL of packed red blood cells contains ~1 mg of iron. Skeletal and cardiac muscle contain **myoglobin**, which uses heme to bind O_2 reversibly (see Chapter 16). Hepatocytes are rich in **cytochromes**, which also contain heme as a prosthetic group.

Inside cells, iron is stored inside **ferritin** in the cytoplasm (Figs. 15.1 and 15.2). Free iron can promote the production of free radicals (see Sections 9.1 and 9.6) that damage cellular constituents. Such undesirable reactions are minimized when iron is stored inside ferritin. T e largest amounts of ferritin iron are found in the liver, the spleen, and the bone marrow (see Fig. 15.1).

Macrophages in the spleen, bone marrow, and, to a lesser degree, the liver (**Kupf er cells**) (Fig. 15.3) degrade heme from engulfed red blood cells and temporarily store the iron inside ferritin. Most of this iron is eventually transferred to red blood cell progenitor cells in the bone marrow, where it is again stored inside ferritin.

Hemosiderin is a membrane-enclosed, insoluble degradation product of ferritin that is normally found in the liver, the bone marrow, and to a lesser extent the spleen. Hemosiderin can be stained with Prussian blue for observation by light microscopy (Fig. 15.4).

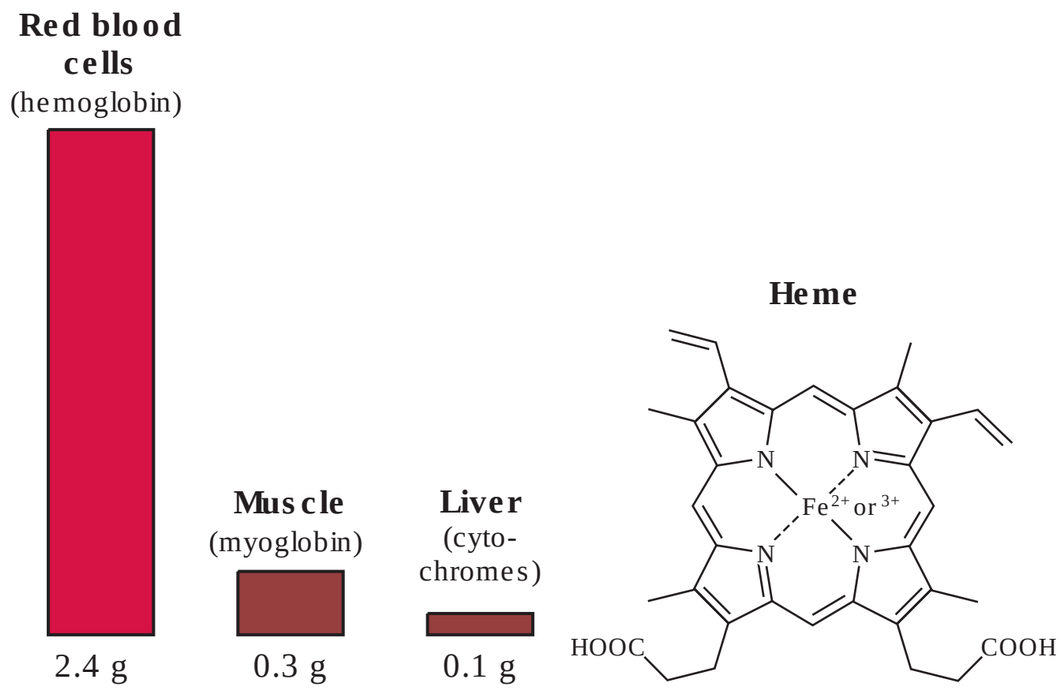


Fig. 15.1 In a healthy human body, most of the iron is inside circulating red blood cells as part of heme in hemoglobin. *Includes iron in hemosiderin.

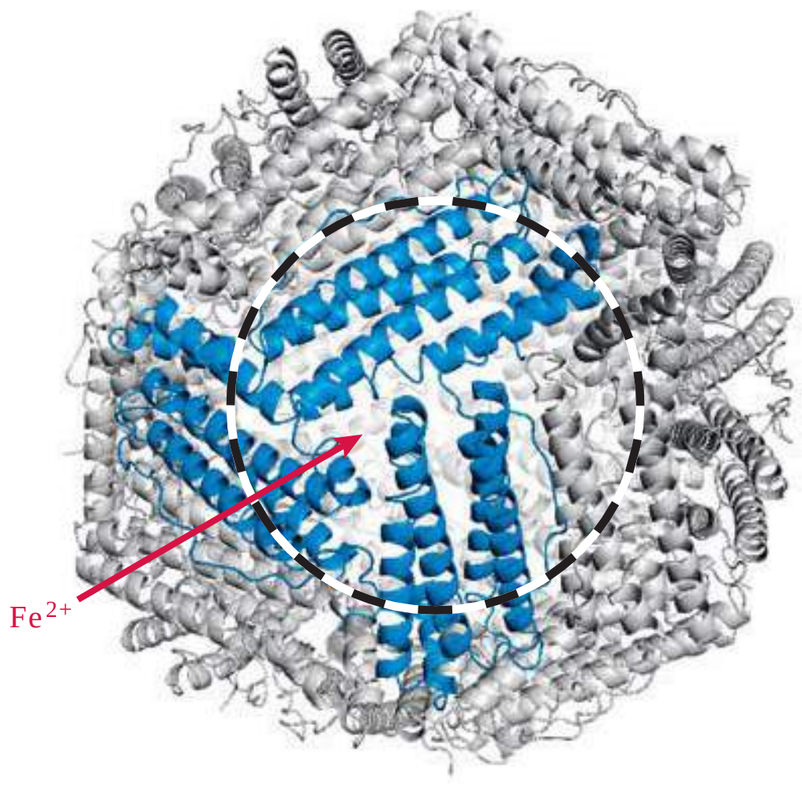


Fig. 15.2 Crystal structure–based model of human H-ferritin. Ferritin consists of H- and L-chains in tissue-specific proportions. Fe^{2+} enters through one of eight channels, is oxidized to Fe^{3+} by O_2, and precipitates inside ferritin. The central cavity (outlined by a black dashed circle) can accommodate a maximum of ~4,000 atoms of Fe^{3+}. The release of iron may require degradation of ferritin by proteasomes or lysosomes. (Based on Protein Data Bank file 3AJO from Masuda T, Goto F, Yoshihara T, et al. The universal mechanism of iron translocation to the ferroxidase site in ferritin, which is mediated by the well conserved transit site. *Biochem Biophys Res Commun.* 2010;400:94-99.)

Iron-sulfur proteins play important roles in the citric acid cycle and oxidative phosphorylation but make up less than 3% of total body iron. Examples of iron-sulfur proteins are aconitase/IRP1 (see Section 3 and Chapter 22) and complexes I, II, and III in oxidative phosphorylation (see Chapter 23).

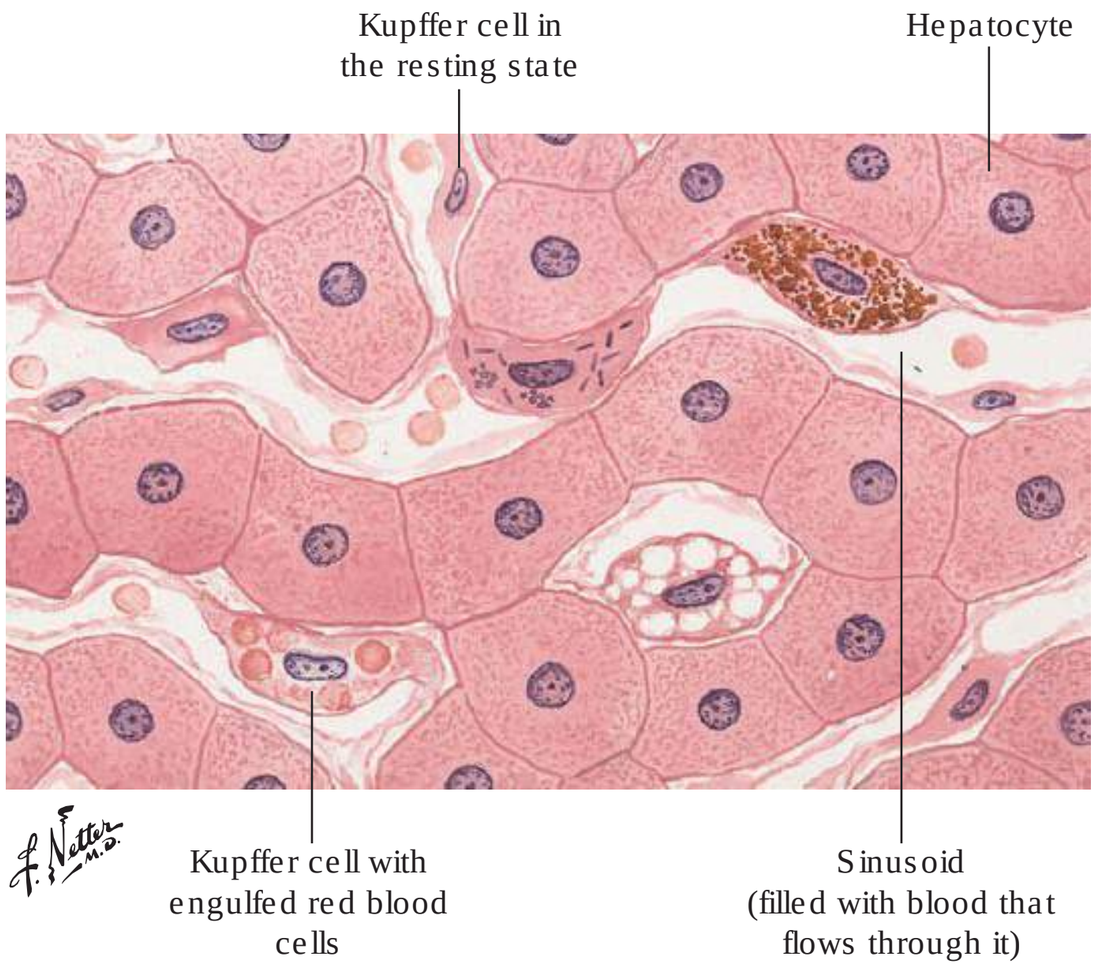


Fig. 15.3 Kupffer cells in liver sinusoids take up old red blood cells and store their iron inside ferritin. Light microscopic image of a hematoxylin-eosin–stained thin section of human liver. The Kupffer cells degrade red blood cells, temporarily store the iron from their heme, and then release it. Normally, ~98% of liver iron is in the hepatocytes. (The dark brown cell is also a Kupffer cell.)

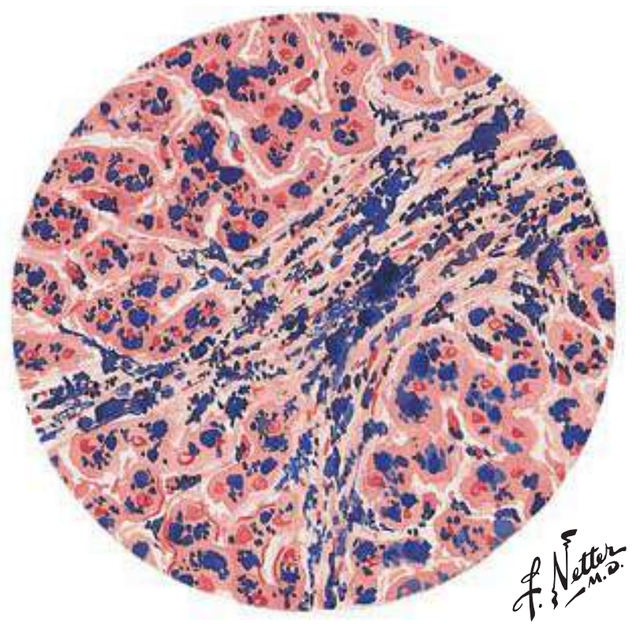


Fig. 15.4 Hemosiderin in an iron-overloaded liver. During histological processing, iron was reacted with acid ferrocyanide to produce dark blue, insoluble Prussian blue. Stainable iron in the liver occurs in proportion to body iron stores. Processing of a sample from a *normal* liver gives rise to no or little Prussian blue (much less than shown here).

Iron-sulfur proteins are also called **nonheme iron proteins** since their iron (Fe^{2+} or Fe^{3+}) is coordinated with inorganic sulfides (S^{2-}) or sulfides of cysteine side chains ($-S^-$).

2. ABSORPTION OF DIETARY IRON

Intestinal epithelial cells take up iron *f*rom the diet either as heme (e.g., *f*rom red meat) or as the *f*errous cation (Fe^{2+}; e.g., *f*rom plant sources or a supplement). T e liver secretes the hormone hepcidin, which regulates the release o*f* iron *f*rom intestinal epithelial cells into the bloodstream.

Iron is absorbed mostly in the duodenum and jejunum, either as **heme**, or as a **ferrous iron ion** (Fe^{2+}; Fig. 15.5). Most

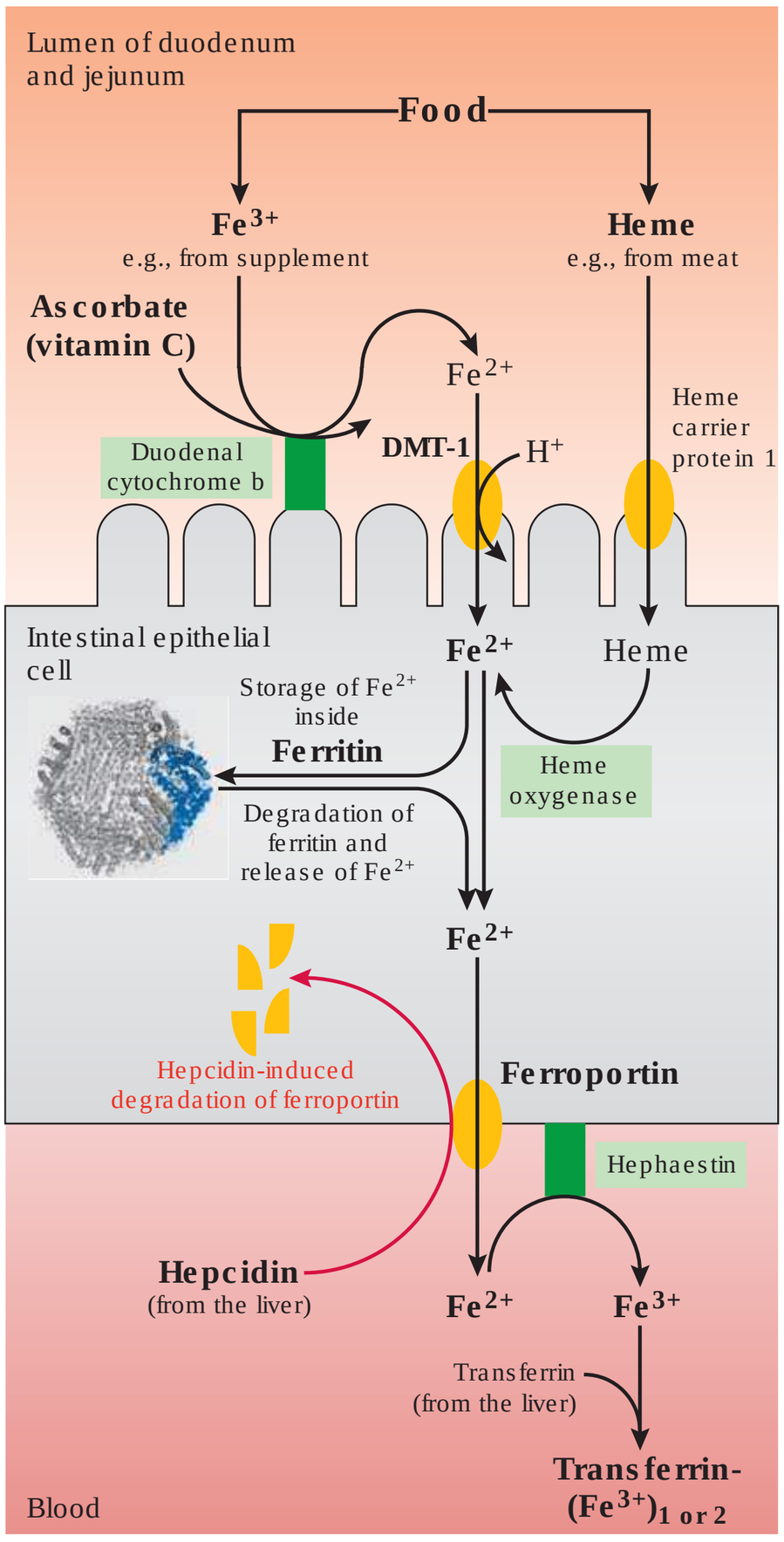


Fig. 15.5 Uptake of iron into enterocytes, temporary intracellular storage, and release of iron into the bloodstream. Hepcidin regulates the release of iron into the bloodstream via ferroportin.

diets contain ~8 mg iron per 1,000 kcal, mostly as nonheme iron. Heme is found in meats. Iron supplements contain iron salts (mostly salts of Fe^{2+}, such as ferrous sulfate, $FeSO_4$). When chelated with **ascorbate** (vitamin C), such iron ions are taken up especially well.

The luminal surface of intestinal epithelial cells contains one transport system for heme and another for Fe^{2+} (see Fig. 15.5). After heme has been taken up into intestinal epithelial cells, it is degraded by heme oxygenase (see Chapter 14), freeing Fe^{2+}. Much of the naturally occurring dietary nonheme iron is in the Fe^{3+} form. The microvilli of the intestinal epithelial cells contain an enzyme (**duodenal cytochrome b**) that reduces ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}). Dietary ferrous iron (Fe^{2+}) is absorbed to a greater extent than dietary ferric iron (Fe^{3+}) because it does not require such reduction. The intestinal epithelial cells take up Fe^{2+} through a **divalent metal transporter** (DMT1), which also transports manganese ions (a required trace metal), and many other metals, including those that are toxic (e.g., cadmium or lead ions).

Intestinal epithelial cells either store Fe^{2+} inside ferritin in the cytosol (after oxidation to Fe^{3+}; see Section 1 and Fig. 15.2) or export it into the blood. Intestinal cells take up much more iron from the gut lumen than they release into the bloodstream. Fe^{2+} leaves intestinal cells via the iron transporter **ferroportin** (also referred to as solute carrier family 40 subfamily A member 1, abbreviated as SLC40A1), the availability of which is regulated by hepcidin (see Section 3). On the basolateral surface of the enterocytes, **hephaestin** or **ceruloplasmin** then oxidize extracellular Fe^{2+} to Fe^{3+}, which binds to **transferrin** (see Section 4). Intestinal epithelial cells are shed when they are ~**2 to 3 days** old, and any iron in them is lost. Intestinal ferritin thus serves as a short-term iron reservoir that evens out changes in the iron content of the diet.

Iron transport in other cells has many similarities to intestinal epithelial cells. Peripheral cells usually acquire their iron from endocytosing transferrin that has iron bound to it, and once it is inside the endosomes, the iron dissociates from the transferrin receptor-transferrin-iron complex. While **DMT1** is in the plasma membrane of intestinal epithelial cells, in other cells it is also in the membrane of the endocytic vacuoles and transports released iron from the vacuoles into the cytoplasm. DMT1 also transports iron across the mitochondrial outer membrane. Finally, **ferroportin** also releases iron from splenic macrophages and Kupffer cells in the liver into the bloodstream. Hephaestin does not seem to be used by splenic macrophages, hepatocytes, and Kupffer cells in the liver to reduce Fe^{2+} to Fe^{3+}; these cells use only ceruloplasmin.

3. REGULATION OF IRON RELEASE INTO THE BLOODSTREAM

Iron-response proteins that bind to iron-responsive elements in mRNAs often regulate the expression of proteins that are involved in iron homeostasis. In addition, the liver secretes the peptide hormone hepcidin, which controls iron transport in the body. Hepcidin inhibits the release of iron into the bloodstream from intestinal epithelial cells, macrophages in the spleen, Kupffer cells in the liver, and macrophages in the bone marrow. Inflammation and high iron stores in the liver stimulate hepcidin secretion and thus diminish the concentration of iron in the blood. In contrast, ineffective erythropoiesis, anemia, and hypoxia minimize hepcidin secretion from the liver and thus enhance the release of iron from the intestine, spleen, or liver into the blood.

The **iron-response proteins IRP1** and **IRP2** regulate the expression of some proteins that are involved in iron transport and storage. In the absence of iron, these proteins bind to **iron-responsive elements** (**IREs**) in mRNA and thereby either block translation or favor the survival of the mRNA. Conversely, in the presence of iron, the iron response proteins no longer have access to mRNA: IRP1 becomes a cytosolic aconitase that cannot bind an IRE, and IRP2 is degraded in proteasomes. Iron-dependent regulation of translation or

degradation is observed for the mRNAs that encode DMT1, ferritin, ferroportin, transferrin receptor 1, or erythroid ALA synthase. Besides iron, the signaling by IRP1 and IRP2 also depends on the local **oxygen** concentration.

Hepcidin is a 25-amino acid peptide that binds to **ferroportin** and thereby causes ferroportin to be internalized and degraded. Hepatocytes synthesize hepcidin and then secrete it into the sinusoids, from where it reaches the central vein of the lobule and finally the systemic circulation. Hepcidin in effect prevents iron from leaving intestinal epithelial cells, macrophages in the spleen, Kupffer cells in the liver, or macrophages in the bone marrow; in this way, hepcidin lowers the concentration of transferrin-bound iron in the blood. The purpose of increased hepcidin secretion appears to be twofold: to prevent an overload of the body with dietary iron and to starve microorganisms of iron, which limits their growth.

The proteins **HFE**, **transferrin receptor 2**, and **hemojuvelin** are involved in regulating hepcidin synthesis. Mutations in these proteins (including hepcidin) are known causes of increased iron absorption (see Sections 9.1 and 9.2).

In healthy individuals, inflammation and high iron stores in the liver stimulate hepcidin secretion and thereby inhibit iron transport via ferroportin (Fig. 15.6). **Chronic inflammation**, via an increased concentration of interleukins (e.g., IL-1 and IL-6) in the blood, increases hepcidin secretion. Inflammation in this context can be caused by AIDS, tuberculosis, rheumatoid arthritis, inflammatory bowel disease, or malignancy, for example. An adverse effect of this regulation is that patients with chronic inflammation become anemic due to insufficient release of iron from stores (see Section 8.2 below). **High iron stores** in hepatocytes also lead to an increased secretion of hepcidin (but the secretion of hepcidin is abnormally low in patients who have hemochromatosis; see Section 9.2 below).

Ineffective erythropoiesis, **anemia**, and **hypoxia** oppose hepcidin secretion (see Fig. 15.6) and thus promote the uptake of dietary iron, as well as the release of iron from macrophages in the spleen, Kupffer cells in the liver, and macrophages in the bone marrow. Patients with chronic ineffective erythropoiesis, anemia, or hypoxia tend to accumulate too much iron because they inhibit hepcidin secretion even when liver iron stores are excessive.

There are clinically significant **limits** to the regulation of iron absorption. Thus, some patients with excessive **blood loss** (e.g., due to a bleeding ulcer or excessive menstrual flow) cannot absorb sufficient iron and develop iron deficiency with or without anemia (see Section 8.1 below). Conversely, patients who ingest iron in very marked excess (acutely or over a long period) do not inhibit iron absorption sufficiently and eventually accumulate too much iron (see Section 9.1 below).

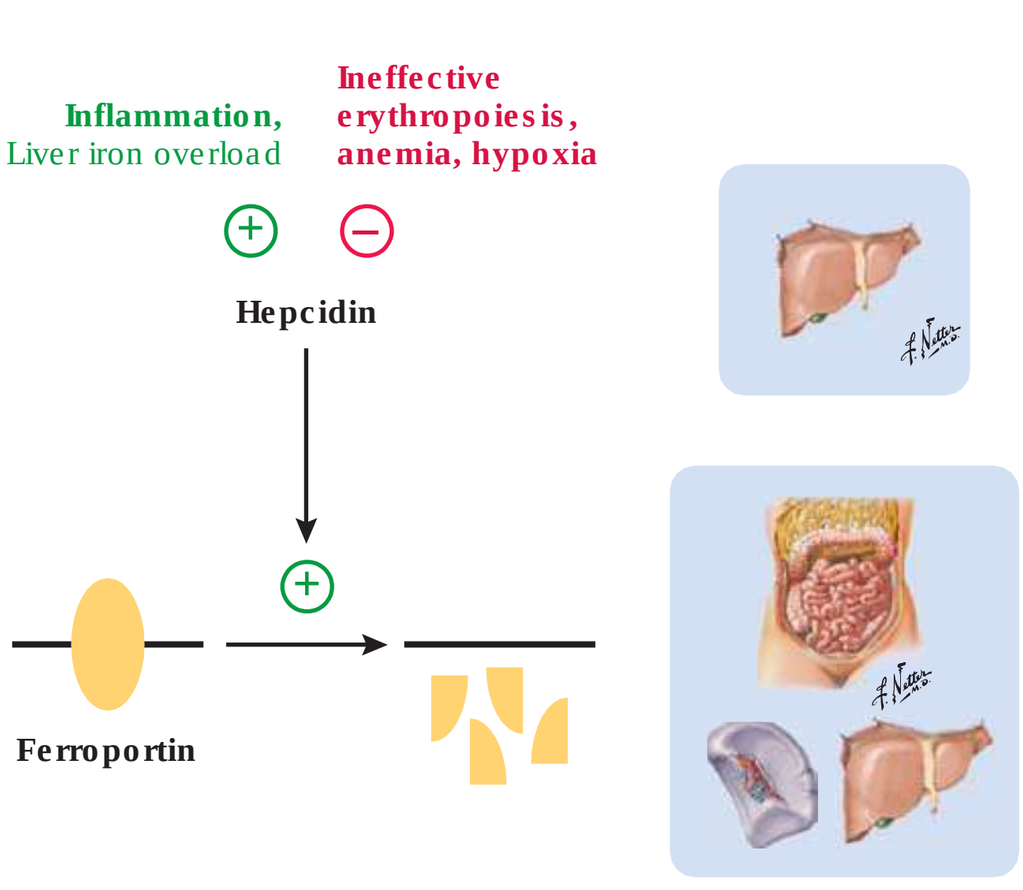


Fig. 15.6 Hepcidin secretion from the liver controls iron transport.

4. TRANSPORT OF IRON IN THE BLOOD

In the blood, iron is bound to transferrin. Cells in need of iron display a transferrin receptor on the surface of their plasma membrane. Transferrin-iron binds to the receptor and the entire complex is endocytosed, followed by liberation of the iron.

In blood plasma, iron is bound to **transferrin**. This lowers the rate of generation of free radicals (see Sections 9.1 and 9.6), prevents loss of iron by filtration in the kidneys, and makes the blood much less hospitable to microorganisms. Transferrin is a ~78,000 molecular weight protein that is synthesized and secreted by hepatocytes. Transferrin has a half-life in the blood of ~1 week. Each molecule of transferrin can bind two Fe^{3+} ions.

The quantitatively most important release of iron into the bloodstream is the release from macrophages in the spleen and Kupffer cells in the liver. Under the control of hepcidin (see Section 3 and Fig. 15.5), these cells export iron as Fe^{2+} (ferrous iron). In the bloodstream, **ceruloplasmin** (synthesized and secreted by hepatocytes) oxidizes Fe^{2+} to Fe^{3+} (ferric iron), and Fe^{3+} then binds to transferrin. The release of iron from intestinal epithelial cells into the bloodstream is comparatively small; it only has to compensate for losses, which are normally small compared with the overall rate of iron recycling.

Almost all cells have **receptors for transferrin**. **Type 1** transferrin receptors (**TfR1**) are ubiquitous; the number of these receptors on the plasma membrane surface is proportional to a cell's iron needs. In contrast, **type 2** transferrin receptors (**TfR2**) are only minimally dependent on a cell's iron stores. TfR2 receptors are found in hepatocytes and, to a lesser degree, in immature erythroid cells, and in the duodenal crypt cells that give rise to the epithelial cells of the villi. TfR2 plays a role in whole body iron homeostasis, but the mechanisms of regulation have not been elucidated.

Cells endocytose transferrin receptor-transferrin-iron complexes. In endosomes, Fe^{3+} is released and reduced to Fe^{2+}, which is transported to the cytosol (via a divalent metal transporter, DMT1; see Section 2). The transferrin receptor-transferrin complex is moved back to the cell surface, and transferrin is released into the blood.

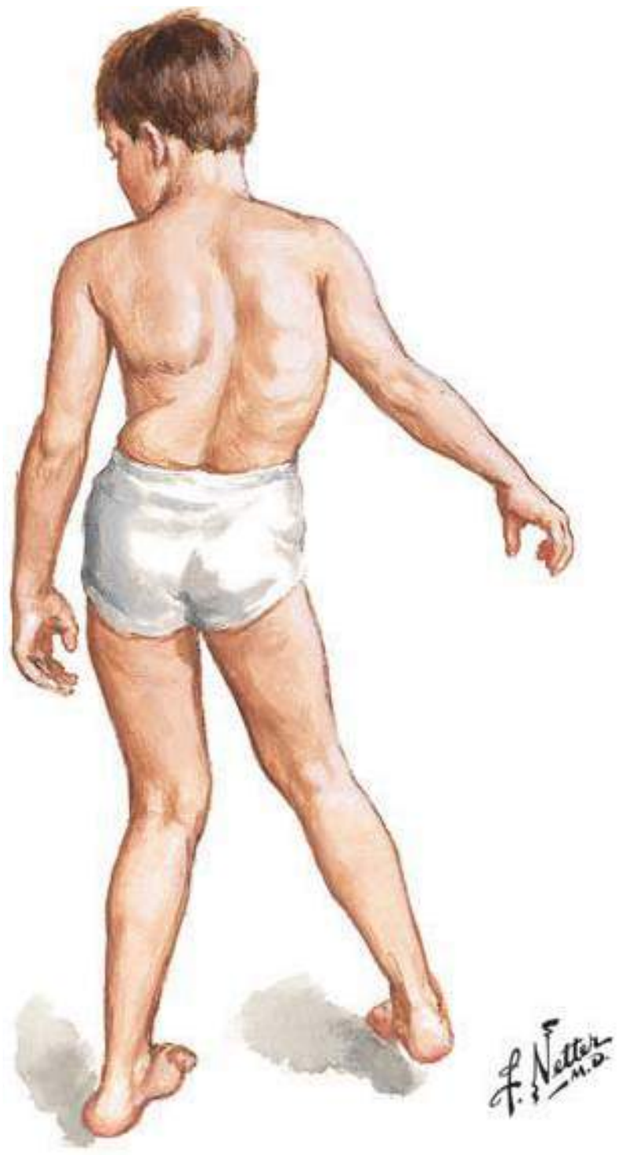


Fig. 15.7 A child who has Friedreich ataxia. The ataxia leads to a wide gait. The patient also has scoliosis. Later, the patient will likely use a wheelchair.

5. IRON IN MITOCHONDRIA

Mitochondria use iron to produce heme and iron-sulfur clusters. Patients who have the neurodegenerative disease Friedreich ataxia carry a trinucleotide repeat expansion in the frataxin gene that greatly reduces the expression of frataxin, a protein that plays a role in iron homeostasis in mitochondria.

DMT1 in the outer mitochondrial membrane and **mitoferrins** in the inner mitochondrial membrane transport iron from the cytosol to the mitochondrial matrix for the production of heme and iron-sulfur clusters. The final steps of **heme** synthesis take place inside mitochondria (see Chapter 14). Heme can be exported from mitochondria and used for the production of hemoglobin, myoglobin, and cytochromes (see Chapter 14). **Iron-sulfur clusters** (2Fe-2S and 4Fe-4S) are part of the active site of the **iron-sulfur proteins**, including some complexes of oxidative phosphorylation and an enzyme of the citric acid cycle (see Section 1 and Chapters 22 and 23). **Frataxin** seems to be a chaperone that helps incorporate iron into iron-sulfur proteins. Mitochondria also store iron inside ferritin.

Patients who have **Friedreich ataxia** (Fig. 15.7) are homozygous (or compound heterozygous) for a mutant frataxin gene. Almost 1% of the population is heterozygous for a pathogenic frataxin allele. Friedreich ataxia has an incidence of about 1:50,000. Pathogenic frataxin alleles contain an expanded **trinucleotide repeat** in intron 1 that leads to reduced transcription of the frataxin gene, which in turn leads to a reduced amount of frataxin protein. Affected patients have muscle weakness, ataxia, and impaired vision, hearing, and speech. The onset of symptoms usually occurs between the ages of 5 and 40 years. Most patients die of dysfunction of the heart (e.g., cardiomyopathy and arrhythmia).

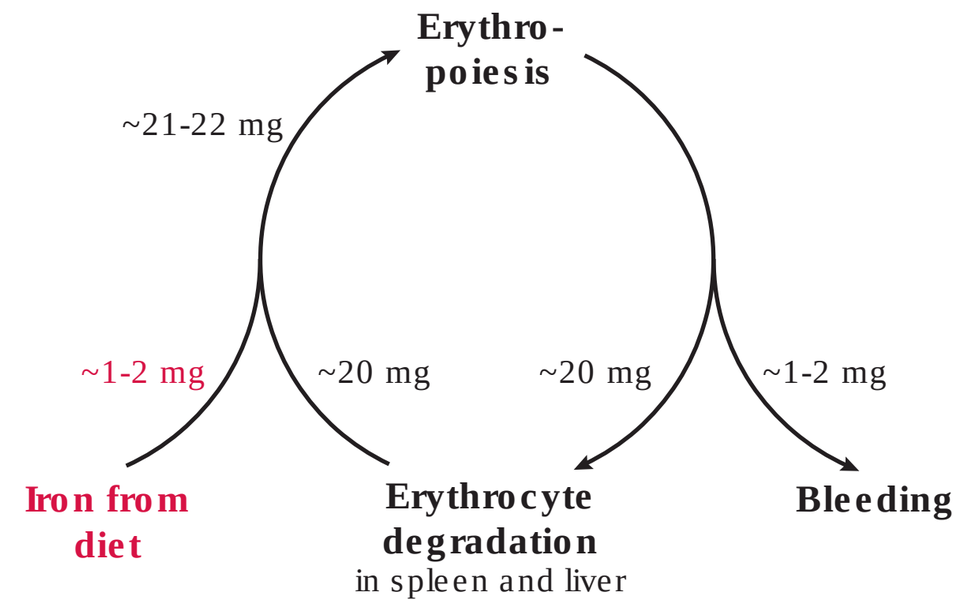


Fig. 15.8 Major iron turnover in the normal human body. Numbers refer only to iron, not its salts.

6. DAILY FLOW OF IRON

Iron is chiefly needed for the biosynthesis of new red blood cells. Iron from ineffective erythropoiesis and old red blood cells is recycled and normally provides ~95% of the iron for new red blood cells. We lose iron chiefly by bleeding into the lumen of the intestine or uterus.

Healthy adults need iron principally for **erythropoiesis**. Every day, 1% of the red blood cells are removed and replaced by new red blood cells. The synthesis of heme for hemoglobin in new red blood cells requires 21 to 22 mg of iron per day (see Fig. 15.8). When old red blood cells are degraded in the bone marrow, spleen, or liver, almost all of their iron is recycled; this yields ~20 mg iron per day. A small amount of iron is lost daily in sweat or blood into the lumen of the intestine (2-4 mL/day) or uterus (~30 mL/menstrual period); the iron contained in this blood cannot be recaptured (in contrast, iron is recovered from bleeding into an internal space, such as muscle or the retroperitoneal space). Hence, erythropoiesis must derive the remaining 1 to 2 mg of iron per day from the diet (see Section 2) or from iron stores (in ferritin or hemosiderin; see Section 1). The liver experiences some turnover of **cytochromes** and their heme, but the near-complete recycling of iron covers the needs of de novo synthesis. **Myoglobin** in muscle contains high levels of heme-iron, but it turns over so slowly that it does not contribute appreciably to the daily turnover of iron.

There are two pathways for the recovery of iron from red blood cells at the end of their lives. In healthy persons, about 90% of red blood cells are degraded by macrophages in the spleen or Kupffer cells in the liver. The remaining ~10% of the red blood cells lyse within the blood stream; there, **haptoglobin** (synthesized and secreted by hepatocytes) binds free hemoglobin, and **hemopexin** (also synthesized and secreted by hepatocytes) binds free heme. Macrophages in the spleen

Table 15.1 Required Daily Iron Intake

Patient Group	Required Daily Net Absorption of Iron (mg)	Required Approximate Daily Dietary Iron Intake (mg)	
		Patients Who Eat Meat	Vegetarians
Postmenopausal women, men	~1.0	~8	~15
Women in reproductive years	~1.5	~18	~30
Pregnant women	~2.5	~27	~50

and Kupfer cells in the liver endocytose haptoglobin-hemoglobin complexes; hepatocytes endocytose hemopexin-heme complexes. After dissociation of these complexes in the lysosomes, haptoglobin and hemopexin are released back into the blood for reuse.

To make up for losses, healthy men and postmenopausal women need to absorb ~1 mg of iron per day from their **diet** (intestinal uptake is described in Section 2 and Fig. 15.5); menstruating or lactating women need to absorb ~1.5 mg per day; and pregnant women need to absorb ~2.5 mg of iron per day (Table 15.1). Most vitamin preparations for expectant mothers provide sufficient iron, but if a pregnant woman becomes anemic, additional iron is given. **Vegetarians** (who consume little heme) need about 80% more iron in their diet than do omnivores.

The iron cost of **pregnancy** is about 1,000 mg of iron, which is equal to about 25% of the normal body iron content. About one-quarter of this iron is transferred to the fetus via the placenta; about another quarter is eventually lost with the placenta and bleeding during a normal vaginal delivery, and about half goes into an expanded red blood cell mass from which the iron is reused after the delivery. Subsequent **lactation** costs ~0.5 mg iron per day.

When needed (e.g., after a major loss of blood), ferritin and hemosiderin iron stores in the body can liberate as much as ~40 mg of iron per day for erythropoiesis.

There is no regulated process of iron excretion.

7. INTERPRETATION OF LABORATORY DATA RELATED TO IRON

Serum iron, total iron-binding capacity, and serum *f*erritin are commonly measured to assess a patient's iron status.

The **total iron-binding capacity (TIBC)** of a patient's serum (often reported in µg/dL) is a measure of the maximal amount of iron that can be bound by transferrin. It is therefore a functional measure of the concentration of transferrin in the serum. The TIBC is increased in iron deficiency (see below) and in pregnancy, and is decreased in iron overload.

Most iron in blood plasma (reported as **serum iron** in µg/dL) is bound to transferrin. Measurements of the TIBC and serum iron allow one to calculate the **transferrin saturation** (serum iron/TIBC, expressed as a percentage). The normal transferrin saturation is 20% to 50% (i.e., Fe^{3+} occupies 20% to 50% of the iron-binding sites on transferrin; the 2:1 stoichiometry for Fe^{3+}: transferrin does not matter in this calculation). The transferrin saturation is decreased in iron deficiency; it is increased in iron overload or acute iron poisoning (see below).

The amount of **ferritin** in the human body is approximately proportional to the body's **iron stores**. An iron-responsive element in ferritin mRNA (see Section 3) regulates ferritin biosynthesis according to need. A small amount of ferritin is normally secreted into the plasma, and additional ferritin can be released into the blood when tissue cells are damaged. The liver removes ferritin from the blood. Even in persons with an iron overload, much of the serum ferritin is apoferritin, which contains no iron. Overall, only ~0.01% of all ferritin is circulating in the blood, and the serum concentration of ferritin reflects the size of the body's iron stores. Normal serum ferritin levels in men are ~100 µg/L and in premenopausal women ~40 µg/L. Elevated serum ferritin levels occur in persons with inflammation or iron overload (see below).

8. IRON DEFICIENCY

Patients become iron deficient mostly through excessive bleeding (i.e., i*f* bleeding amounts to more than ~9 mL per day) and through pregnancy, occasionally also as a consequence o*f* bariatric surgery, chronic in*f*ection, or chronic in*fl*ammation. Persons with advanced iron deficiency develop anemia due to decreased synthesis o*f* heme and hemoglobin as well as ine*ff*ective erythropoiesis. Iron-deficiency anemia is associated with an elevated *T*IBC, elevated trans*f*errin saturation, elevated red cell distribution width (anisocytosis), decreased mean corpuscular volume (MCV), and decreased mean corpuscular hemoglobin concentration (MCHC). Iron deficiency is treated with oral iron supplements (e.g., *f*errous sul*f*ate) or with special parenteral iron preparations.

8.1. Iron-Deficiency Anemia

Worldwide, iron deficiency (Fig. 15.9) is the most common cause of **anemia**. Folate deficiency is another common cause of anemia (see Chapters 36 and 37). Iron deficiency leads to diminished heme synthesis (see Chapter 14) in reticulocytes

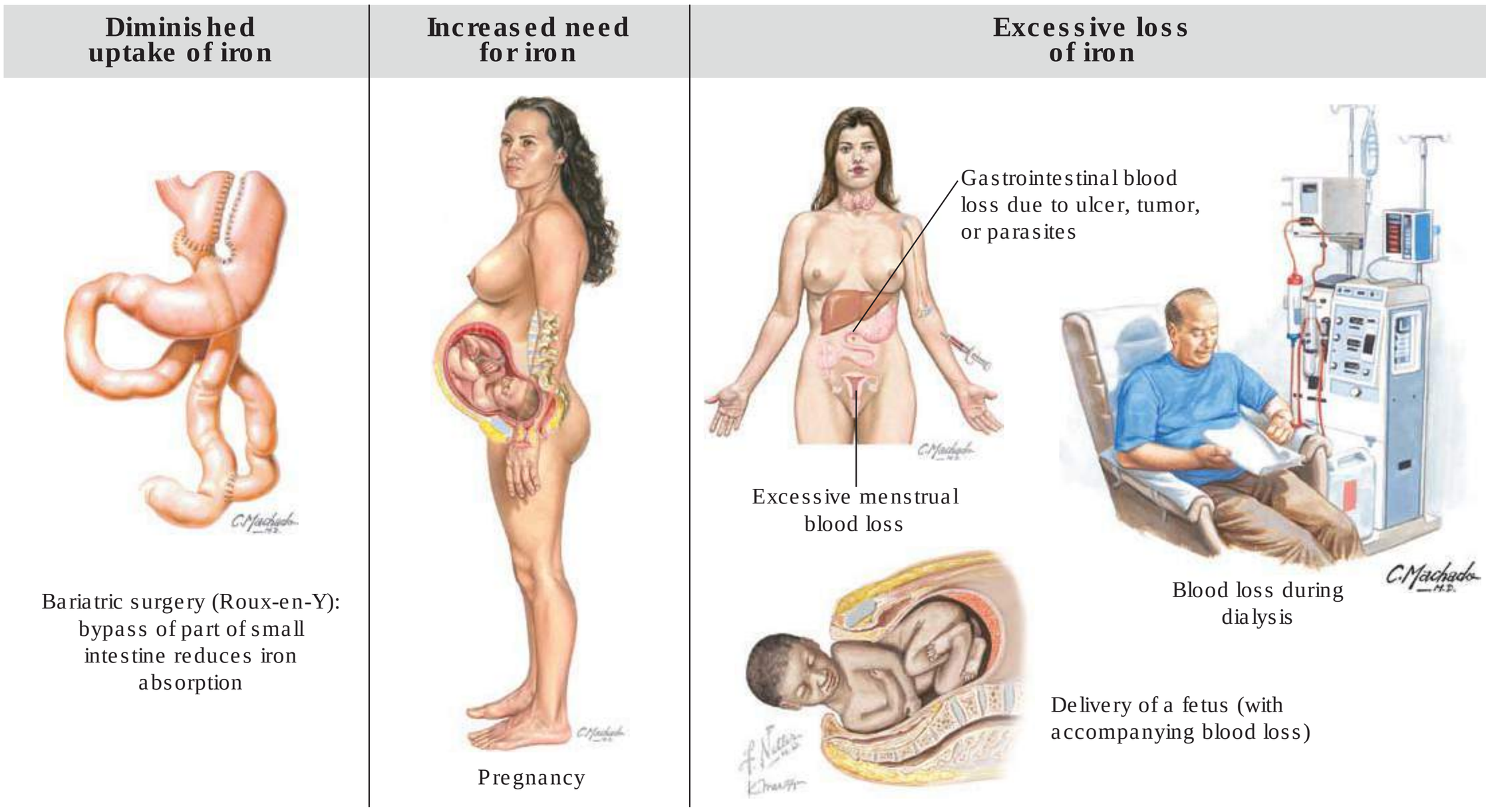


Fig. 15.9 Common causes of iron-deficiency anemia.

and their precursors, which decreases hemoglobin production and the rate of erythropoiesis, causing anemia. Women are more likely to be iron deficient than men due to lower food intake and higher iron losses associated with pregnancy, lactation, and menstruation. Excessive **gastrointestinal** or **menstrual blood loss** is the most common cause of iron-deficiency anemia in the Western world. Excessive gastrointestinal blood loss may be due to **ulcers**, **tumors**, **infection** with hookworms (which suck blood in the intestine), infection with Schistosoma (which causes bleeding), or several other intestinal diseases. Patients on **hemodialysis** lose blood during the dialysis procedure and often also have reduced intestinal iron absorption. Patients who have had **bariatric surgery** (a treatment for obesity) often develop iron deficiency because dietary iron bypasses the duodenum, the principal site of iron absorption in the small intestine. Similarly, patients who use a **proton pump inhibitor** to inhibit acidification of stomach contents have compromised iron absorption, such that they are often unable to take up sufficient iron if they have recurrent, increased losses of blood (e.g., heavy periods or blood donations).

Iron deficiency leads to a gradual depletion of iron in brain and muscle, and finally in the red bone marrow. Depletion of iron from the brain can lead to fatigue. Anemia is a late consequence of iron deficiency and also leads to fatigue.

Iron-deficiency anemia is associated with low blood hemoglobin and low hematocrit, abnormally small (microcytic) red blood cells (i.e., low MCV; see Chapter 16), hypochromic red blood cells (i.e., low MCHC), low mean red blood cell hemoglobin content (i.e., low MCH), increased red cell distribution width (anisocytosis, a measure of the size heterogeneity of red blood cells), low transferrin saturation (less than ~16%), low serum ferritin (less than ~12 µg/L), and increased free red blood cell protoporphyrin (protoporphyrin + Fe^{2+} → heme; see Chapter 14).

Iron deficiency can be treated with **oral iron** supplements or **parenteral iron** preparations. In the latter preparations, the toxicity of iron is reduced by complexing the iron with gluconate, dextran, sucrose, or another carbohydrate.

8.2. Anemia of Inflammation

Anemia of inflammation is a result of a chronic inhibition of iron transport by inflammation. As described in Section 3, chronic inflammation increases the secretion of **hepcidin**, which in turn inhibits iron release into the bloodstream from macrophages in the spleen, Kupf er cells in the liver, and epithelial cells in the intestine. T e resulting lower concentration of iron in the blood is less hospitable to bacteria. Chronic inflammation, via long-term lowering of serum iron (see Section 8.1.), leads to anemia.

9. IRON OVERLOAD

In iron-overloaded cells, iron participates in generating reactive radicals. Overt symptoms occur when total body iron stores are about 10 times the normal amount. Excess iron accumulates in the liver, heart, certain endocrine cells, skin, joints, and sometimes also in the central nervous system. Patients with hereditary hemochromatosis absorb too much

dietary iron. Other patients develop iron overload due to ineffective erythropoiesis, frequent red blood cell transfusions, or excessive treatment with intravenous iron preparations. Acute iron poisoning from excess intake of iron pills is seen chiefly in small children. Iron overload is treated by phlebotomy or with an iron chelator, depending on the etiology.

9.1. General Comments on Iron Overload

In patients who have iron overload, iron accumulates predominantly in the liver, pancreas, anterior pituitary, and (sometimes) the heart. Most of the excess iron is stored inside ferritin or hemosiderin. Severe systemic iron overload is associated with **cirrhosis**, **impaired insulin secretion, hypogonadism,** and **cardiomyopathy**. Some patients also develop a distinctive type of arthropathy and a hyperpigmentation of the skin (either brownish or grayish). Liver iron overload also greatly increases the risk of developing primary **liver cancer**. Men are much more likely to have iron overload than women. In men, higher testosterone levels reduce hepcidin secretion and thus increase iron absorption. Additionally, women, unlike men, lose extra iron with menstruation and pregnancy. Iron overload should be diagnosed before symptoms develop because most of the concomitant pathologic processes are irreversible by phlebotomy as a means of iron depletion.

Overt symptoms of systemic iron overload develop when the body contains ~15 to 40 g of iron instead of the normal 2 to 4 g. As patients with an iron overload develop excessive ferritin iron stores and associated liver damage, laboratory assays reveal increasing serum concentrations of **ferritin** and **liver enzymes**.

Iron overload may be toxic due to increased levels of free iron. Free iron is toxic, because Fe^{2+} and Fe^{3+} may generate highly reactive **radicals** (e.g., $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- +$ •OH; see Chapter 21). The radicals may react with proteins, lipids, and DNA in mitochondria and other subcellular structures, thus damaging them (see Section 9.6). For instance, •OH, the hydroxyl radical, may react with polyunsaturated fatty acids and create an avalanche of lipid peroxyl radicals (see Chapter 21).

Possible causes of iron overload are briefly explained in Table 15.2. The three major causes of iron overload are **hemochromatosis** (increased iron absorption), **frequent transfusion of erythrocytes**, and **ineffective erythropoiesis** (e.g., β-thalassemia major). **Excessive intravenous (IV) iron** and

Table 15.2 Causes of Iron Overload

Cause	Explanations
Excessive uptake of iron from diet	
Ineffective erythropoiesis Hemolytic anemia Chronic hypoxia	Hepcidin release is inhibited even when the liver is overloaded with iron (see Section 3). Hence, intestinal epithelial cells can release too much iron into the bloodstream.
Excessive dietary iron	When the diet contains >100 mg iron/day, the body's controls do not sufficiently reduce intestinal iron uptake.
Hemochromatosis	Hereditary excessive uptake of iron from the diet as a consequence of inadequate hepcidin synthesis and secretion or as a result of hepcidin-unresponsive ferroportin.
Adult onset	Due to mutant HFE protein or mutant TfR2 transferrin receptors (which participate in iron sensing), or due to mutant ferroportin (which exports iron from cells).
Juvenile onset	Symptoms at 10 to 30 years of age. Caused by mutant hepcidin or mutant hemojuvelin. Hemojuvelin is required for a regulated increase in transcription of the hepcidin gene.
Frequent blood transfusions	Heme iron in transfused blood bypasses the controls on the intestinal iron uptake. One unit of packed RBCs (volume is up to 250 mL) contains up to **250 mg** iron (i.e., up to 250 days' worth of normal intestinal iron uptake). Patients with severe hemolytic anemia, such as β-thalassemia major (see Chapter 17), are at greatest risk. Making matters worse in these patients, chronic anemia (by reducing hepcidin secretion) also increases iron uptake from the diet. (Note: Transfusions that replace erythrocytes lost during surgery are not harmful because they only replace lost erythrocytes and thus iron. Nonetheless, risks for infectious and noninfectious adverse events occur with every unit of transfused erythrocytes.)
Excessive infusion of iron	This is an iatrogenic disease due to excess iron given to anemic patients along with erythropoietin, such as to patients who receive regular hemodialysis.

chronic excessive uptake of iron from oral supplements are uncommon causes of iron overload.

9.2. Hemochromatosis

Hemochromatosis is a genetic condition of increased iron absorption that can result in an iron overload. Causes for hemochromatosis are listed in Table 15.2. Most patients with hemochromatosis take up only milligram amounts of excessive iron each day so that the common type of hemochromatosis (HFE hemochromatosis) typically manifests in 40- to 60-year-olds. A subgroup of patients with hemochromatosis, patients who have **juvenile hemochromatosis**, take up even larger amounts of dietary iron and therefore have an earlier onset of disease (as early as the first decade of life).

Since a patient with hemochromatosis releases too much iron into the blood, both the serum **iron** concentration and the **transferrin saturation** are already increased in the presymptomatic stage of the disease. The regulation of tissue iron uptake via the transferrin receptor 1 (TfR1) does not prevent the excessive uptake of iron from the blood into tissues. The liver, in particular, acts as a sink for excess iron, probably because it expresses the TfR2 transferrin receptor, which takes up transferrin-bound iron even when intracellular iron stores are already large.

The most common form of hemochromatosis is due to homozygosity for a missense mutation in the HFE gene. The mutant HFE protein fails to stimulate **hepcidin** synthesis and secretion adequately, leading to the excessive transfer of dietary iron from the intestine into the blood and organs in the body (Fig. 15.10). Most patients are of Northwest-European origin. Although there are many different HFE mutations, most (~85%) patients with HFE hemochromatosis are homozygous for a **C282Y** mutation in the HFE gene; a minority (~15%) is compound heterozygous for an **H63D** and a C282Y mutation, and ~2% are homozygous for H63D.

Some 5% to 10% of all European and U.S. whites are heterozygous for the C282Y mutation, and about 0.1% to 0.4% (i.e., up to 1 in 250) are homozygous. Heterozygosity for C282Y in the absence of H63D is usually benign. Although almost all homozygotes for the C282Y HFE allele accumulate too much iron, only 20% to 50% of these patients develop organ damage. Gender, alcohol consumption, iron intake, and genes other than HFE appear to modify the expression of the disease.

About 2% of Western European whites are homozygous for HFE H63D, and another 2% are compound heterozygous for C282Y and H63D. These persons rarely exhibit an iron overload. If they do develop an iron overload, they usually have concomitant alcoholic liver disease, nonalcoholic fatty liver, or chronic hepatitis (especially hepatitis C). It is thought that the combination of these HFE genotypes and one of these liver conditions leads to decreased hepcidin secretion and therefore increased iron absorption.

Persons can be tested for the C282Y and the H63D mutations as a means of diagnosing the hemochromatosis risk before iron overload and associated symptoms occur.

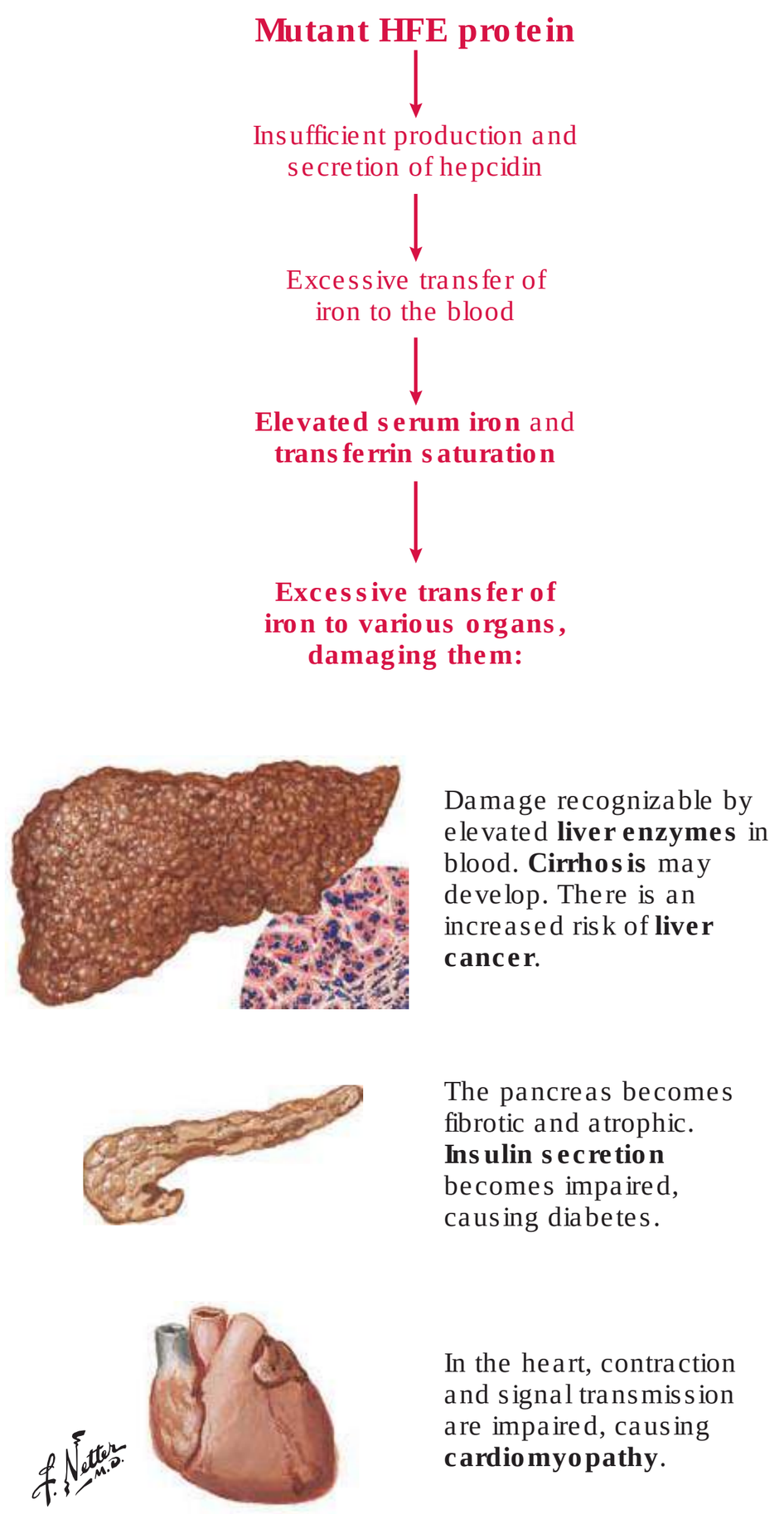


Fig. 15.10 Abnormal regulation of iron metabolism in hereditary HFE **hemochromatosis.**

The most common form of **juvenile hemochromatosis** is due to mutations in the gene (HJV) that encodes **hemojuvelin**. The disease is inherited in autosomal recessive fashion. Like mutant HFE protein, mutant hemojuvelin does not permit adequate stimulation of hepcidin synthesis, which in turn leads to the excessive transfer of dietary iron from the intestine into the blood.

With the exception of patients who have anemia, hereditary hemochromatosis is usually treated with weekly **phlebotomy**. At each session, ~500 mL of blood is removed, the erythrocytes of which contain 200 to 250 mg of heme iron. Progress is followed by monitoring serum ferritin.

9.3. Iron Overload With Blood Transfusions

Patients who need frequent **erythrocyte transfusions** due to insufficient erythropoiesis often develop a secondary iron

overload. For instance, patients who have β-thalassemia major need regular transfusions of erythrocytes to survive. Patients who have sickle cell anemia occasionally also need packed red blood cell transfusions. As senescent transfused cells are degraded, all of their iron is recovered, stored, and sometimes reused. In patients with β-thalassemia major, iron accumulation from transfusions and excessive absorption from the diet (stimulated by inef ective erythropoiesis) is so large that it presents a major challenge for treatment. In patients who have iron overload due to blood transfusions, the iron is removed by **chelation** therapy (see Section 9.4).

9.4. Iron Chelation Therapy

T ree major drugs are used to chelate (i.e., bind) iron and excrete a drug-iron complex. **Desferrioxamine** (**deferoxamine**) is usually given intravenously, often overnight and via a small battery-driven pump. **Deferasirox** and **deferiprone** are administered orally. All three drugs bind Fe^{3+} in blood plasma, and the resulting complex is excreted via the urine and/or the bile and feces.

9.5. Hemosiderosis and Siderosis

T e term **hemosiderosis** is used to describe pathologic amounts of **hemosiderin** in cells. Hereditary and secondary hemochromatosis are associated with hemosiderosis of the liver, heart, and pancreas. **Pulmonary hemosiderosis** is due to iron accumulation in macrophages from chronic local bleeding, a disease that is very dif erent from the hemochromatoses described above.

T e term **siderosis** is used for a disease that results from the **inhalation** of pathogenic amounts of iron fumes or iron dust, commonly by welders or miners. **Siderosis** is uncommon and usually benign.

9.6. Acute Iron Poisoning

Clinically apparent, acute iron poisoning occurs with the intake of greater than 20 mg of Fe/kg body weight. Iron poisoning is the most common cause of lethal poisoning in children under the age of 6 years. As little as 550 mg iron can be fatal to a small child. Supplements and chewable multivitamin preparations are the usual sources of iron. A single tablet of ferrous sulfate often contains ~60 mg iron. A high luminal concentration of iron damages the gastrointestinal mucosa.

Symptoms of systemic toxicity occur when the total serum iron exceeds the iron-binding capacity of transferrin (i.e., above a serum iron of about 300 µg/dL). A high serum iron concentration (usually more than 500 µg/mL) may be associated with metabolic acidosis, cardiovascular collapse, coma, and liver failure.

Treatment of iron poisoning often involves gut decontamination and chelation of iron in blood with intravenous **desferrioxamine**.

SUMMARY

- In healthy human beings, red blood cells contain the body's largest store of iron (~2.4 g, in the form of heme-iron). Additional iron (~1.4 g) is found in the liver, spleen, and muscle as heme-iron or as iron stored inside macrophages as ferritin or hemosiderin.
- Most iron is needed for the synthesis of heme by reticulocytes and their precursors. When red blood cells lyse in the circulation or when they are degraded, macrophages in the spleen and Kupf er cells in the liver recover the iron from the heme. T is iron is shuttled back to cells that actively synthesize heme.
- T ere are no regulated means of iron excretion; iron is lost mainly through bleeding.
- Men need to absorb ~1 mg iron per day, menstruating or lactating women ~1.5 mg iron per day, and pregnant women ~2.5 mg iron per day to maintain a normal iron balance. T e iron content of the daily diet needs to be 10 to 20 times larger than the daily need.
- Iron is absorbed mostly in the duodenum and jejunum and stored temporarily in the cytosol in a ferritin complex. From there, iron is released into the bloodstream through the iron transporter ferroportin, unless the hormone hepcidin inhibits this transport by inducing the degradation of ferroportin.
- T e liver secretes hepcidin in response to inflammation and high liver iron stores. Hepcidin inhibits the release of iron not only from intestinal epithelial cells, but also from Kupf er cells, macrophages in the spleen, and macrophages in the bone marrow.
- Inef ective erythropoiesis, anemia, and anoxia inhibit hepcidin release. T ese conditions favor the absorption of iron from the diet.
- In blood plasma, iron is bound to transferrin. Peripheral cells endocytose the transferrin-iron complex via specific surface transferrin receptors, retrieve the iron, and release transferrin back into the circulation.
- Determinations of the total iron-binding capacity of serum, the iron content of serum, and the concentration of ferritin in serum allow assessment of the iron status of a patient.
- Iron deficiency causes anemia through inadequate heme and hemoglobin synthesis. Iron deficiency is most often caused by gastrointestinal or menstrual bleeding but can also be caused by excessive blood donation. Patients with chronic inflammation secrete too much hepcidin and thus release too little iron from the intestine, liver, and spleen; eventually, this leads to anemia.
- Hemochromatosis is a hereditary condition of excessive iron absorption that sometimes leads to iron accumulation in the liver, pituitary, pancreas, and the heart. Hereditary hemochromatosis is usually associated with abnormally low concentrations of circulating hepcidin. Most often, HFE is mutated. Normal HFE protein plays a role in stimulating hepcidin synthesis and secretion in response to elevated liver iron stores. Abnormally low concentrations of

circulating hepcidin may also be the result of chronic ineffective erythropoiesis, anemia, or hypoxia.
- Iron overload can be secondary to frequent erythrocyte transfusions that are needed to treat diverse types of chronic anemia.
- Large, excessive amounts of supplemental iron, especially in small children, damage the gastrointestinal mucosa and increase serum iron to levels that exceed the iron-binding capacity of transferrin. Unbound iron then damages the vasculature, brain, and liver, which may be lethal.

FURTHER READING

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Review Questions

1. A 52-year-old woman presents with arthritis in her hands. Her serum ferritin is 510 µg/L (normal, 15-200 µg/L), her serum iron is 180 µg/dL (normal, 50-170 µg/dL), and her total iron-binding capacity is 240 µg/dL (normal, 220-420 µg/dL). Further testing reveals that she is homozygous for the C282Y mutation of the HFE gene. Radiographs of her hands are consistent with an abnormality of iron homeostasis as the cause of arthritis. Which of the following would be the most appropriate treatment for this patient?

A. IV deferoxamine (= desferrioxamine)
B. IV iron dextran
C. phlebotomy
D. Oral deferasirox
E. Oral iron sulfate

2. A 49-year-old woman presents with hypermenorrhea (menorrhagia; excessive menstrual flow). Her past history is significant for gastric bypass surgery three years prior. Laboratory analysis of a blood sample reveals the following:

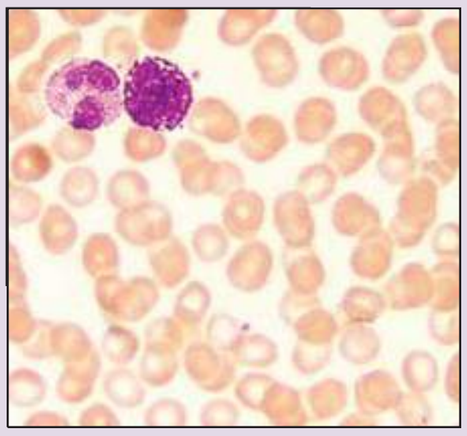
- Hemoglobin (g/dL): 7.8
- MCV (fL): 67
- Serum iron (µg/dL): 20 (normal, 50-170)
- Total iron-binding capacity (µg/dL), 400 (normal, 220-420)
- Serum ferritin (ng/mL): 20 (normal, 12-150)
- Serum folate (ng/mL): 10 (normal, 2.5-20)
- Serum cobalamin (B_{12}; pg/mL): 650 (normal, 200-900)

Which of the following is the most likely explanation for the abnormal laboratory findings?

A. Excessive dietary iron intake
B. Folate deficiency
C. Hemochromatosis
D. Iron deficiency
E. Pernicious anemia

3. Serum hepcidin is not yet measured in routine clinical care, but it is measured for research purposes. Serum from patients with which one of the following diseases shows the highest concentration of hepcidin?

A. Untreated juvenile hemochromatosis
B. Inflammation
C. Treated HFE hemochromatosis
D. Iron-deficiency anemia



Chapter 16 Erythropoiesis, Hemoglobin Function, and the Complete Blood Count

SYNOPSIS

- Erythropoiesis is the production of new red blood cells (erythrocytes). Erythrocytes are made in the bone marrow under the regulation of the hormone erythropoietin, which is secreted from the kidneys, depending on the local concentration of oxygen.
- Hemoglobin inside erythrocytes carries oxygen from the lungs to peripheral cells. The binding of oxygen to hemoglobin is regulated by pH, the concentration of carbon dioxide, and the concentration of 2,3-bisphosphoglycerate inside red blood cells.
- Blood loss and diseases that affect the lungs, kidneys, bone marrow, heart, or erythrocytes, may affect oxygen delivery to tissues and the production of red blood cells (Fig. 16.1).
- Important information regarding a patient's health status can be derived from the complete blood count (CBC) and the fraction of immature erythrocytes. The quality of tissue oxygenation can also be judged by the color of a patient's skin.

LEARNING OBJECTIVES

For mastery of this topic, you should be able to do the following:

- Describe erythropoiesis and its regulation, and list commonly measured laboratory values that provide insight into this process.
- List treatment options to increase the rate of erythropoiesis in an anemic patient.
- Explain how hemoglobin binds oxygen in the lungs and how it delivers oxygen to tissues, thereby paying attention to pH, partial CO_2 pressure, and 2,3-bisphosphoglycerate.
- Explain how oxygen is delivered from the mother to the fetus, taking into account the structural differences between adult and fetal hemoglobin and O_2 binding affinity.
- Compare and contrast the structure and the O_2 binding properties of hemoglobin and methemoglobin. Identify means to lower a patient's elevated fraction of methemoglobin.
- Explain the deleterious effects of CO on hemoglobin function. Describe how an increase in environmental CO can lead to tissue hypoxia.
- Identify the effects of smoking on blood and hemoglobin. List smoking-induced changes in typical blood laboratory values.
- Explain the role of amyl nitrite and sodium nitrite in the treatment of cyanide poisoning.
- Use a patient's skin color to infer an abnormality in the fraction of deoxyhemoglobin in the patient's blood.

1. ERYTHROPOIESIS

Under the influence of growth factors (e.g., erythropoietin), stem cells in the bone marrow give rise to erythroblasts that lose their nuclei to become reticulocytes. The reticulocytes are released into the bloodstream where they continue to synthesize hemoglobin until they lose their RNA, mitochondria, and endoplasmic reticulum to become mature red blood cells. The kidneys secrete erythropoietin depending on the renal oxygen concentration. Renal oxygen sensing involves the oxygen-dependent hydroxylation of hypoxia-inducible factors (HIFs) that regulate transcription. With certain patients, exogenous recombinant erythropoietin effectively stimulates erythropoiesis, so that these patients do not need blood transfusions.

1.1. Location of Erythropoiesis

Except during embryonic and early fetal life, the **bone marrow** produces virtually all red blood cells (Fig. 16.2). In young children, all bones produce red blood cells. In normal adults, erythropoiesis (the production of erythrocytes) is restricted to the axial skeleton and the proximal ends of the humeri and femora. Adult patients with a markedly increased rate of erythropoiesis have an increased amount of bone marrow and also produce red blood cells in additional bones. Children with an increased rate of erythropoiesis may develop **bone deformities,** such as tower skull and frontal bossing.

1.2. Major Stages in Erythropoiesis

Stem cells divide asymmetrically (one daughter cell remains a stem cell, the other becomes partially differentiated), and their offspring eventually divide symmetrically to produce erythroblasts (Fig. 16.3). **Hematopoietic stem cells** in the bone marrow (through asymmetric division) give rise to **progenitor cells**, which can circulate in the blood and populate new sites in the bone marrow. The original progenitor cells can give rise to progenitor cells that have a more restricted cell fate. Erythroid progenitor cells eventually give rise to **proerythroblasts**, which in turn give rise to **erythroblasts** (i.e., **normoblasts**).

Erythroblasts begin synthesizing **hemoglobin**. Over a period of a few days, erythroblasts become smaller and lose their nucleus to become marrow **reticulocytes**, which are released into the bloodstream. In the bloodstream, a reticulocyte synthesizes about one-third of the final amount of hemoglobin of a mature red blood cell (globin mRNAs are synthesized 2 to 3 days earlier, before enucleation; proteins bind specifically to the 3′-untranslated region of α- and β-globin mRNA and thereby protect the RNA from degradation; see also Chapter 6). After ~1 day in the circulation, reticulocytes lose their mRNA, endoplasmic reticulum, and mitochondria, and the reticulocytes become **mature erythrocytes**. A mature red blood cell stays in circulation for about 120 days. Then, macrophages in the spleen or liver degrade it (for the degradation of heme, see Chapter 14; for the reuse of iron, see Chapter 15).

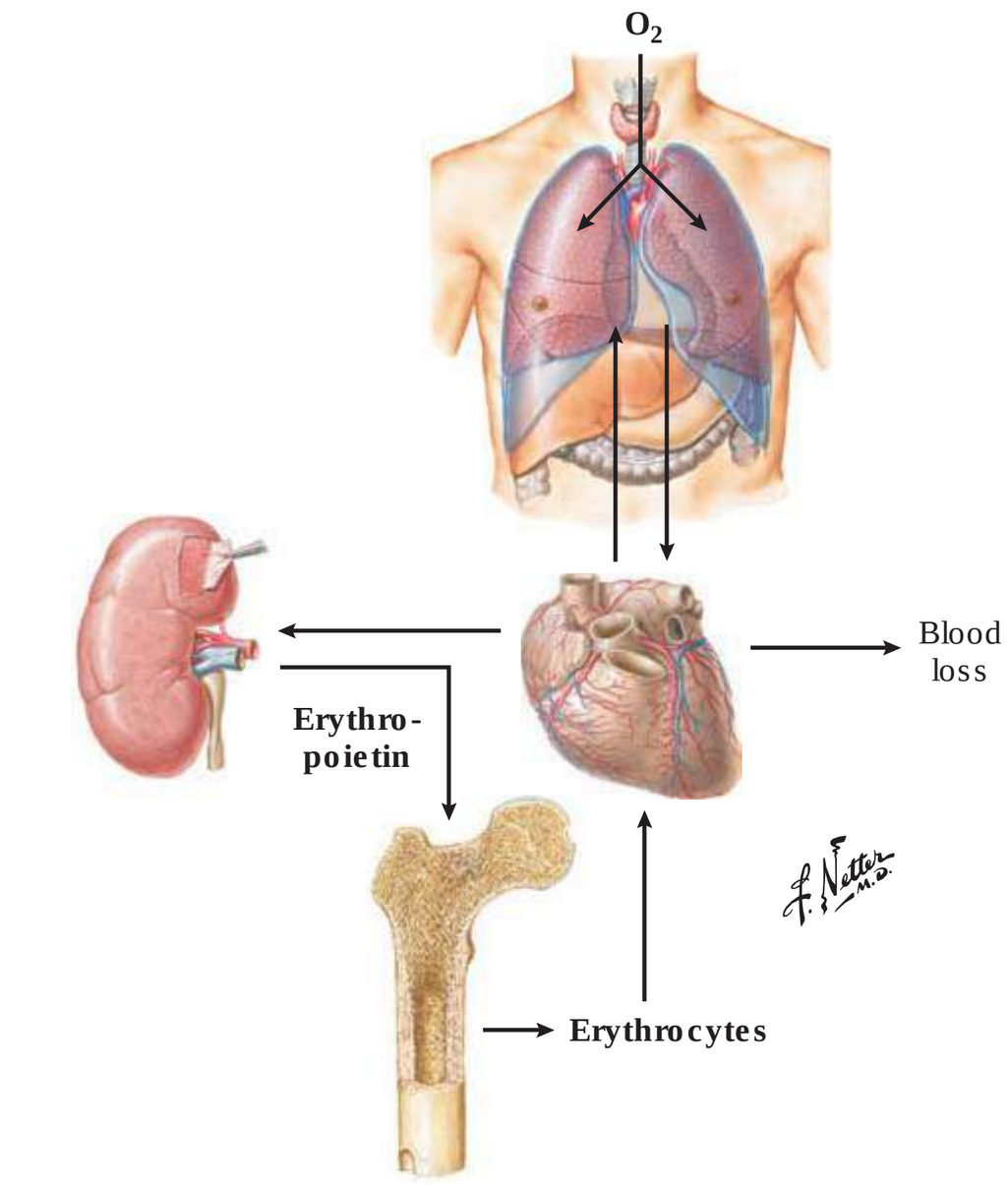


Fig. 16.1 **Factors that influence oxygen transport in the human body.**

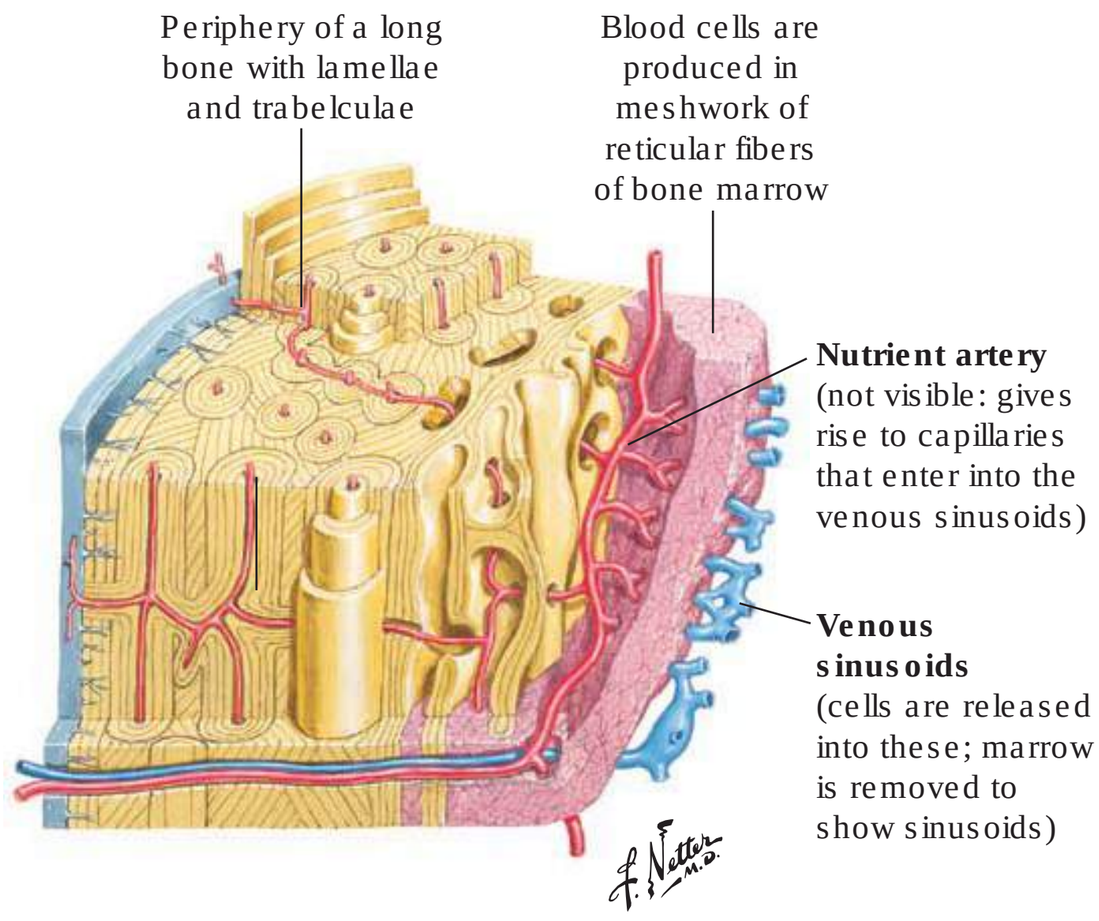


Fig. 16.2 **Architecture of bone and bone marrow.**

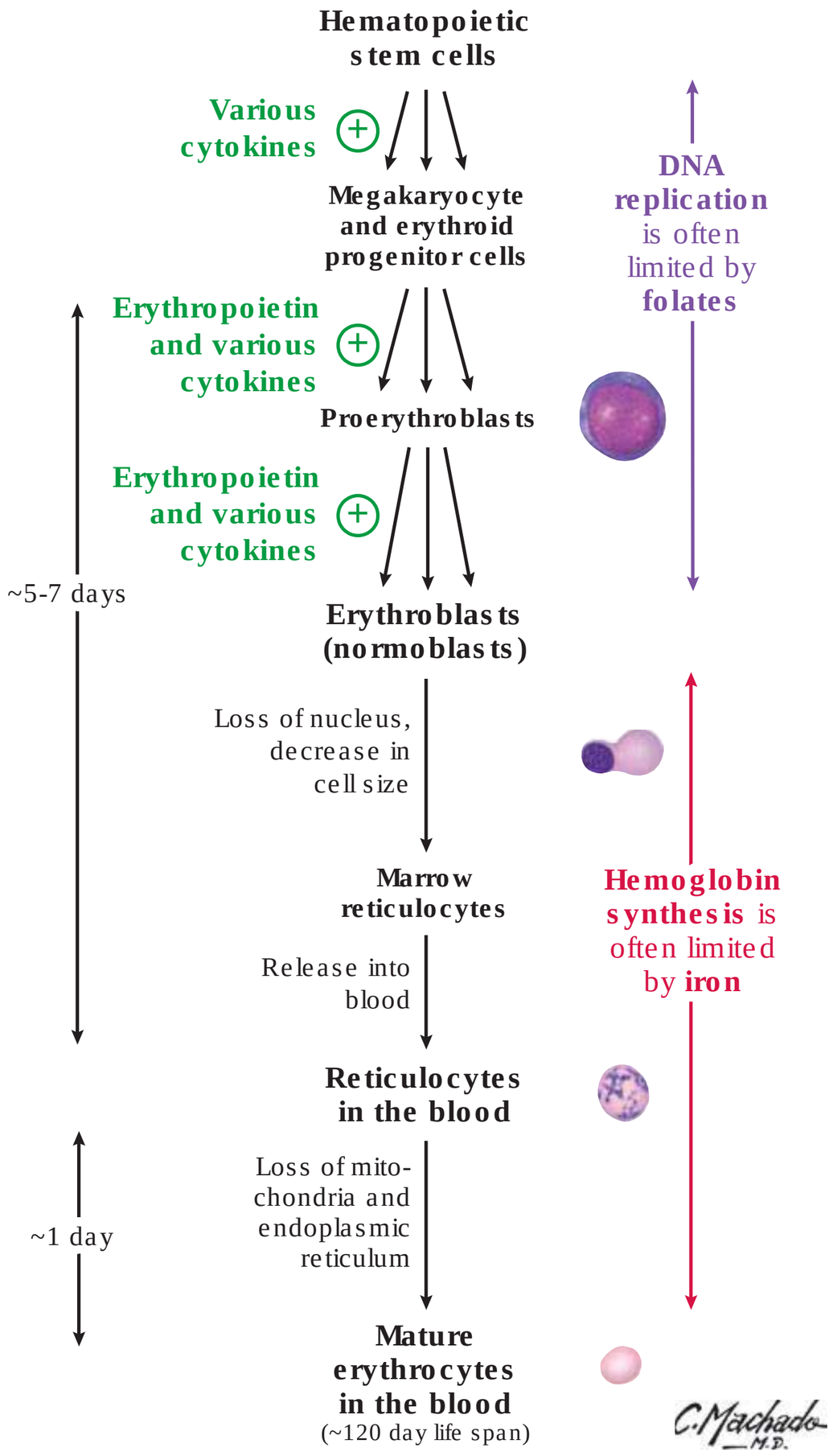


Fig. 16.3 **Erythropoiesis in bone marrow.**

In most patients, supplements of a folate and iron lead to a modest increase in hematocrit. Folates are required for adequate DNA replication in dividing cells (see Fig. 16.3 and Chapters 36 and 37). Iron is required for heme synthesis (see Chapters 14 and 15). In patients with a pronounced deficiency of either folates or iron, red blood cell precursors die at an abnormally high rate.

1.3. Role of Erythropoietin in the Control of Red Blood Cell Production

Red blood cell production is controlled by a large number of cytokines, among them **erythropoietin**, granulocyte-macrophage colony-stimulating factor (GM-CSF), and insulin-like growth factor 1 (IGF-1). Cytokines are an ill-defined, large family of proteins that control cell growth and differentiation. In contrast to hormones, cytokines are not secreted by a discrete gland (e.g., the pancreas), but by a number of cells in the body. Erythropoietin is the cytokine that usually exerts the greatest control over the rate of red blood cell production.

Cells in the cortex and outer medulla of the **kidneys** (Fig. 16.4) synthesize erythropoietin and release it into the bloodstream. These cells are in a somewhat oxygen-poor region with an oxygen concentration that is rarely affected by physiological changes in blood flow. Erythropoietin is secreted from endothelial cells in capillaries around tubules and/or

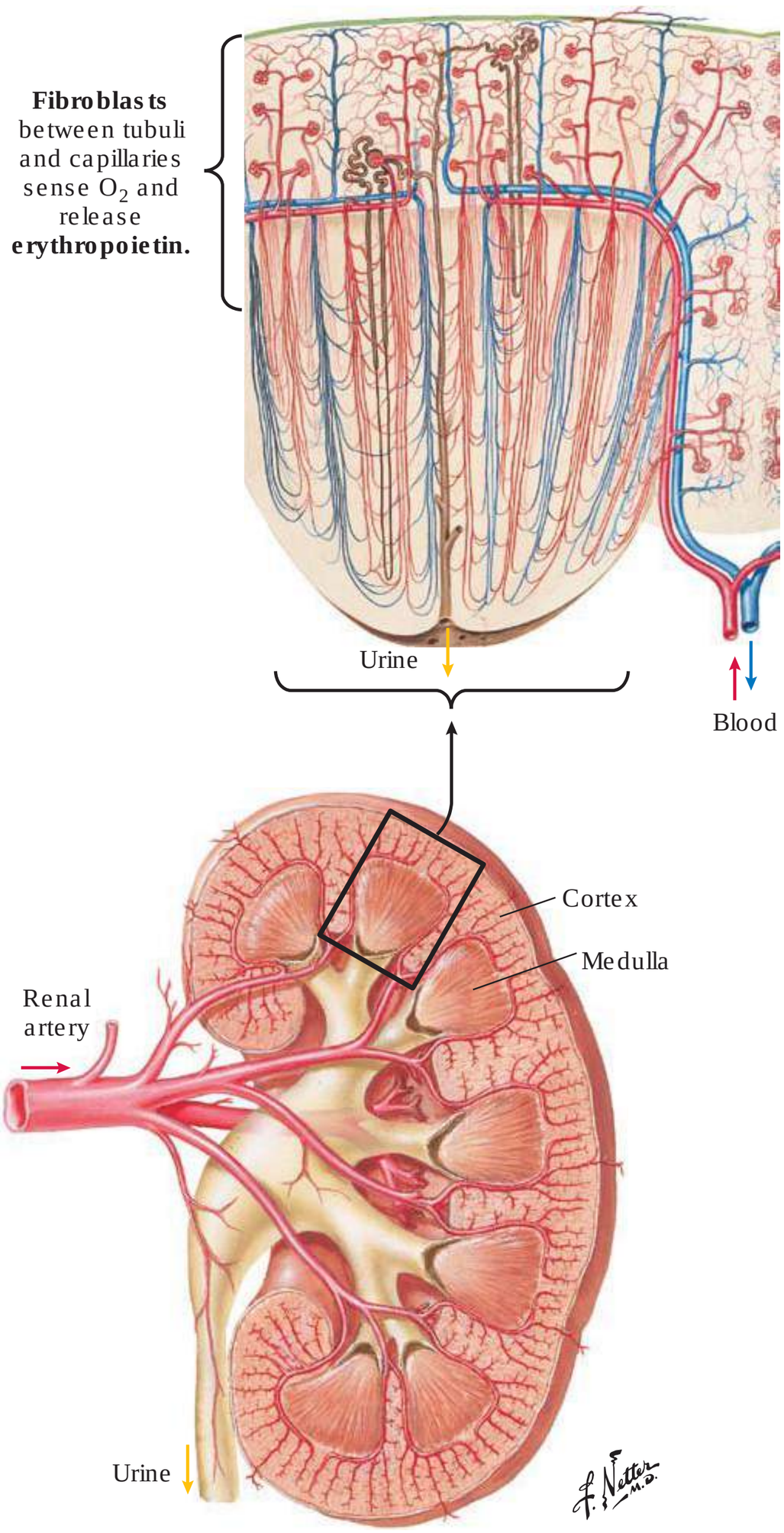


Fig. 16.4 Peritubular cells in the kidneys secrete erythropoietin into the bloodstream in an oxygen-dependent fashion.

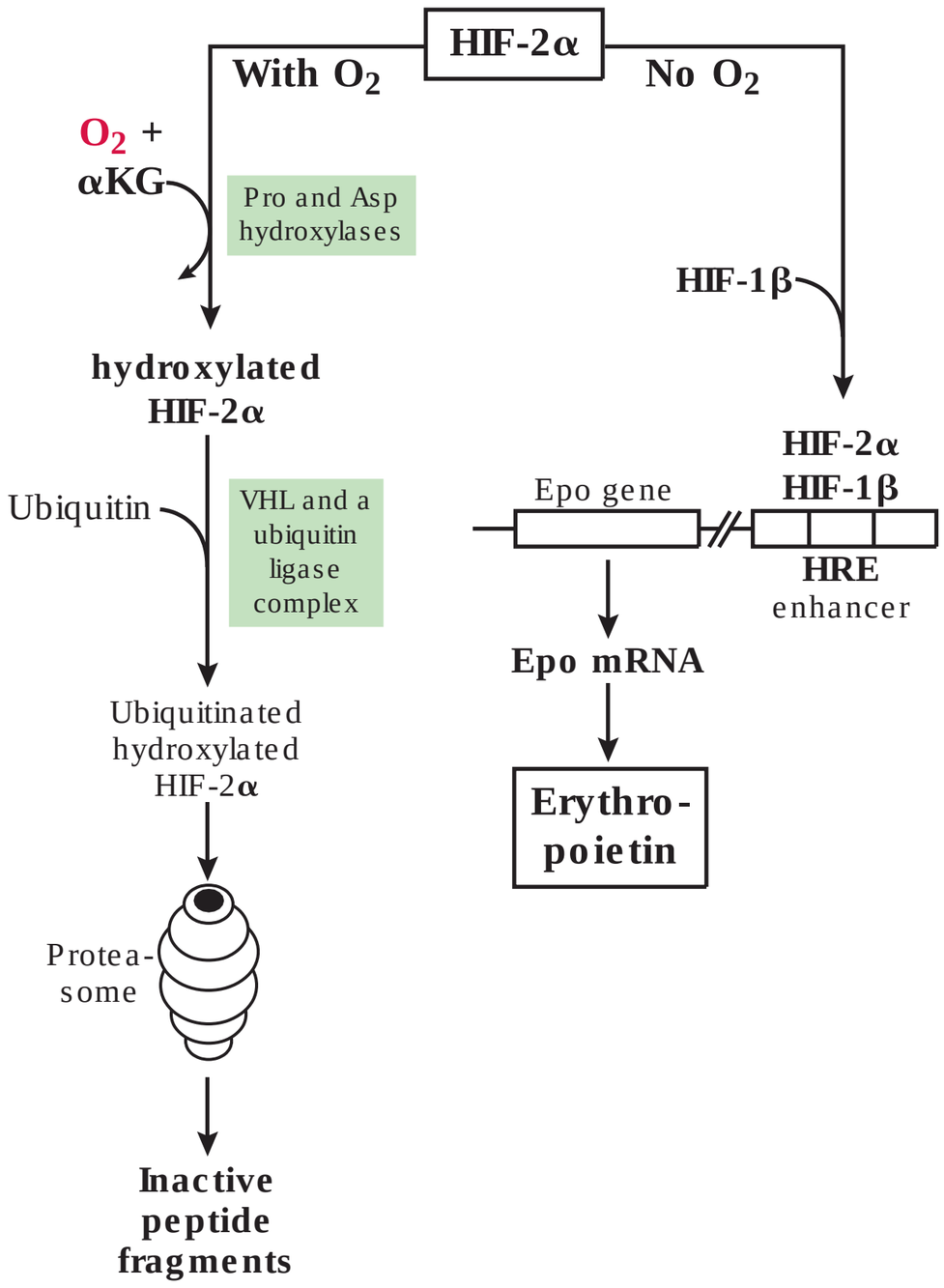


Fig. 16.5 Role of hypoxia-inducible factor (HIF) and von Hippel-Lindau factor (VHL) in the O_2-dependent regulation of transcription of the erythropoietin (Epo) gene. HRE, hypoxia-response element.

fibroblasts between capillaries and tubules. T e rate of erythropoietin secretion depends on the concentration of **oxygen** in these peritubular cells (see below). In patients with severe hypoxia (a condition of insufficient oxygen in tissues), the **liver** also synthesizes and releases some erythropoietin.

1.4. Oxygen-Dependent Secretion of Erythropoietin

Oxygen sensing in the kidneys and other cells involves the O_2-dependent modification of HIF-1α, HIF-2α, or HIF-3α transcription factors. **HIF** stands for **hypoxia-inducible factor**. HIF-α factors are constantly synthesized in the cytoplasm (Fig. 16.5). HIF-2α regulates the transcription of the erythropoietin gene. In the absence of **oxygen**, HIF-2α moves into the nucleus, binds to HIF-1β, and stimulates transcription of the erythropoietin gene. In the presence of a normal concentration of oxygen, almost all HIF-2α is destroyed in a pathway that involves hydroxylation by an **O_2-dependent prolyl hydroxylase** or **asparaginyl hydroxylase** and ubiquitination by a protein complex that includes the **von Hippel-Lindau factor** (**VHL**). Mutant, nonfunctional VHL favors tumorigenesis, as does the inhibition of prolyl hydroxylases by the citric acid cycle intermediates **succinate** and **fumarate,** and of asparaginyl hydroxylases by **citrate** or **oxaloacetate** (see Chapter 22).

Besides a role in regulating the transcription of the erythropoietin gene in the kidneys, HIF-1α and HIF-2α modify the transcription of more than 1000 other genes in a variety of organs. Increased HIF-dependent transcription favors anaerobic glycolysis and angiogenesis, for example. HIF dimers mainly work through a **hypoxia-response element** (**HRE**) in the promoter or enhancer region of a target gene.

1.5. Clinical Uses of Recombinant Erythropoietin

Recombinant erythropoietin can be injected into a patient to stimulate erythropoiesis; this therapy decreases the need for

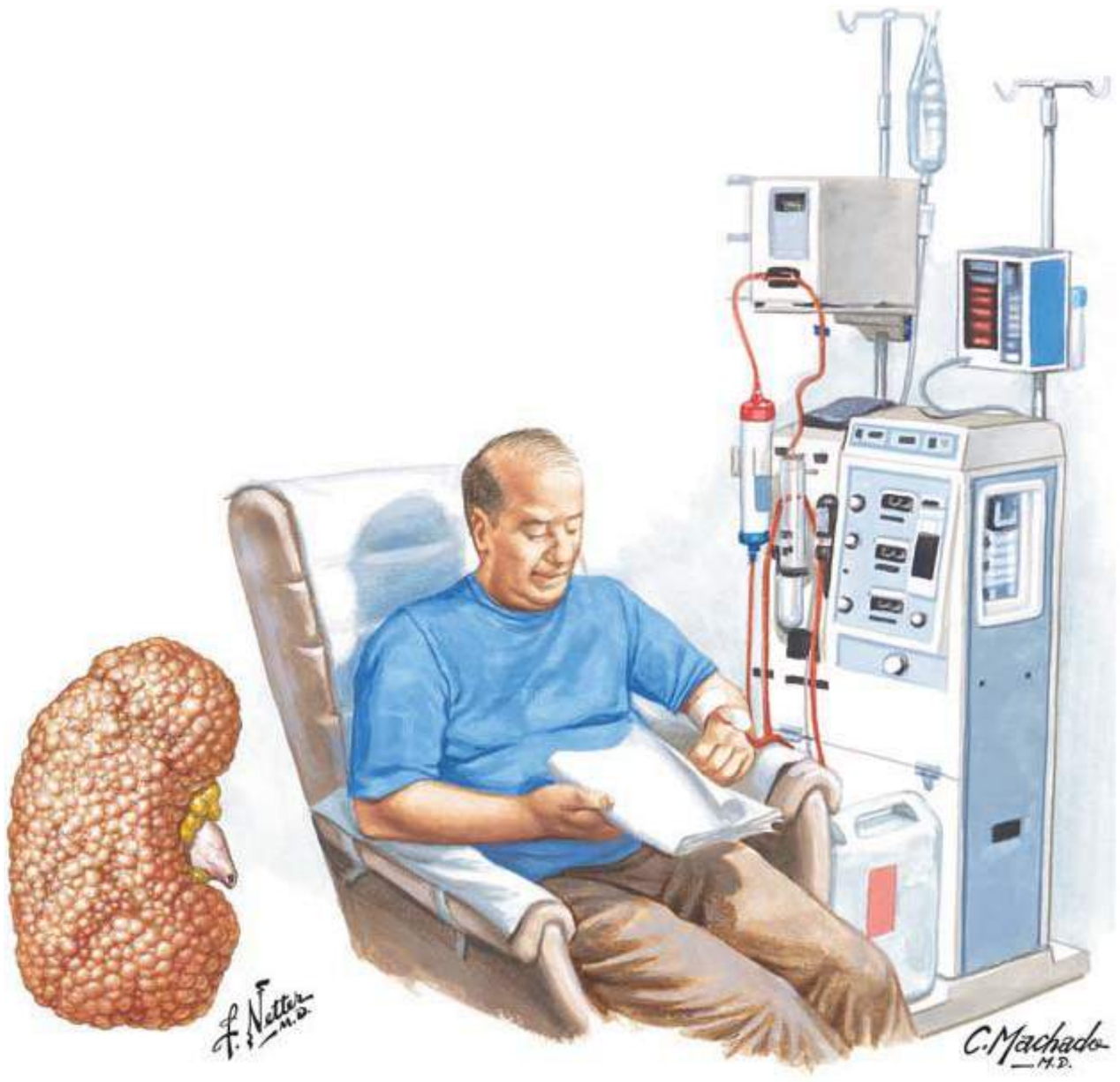


Fig. 16.6 **Erythropoietin can greatly reduce the need for blood transfusion in patients with late-stage chronic glomerulonephritis.**

blood transfusions. Patients who have lost most of their **kidney function** and receive dialysis (Fig. 16.6) no longer secrete enough erythropoietin and become deficient in red blood cells (by itself, the liver does not synthesize sufficient erythropoietin). T ese patients are often treated with injections of **recombinant erythropoietin**, which is isolated from cultured mammalian cells that express the human erythropoietin gene. Erythropoietin can also be used to reduce the need for blood transfusion in other clinical settings, such as radiation therapy or chemotherapy for cancer.

2. PROTEIN COMPOSITION OF HEMOGLOBIN

T e normal hemoglobins are tetramers o*f* two di*ff*erent types o*f* globins. Each globin binds heme as a prosthetic group. T e most commonly encountered normal hemoglobins are hemoglobin A (composition: $\alpha_2\beta_2$) and hemoglobin F (composition: $\alpha_2\gamma_2$).

T ere are several types of **hemoglobin**, each with a dif erent **globin** composition. All types of hemoglobin contain four globins per molecule of hemoglobin (Fig. 16.7). Globins are 142- to 147-amino acid proteins. Each globin molecule has a hydrophobic pocket that binds one **heme** molecule (see Chapter 14) noncovalently. Globins constrain the reactivity of heme so that the heme-Fe^{2+} atom can reversibly bind O_2. Globins and heme are synthesized during the approximately week-long dif erentiation of erythroblasts into erythrocytes (see Fig. 16.3). Mature red blood cells cannot synthesize

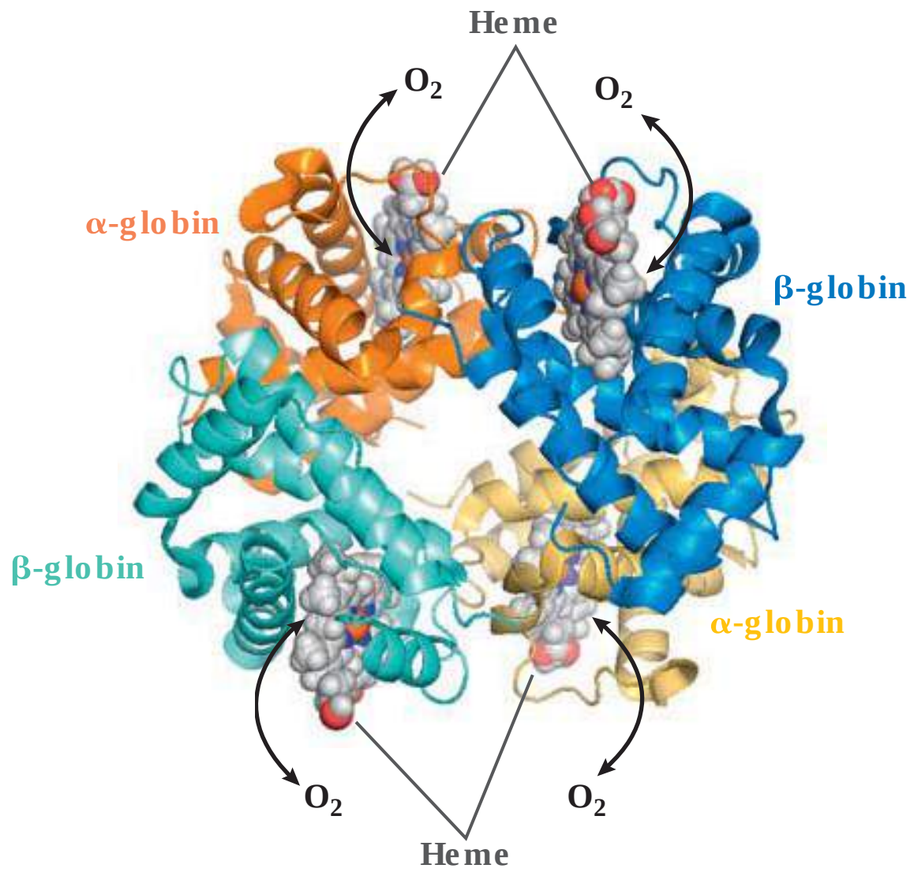


Fig. 16.7 **X-ray crystallographic structure of deoxygenated hemoglobin A.** Heme atoms are shown as spheres. Based on Protein Data Bank file 1HBB. (From Kavanaugh JS, Rogers PH, Case DA, Arnone A. High-resolution x-ray study of deoxyhemoglobin Rothschild 37 beta Trp—Arg: a mutation that creates an intersubunit chloride-binding site. *Biochemistry*. 1992;31:4111-4121.)

globins. T ere are four clinically important types of globin, all derived from dif erent genes: α, β, γ, and δ. T ere are two genes for α-globin, two genes for γ-globin, and there is one gene each for β- and δ-globin. All normal hemoglobins of newborns and adults consist of two α-globins that are paired

Table 16.1 Hemoglobins in Normal Blood

Name	Globin Composition	Comments
Hemoglobin **A** (HbA)	$\alpha_2\beta_2$	Usually makes up ~**96%** of the hemoglobin in an adult and ~25% of the hemoglobin of a newborn (these numbers include HbA_{1c}; see below).
Hemoglobin **A_{1c}** (HbA_{1c})	$\alpha_2\beta_2$ (glycated)	HbA_{1c} is **glycated** HbA. The glycation (a reaction of glucose with the N-terminal valine of β-globin) is a slow, nonenzymatic reaction (see Section 8.3 in Chapter 39). Its rate depends on the concentration of **glucose** in the blood. HbA_{1c} usually makes up about 5% of the hemoglobin in an adult. Patients with **diabetes** usually have an elevated HbA_{1c}, while patients who lose their red blood cells prematurely have a decreased HbA_{1c}.
Hemoglobin **A_2** (HbA_2)	$\alpha_2\delta_2$	Usually makes up 2%–3% of the hemoglobin in an adult. A higher percentage is found in patients with **β-thalassemia**.
Hemoglobin **F** (HbF)	$\alpha_2\gamma_2$	Usually makes up ~**75%** of the hemoglobin in a **newborn** (at term). Inside red blood cells, it has a higher affinity for O_2 than HbA. This helps the fetus extract oxygen from the mother's blood. HbF makes up ~1% of the hemoglobin in a healthy adult.

The hemoglobins in this table have a molecular weight of about 64,500. The abnormal hemoglobins C, E, H, and S are discussed in Chapter 16.

with two β-, γ-, or δ-globins. Table 16.1 lists these normal hemoglobins.

3. OXYGEN BINDING BY HEMOGLOBIN AND MYOGLOBIN

Each hemoglobin molecule can bind up to *four* molecules *of* oxygen. In the lungs, hemoglobin is almost saturated with oxygen. In peripheral tissues at rest, hemoglobin releases about one-*fourth* o*f* its oxygen. Hemoglobin releases much more oxygen in tissues that have a low concentration o*f* oxygen, a low pH, or a high concentration o*f* CO_2. On a time scale o*f* days, changes in the concentration o*f* bisphosphoglycerate inside red blood cells adjust the a*ffi*nity o*f* hemoglobin *f*or oxygen. Myoglobin stores O_2 inside muscle cells.

3.1. Cooperative Binding of O_2 to Hemoglobin

Hemoglobin carries oxygen from an environment of relatively high concentration (i.e., the alveoli, or the placenta) to an environment of an intermediate concentration (i.e., the capillaries of the tissues). Oxygen has a relatively low solubility in water. T anks to the presence of hemoglobin inside red blood cells, whole blood may contain ~70 times more oxygen than blood plasma. Oxygen (O_2) moves across cells and cell membranes by simple passive dif usion (i.e., without a transporter). T e high surface/volume ratio of the disk shape of red blood cells facilitates the equilibration of solutes between the cytosol and the extracellular space.

Although the terms oxidation and oxygenation sound similar, they have very dif erent meanings. **Oxidation** refers to a change in redox state. Oxidation of hemoglobin usually refers to the process of oxidizing heme-Fe^{2+} to heme-Fe^{3+}, forming methemoglobin (see Section 5.2). **Oxygenation** refers to the addition of O_2; oxygenated hemoglobin is called **oxyhemoglobin**. In oxyhemoglobin, O_2 reversibly binds to heme-Fe^{2+} atoms in hemoglobin. Each hemoglobin can bind up to four molecules of O_2 (one O_2 per heme; see Fig. 16.7). T e release of O_2 from a molecule is called **deoxygenation**; deoxygenated hemoglobin is called **deoxyhemoglobin**.

As 1, 2, or 3 molecules of O_2 bind to hemoglobin, conformational changes in the protein facilitate binding of a subsequent molecule of O_2. T e process by which occupation of one binding site af ects the ligand affinity of a neighboring binding site is referred to as **cooperativity**. For proteins with cooperativity toward the binding of a ligand (e.g., hemoglobin and O_2), a graph of the ligand concentration versus the fraction of liganded protein is S shaped (Fig. 16.8). For enzymes with a cooperativity toward a substrate, a curve of substrate concentration versus enzyme activity is likewise S shaped (see Fig. 10.4).

Hemoglobin is oxygenated in the capillary bed of the alveoli (Fig. 16.9). In humidified air at sea level and 37°C, the **partial pressure of oxygen**, the **pO_2**, is ~150 mm Hg (or 150 torr, see Fig. 16.8; the total air pressure is ~760 mm Hg; the pO_2 can be thought of as a concentration of O_2). In the alveolar gas space, the concentration of O_2 is ~100 mm Hg; it is lower than that of humidified air due to the competition between blood that removes O_2 and ventilation that adds O_2.

T e **P_{50}** value of hemoglobin for O_2 is the partial pressure of oxygen (pO_2), at which hemoglobin is half-maximally saturated with oxygen (see Fig. 16.8). As red blood cells move between tissues, the P_{50} changes in response to dif erences in temperature and the concentrations of H^+ and CO_2 (see Section 3.2). A low P_{50} enhances O_2 loading in the lungs, while a high P_{50} enhances O_2 unloading in the tissues. (In a matter of days, the concentration of 2,3-bisphosphoglycerate also regulates the P_{50}; see Section 3.3.)

T e pO_2 of heavily exercising muscle is about 25 mm Hg.

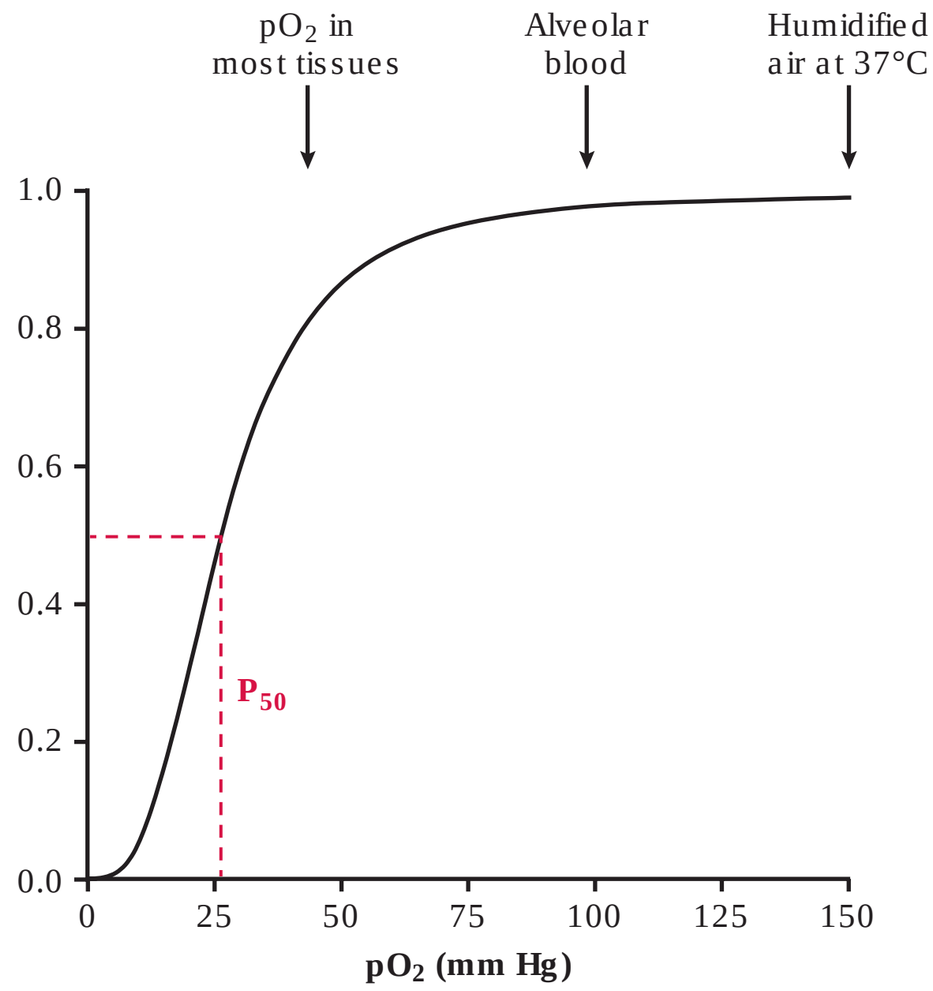


Fig. 16.8 Cooperative binding of O_2 to hemoglobin near sea level. The curve is representative of the conditions in the pulmonary venous effluent blood. The P_{50} value of hemoglobin for oxygen binding is the pO_2 at which hemoglobin is half-maximally saturated with O_2. 1 mm Hg = 1 torr.

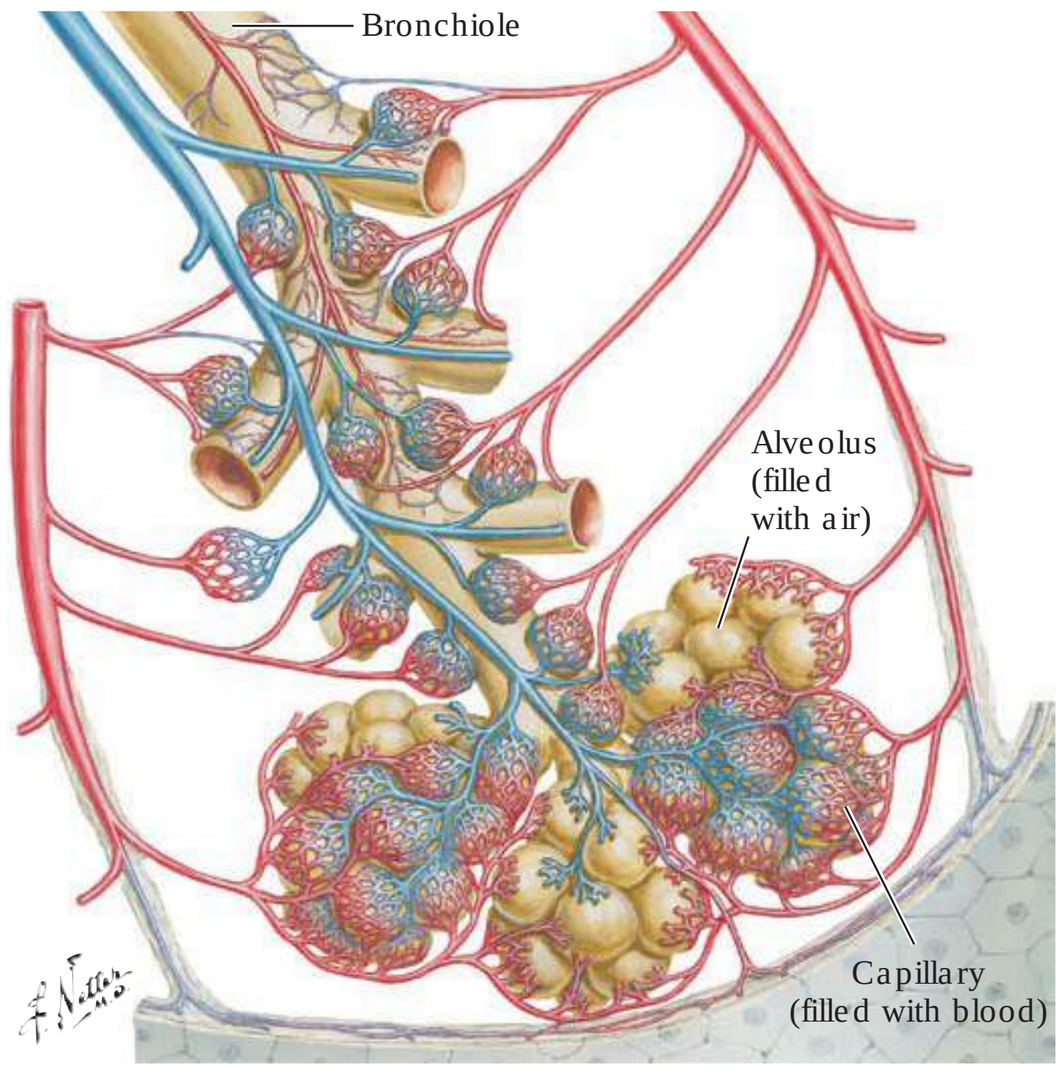
3.2. Short-Term Regulation of the O_2 Affinity of Hemoglobin

In a time frame of microseconds, the affinity of hemoglobin for O_2 is regulated by temperature, the concentration of hydrogen ions, and the concentration of CO_2. An increase in any of these parameters favors unloading of O_2 from oxyhemoglobin (Fig. 16.10).

A 4°F (2°C) increase in **temperature** (e.g., in exercising muscle or during illness) increases the P_{50} by ~15%, thus promoting the release of oxygen.

As blood flows through tissues, the **pH** drops, which decreases the binding of O_2 to hemoglobin (see Fig. 16.10). All tissues produce acids such as H_2CO_3 (from CO_2, which is mostly produced by the citric acid cycle; see Chapter 22) and lactic acid (mostly from anaerobic glycolysis; see Chapter 19). In the extended fasting state, the liver also releases significant quantities of ketone bodies (i.e., acids) into the blood (see Chapter 27). When tissues release acids into the blood, the acids dissociate to form H^+. As the pH of blood decreases (i.e., as the concentration of H^+ increases), certain amino acid residues on hemoglobin (e.g., the side chain of His-146 on β-globin and the amino terminus of α-globin) become protonated, and the affinity of hemoglobin for oxygen decreases. T is ef ect is called the **Bohr ef ect**.

T e increased concentration of **CO_2** in the peripheral circulation lowers the affinity of hemoglobin for O_2. CO_2 can reversibly carboxylate the N-terminus of β-globin (and less frequently of α-globin) to form **carbamino-hemoglobin** (globin-NH_3^+ + CO_2 ↔ globin-NH-COO^- + $2H^+$). T e H^+



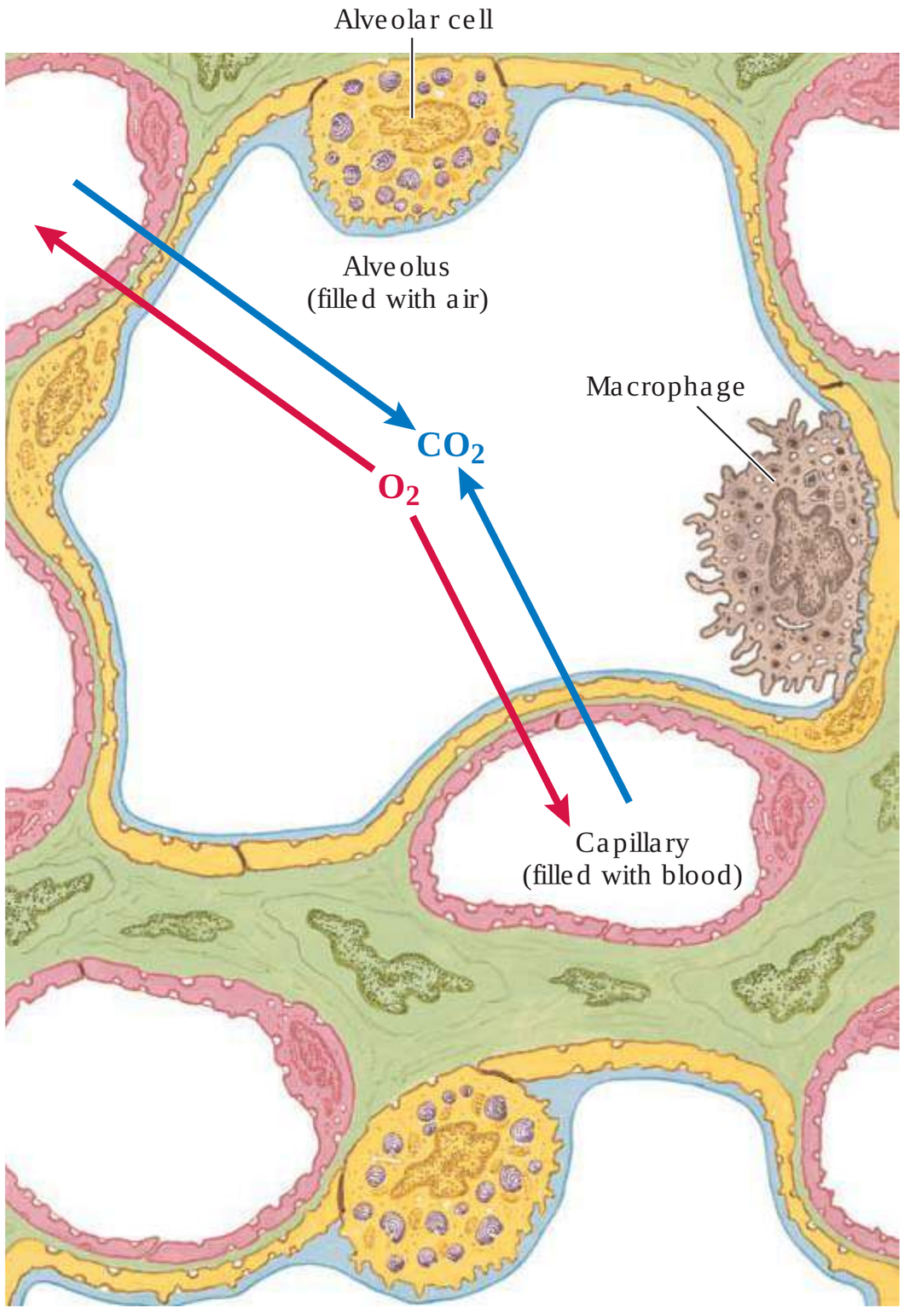


Fig. 16.9 Passive diffusion of O_2 and CO_2 between the alveoli and the capillaries in the lungs. Arrows indicate the normal direction of net diffusion of O_2 and CO_2.

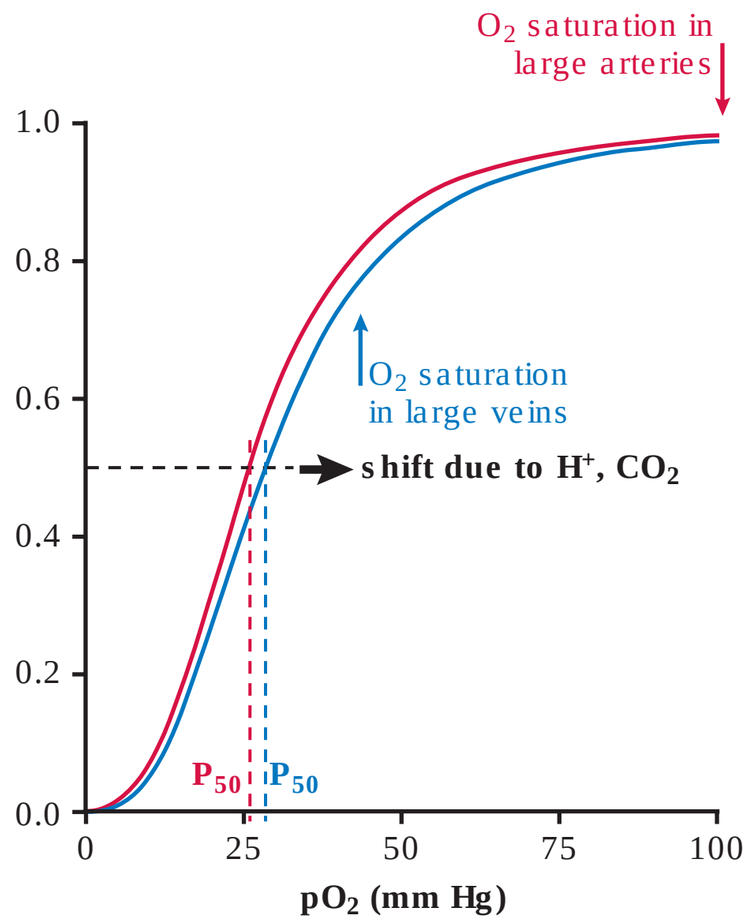


Fig. 16.10 Relationship between the pO_2 and the oxygen saturation of hemoglobin A in a healthy patient at sea level. The drop in pO_2 and the increase in P_{50} occur in the capillaries. In a healthy person at rest, hemoglobin loses about one-fourth of its oxygen as red blood cells pass through the capillaries of peripheral tissues.

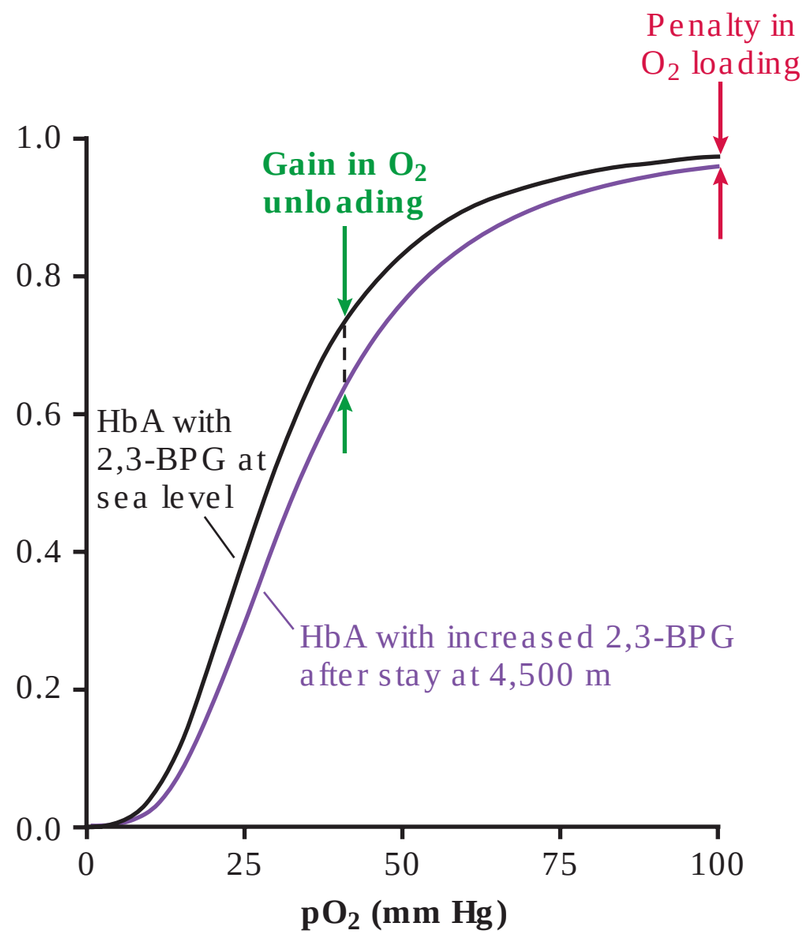


Fig. 16.11 An altitude-induced increase in the concentration of 2,3-bisphosphoglycerate (2,3-BPG) decreases the affinity of hemoglobin for O_2. Data are based on changing elevation from 0 to 4,500 m for 5 days and assuming a Hill coefficient of 2.9.

release by this carboxylation reaction contributes to the Bohr effect. Carboxylation decreases the affinity of hemoglobin for O_2 (see Fig. 16.10). Conversely, in the lungs, the oxygenation of hemoglobin decreases the affinity of hemoglobin for CO_2 (i.e., the N-terminus of β-globin is harder to carboxylate). This effect is called the **Haldane effect**.

Of all the CO_2 transported from the tissues back to the lungs, only ~15% is transported via carboxylated hemoglobin, while ~85% of the CO_2 is transported as CO_2, H_2CO_3, and HCO_3^- in solution.

The Bohr effect (pH) and the Haldane effect (CO_2) cause a lowering of the affinity of hemoglobin for O_2 in the peripheral tissues compared with the lungs (i.e., the P_{50} of hemoglobin for O_2 is higher in the peripheral tissues than in the lungs; see Fig. 16.10). This favors the loading of O_2 in the lungs and unloading of O_2 in the tissues.

In a patient who is at rest, the pH and CO_2 have a small effect on O_2-unloading in the capillaries (see Fig. 16.10). In maximally exercising muscle on the other hand, or when blood flow to a tissue is impaired severely, pH and CO_2 can cause a more substantial decrease in the affinity of hemoglobin for O_2. Under extreme circumstances, almost all O_2 dissociates from hemoglobin.

H^+, CO_2, and 2,3-bisphosphoglycerate (see below) each affect the affinity of hemoglobin for O_2 via a site that is different from the O_2 binding site; such effects are called **allosteric effects**. Other proteins and enzymes are also subject to allosteric effects (see Chapter 10). Cooperativity usually implies the presence of allosteric effects (e.g., as one globin subunit in deoxyhemoglobin binds O_2, it undergoes a change of conformation that is transmitted to other globins).

3.3. Long-Term Regulation of the O_2 Affinity of Hemoglobin

The concentration of **2,3-bisphosphoglycerate (2,3-BPG)** optimizes the O_2 affinity of hemoglobin in the long run (i.e., in a matter of days). This is important because inefficient O_2 loading or unloading leads to an increase in the total mass of red blood cells, the viscosity of blood, cardiac work output, and the risk of thrombosis. 2,3-BPG is produced from 1,3-BPG, a metabolite in glycolysis (see Chapter 19). 2,3-BPG binds into a cavity between the amino termini of the β-globins of hemoglobin and thereby lowers the affinity of hemoglobin for oxygen. 2,3-BPG has a much greater affinity for deoxyhemoglobin than for oxyhemoglobin.

Persons who live at a high **altitude** have a higher concentration of 2,3-BPG in their red blood cells than people who live at sea level; the increased concentration of 2,3-BPG in red blood cells improves O_2 delivery. While the pO_2 of humidified air at sea level is ~150 mm Hg, it is only ~100 mm Hg at an elevation of 3,000 m (~10,000 ft). As is evident from Fig. 16.11, a 2,3-BPG-induced change in pO_2 has a greater effect on hemoglobin oxygenation in the steep portion of the curve than in the flat portion. Such a change affects O_2 unloading more than O_2 loading. In addition, increased ventilation at higher elevations makes up for decreased O_2 loading in the lungs. Upon changing altitude, it takes ~20 hours for a half-maximal change in the P_{50} of hemoglobin for O_2 to occur. The trade-off for altitude-induced deoxygenation of hemoglobin is a decreased oxygen reserve in blood.

An increase in the concentration of 2,3-BPG inside red blood cells (and a corresponding decrease in the oxygen affinity of hemoglobin) is also seen in patients with **hypoxia** and in patients with (usually partial) **blocks in glycolysis** distal to

1,3-BPG and 2,3-BPG (e.g., pyruvate kinase deficiency; see Chapter 19).

A decrease in the concentration of 2,3-BPG inside red blood cells (and a corresponding increase in the oxygen affinity of hemoglobin) is observed in patients with a **low blood pH** (e.g., lactic acidosis or ketoacidosis), in patients with (usually partial) **blocks in glycolysis** proximal to 1,3-BPG and 2,3-BPG (e.g., **hypophosphatemia),** and in patients with 2,3-BPG mutase deficiency (rare; see Chapter 19).

Chronic mountain sickness is characterized by a low oxygen saturation of hemoglobin, high hematocrit, hypertrophy of the right side of the heart, and pulmonary hypertension. The disease develops over a period of years of living at high altitude (usually greater than 3,000 m or 9,800 ft above sea level). The disease has a prevalence of up to 15% in the South American highlands. Affected patients have less than about 85% oxygen saturation of hemoglobin in the arterial blood. The hypoxia drives erythropoiesis so that patients have more than about 20 g hemoglobin/dL blood (equal to a hematocrit of >60%). The hypoxia also increases the output of the heart, blood pressure in the lungs, and the rate of breathing. Long-term treatment involves moving to a lower altitude.

Acute mountain sickness is seen in persons who move to a higher altitude and shortly afterward complain of fatigue, headache, and nausea.

High-altitude pulmonary edema is accompanied by excess fluid around the lungs, which reduces a person's ability to breathe. The edema progressively worsens the condition. If present, pulmonary edema usually develops after a 1- to a 4-day stay at more than about 2,500 m (8,200 ft) above sea level. The arterial oxygen saturation is often around 50% to 75%. Treatment involves immediate descent to a lower altitude. Administration of pure oxygen also helps. Without treatment, the affected person may die within hours.

High-altitude cerebral edema is accompanied by excess fluid in the brain, impairing brain function. High-altitude cerebral edema occurs in a similar setting as high-altitude pulmonary edema, is treated in a similar fashion, and is equally dangerous.

3.4. Maternal-Fetal Exchange of O_2

In the placenta, maternal blood oxygenates the fetal blood, which has a considerably lower oxygen concentration than the maternal blood. In the presence of physiological concentrations of 2,3-BPG, the P_{50} for HbF is about 25% lower than the P_{50} for HbA. While the mother's blood in the placental vein has a pO_2 of ~40 mm Hg, the fetus' blood in the umbilical vein (i.e., oxygenated blood exiting from the placenta) has a pO_2 of ~35 mm Hg. The fetus' blood in the umbilical artery (i.e., blood returning to the placenta for oxygenation) has a pO_2 of ~20 mm Hg.

3.5. Binding of O_2 to Myoglobin

Myoglobin is a heme-containing protein the size of a single globin subunit of hemoglobin. Each molecule of myoglobin can reversibly bind one molecule of O_2; the P_{50} is ~4 mm Hg. Myoglobin shows no cooperativity in binding O_2. The affinity of myoglobin for O_2 is not influenced by 2,3-BPG. Myoglobin may have a role in storing O_2. It is found chiefly in the cytoplasm of cardiac and skeletal muscle.

4. TRANSPORT AND BUFFERING FUNCTION OF CO_2 AND HCO_3^- IN BLOOD

CO_2 and bicarbonate *form* the major pH buffer in blood.

In peripheral tissues, carbon dioxide (CO_2) passively diffuses across the plasma membrane bilayer into erythrocytes, and bicarbonate (HCO_3^-) enters erythrocytes via an anion exchanger (Fig. 16.12). As mentioned above, most of the CO_2 produced by tissues is carried back to the lungs as bicarbonate ions dissolved in water. A small fraction of the CO_2 binds to hemoglobin and allosterically decreases its affinity for oxygen. The plasma membrane of red blood cells contains an anion exchanger, band 3, that exchanges HCO_3^- for chloride (Cl^-) across the lipid bilayer; this transporter can move HCO_3^- in either direction.

The drug **acetazolamide** inhibits carbonic anhydrase in several tissues. The drug is used in the treatment of a variety of diseases, such as high-altitude sickness, glaucoma, and congestive heart failure. In the kidneys, acetazolamide leads to decreased excretion of protons and an increased loss of HCO_3^-; as a consequence, the pH of blood decreases.

CO_2 and HCO_3^- form the major pH buffer in blood plasma and red blood cells. In water, CO_2 is spontaneously hydrated to carbonic acid according to the reaction $CO_2 + H_2O \leftrightarrow H_2CO_3$. At equilibrium, there is much more CO_2 than H_2CO_3. Inside red blood cells, **carbonic anhydrase** catalyzes this reversible hydration of CO_2 and thereby shortens the time needed to establish equilibrium. H_2CO_3 spontaneously dissociates: $H_2CO_3 \leftrightarrow HCO_3^- + H^+$. The combination of these two reactions, the system $CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+$,

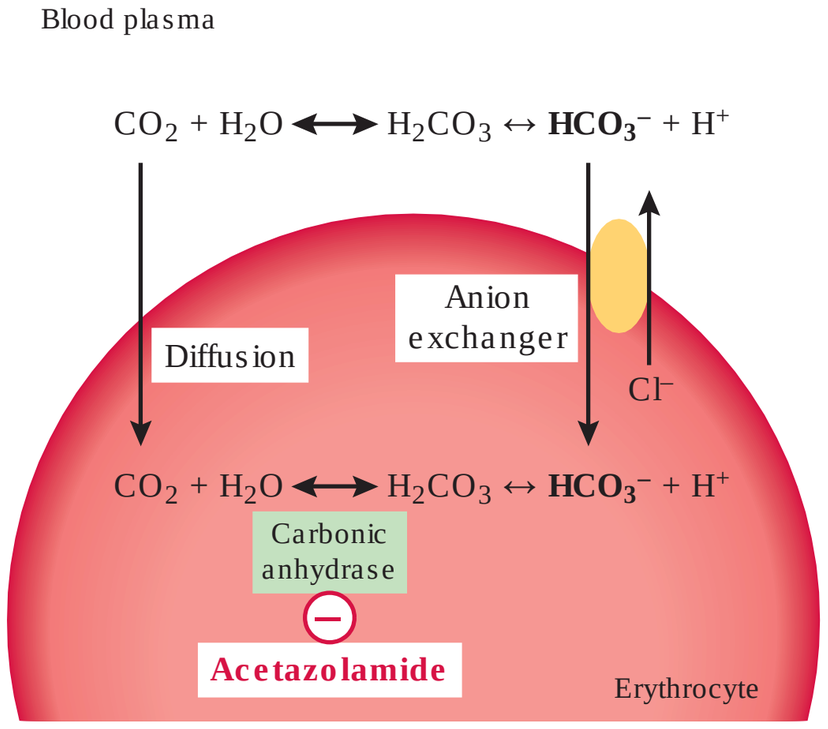


Fig. 16.12 Transport of CO_2 and HCO_3^- across the red blood cell plasma membrane in the peripheral capillary bed. In the lungs, the flux is reversed.

forms the major pH buf er of blood. T e pH is determined by the ratio of the concentrations of HCO_3^- and CO_2 according to the **Henderson-Hasselbalch** equation:

$$pH = pK_{CO2} + \log([HCO_3^-]/[CO_2])$$

pK_{CO2} is about 6.1 at 37°C. As an example, at pH 7.1, the term $\log[(HCO_3^-)/(CO_2)]$ equals 1; hence, there is 10 times more HCO_3^- than CO_2.

Analyses of blood for **blood gasses** are most often carried out with arterial blood at 37°C. T e following parameters are often measured: pH, pO_2 (partial pressure of O_2), pCO_2 (partial pressure of CO_2), the concentration of HCO_3^-, and saturation of hemoglobin with oxygen (SaO_2 for arterial blood, SvO_2 for venous blood). Approximate normal values for these parameters in arterial blood are pH, 7.35 to 7.45; pO_2, 75 to 105 mm Hg; pCO_2, 33 to 45 mm Hg; and concentration of HCO_3^-, 22 to 28 mEq/L. T e saturation of hemoglobin with oxygen decreases with altitude; SaO_2 is usually greater than 90%. If the pO_2 of peripheral blood is abnormally low, the patient has **hypoxia**.

If the pH of the blood is abnormally low, the patient has an **acidemia**; if the pH is abnormally high, the patient has an **alkalemia**.

Patients who have a **metabolic acidosis** have a low blood pH and low concentrations of HCO_3^- and CO_2 in the blood. Tissues of these patients release H^+ into the blood at an abnormally high rate. T e H^+ may stem, for instance, from the release of lactic acid or the ketone bodies acetoacetic acid and β-hydroxybutyric acid (see Chapter 27). In the blood, the concentration of HCO_3^- becomes low because HCO_3^- is consumed by buf ering H^+ ($HCO_3^- + H^+ \rightarrow H_2CO_3 \rightarrow CO_2$). T e low pH stimulates breathing and concomitant exhaling of CO_2 to a lower than normal blood pCO_2. T is is an attempt to normalize the pH by defending the ratio $[HCO_3^-]/[CO_2]$ of the above Henderson-Hasselbalch equation. At a lower rate than the lungs, the kidneys can also rid the blood of acid by deprotonating H_2CO_3, excreting H^+ as part of NH_4^+, and returning HCO_3^- to the blood (see Chapter 35); however, in metabolic acidosis, the rate of renal HCO_3^- production is much smaller than the rate of HCO_3^- consumption by buf ering.

5. CARBON MONOXYHEMOGLOBIN AND METHEMOGLOBIN

Carbon monoxide is a poisonous gas that binds tightly to hemoglobin and impairs oxygen delivery. Other chemicals, such as oxidizing drugs or the vasodilator nitric oxide (NO), can oxidize the heme iron in hemoglobin, generating methemoglobin, which is not suitable *f*or tissue oxygenation either. An enzyme inside red blood cells reduces the heme-iron o*f* methemoglobin and thus restores hemoglobin. Since methemoglobin binds cyanide, methemoglobin is sometimes generated pharmacologically in patients who have cyanide poisoning.

5.1. Carbon Monoxyhemoglobin

Carbon monoxide (CO) binds to heme-Fe^{2+} in hemoglobin with ~200-fold greater affinity than O_2 (i.e., at 0.1% CO and 20% O_2, CO and O_2 bind to hemoglobin about equally well). CO stems from incomplete combustion of carbon-containing substances; it is odorless and colorless. Hemoglobin with CO bound to all four heme irons is called **carbon monoxy-hemoglobin** (hemoglobin with less than 3 CO is called **partial** carbon monoxy-hemoglobin or **hetero**-carbon monoxy-hemoglobin). Carbon monoxy-hemoglobin is also called **carboxy-hemoglobin**. Partial carbon monoxy-hemoglobin has an abnormally high affinity for O_2 such that it cannot be used for physiological oxygen delivery.

Elevated amounts of CO bound to hemoglobin are found in patients who smoke or are otherwise exposed to elevated concentrations of CO. **Smoking** 20 cigarettes per day leads to a mean CO saturation of hemoglobin of ~7% before bedtime; the CO saturation decreases to ~2% to 3% by waking. In a **pregnant** woman who is a heavy smoker, fetal and maternal blood contain the same amounts of carbon monoxy-hemoglobin because CO dif uses from the maternal blood across the placenta to the fetal blood.

Carbon monoxide poisoning decreases a patient's capacity for O_2 delivery to the tissues. In nonsmokers, a carbon monoxy-hemoglobin fraction of greater than 3% to 4% indicates CO poisoning; in smokers, the threshold is about 10%. To favor the binding of O_2 to heme in place of CO, CO-poisoned patients are treated with pure O_2 at normal pressure or in a pressure chamber (commonly at about three times the normal pressure). CO is physiologically produced in the human body and functions as a signaling molecule. CO signaling pathways contribute to the pathology of carbon monoxide poisoning in a yet poorly understood fashion. In the United States about 3% of CO-poisoned patients who get to a hospital die from the poisoning.

5.2. Oxidation of Hemoglobin to Methemoglobin

In oxygenated hemoglobin, the heme-Fe^{2+} (with O_2 bound to it) can be oxidized to the Fe^{3+} state, producing oxidized hemoglobin, which can no longer be used for the delivery of oxygen. Hemoglobin with four heme-Fe^{3+} is called **methemoglobin**, while hemoglobin with 1 to 3 heme-Fe^{3+} is called **partial** methemoglobin, or **hetero**-methemoglobin. Oxygenated hemoglobin auto-oxidizes to generate partial methemoglobin and superoxide (O_2•) at a rate of ~3%/day. Like partial carbon monoxy-hemoglobin (see Section 5.1), partial methemoglobin has an abnormally high affinity for O_2 such that it cannot be used for O_2 delivery to tissues. T e heme-Fe^{3+} in methemoglobin cannot bind O_2.

NO is a short-lived gas that is mainly produced in endothelial cells, neurons, and neutrophils, from which it can dif use to adjacent cells. In blood vessels, NO inhibits platelet activation and relaxes smooth muscle. To regulate blood flow in the arterioles, endothelial cells synthesize NO in response to shear stress. To this end, **NO synthase** catalyzes the reaction

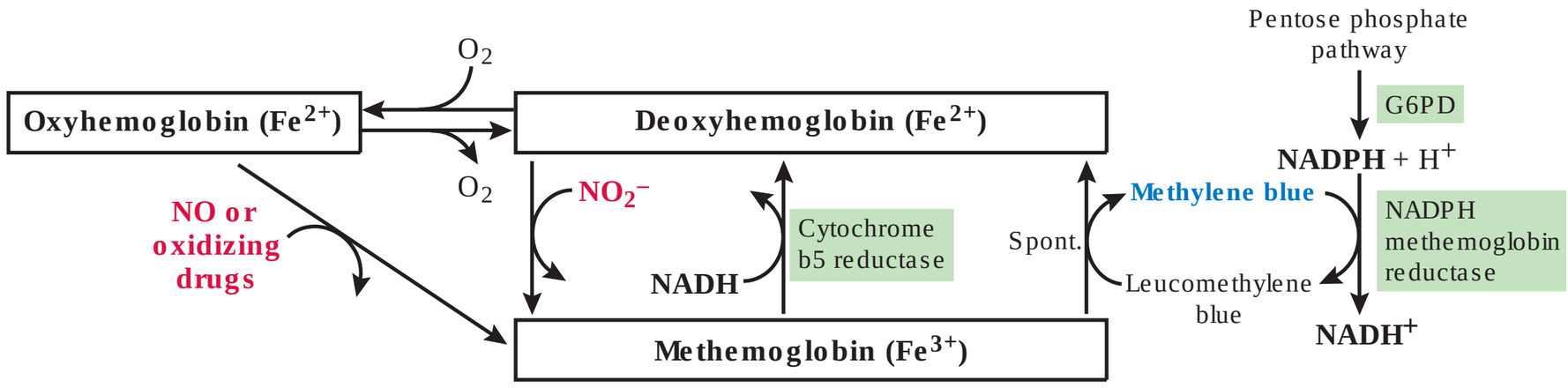


Fig. 16.13 Causes and treatment of acquired methemoglobinemia. G6PD, glucose-6-phosphate dehydrogenase; NADH, reduced nicotinamide adenine dinucleotide; NADPH, NADH phosphate; NO, nitric oxide.

arginine + 2 NADPH + 2 H^+ + 2 O_2 → citrulline + NO + 2 $NADP^+$ + 2 H_2O. NO signals in two major ways: (1) NO reacts with **heme-iron**, for instance in soluble guanylyl cyclase, which in turn produces cyclic guanosine monophosphate (cGMP) that activates protein kinase G; and (2) NO also reacts with the –SH group of cysteine residues, forming an S-nitrosothiol (-SNO) in a process called **S-nitrosylation** that modifies the properties of a protein.

Erythrocytes participate in both the production and removal of NO. T ese processes are incompletely understood. On one hand, deoxyhemoglobin converts circulating nitrite (NO_2^-) to NO. T is reaction likely plays a role in producing NO-induced vasodilation during local hypoxia. On the other hand, deoxyhemoglobin also binds NO to form S-nitroso-hemoglobin (SNO-hemoglobin), which acts as a relatively long-lived reservoir of NO. Furthermore, oxyhemoglobin can oxidize NO to nitrate (NO_3^-), thereby producing partial **met-hemoglobin** (see Fig. 16.13).

Methemoglobinemia is often due to an ingested toxin (e.g., **nitrite**) or due to **oxidizing drugs** (e.g., uricases, benzocaine, lidocaine, metabolites of prilocaine, EMLA cream, sulfonamides, primaquine, dapsone, or nitrofurantoin). Methemoglobinemia can also be due to an unstable variant hemoglobin (e.g., **hemoglobin M**) and, less frequently, due to a **deficiency of cytochrome b5 reductase** (Fig. 16.13).

A methemoglobinemia-induced **cyanosis** due to impaired oxygen delivery is seen when more than ~20% of all heme-iron is heme-Fe^{3+}. A fraction of more than 70% heme-Fe^{3+} is usually fatal.

Red blood cells contain the enzyme **cytochrome b5 reductase** (also called **methemoglobin reductase**; see Fig. 16.13), which uses NADH produced in glycolysis to reduce the heme-Fe^{3+} of methemoglobin to heme-Fe^{2+}, thereby restoring hemoglobin. Normal human blood contains less than 3% methemoglobin.

Methylene blue helps convert methemoglobin to deoxyhemoglobin (see Fig. 16.13). T is process uses NADPH and NADPH methemoglobin reductase, an enzyme that normally plays little role in methemoglobin reduction. NADPH stems from the oxidative branch of the pentose phosphate pathway (see Chapter 21). Methylene blue is inef ective in patients who have a severe **glucose 6-phosphate dehydrogenase deficiency** (see Chapter 21) because they cannot provide NADPH at an adequate rate.

Cyanide-poisoned patients can be given **amyl nitrite** (via inspired air) and/or **sodium nitrite** (by vein) to produce about 10% to 30% **methemoglobin**, which traps cyanide (CN^-). Induction of methemoglobinemia is contraindicated in patients who also have carbon monoxide poisoning (see Section 5.1). Other methods of treatment involve binding cyanide with hydroxocobalamin and facilitating the removal of cyanide with thiosulfate (see Section 1.3 in Chapter 23). T e toxicity of cyanide is primarily due to its inhibition of complex IV of oxidative phosphorylation (see Chapter 23). Methemoglobin has a higher affinity for cyanide than does complex IV. T e temporary binding of cyanide by methemoglobin buys the patient time to react cyanide with thiosulfate, producing thiocyanate, which is excreted in the urine. T e half-life of cyanide in plasma is 20 to 60 min.

6. CLINICALLY IMPORTANT LABORATORY DATA ON RED BLOOD CELLS

Laboratory analyses o*f* blood to determine the volume and number o*f* red blood cells, the concentration o*f* hemoglobin, and the number o*f* reticulocytes provide essential data on a patient's state o*f* health.

A **complete blood count** (**CBC**) refers to the following set of parameters that are measured in a blood sample: hematocrit, hemoglobin, red blood cell count, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, red blood cell distribution width, white blood cell count, and platelet count. A CBC is one of the most frequently performed blood tests. A **reticulocyte count** must be requested separately. A request for a CBC with a **dif erential count** of white blood cells also includes counts for lymphocytes, monocytes, neutrophils, eosinophils, and basophils.

In modern clinical laboratories, CBCs are performed on automated machines that use a combination of spectrophotometry (to measure hemoglobin), antibody staining (to identify platelets), flow cytometry (to count cells and infer sizes), and/or impedance measurements when a cell passes through an aperture (to count cells and infer sizes). Abnormal values are often investigated with additional less-automated methods.

T e **hematocrit** is the ratio of the volume of packed (as in a centrifuge) red blood cells/volume of blood (Fig. 16.14). It

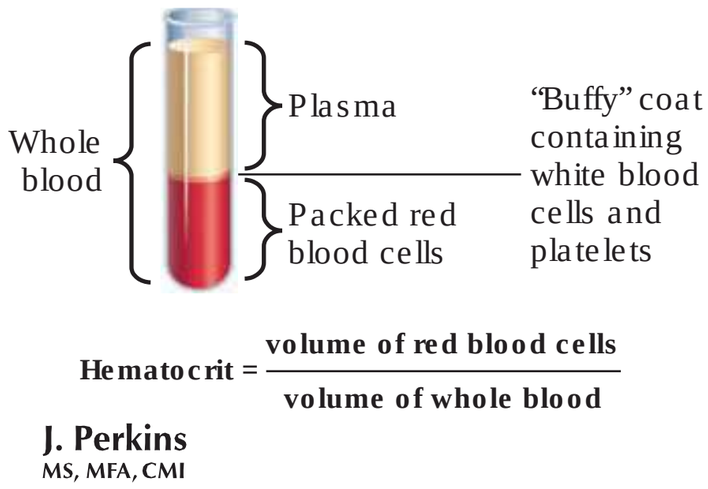


Fig. 16.14 Relationship of the hematocrit to the packed red blood cell fraction of centrifuged blood.

is typically reported as a percentage, and in people living close to sea level, it is usually about 45% (typical normal ranges are 36%–46% in women and 41%–53% in men). In well-equipped laboratories, the hematocrit is no longer measured but estimated from other values.

Blood plasma is the fluid that surrounds red blood cells, white blood cells, and platelets; blood **serum** is the fluid that can be recovered from coagulated, centrifuged blood. Compared with plasma, serum is devoid of proteins that form a clot.

The concentration of **hemoglobin** in whole blood (g/dL) is usually about 15 g/dL in patients living close to sea level (typical normal ranges are 12.0–16.0 g/dL in women and 13.5–17.5 g/dL in men).

In practice, hematocrit and hemoglobin concentration are well correlated. As a rule of thumb, the numerical value of the hematocrit (%) is three times larger than the numerical value of hemoglobin (g/dL). For example, at a hemoglobin concentration of 10 g/dL, a hematocrit of 30% is expected.

Transfusion of one unit of packed red blood cells into a patient who does not actively bleed increases the concentration of hemoglobin in whole blood by ~1 g/L and the hematocrit by ~3 to 4 percentage points (e.g., from 15% to ~18%–19%).

The **red blood cell count** is the number of red blood cells per µL (mm^3) of blood. In patients living close to sea level, the red blood cell count is usually about 5 million/µL (typical normal ranges are 3.5–5.5 million/µL in women and 4.3–5.9 million/µL in men).

A patient with an abnormally low hematocrit, concentration of hemoglobin in the blood, or number of red blood cells per microliter of blood is said to have **anemia**. In most markedly anemic patients all three of these parameters are abnormally low. Anemia is caused by insufficient red blood cell production, premature loss of red blood cells, or a combination of these processes.

An abnormally high hematocrit, hemoglobin concentration in the blood, or number of red blood cells per microliter of blood often indicates that the patient is **dehydrated** (this would go along with elevated blood urea nitrogen; see Chapter 35) or a heavy **smoker** (carbon monoxide in smoke binds to hemoglobin and decreases its capacity for O_2 transport; hence, more red blood cells are needed for adequate oxygenation of tissues; see Section 5.1). More rarely, it is due to excessive synthesis of red blood cells, in which case it is a true **polycythemia** (also called **erythrocytosis**). A true primary **polycythemia** is due to an inherited or acquired defect in the stem cells that give rise to red blood cells. A secondary **polycythemia** is due to an elevated concentration of circulating cytokines (most often erythropoietin in response to renal hypoxia). Healthy persons who have normally adjusted to living at **high altitude** have an increased concentration of hemoglobin, hematocrit, and red blood cell count compared with persons living at sea level; obviously, a different, elevation- or location-appropriate set of reference values must be used for these patients.

The mean volume of individual red blood cells is called the **mean corpuscular volume** (**MCV**). The MCV is normally 80 to 100 fL (1 fL = 10^{-15} L 1 µm^3). If the MCV is abnormally high, the red blood cells are said to be **macrocytic**. This is usually due to a problem with **DNA replication** and cell proliferation (see Fig. 16.3 and Chapter 37). If the MCV is abnormally low, the red blood cells are said to be **microcytic**. This is usually due to a problem with **hemoglobin synthesis** (this includes iron deficiency; see also Fig. 16.3 and Chapters 15 and 17).

The mean amount of hemoglobin inside red blood cells is called the **mean corpuscular hemoglobin** (**MCH**). The MCH is normally 25 to 35 pg.

The mean concentration of hemoglobin inside red blood cells is called the **mean corpuscular hemoglobin concentration** (**MCHC**). The MCHC is usually about 31 to 36 g/dL. Erythrocytes with a normal MCHC are **normochromic**, those with an abnormally low MCHC are **hypochromic**, and those with an abnormally high MCHC are **hyperchromic**. A severe impairment of hemoglobin synthesis (e.g., in thalassemia [see Chapter 17] or iron deficiency [see Chapter 15]) leads to both microcytosis (low MCV) and hypochromia (low MCHC). A severe impairment of DNA synthesis often leads to macrocytosis and hypochromia.

The **red blood cell distribution width** (**RDW**) is a measure of the variation in red blood cell size. Under normal circumstances, the distribution of red blood cell size is nearly Gaussian, and the RDW is about 13%. An elevated RDW indicates abnormal variation in the size of red blood cells and is referred to as **anisocytosis**. An increased RDW is observed, for instance, in iron deficiency (see Chapter 15) and in folate deficiency (see Chapter 36).

The **platelet count** indicates the number of platelets per microliter (µL or mm^3) of blood. The normal range is 150,000 to 400,000/µL.

The white blood cell count is the number of white blood cells per microliter of blood. It is usually about 7,000/µL. An abnormally low white blood cell count is referred to as **leukopenia**; it can be due to stem cell disorders, chemotherapy, radiation therapy, or a decreased life span in the bloodstream. An abnormally high white blood cell count is referred to as **leukocytosis**; it is a classic sign of infection or leukemia.

The **white blood cell differential** is an account of the fraction (%) of different types of white blood cells: lymphocytes, monocytes, neutrophils, basophils, and eosinophils.

The percentage of **reticulocytes** in the whole blood is a clinically useful indicator of red blood cell production. Reticulocytes can be discerned from mature red blood cells by their RNA content. In flow cytometers, reticulocytes are identified after staining of nucleic acids with a fluorescent dye. Normally, reticulocytes make up 0.5% to 1.5% of all circulating red blood cells. An abnormally high reticulocyte count usually indicates a compensatory increase in hematopoiesis due to the premature loss of red blood cells.

7. COLOR OF HEMOGLOBINS

The color of a patient's skin reflects the ligands and oxidation state of heme-iron in hemoglobin. With a pulse oximeter, one can measure a patient's pulse rate as well as the apparent O_2 saturation of hemoglobin.

7.1. Effect of Hemoglobin on Skin Color

The color of heme in blood capillaries near the surface affects the color of the skin; assessment of skin color is part of the physical examination of a patient. The oxidation state and ligand of the heme-iron in hemoglobin affect the absorption spectrum of hemoglobin. In sunlight, oxygenated hemoglobin looks red, whereas deoxygenated hemoglobin looks dark purplish red. Patients with excessively oxygenated hemoglobin (i.e., patients who do not unload O_2 normally, such as from increased affinity of hemoglobin for O_2) have a **ruddy** appearance. Patients with poorly oxygenated blood (e.g., patients with heart and lung disease) look **cyanotic** (Fig. 16.15).

Patients with lethal **carbon monoxide** (CO) poisoning may have a **cherry-red** appearance because carbon monoxyhemoglobin (i.e., hemoglobin with CO bound to it) has a cherry-red color. However, most patients who present with carbon monoxide poisoning have lower carboxyhemoglobin concentrations and do not have a cherry-red appearance.

In patients with **cyanide** poisoning, venous blood looks unusually red because cyanide primarily inhibits oxygen consumption by mitochondria, which in turn leaves capillary blood well oxygenated.

Although **methemoglobin** (i.e., oxidized hemoglobin, Fe^{3+}) imparts a brownish color to drawn blood, patients with more than about 15% methemoglobin have a cyanotic appearance due to increased oxygen unloading from normal hemoglobin (Fig. 16.16).

Emphysema

Chronic bronchitis

Fig. 16.15 Oxygen saturation of blood affects skin color. Extremes of men who have chronic obstructive pulmonary disease (COPD). Most COPD is induced by smoking. Emphysema is characterized by pursed-lip breathing, which keeps the bronchi from collapsing. Chronic bronchitis is characterized by inadequate oxygen saturation of blood in the lungs, leading to secondary heart failure, which exacerbates hypoxia.

7.2. Pulse Oximeter

In intensive care, respiratory care, surgical wards, and obstetric wards, the **pulse rate** and the degree of **oxygenation of hemoglobin** of patients are often monitored noninvasively with a two-wavelength or multiwavelength pulse oximeter. Red and infrared light, which travel well through tissues, are shone through the nail bed of a finger or toe or through the earlobe, and transmitted light is analyzed. Reflected light can also be measured (e.g., from the forehead, or from the cheek of a fetus during labor and delivery).

Oscillations in the amount of transmitted or reflected light of different wavelengths are used to calculate the pulse rate and the O_2 saturation of hemoglobin. For a pulse oximeter to work, the volume of the arterial bed of the tissue must oscillate with the heartbeat (Fig. 16.17). The ratio of the pulsatile portions of red and infrared light depends on the state of

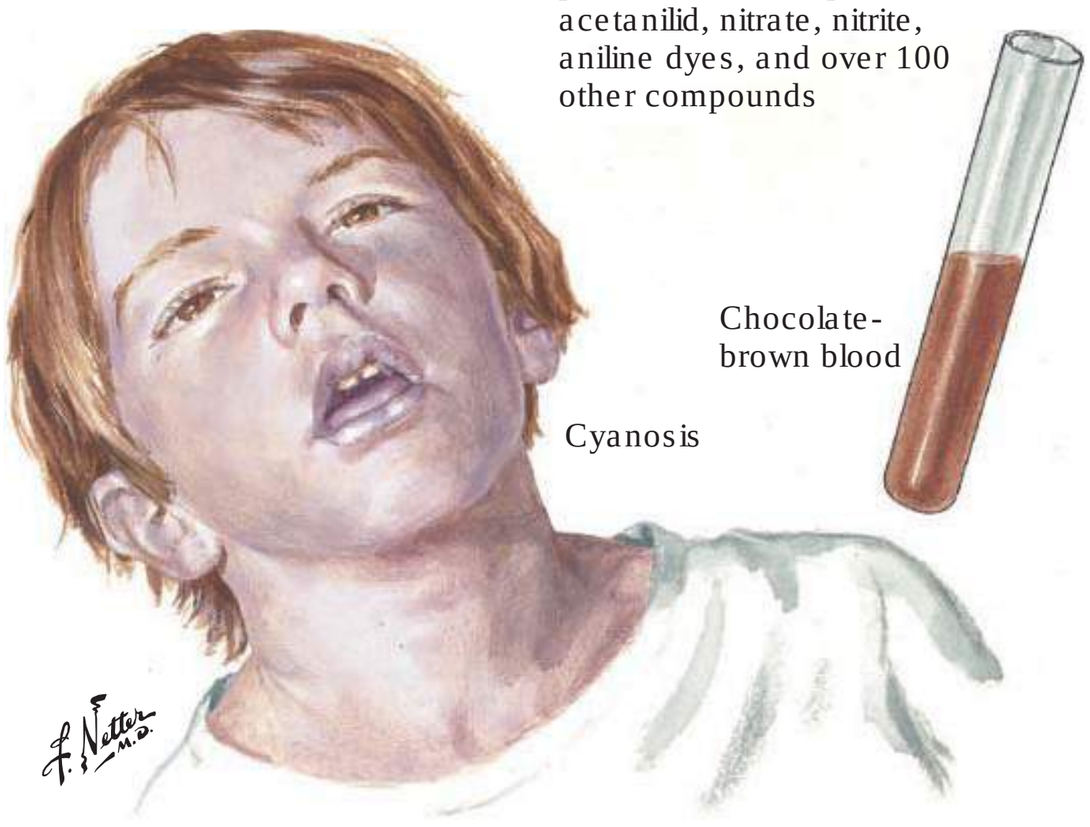


Fig. 16.16 **Methemoglobinemia.**

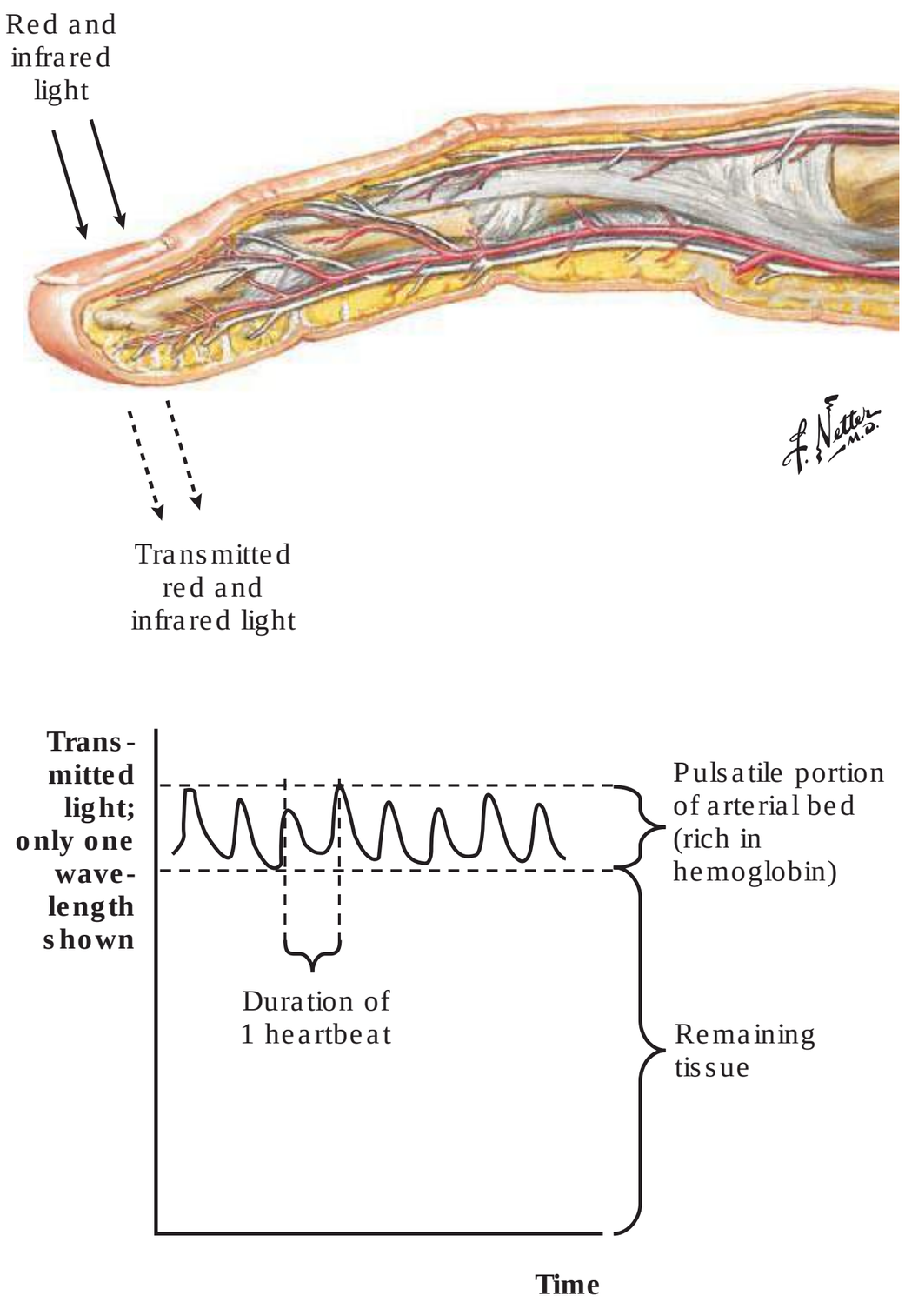


Fig. 16.17 **Principle of pulse oximetry with a measurement of transmitted red and infrared light.**

oxygenation of hemoglobin (the nonpulsatile portion of the signal is not analyzed). T e pulse oximeter compares actual measurements to reference measurements that were made on volunteers who simultaneously had arterial blood drawn and analyzed in a laboratory by a dif erent setup. A two-wavelength pulse oximeter with a probe is calibrated to provide a readout of apparent oxygen saturation of normal hemoglobin; this readout may be wrong in the presence of **methemoglobin** or **carbon monoxy-hemoglobin**. Multiwavelength pulse oximeters can be constructed so that they show the concentrations of oxygenated hemoglobin, carbon monoxy-hemoglobin, and methemoglobin).

SUMMARY

- Under the control of erythropoietin from the kidneys, stem cells in the bone marrow give rise to erythroblasts. Over the course of ~1 week, erythroblasts dif erentiate into reticulocytes, which are released into the bloodstream. For ~1 day, the reticulocytes continue to synthesize hemoglobin. T en, they lose the last of their mitochondria, endoplasmic reticulum, and mRNA, and thus become mature erythrocytes. T e lifetime of a mature erythrocyte in the bloodstream is ~120 days.
- In a healthy patient, the reticulocytes make up ~1% of all red blood cells.
- T e transcription of the erythropoietin gene and many other genes is regulated by the HIF/hydroxylase/VHL system in an oxygen-dependent fashion. Patients who have a major loss of kidney function no longer secrete sufficient erythropoietin and are commonly treated with recombinant erythropoietin.
- T e most common fetal hemoglobin is hemoglobin F, which has an $\alpha_2\gamma_2$ globin composition. T e most common adult hemoglobin is hemoglobin A, which has an $\alpha_2\beta_2$ subunit composition. HbA_{1c} is glycated hemoglobin A. T e fraction of HbA_{1c} is indicative of the average concentration of glucose in the blood, which is of clinical interest in patients who have diabetes.
- Hemoglobin cooperatively binds O_2, and this process is allosterically regulated by H^+, CO_2, and 2,3-BPG. Hemoglobin releases O_2 in tissue capillaries as a consequence of a lower local pO_2 and pH, and a higher pCO_2. 2,3-BPG regulates the affinity of hemoglobin for O_2 from day to day. T e concentration of 2,3-BPG increases with altitude and decreases when hypophosphatemia develops.
- Under physiological conditions, hemoglobin F has a higher affinity for O_2 than does hemoglobin A; this facilitates O_2 delivery from the mother to her fetus.
- CO_2 and HCO_3^- form the major pH buf er of blood. Most of the CO_2 produced in tissues is carried back to the lungs as HCO_3^- dissolved in erythrocyte cytosol and blood plasma. T e remainder (~15%) of the CO_2 produced in tissues binds to the N-terminus of β-globin and thus lowers the affinity of hemoglobin for O_2, which increases O_2 delivery to the tissues.

- The ratio of $[HCO_3^-]/[CO_2]$ determines the pH of blood, and the pH of blood is a major regulator of breathing. Patients with a metabolic acidosis neutralize excess H^+ with HCO_3^-, forming CO_2, which they give off through the lungs. The process leads to blood with a low concentration of HCO_3^- and a low pCO_2.
- Carbon monoxide (CO), which is poisonous, binds to heme-Fe^{2+} of hemoglobin with greater affinity than O_2 and increases the oxygen affinity of the remaining globin-hemes in the same hemoglobin molecule. As a result, carbon monoxyhemoglobin and partial carbon monoxyhemoglobin cannot be used for O_2 delivery. Hemoglobin in the blood of smokers contains a few percent of carbon monoxyhemoglobin. CO crosses the placenta and impairs O_2 delivery in the fetus.
- NO is an endogenous vasodilator and platelet inhibitor. Hemoglobin can produce, store, and remove NO. NO, and oxidizing drugs can oxidize hemoglobin to methemoglobin or partial methemoglobin (≥1 heme-Fe^{2+} is heme-Fe^{3+}). These methemoglobins cannot be used for physiological O_2 delivery. Cytochrome b5 reductase normally keeps methemoglobin at less than 3% of all hemoglobin. Cyanide-poisoned patients are sometimes treated with a nitrite to produce methemoglobin, which binds cyanide.
- Patients who have unintended methemoglobinemia (e.g., due to ingestion of a nitrite or an oxidizing agent) can be treated with methylene blue. Methylene blue gets reduced to leucomethylene blue by an enzyme that uses NADPH. Leucomethylene blue in turn spontaneously reduces heme-Fe^{3+} in methemoglobin to heme-Fe^{2+}. Methylene blue is ineffective in patients who have a severe G6PD deficiency.
- Analysis of a patient's blood often includes determinations of hematocrit, hemoglobin concentration, red blood cell count, reticulocyte count, and mean corpuscular volume. Anemia is characterized by a lack of red blood cells and hemoglobin. An excess of red blood cells and hemoglobin is often caused by dehydration, heavy smoking, or a malignancy. Microcytic and macrocytic red blood cells, respectively, are cells with an abnormally small and an abnormally large mean corpuscular volume. Hypochromic and hyperchromic red blood cells, respectively, are cells with an abnormally low and an abnormally high mean corpuscular hemoglobin concentration. Microcytosis and hypochromia usually occur together as a result of a problem with hemoglobin synthesis. Macrocytosis is generally due to a problem with DNA replication. Micro- and macrocytosis are often accompanied by anemia.
- Deoxygenated and oxygenated hemoglobin, methemoglobin, and carbon monoxy-hemoglobin have different colors. Patients with a markedly increased fraction of deoxygenated hemoglobin look cyanotic. In pulse oximetry, variations in the amount of light passing through a pulsating arterial bed (e.g., fingernail, toenail, or earlobe) are used to estimate the pulse rate and the arterial O_2 saturation of blood. With a two-wavelength pulse oximeter, the presence of abnormal hemoglobins or hemoglobin ligands may lead to an erroneous readout.

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Review Questions

1. A 12-year-old boy has a mutant HIF-2α (hypoxia-inducible factor-2α) that is only poorly hydroxylated and therefore has an unusually long lifetime. This mutation is expected to have which one of the following effects?

   A. Decreased tissue oxygenation (hypoxia)
   B. High hematocrit
   C. Low MCV
   D. Low reticulocyte count

2. In the emergency department, a 36-year-old male patient who was rescued from a fire in an industrial plant receives amyl nitrite by a mask. Which one of the following conditions is this patient most likely receiving treatment for?

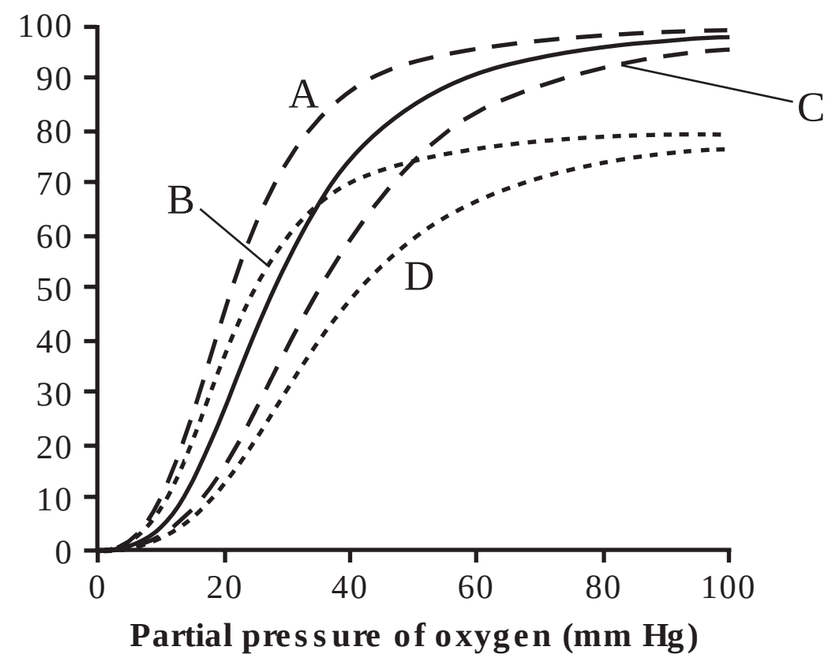
   A. Carbon monoxide poisoning
   B. Cyanide poisoning
   C. Hypoxia
   D. Methemoglobinemia

3. Which one of the options below correctly describes effects on the P_{50} of hemoglobin for oxygen inside red blood cells?

Option	Effect of a Decrease in pH	Effect of an Increase in pCO_2	Effect of an Increased Concentration of 2,3-BPG
A.	P_{50} decreases	P_{50} decreases	P_{50} decreases
B.	P_{50} decreases	P_{50} increases	P_{50} increases
C.	P_{50} increases	P_{50} decreases	P_{50} decreases
D.	P_{50} increases	P_{50} increases	P_{50} increases

4. A woman who used to live at sea level moved to a place 6,000 ft above sea level, where the air pressure is only 80% of that at sea level. After 1 week of residence at 6,000 ft, a blood sample was taken from the woman, and the O_2 saturation of hemoglobin (in intact red blood cells) was measured as a function of the partial pressure of oxygen. Which one of the curves at right most likely represents the data for this woman's red blood cells? (T e solid curve is the reference for sea level.)

A. A
B. B
C. C
D. D



Chapter

17 Hemoglobinopathies

SYNOPSIS

- Hemoglobinopathies are caused by heritable mutations that affect the expression of globins, the amino acid sequence of globins, or both.
- More than 5% of the world's population are carriers of hemoglobin disorders. These are predominantly found among patients with geographic ancestry in areas in which malaria has been endemic.
- The most common hemoglobinopathies are hemoglobin H disease, β-thalassemia, sickle cell anemia, hemoglobin C disease, and hemoglobin E disease. The most severe symptoms are experienced by those with β-thalassemia major (who require frequent blood transfusions and iron chelation therapy), and those with sickle cell anemia (who experience acute painful episodes and organ failure as a result of local hypoxia).

LEARNING OBJECTIVES

For mastery *of* this topic, you should be able to do the *f*ollowing:

- Compare and contrast the genetic basis of the common forms of α- and β-thalassemia.
- Compare and contrast the pathology and major complications of a patient who has hemoglobin H disease and a patient who has β-thalassemia major (considering, in particular, circulating bilirubin, the need for blood transfusions, and the risk of hemochromatosis).
- Describe the pathogenesis of sickle cell anemia, paying special attention to patient age.
- Compare and contrast the major complications of sickle cell anemia and β-thalassemia major.
- Identify the causes and inheritance patterns of α-thalassemia trait, hemoglobin H disease, hemoglobin Barts hydrops fetalis syndrome, β-thalassemia, sickle cell trait, and sickle cell anemia, along with other forms of sickle cell disease.
- Compare and contrast the hemoglobin composition in the blood of a healthy adult and a healthy baby in the months after birth.
- Use laboratory data on hemoglobin composition to determine whether a patient has β-thalassemia minor, β-thalassemia major, sickle cell trait, sickle cell anemia, sickle cell hemoglobin C disease, hemoglobin C disease, or hemoglobin E disease.

1. LINK BETWEEN MALARIA AND SOME HEMOGLOBINOPATHIES

T e most common hemoglobinopathies are *f*ound in populations that have been exposed to malaria *f*or an extended period.

Malaria is the result of an infection with the protozoans Plasmodium vivax, P. malariae, P. ovale, or P. *f*alciparum, all of which are transmitted by mosquitoes. T e parasites initially develop in the liver. T en they invade red blood cells and multiply within them. As parasite-laden red blood cells lyse, they release toxins that lead to fever and inflammation of multiple tissues. Malaria may be accompanied by anemia, coma, and death.

Hemoglobins C, E, and S, as well as the impaired synthesis of α- and β-globin, are predominantly found in populations from geographic areas in which malaria has been endemic for generations. Patients who are heterozygous for α-thalassemia or sickle cell anemia (described below) are less likely to develop severe malaria than patients who are homozygous for normal hemoglobin. Furthermore, patients who are homozygous for hemoglobin C have a greatly reduced risk of developing malaria (Fig. 17.1).

2. THALASSEMIAS

T e most common thalassemias are the result o*f* impaired production o*f* the normal adult hemoglobin (HbA) and the noxious e*ff*ects o*f* excess α- or β-globins. A decreased number o*f* α-globin alleles (usually due to large-scale deletions) is the cause o*f* most cases o*f* α-thalassemia trait, hemoglobin H disease, and hemoglobin Barts hydrops *f*etalis syndrome. β-T alassemia is usually due to point mutations in the β-globin gene or its promoter.

2.1. General Comments About the Thalassemias

T e most common thalassemias are characterized by an impaired synthesis of normal α- or β-globin with concomitant aggregation of excess β-, γ-, or α-globin. When the synthesis of α- or β-globin is compromised or absent, most of the excess globins of the complementary type (α, β, or γ) are destroyed. However, some of these excess globins form **homotetramers** (α_4, β_4, or γ_4), which are useless as O_2 carriers and impair red blood cell production and survival. Precipitated, aggregated, and modified globin homotetramers may be visible by light microscopy as **inclusions** in red blood cells.

Impaired globin synthesis leads to **microcytosis** and **anemia** (see Chapter 16). T e less efficient globin synthesis is, the more pronounced is the microcytosis of the red blood cells. T e anemia leads to a compensatory expansion of the erythropoietic bone marrow, which may even lead to skeletal abnormalities. However, the expanded bone marrow cannot fully compensate for the anemia.

T e terms **thalassemia minor** and **thalassemia major** describe the clinical severity of a thalassemia and stem from a time when the molecular defects were unknown. Typically, patients with thalassemia minor are asymptomatic but have

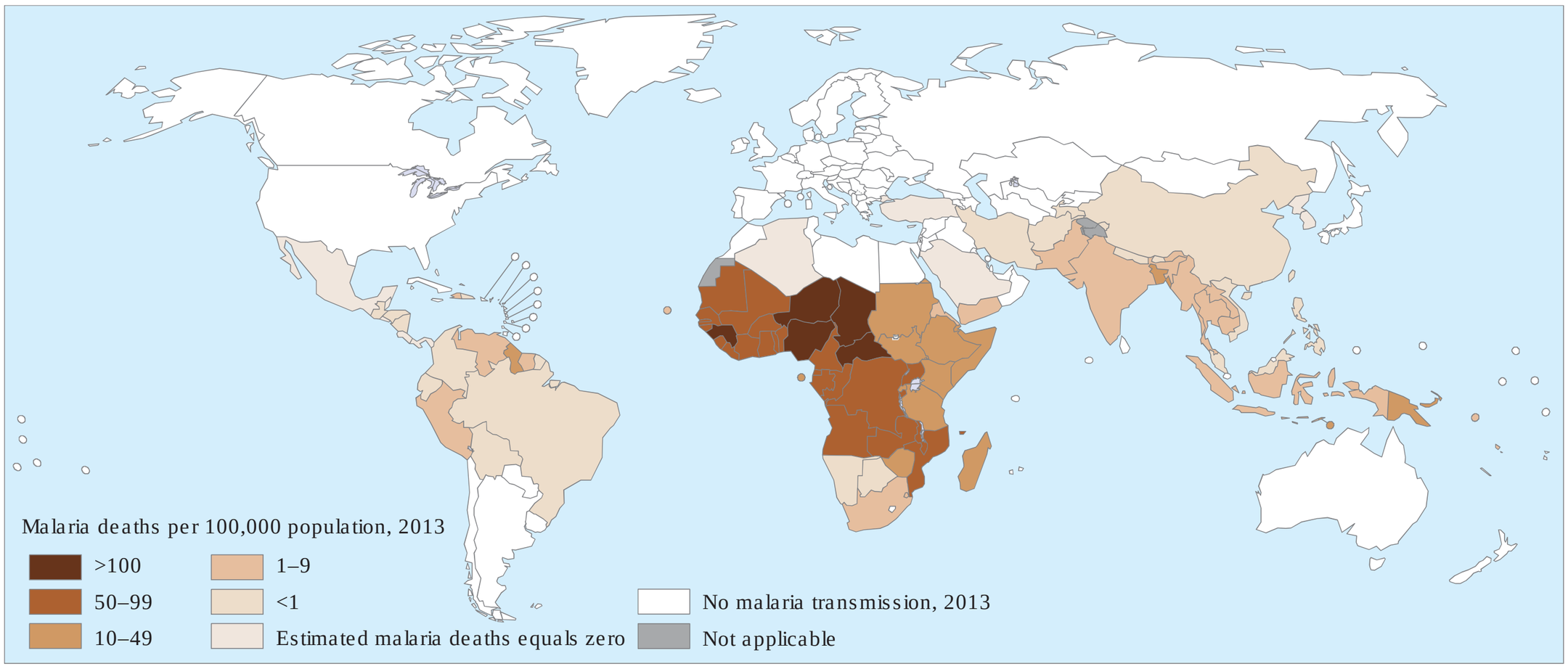


Fig. 17.1 Malaria deaths in countries with ongoing transmission of the disease in 2013. Note that in the past, malaria was also endemic in the Mediterranean basin. (From *World Malaria Report 2014*. World Health Organization, Geneva, Switzerland; p. 37. Available at http://www.alma2015.org/sites/default/files/reference-document/world_malaria_report_2014.pdf.)

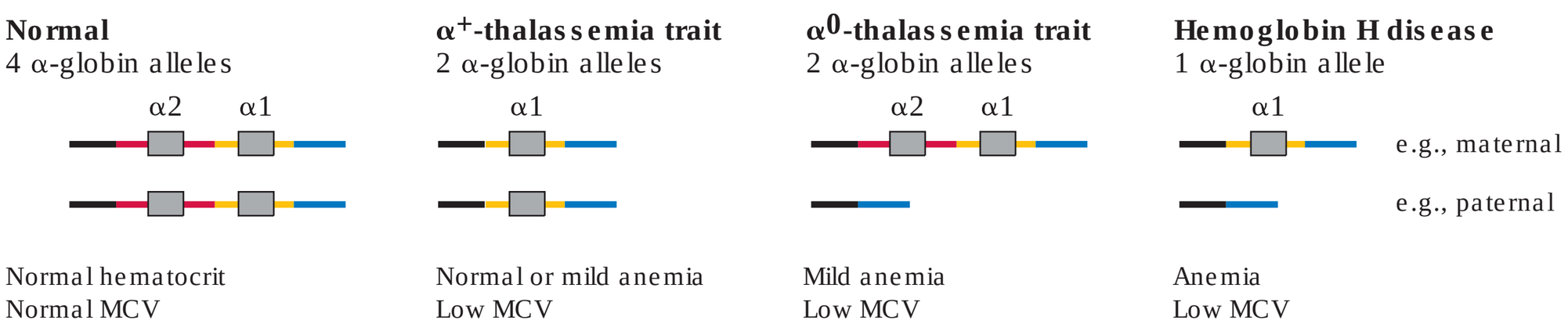


Fig. 17.2 Examples of deletions of α-globin genes in common forms of α-thalassemia. The diagram shows maternal and paternal chromosomes, each of which normally carries two α-globin genes (α1 and α2). Shaded boxes denote genes, and colored lines represent the DNA that flanks these genes. Deletions are typically larger than the affected gene itself (not to scale). There is some variation in the borders of the deletions.

microcytic red blood cells. Patients with thalassemia major are symptomatic and anemic, and their red blood cells have pronounced microcytosis.

2.2. α-Thalassemia Trait, Hemoglobin H Disease, and Hemoglobin Barts Hydrops Fetalis Syndrome

α-Thalassemia trait, hemoglobin H disease, and hemoglobin Barts hydrops fetalis syndrome develop in response to an insufficient production of α-globin; patients with these disorders have, respectively, only 2, 1, and 0 functional α-globin alleles instead of the normal 4.

There are **two different α-globin genes** (α1 and α2; Fig. 17.2); both genes encode the same α-globin amino acid sequence. The two α-globin genes are next to each other on chromosome 16. Normally, the α2 globin gene gives rise to ~70% of all α-globin. Most people have four α-globin alleles (genotype αα/αα; the α2 gene is written to the left of the α1 gene; the slash separates the two homologous chromosomes).

In most cases, loss of function of one or more α-globin alleles is due to **large deletions** involving one or both α-globin genes. In the shorthand for a genotype, a deletion of a single α-globin allele is denoted with a dash (e.g., αα/α–). The common deletions may have arisen from misalignment of chromatids at meiosis (α1 aligning with α2), followed by crossover, resulting in a deletion of one α-globin gene on one chromatid (whereby the sister chromatid gains one α-globin gene).

Patients with only three functional α-globin genes (genotypes –α/αα or α–/αα) are **silent carriers** of α-thalassemia. Their red blood cell indices are in the normal range.

Patients with only two functional α-globin genes (genotypes –α/–α, –α/α–, α–/α–, α–/–α, or ––/αα) have **α-thalassemia trait** (also called **α-thalassemia minor**; see Fig. 17.2). Most of these patients are asymptomatic, but their red blood cells are **microcytic** (mean corpuscular volume

[MCV] ~70 fL; normal erythrocytes have an MCV of 80–100 fL; see Chapter 16). Mild anemia may be present. Patients with the haplotypes α– and –α are sometimes said to have a form of **α⁺-thalassemia**, while patients with a haplotype of –– are said to have a form of **α⁰-+thalassemia**. With β-thalassemia, the superscripts are used differently (see below). Patients with α^+-thalassemia produce more α-globin than patients with α^0-thalassemia.

Patients with only one functional α-globin gene (genotypes ––/–α or ––/α–) have **hemoglobin H disease** (see Fig. 17.2). Hemoglobin H consists of **β_4 globin tetramers**, which arise from β-globin that was synthesized in excess of α-globin, but escaped intracellular degradation. As red blood cells age in the bloodstream, hemoglobin H forms aggregates (inclusions) that can be seen under a light microscope after staining with brilliant cresyl blue. The spleen removes red blood cells with hemoglobin H aggregates before the cells are 120 days old. The red blood cells of patients with hemoglobin H disease are **microcytic** (MCV: ~65 fL) and **hypochromic** (mean corpuscular hemoglobin concentration [MCHC] is low). The patients are **anemic**, mostly because their red blood cells are removed prematurely from the bloodstream (this is accompanied by splenomegaly). A concomitant compensatory bone marrow expansion may cause skeletal changes, typically in the skull. Most patients do not require chronic blood transfusions.

In the absence of α-globin genes (not shown), **hemoglobin Barts hydrops fetalis** syndrome develops, which usually results in fetal or perinatal death. Hydrops is an abnormal accumulation of fluid in at least two compartments of the fetus. A fetus with hemoglobin Barts hydrops fetalis poses a substantial risk to the mother's life, and early detection is important.

In contrast to the above, there are also **point mutations** in the α-globin gene that can lead to a severe form of hemoglobin H disease. These mutations are sometimes referred to as **nondeletional variants**. The most common of these is **Constant Spring** in the α2-globin gene (α^{CS}), which is due to a point mutation in the stop codon that results in a 9-amino acid extension at the C-terminus. Most affected patients have the genotype ––/α^{CS}–, and this disorder is often referred to as **hemoglobin Constant Spring (HCS) disease**. α-Globin^{CS} is unstable, and patients have ongoing mild hemolytic anemia. During illness with high fever, affected patients may develop life-threatening anemia.

2.3. β-Thalassemia

β-Thalassemia is caused by inadequate production or function of **β-globins**. There is only one β-globin gene. A wide variety of **mutations** can give rise to β-thalassemia. Most of these involve one or a few bases in the promoter of the β-globin gene or in the β-globin gene itself that leads to altered RNA synthesis or processing, altered mRNA stability, altered protein sequence, altered hemoglobin stability, or a combination of these. Examples are shown in Fig. 17.3. The shorthand **β^0** denotes a **lack of expression** of β-globin, **β^+** indicates a **reduced expression**, and **β** denotes a **normal** expression. (The "0" and "+" terminology of β-thalassemia is different from the one used for α-thalassemia; see Section 2.2.)

Patients with **β-thalassemia minor** are usually asymptomatic, although mild anemia may occur, especially during **pregnancy**. These patients have the genotype **β/β^+** or **β/β^0**. The patients' red blood cells are microcytic (MCV <75 fL; normal

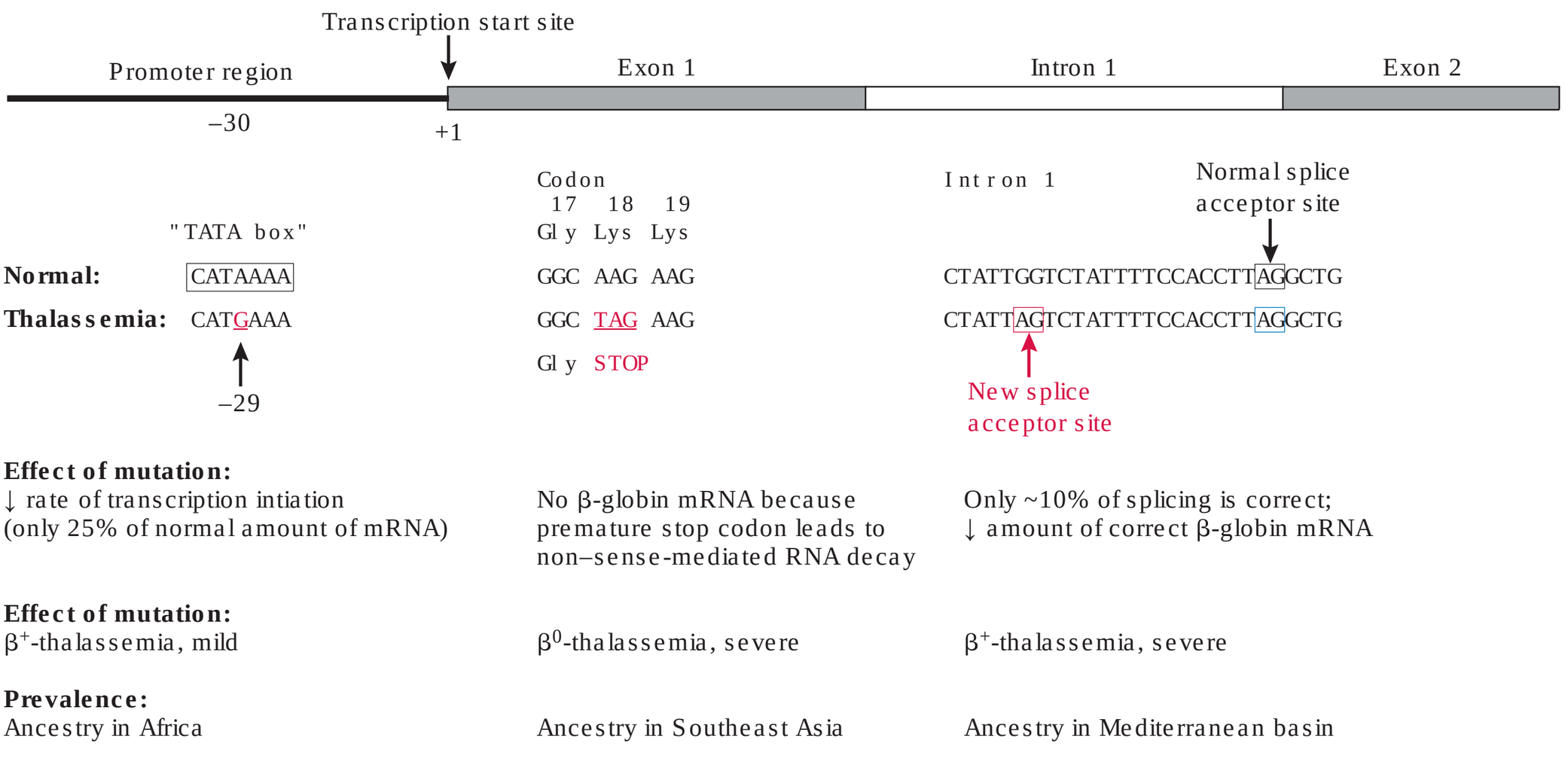


Fig. 17.3 Examples of mutations that cause β-thalassemia. Three commonly observed alleles and the population in which they commonly occur are shown. The phenotype applies to patients who are homozygous for the given mutation. The β-globin gene contains two introns and three exons; the normal stop codon is in exon 3. The structure of the gene is not drawn to scale.

erythrocytes have an MCV of 80 to 100 fL) and hypochromic, but this is often compensated by an increased red blood cell count.

By definition, patients with **β-thalassemia intermedia** have symptomatic anemia but maintain a hemoglobin concentration of greater than 6 to 7 g/dL blood (i.e., about half the normal value or more) without transfusions. These patients have the genotype β^+/β^+ or β^+/β^0. The MCV is about 50 to 75 fL and the MCH about 16 to 24 pg.

Patients with **β-thalassemia major** chronically require **blood transfusions**. These patients have the genotype β^0/β^0 or, occasionally, β^+/β^0 or β^+/β^+. Untransfused patients show a massive expansion of the erythropoietic bone marrow, and even the liver and spleen may produce red blood cells. **α_4 Globin tetramers** accumulate, precipitate, give rise to noxious products, and thus lead to the premature destruction of red blood cell precursors in the bone marrow (i.e., **ineffective erythropoiesis**) and of mature red blood cells in the bloodstream. When red blood cells of these patients are stained with methyl violet, aggregates of α_4 globin tetramers are visible by light microscopy. The red blood cells of patients with β-thalassemia major contain an increased fraction of **hemoglobin F** ($\alpha_2\gamma_2$ globin subunit structure) and **hemoglobin A_2** ($\alpha_2\delta_2$ globin subunit structure). Due to a high rate of destruction of hemoglobin-containing immature and mature red blood cells, untransfused patients with β-thalassemia major are jaundiced (see Chapter 14), and their liver and spleen are enlarged. The MCV is less than 70 fL and the MCH less than 20 pg.

Patients with β-thalassemia major are at risk for complications such as folate deficiency, gout, iron overload, and gallstones. **Folate deficiency** develops as a result of the high rate of erythropoiesis and the concomitant need for nucleotides (see Chapter 37). **Gout** results from the high rate of destruction of purine nucleotides in immature and mature red blood cells (see Chapter 38). **Iron overload** is a consequence of transfusions of red blood cells and excessive iron uptake from the intestine (stimulated by ineffective erythropoiesis and anemia; see Chapter 15). Overload occurs particularly in the liver, heart, and endocrine glands. Excess iron can be removed by chelation therapy, often with nightly intravenous desferrioxamine. Prevention of organ damage due to iron overload is currently the most challenging part of managing β-thalassemia major. Black pigment **gallstones** form in large part due to the precipitation of calcium salts of bilirubin monoglucuronide, which is formed at an elevated rate when red blood cell precursors are destroyed. (β-Thalassemia intermedia and other hemolytic anemias are also associated with an increased incidence of gallstones.)

Because β-globin is a part of adult hemoglobin A but not the fetal hemoglobin F, β-thalassemia becomes clinically apparent only around **6 months of age** (Fig. 17.4; a similar time course is seen with **sickle cell anemia**, a disease due to the production of a mutant β-globin; see Section 3).

Parents who both have β-thalassemia minor (genotypes β/β^+ or β/β^0) may produce **offspring** with β-thalassemia major (genotypes β^0/β^0, β^+/β^0, or β^+/β^+).

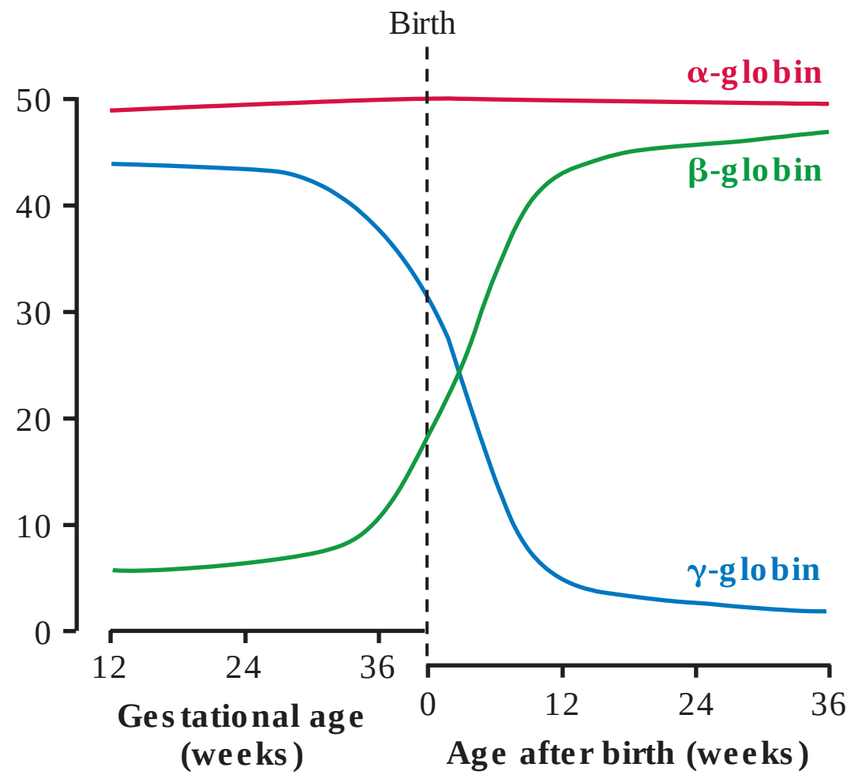


Fig. 17.4 Globin synthesis during normal fetal and postnatal development. The globin composition of blood lags temporally due to the ~120-day life span of mature red blood cells. (Modified from Wood WG. Haemoglobin synthesis during human fetal development. *Br Med Bull.* 1976;32:282.)

The β-thalassemia allele has a geographic distribution akin to past endemic malaria (see Fig. 17.1), but among these areas, it is least common in Africa.

Carriers for β-thalassemia can often be detected based on an elevated fraction of HbA_2 (Table 17.2).

3. SICKLE CELL ANEMIA AND HEMOGLOBIN S

Sickle cell anemia is caused by homozygosity *for* the mutant β^S-globin allele. Deoxygenated sickle cell hemoglobin ($\alpha_2\beta^S_2$) polymerizes, changes the shape *of* red blood cells, and alters the properties *of* red blood cell membranes. Patients with sickle cell anemia suffer *from* recurrent acute painful vaso-occlusive episodes, anemia, and progressive organ *failure*.

3.1. Cause and the Genetics of Sickle Cell Anemia

Sickle cell anemia is due to a hereditary presence of sickle hemoglobin associated with acute painful vaso-occlusive episodes and subsequent organ damage (see Section 3.3). **Sickle hemoglobin** (**hemoglobin S**) has an $\alpha_2\beta^S_2$ composition; **β^S-globin** is β-globin in which the negatively charged amino acid Glu at residue 6 is replaced by the uncharged, hydrophobic amino acid Val. Patients who are homozygous for the β^S-globin allele (genotype β^S/β^S) have **sickle cell anemia.** Patients who are heterozygous for the β^S-globin allele (genotype β/β^S) have **sickle cell trait**, which is associated with few symptoms (see below). However, patients who have one β^S-globin allele and one β-globin allele for another hemoglobinopathy may have vaso-occlusive episodes (see below).

Sickle cell trait offers carriers some protection against Plasmodium *falciparum* **malaria**. However, patients with sickle cell anemia have no such protection.

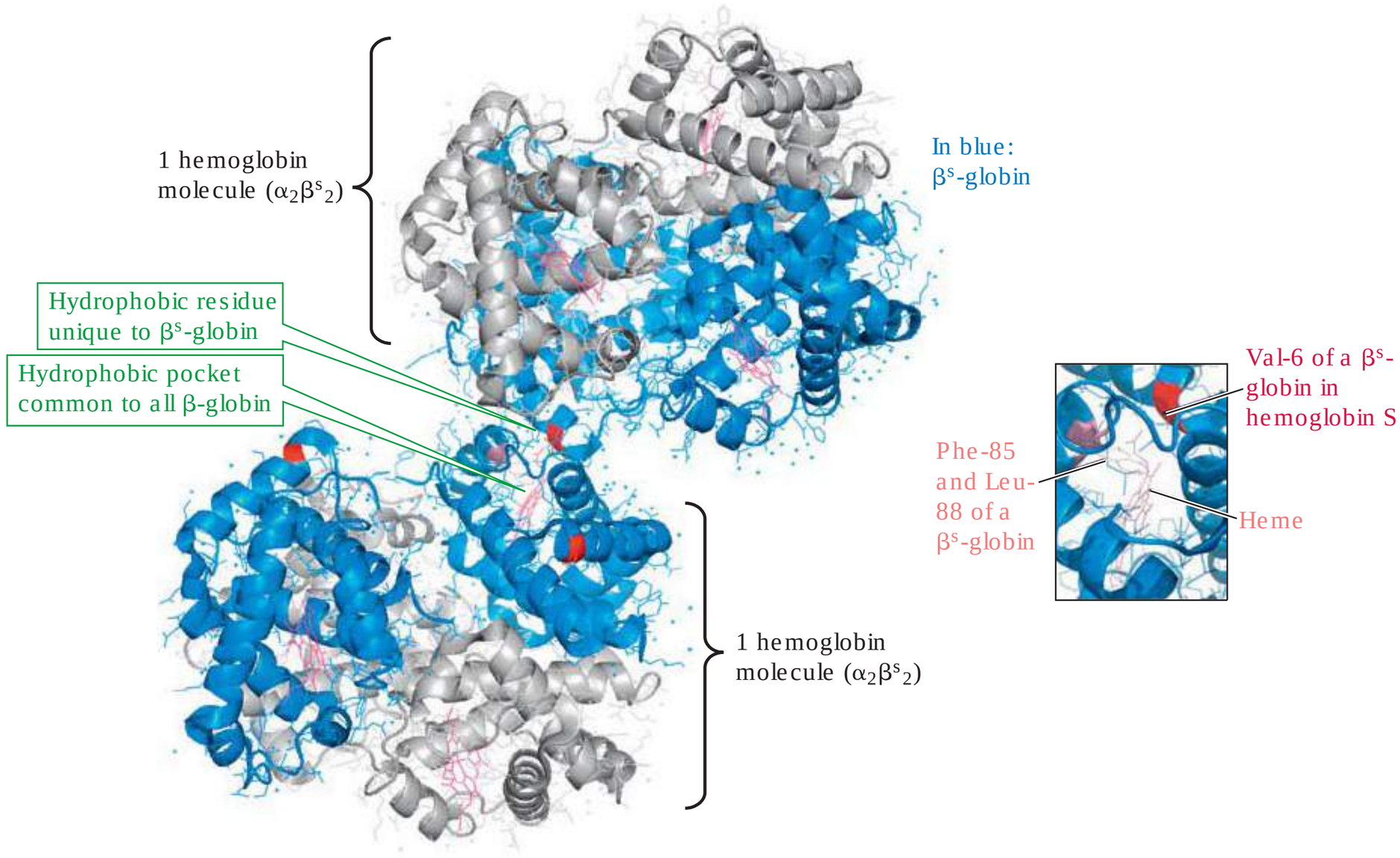


Fig. 17.5 Model of a human deoxyhemoglobin S dimer and mechanism of polymerization. Two hemoglobin molecules are shown, each with 2 α- and 2 β^S-globins. Although both β^S-globins in a hemoglobin S molecule have a Val as the sixth residue (shown in red), Val of only one β^S-globin per hemoglobin molecule binds into a hydrophobic pocket on a β-globin of a neighboring hemoglobin molecule. The hydrophobic pocket consists of Phe-85, Leu-88, and heme. (Based on Protein Data Bank [www.rcsb.org] file 2HBS by DJ Harrington DJ, Adachi K, Royer WE Jr. The high resolution crystal structure of deoxyhemoglobin S. *J Mol Biol.* 1997;272:398-407.)

In general, the sickle cell mutation is common in populations that have been exposed to malaria for many generations. Sickle cell anemia is most prevalent in patients with ancestry in **Africa**, and less so in the **Mediterranean** region or **India**. In the **United States**, about 10% of African Americans have sickle cell trait, and about 0.2% have sickle cell anemia. Genes outside the β-globin locus influence the course of sickle cell anemia (an example is increased expression of fetal hemoglobin; see Section 3.3).

Affected children do not develop symptoms of sickle cell anemia until 2 to 4 months of age. This time frame is due to the normal increase in expression of the β-globin gene, the normal decrease in expression of the γ-globin genes around the time of birth (see Fig. 17.4), and the life span of red blood cells. **Newborns** can be **screened** for sickle cell anemia by analyzing their hemoglobin by electrophoresis, isoelectric focusing, or high-pressure liquid chromatography; by analyzing their DNA; or by a combination of these techniques (see Section 6).

3.2. Polymerization of Deoxyhemoglobin S

When deoxygenated hemoglobin S polymerizes, the mutant Val6 residue of β^S-globin binds into a hydrophobic pocket of another hemoglobin molecule. The hydrophobic pocket is formed by heme and the hydrophobic sidechains of Phe85 and Leu88 of a β-globin (Figs. 17.5 and 17.6). This hydrophobic pocket is present for instance in normal β-globin, in β^S-globin, and in β^C-globin (see Section 4). For polymerization into multimers, a single β^S-globin chain per hemoglobin is sufficient. Hence, deoxygenated $\alpha_2\beta^S{}_2$, $\alpha_2\beta\beta^S$, and $\alpha_2\beta^C\beta^S$ tetramers can all polymerize.

The polymerization of hemoglobin S has a **lag time** (delay time) that changes considerably with even a **small change in the concentration** of deoxyhemoglobin S. The lag time depends on the concentration of deoxyhemoglobin S approximately to the **power** of 30. Normally, red blood cells spend less than about 4 seconds in the capillary bed, which is not sufficient for appreciable polymerization. When deoxygenated sickle cells stay in a capillary for a considerably longer time, they may sickle and occlude the microcirculation, causing a vaso-occlusive episode.

In patients with sickle cell anemia, a low affinity of hemoglobin S for oxygen and impaired lung function both favor the deoxygenation of hemoglobin S and thus contribute to polymerization. Damage to the **lungs** is probably due to repeated episodes of vaso-occlusion because red blood cells normally reside in the capillary bed of the alveoli for several seconds. As a result, many patients have an **arterial oxygen saturation** of less than 85% to 90%, which further contributes to sickling.

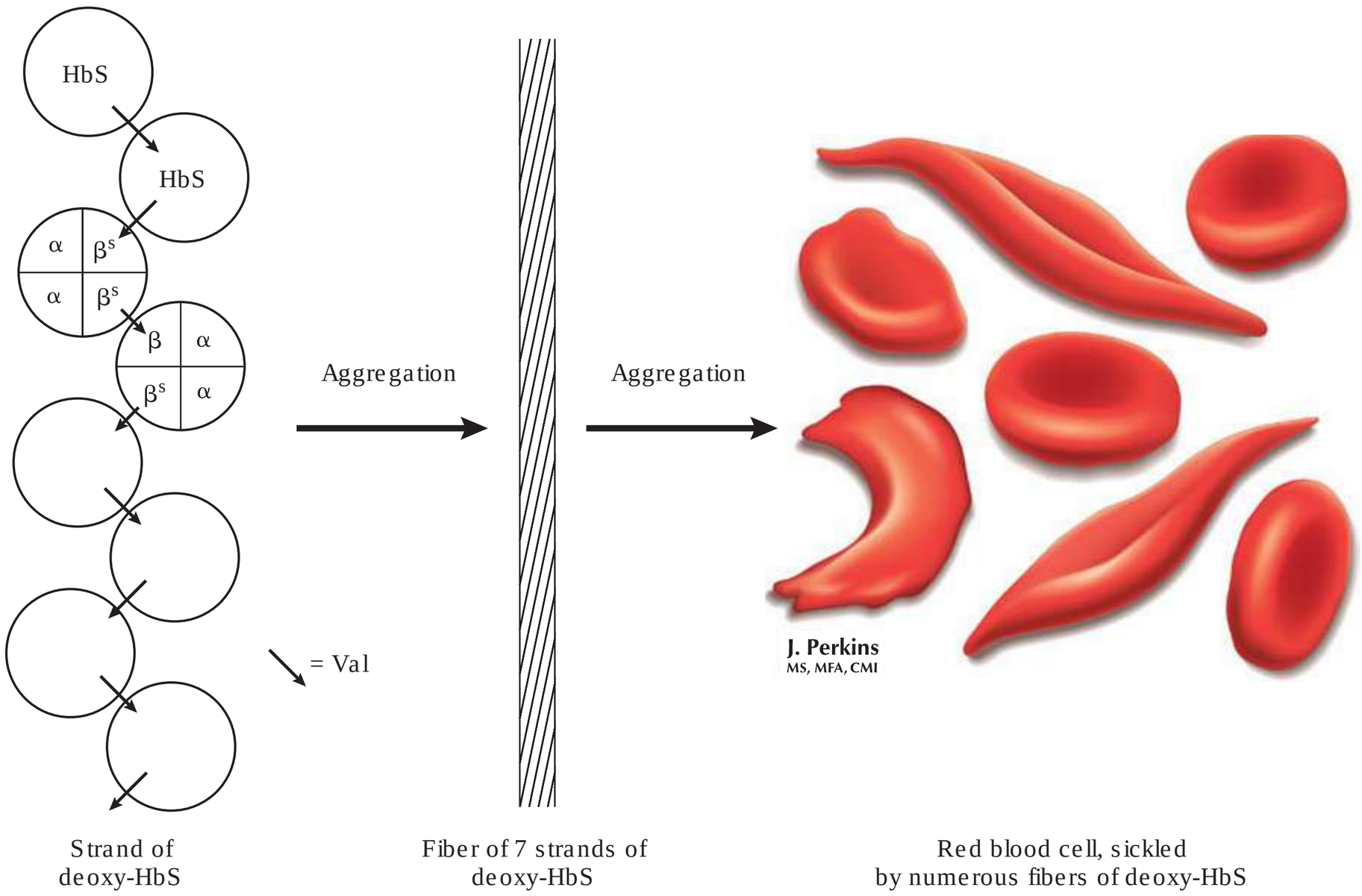


Fig. 17.6 Polymerization of deoxyhemoglobin S. Both hemoglobin S (subunit composition $\alpha_2\beta^S{}_2$) and hybrid hemoglobin of the type $\alpha_2\beta\beta^S$ can polymerize as shown in Fig. 17.5, thereby forming a strand. Seven such strands can be twisted into a fiber, and the fibers in turn can aggregate into large structures that distort and damage red blood cells.

3.3. Vaso-Occlusive Episodes in Sickle Cell Anemia

Vaso-occlusive episodes are caused by red blood cells occluding the microcirculation, which in turn gives rise to local hypoxia. T ese episodes may be extremely painful and last hours to weeks. Temporary vaso-occlusion may also be debilitating because of damage to the lungs, heart, bones, joints, kidneys, spleen, and brain.

Hemoglobin S alters the properties of red blood cells and the luminal lining of blood vessels (i.e., the endothelium). T e Fe^{2+} in the hemes in hemoglobin S is oxidized to Fe^{3+} at a higher rate than in hemoglobin A, and heme dissociates more rapidly from hemoglobin S than from hemoglobin A. Both of these abnormalities give rise to increased **oxidative damage** (see Chapter 21). Oxidative damage and the ef ects of polymerization of deoxyhemoglobin S alter the **properties of red blood cells**. Compared with normal red blood cells, the red blood cells of patients with sickle cell anemia (1) become more dehydrated with age, (2) stick more to the blood vessel endothelium, (3) increase the viscosity of blood, (4) are less deformable, (5) are more likely to initiate vaso-occlusion (in turn causing infarction), and (6) have a decreased life span (causing **anemia**). In patients with sickle cell anemia, as much as 30% of all red blood cells hemolyze in the bloodstream rather than in the spleen and liver (in unaf ected patients, only ~10% of the red blood cells lyse in the bloodstream). Not all hemoglobin S lost into blood plasma can sufficiently be removed by **haptoglobin** (a protein in blood plasma that binds hemoglobin and carries it to the liver; see Chapter 14); remaining hemoglobin S may thus scavenge the vasodilator **nitric oxide** (NO) prematurely (see Chapter 16), thereby contributing to vaso-occlusion.

Several factors influence the incidence of vaso-occlusive episodes; among them are **temperature** and the concentrations of **hemoglobin F** and hemoglobin S. Extremes of both high and low temperature increase the likelihood of acute painful episodes. T us an increase in temperature decreases the oxygen affinity and also favors the polymerization of hemoglobin S, whereas a decrease in temperature leads to a decrease in blood flow through peripheral tissues and thus to greater deoxygenation of hemoglobin S. **Infection**, besides af ecting body temperature, often increases the adhesion of red blood cells to the endothelium. **Exertion** and **anemia** both lead to greater deoxygenation of hemoglobin, and **dehydration** increases the concentration of hemoglobin S inside red blood cells. T ese conditions favor the polymerization of deoxyhemoglobin S. Patients with a high fraction of hemoglobin F ($\alpha_2\gamma_2$) and F cells (red blood cells that contain about 20% HbF) often have fewer vaso-occlusive episodes because hybrid hemoglobin $\alpha_2\beta^S\gamma$ delays and inhibits polymerization and because a lower concentration of hemoglobin S prolongs the lag time for polymerization.

T e chemotherapeutic drug and inhibitor of nucleotide reductase **hydroxyurea** (see Chapter 37) increases the fractions of hemoglobin F and F cells. Some patients with frequent

vaso-occlusive episodes respond favorably to treatment with hydroxyurea.

Sickle cell anemia is a **chronic hemolytic anemia**. In patients with sickle cell anemia, red blood cells have a shortened life span that can be as low as 10 to 25 days (the normal life span of red blood cells is ~120 days). At a few years of age, children with sickle cell anemia tend to lose much of the function of their spleen to sickle cell–induced infarction, a process called **autosplenectomy**. In patients without a functioning spleen, the liver removes red blood cells. Functionally asplenic children have decreased immunity and therefore routinely receive prophylactic treatment with **vaccines** and **antibiotics**.

Patients with sickle cell anemia eventually show damage to various organs. Children are at a high risk of cerebrovascular infarcts. Adults commonly suf er from **acute chest syndrome**, which is due in part to infarction of lung tissue and bone. Chronic damage to the lungs leads to pulmonary hypertension. Kidney damage stems from infarcts that occur in the peritubular capillaries, presumably due to the physiologically especially low pH and pO_2 in the renal medulla. As a result, patients with sickle cell anemia may experience **hematuria**, and they usually cannot concentrate urine normally when stressed by water deprivation (i.e., they have **hyposthenuria)**.

Patients with **sickle cell trait** (genotype β/β^S) are usually free of obvious vaso-occlusive acute painful episodes because the concentration of deoxyhemoglobin S and the duration of the deoxygenated state of hemoglobin S do not permit polymerization. However, with pronounced hypoxia, the red blood cells of patients with sickle cell trait do sickle. In fact, most patients with sickle cell trait have kidney damage as they have hyposthenuria and experience hematuria at one time or another. Furthermore, heat illness and death during high-intensity exercise in a hot environment are much more common in patients who have sickle cell trait than in the general population.

Patients who have **sickle-β^0-thalassemia** have a sickle cell disease that resembles sickle cell anemia. If there is some expression of a β-globin with normal function (as in **sickle-β^+-thalassemia**), the severity of the symptoms is reduced.

4. HEMOGLOBIN C, HEMOGLOBIN SC, AND HEMOGLOBIN E DISEASE

Patients with hemoglobin C, SC, or E disease produce mutant β-globins. Patients with hemoglobin C or E disease may have mild anemia. Patients with hemoglobin SC disease can have vaso-occlusive acute pain*f*ul episodes, similar to patients with sickle cell anemia. Patients who are heterozygous *f*or both hemoglobin E and β-thalassemia may be severely anemic.

Hemoglobin C shortens the life span of red blood cells, but in the absence of other pathogenic hemoglobins, hemoglobin C causes only mild disease. Hemoglobin C has an $\alpha_2\beta^C_2$ composition, where β^C-globin is β-globin in which Glu (negatively charged) in residue 6 is replaced by Lys (positively charged; in β^S-globin the same residue is replaced by Val). Hemoglobin C forms intracellular aggregates and crystals that lead to the premature removal of red blood cells. Patients with the genotype β/β^C have **hemoglobin C trait** and are asymptomatic. Patients with the genotype β^C/β^C have **hemoglobin C disease**; they show microcytosis, mild hemolysis, mild anemia, and moderate splenomegaly (a response to an increased rate of red blood cell degradation)**.** Hemoglobin C in the red blood cells of patients with hemoglobin C disease has a decreased affinity for O_2. T is leads to the release of a larger fraction of bound O_2 and thus ensures normal tissue oxygenation despite mild anemia.

T e red blood cells of patients with **hemoglobin SC disease** (i.e., sickle cell hemoglobin C disease) can **sickle in vivo**, causing **vaso-occlusive acute painful episodes**. T ese patients have the genotype β^C/β^S and produce some β-globins with a Glu6Lys and others with a Glu6Val amino acid substitution. Red blood cells from patients with hemoglobin SC disease contain a higher fraction of HbS than red blood cells from patients with sickle cell trait (genotype β/β^S**)**. Patients with hemoglobin SC disease have less severe and fewer painful vaso-occlusive episodes than patients with sickle cell anemia (genotype β^S/β^S).

Hemoglobin E has an $\alpha_2\beta^E_2$ composition; β^E-globin is β-globin in which Glu (negatively charged) in residue 26 is replaced by Lys (positively charged). Hemoglobin E has normal oxygen transport properties. About half of the β^E-globin RNA is **spliced** to an mRNA that is destroyed via **nonsense-mediated RNA decay** (see Chapter 7). Homozygotes and heterozygotes for hemoglobin E are both asymptomatic, but they have microcytosis (MCV: 55 to 65 fL; normal MCV: 80 to 100 fL) and hypochromia. Homozygotes may also have mild anemia. T e β^E-globin allele is common among patients with Southeast Asian ancestry (allele frequency up to 15%). Patients with a β^E/β^+ or β^E/β^0 genotype (i.e., heterozygotes for both **β-thalassemia** and hemoglobin E) can have symptoms that range from mild anemia to severe anemia that needs to be treated with blood transfusions.

5. SUMMARY OF THE CAUSES AND MANIFESTATIONS OF THE MOST COMMON HEMOGLOBINOPATHIES

See Table 17.1.

6. HEMOGLOBIN ANALYSIS FOR THE DIAGNOSIS OF HEMOGLOBIN DISORDERS

Electrophoresis, isoelectric *f*ocusing, and/or chromatography are o*ft*en used to characterize and quanti*f*y hemoglobin species in a patient's red blood cells. Interpretation o*f* these data is up to the health care provider.

Analyses of hemoglobin species are often performed as part of newborn screening, follow-up of abnormal laboratory results, or genetic counseling, as well as before

Table 17.1 Causes and Manifestations of the Most Common Hemoglobinopathies

Disease	Genotype (Molecular Cause of Disease)	Clinical Observations
α-Thalassemia		
Silent carrier	αα/α– or αα/–α (impaired synthesis)	None or mild microcytosis (asymptomatic)
Trait/minor	α–/α–, α–/–α, –α/–α, –α/α–, or – –/αα (impaired synthesis)	Microcytosis (asymptomatic)
Hemoglobin H disease	– –/–α or – –/α– (impaired synthesis of α-globin; excess β-globin)	Anemia, microcytosis, skeletal changes
Hb Barts hydrops fetalis	– –/– – (no α-globin synthesis; excess γ- and β-globin)	Hydrops fetalis, perinatal death
β-Thalassemia		
Minor	β/β^{+} or β/β^{0} (impaired synthesis)	Microcytosis, hypochromia, increased red blood cell count (asymptomatic)
Major	β^{0}/β^{0} or β^{+}/β^{0} or β^{+}/β^{+} (impaired synthesis; excess α-globin)	Anemia after ~6 mo of age, microcytosis, ineffective erythropoiesis, skeletal changes
Sickle cell trait	β/β^{S} (Glu6Val; impaired synthesis)	Hematuria, hyposthenuria
Sickle cell anemia	β^{S}/β^{S} (Glu6Val; impaired synthesis; sickling and impaired survival of red blood cells)	Anemia starting at 2–4 mo of age, vaso-occlusive episodes, skeletal changes, autosplenectomy, organ failure, hematuria, hyposthenuria
Hemoglobin C disease	β^{C}/β^{C} (Glu6Lys; impaired synthesis; impaired stability of red blood cells)	Microcytosis, mild anemia, mild hemolysis, moderate splenomegaly
Hemoglobin SC disease	β^{S}/β^{C} (Glu6Val and Glu6Lys; impaired synthesis; sickling and impaired survival of red blood cells)	Anemia, vaso-occlusive episodes, splenomegaly or autosplenectomy
Hemoglobin E disease	β^{E}/β^{E} (Glu26Lys; impaired synthesis)	Microcytosis, mild anemia, hypochromia (asymptomatic)
Hemoglobin E/β-thalassemia	β^{E}/β^{+} or β^{E}/β^{0} (β^{E}: Glu26Lys with impaired synthesis, β^{0}: impaired synthesis)	Microcytosis, anemia, hypochromia

medical interventions that present increased risk to patients who have a hemoglobinopathy or carry an allele for a hemoglobinopathy.

T e **hemoglobin composition** in red blood cells is currently determined by electrophoresis, isoelectric focusing, or high-pressure liquid chromatography, and final diagnoses of hemoglobinopathies increasingly rely on analyzing globin mRNA in the red blood cell fraction and/or DNA in the white blood cell fraction of a blood sample. For most lab assays, the same volume of red blood cells is used for each patient, regardless of **hematocrit**. T e results of the electrophoresis are reported for each hemoglobin as a **fraction of all hemoglobins** present in the sample (e.g., 96% HbA); these results are independent of the hematocrit. Sometimes, blood samples from **family members** are analyzed as part of a final diagnosis of a hemoglobinopathy (e.g., homozygosity for HbS vs. compound heterozygosity for HbS and β^{0}-thalassemia).

Results of hemoglobin analyses are typically reported as idealized fractions of hemoglobins rather than as fractions of individual globins. Commonly reported hemoglobins are A, A_2, C, E, F, S, and Barts. Physiologically, additional forms of hemoglobin exist. For instance, the red blood cells of patients who have sickle cell trait contain not only pure hemoglobin A and S, but also hemoglobin with the composition $\alpha_2\beta^{S}\beta$. If an analysis method cannot distinguish certain hemoglobins, these are reported in aggregation (e.g., hemoglobin A_2 + C + E after electrophoresis on cellulose acetate at pH ~8.5; see Table 17.2).

SUMMARY

- Patients who produce either α- or β-globin at an abnormally low rate and patients who are heterozygous for

Table 17.2 Typical Results of Hemoglobin Analyses

Hemoglobin Disease	HbA ($\alpha_2\beta_2$)	HbA_2 ($\alpha_2\delta_2$)	HbF ($\alpha_2\gamma_2$)	Other Hemoglobins
Normal newborn	~20%	<1%	~80%	
Normal adult	~96%	~2.5%	<1%	
α^0-Thalassemia minor	~Normal	~Normal	~Normal	At birth: ~6% Hb Barts (γ_4)
Hemoglobin H disease	~80% (adult)	<2%*	~10%	At birth: ~35% Hb Barts (γ_4)* Adult: ~9% HbH (β_4)*
Hb Barts hydrops fetalis	0%	0%	0%	~80% Hb Barts (γ_4)*, ~20% embryonic Hb, ~0% HbH (β_4)
β^0-Thalassemia carrier	~93%	>3.5%*	~2%	
β^0-Thalassemia major	0%*	>3.5%*	~96%*	
Sickle cell trait	~60%	~Normal	~1%	~40% HbS
Sickle cell anemia	0%*	~Normal	~5%	~90% HbS*
Hemoglobin C disease	0%*	~Normal	~1%	~95% HbC*
Hemoglobin E disease	0%*	~Normal	~1%	~95% HbE*

*These abnormal values are of special diagnostic value.

hemoglobin C, E, or S are more likely to survive malaria than patients who produce normal amounts of hemoglobin A.

- Thalassemia typically results from the deficient production of α- or β-globin. Patients with thalassemia major are anemic, and their red blood cells are microcytic. The red blood cells contain noxious homotetramers of excess complementary globins, such as α_4, β_4, or γ_4, often as part of inclusions.
- Humans have two α-globin genes. Patients with three or two normal α-globin alleles are usually asymptomatic. Patients with only one normal α-globin allele have hemoglobin H disease. Hemoglobin H disease is apparent at birth by the presence of hemoglobin Barts (γ_4) and later in life by the presence of hemoglobin H (β_4). Patients with hemoglobin H disease are anemic and have microcytic erythrocytes of decreased life span. Patients who have only one α^{CS} allele (carrying a point mutation) have a particularly severe form of the disease called hemoglobin HCS disease. A complete deficiency of functioning α-globin genes leads to hemoglobin Barts hydrops fetalis and death before or shortly after birth; furthermore, it threatens the mother's health.
- Patients with β-thalassemia minor have one normal and one abnormal β-globin allele. Their red blood cells are microcytic and hypochromic, yet the patients usually remain asymptomatic. Patients with β-thalassemia major have no normal β-globin allele, and they have pronounced anemia (more severe than patients with hemoglobin H disease). The anemia is due to ineffective erythropoiesis and decreased survival of circulating red blood cells (α_4 is noxious). The anemia develops around 6 months of age. Patients with β-thalassemia major depend on blood transfusions, and they need chelation therapy to remove excess iron from their body, which they acquire both enterally and via blood transfusions.
- Patients with sickle cell anemia are homozygous for β^S-globin (Glu6Val substitution). Deoxyhemoglobin S can polymerize and damage red blood cells. Erythrocytes from patients with sickle cell anemia adhere excessively to the vascular endothelium, and the spleen or liver removes them long before they are 120 days old. The polymerization of deoxyhemoglobin S has a lag time so that polymerization takes place only during a minority of oxygenation/deoxygenation cycles. Patients with sickle cell anemia suffer from occasional and potentially life-threatening acute painful episodes, which are the result of local vaso-occlusion. Due to the extreme concentration dependence of the polymerization reaction, both a small decrease in the concentration of deoxyhemoglobin S and an increase in the fraction of hemoglobin F (induced by treatment with hydroxyurea) can drastically decrease the incidence of vaso-occlusive crises. The red blood cells of patients with sickle cell trait do not sickle under physiological conditions.
- β^C-globin is Glu6Lys- and β^E-globin is Glu26Lys-substituted β-globin. Patients who are homozygous for β^C-globin have hemoglobin C disease and mild hemolytic anemia due to aggregation and crystallization of hemoglobin C that shortens the life span of red blood cells. Patients with

hemoglobin SC disease have symptoms that are intermediate between sickle cell trait and sickle cell anemia. Patients with hemoglobin E disease are homozygous for β^E-globin. They have microcytic red blood cells and mild anemia due to an impaired production of β^E-globin mRNA, which in turn impairs globin synthesis.

- Hemoglobin analyses provide clues to hemoglobinopathies. If an adult patient has mostly hemoglobin F, the patient may have β^0-thalassemia, and family members with an elevated fraction of hemoglobin A_2 may be β^0-thalassemia carriers. The presence of hemoglobin Barts at birth and hemoglobin H after that are characteristic of hemoglobin H disease. If hemoglobin C, E, or S account for the majority of the hemoglobin in a patient's red blood cells, the patient likely has hemoglobin C disease, hemoglobin E disease, or sickle cell anemia, respectively.

FURTHER READING

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Review Questions

1. A woman and a man consider having children. The woman has hemoglobin H disease. The man has α^0-thalassemia trait. What is the risk that their child will have the genotype for hemoglobin H disease?

   A. 0%
   B. 12.5%
   C. 25%
   D. 50%
   E. 100%

2. On her first visit to an obstetrician, a 33-year-old pregnant patient is screened for hemoglobin disorders. Hemoglobin electrophoresis yields the following result: HbA: 93%, HbS: 0%, HbF: 2%, HbA_2+HbC+HbE: 5% (normal: 2.5%). The patient's hemoglobin is 11.2 g/dL and the MCV is 70 fL. The patient's total iron-binding capacity is normal. She has no history of recent bleeding or blood donation. Based on these laboratory values and her history, the most likely diagnosis is which of the following?

   A. β-thalassemia minor
   B. Hemoglobin C trait
   C. Hemoglobin E trait
   D. Hemoglobin SC disease
   E. Sickle cell trait

3. Preoperative screening of a 45-year-old African-American woman for sickle cell disease reveals the following: 60% HbA, 2.5% HbA_2+HbC, 1% HbF, 36% HbS. This patient most likely has which of the following?

   A. β^+-thalassemia
   B. Diabetes
   C. Sickle cell anemia
   D. Sickle cell trait
   E. Sickle cell-hemoglobin C disease

4. A woman and a man consider having children. The woman has α^0-thalassemia trait, and the man has α^+-thalassemia trait. What is the risk that their child will have hemoglobin H disease?

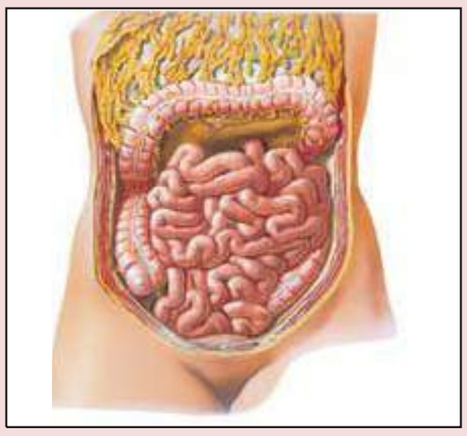
   A. 0%
   B. 25%
   C. 50%
   D. 100%

5. A patient who has sickle cell disease could have which one of the following genotypes?

   A. β-globin (normal)/β-globin^0
   B. β-globin (normal)/β-globin^S
   C. β-globin^C/β-globin^C
   D. β-globin^C/β-globin^S

6. A patient with which one of the following sets of laboratory data is most likely to have a hemoglobinopathy?

   A. Direct bilirubin/total bilirubin = 25%, normal ferritin
   B. ↓ Serum iron, ↓ MCV, ↓ hemoglobin
   C. ↑ Red blood cell count, ↑ hematocrit, normal bilirubin
   D. ↑ Total bilirubin, ↑ serum lactate dehydrogenase (LDH), ↓ MCV
   E. ↑ Total bilirubin, ↑ serum lactate dehydrogenase (LDH), ↑ MCHC



Chapter

18 Carbohydrate Transport, Carbohydrate Malabsorption, and Lactose Intolerance

SYNOPSIS

- Carbohydrates in the form of monosaccharides, disaccharides, and starches make up a large portion of our diet.
- The pancreas secretes amylase into the intestine. The outer surface of the microvilli of intestinal epithelial cells contains disaccharidases. Together, these enzymes degrade starches and disaccharides into monosaccharides, principally glucose, galactose, and fructose.
- Intestinal epithelial cells take up monosaccharides and then release them into the bloodstream.
- If intestinal digestive enzymes have reduced activity, or if monosaccharides are not properly taken up, carbohydrates reach the colon. There, bacteria degrade the carbohydrates to acids and to gases that may cause flatulence and abdominal pain.
- Certain dietary carbohydrates cannot be digested by humans. An enzyme supplement can aid in digestion and thus lessen the adverse effects of metabolism by bacteria.
- Lactose malabsorption is common among adults and is usually due to the normal hereditary inactivation of the expression of lactase after the first few years of life.
- A variety of transporters facilitate the recovery of monosaccharides from the glomerular filtrate and the uptake of monosaccharides from the blood into peripheral cells. In adipose tissue and skeletal muscle, insulin regulates monosaccharide transport.
- Inhibitors of renal glucose transporters and intestinal carbohydrate-degrading enzymes are used in the treatment of diabetes.

LEARNING OBJECTIVES

For mastery *of* this topic, you should be able to do the *f*ollowing:

- Describe the digestion of dietary starch and disaccharides and list transporters for monosaccharides, paying attention to tissue location.
- Interpret biopsy reports about disaccharidase activities.
- Explain the symptoms of lactose restriction (also called lactose intolerance, lactase deficiency, or hypolactasia) and describe an appropriate strategy for the prevention of symptoms.
- Describe the function and use of α-galactosidase (Beano).
- Describe the functions and uses of lactulose and sorbitol.
- Compare and contrast the characteristics of glucose transporters.
- Describe the effect of SGLT2 inhibitors on plasma glucose concentrations.
- Describe the effects of α-glucosidase inhibitors on carbohydrate digestion and list carbohydrates that can be absorbed even in the presence of an α-glucosidase inhibitor.

1. CLASSIFICATION OF CARBOHYDRATES

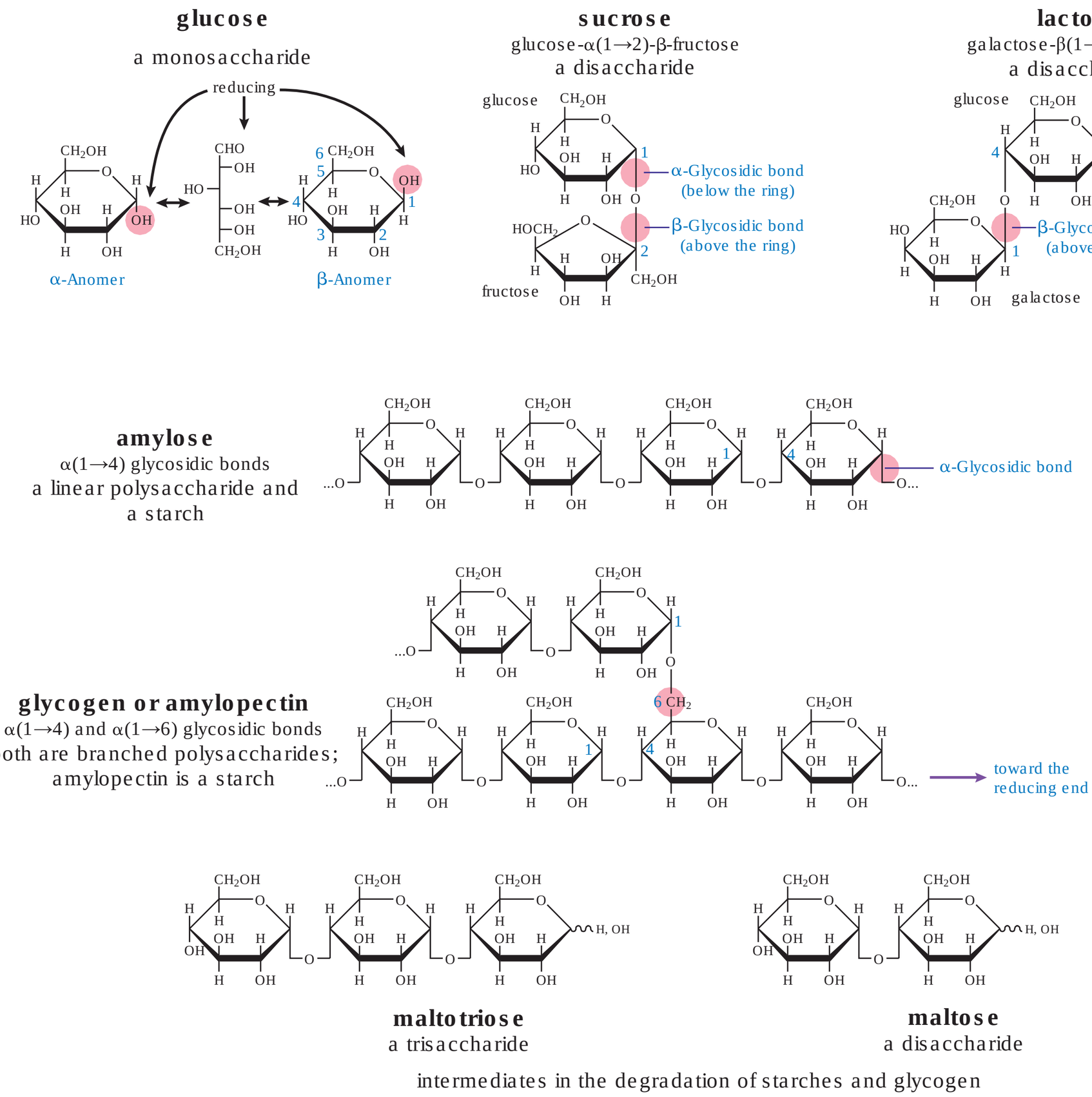
T e most important dietary carbohydrates are glucose, *f*ructose, and di-, oligo-, and polysaccharides that are made up o*f* glucose, *f*ructose, and galactose. Fiber contains carbohydrates that cannot be digested by human enzymes in the small intestine.

Carbohydrates were originally defined as compounds of the summary chemical formula $C_n(H_2O)_n$, but now the term also includes their derivatives, even if they have a slightly different formula. T e physiologically important carbohydrates are mono-, di-, oligo-, and polysaccharides. **Saccharide** is an ill-defined term that derives from the Latin saccharum (meaning sugar) and refers to carbohydrates that resemble chemically the sugars found in food (Fig. 18.1). **Monosaccharides** typically have five to seven carbon atoms; pentoses contain five carbons, hexoses six, and heptoses seven. T e most common monosaccharide is glucose ($C_6H_{12}O_6$). **Disaccharides** consist of two monosaccharides that are joined by a glycosidic bond (e.g., sucrose). **Oligosaccharides** typically consist of 3 to 10 monosaccharides (e.g., maltotriose). **Polysaccharides** consist of more than 10 monosaccharides (e.g., **glycogen**, which serves as an energy store in humans and animals; see Chapter 24). **Starches** are polysaccharides that serve as energy stores in plants; they consist of amylose and amylopectin.

In di-, oligo-, and polysaccharides, the monosaccharides are typically joined by glycosidic bonds that contain oxygen; such bonds are called **O-glycosidic** bonds. **N-Glycosidic** linkages, by contrast, contain nitrogen; these bonds are found in nucleotides, linking the ribose and the base (see Chapters 1, 37, and 38).

T e quantitatively most important sugars in the diet are glucose, galactose, and fructose. **Glucose** makes up the majority of dietary carbohydrates. Glucose is found as a monosaccharide in honey and fruits, as part of the disaccharides sucrose (table sugar) and lactose (milk sugar), and as the constituent of the glucose polymers amylose, amylopectin, and glycogen (see Fig. 18.1 and Chapter 24). **Galactose** is found almost exclusively in milk products as part of lactose (see Chapter 20). **Fructose** is found chiefly in dietary sucrose, in biochemically made high-fructose corn syrup, and as a monosaccharide in honey and many fruits (see Table 20.1).

Glucose and galactose exist in solution as mixtures of their **α- and β-anomers**, which dif er in the orientation of their –H and –OH groups at carbon number 1 (C-1) relative to the sugar ring structure. Since the sugar ring opens and closes frequently between the ring oxygen and C-1, α-anomers of these monosaccharides can rapidly become β-anomers and vice versa. One can indicate this rapid anomerization by writing “H, OH” next to a squiggly line that emanates from the anomeric carbon; examples are given in Fig. 18.1 with lactose, maltotriose, and maltose. T e clinically used term **dextrose** refers to crystals of α-glucose. Once dextrose is



intermediates in the degradation of starches and glycogen

Fig. 18.1 Chemical structures of common dietary carbohydrates. The branches of amylopectin are ~25 residues long. Glycogen usually has shorter branches and a larger molecular weight than amylopectin. Amylose, amylopectin, and glycogen typically contain a hundred to many thousands of glucose residues. Blue numbers indicate the chemical numbering of carbon atoms.

dissolved in water, some α-glucose rapidly isomerizes to β-glucose, so that comparable amounts of α- and β-glucose are present in solution.

Reducing sugars in stools are sometimes measured in the diagnostic evaluation of diarrhea. Reducing sugars have an aldehyde carbon (–CH=O) available for a redox reaction with Cu^{2+} or Ag^{+} in Fehling or Benedict solution, respectively. Glucose, lactose, galactose, and fructose are reducing sugars. When the aldehydes of glucose, galactose, or fructose are part of a glycosidic bond, they are no longer reducing. Hence, sucrose is not a reducing sugar. Starches contain only a single, terminal monosaccharide that is reducing, which pales compared with a large number of nonreducing glycosidic bonds (see Fig. 18.1).

T e term **fiber** encompasses polysaccharides (see Section 4 below) and **lignins** (polymers containing various methoxyphenols) that cannot be digested by human enzymes. Fiber is made by plants. **Soluble fiber** dissolves in water and forms a gel that slows the rate of digestion and the absorption of starch. Soluble fiber consists of **pectic substances** (branched polysaccharides rich in a derivative of galactose) and **gums**, which are found in fruits, legumes, and oats. Bacteria in the colon degrade soluble fiber to various short-chain acids and gases. **Insoluble fiber** does not dissolve in water and is poorly degraded by intestinal bacteria. Insoluble fiber is typically found in the outer layers of grains (i.e., in "**bran**") and consists mostly of lignins and the polysaccharides **cellulose** and **hemicellulose**. Insoluble fiber increases the bulk of feces.

2. DIGESTION OF POLYSACCHARIDES AND DISACCHARIDES IN THE SMALL INTESTINE

T e pancreas secretes amylase into the duodenum. Amylase partially hydrolyzes starches and glycogen. T e outer sur*f*ace o*f* small-intestinal epithelial cells contains disaccharidases

that hydrolyze disaccharides, as well as branch points in polysaccharides. The main disaccharidases of the small intestine are lactase-glycosylceramidase, sucrase-isomaltase, maltase-glucoamylase, and trehalase.

2.1. Structure of the Small Intestine

The small intestine consists of the duodenum, jejunum, and ileum. The duodenum and jejunum, but less so the ileum, contain many **folds** (plicae; Fig. 18.2). The folds and the remainder of the intestinal wall are covered by numerous 1-mm-long **villi**. The villi are fingerlike projections with central blood capillaries and a surface made up of many absorptive epithelial cells. Each absorptive epithelial cell has a plasma membrane that forms numerous **microvilli** (there are ~200 million microvilli per square centimeter of villus). As a result, the small intestine has a surface area of about 200 m^2. The wells at the base of (and between) the villi are called **crypts**. **Stem cells** near the bottom of a crypt give rise to absorptive **epithelial cells** that migrate to the tip of the villus; there, epithelial cells are shed approximately 2 to 3 days after their creation. **Goblet cells**, which migrate like absorptive cells, secrete mucins that give rise to **mucus** that covers the intestinal epithelium. **Paneth cells** at the base of the crypts play a role in immune surveillance and in maintaining the stem cells.

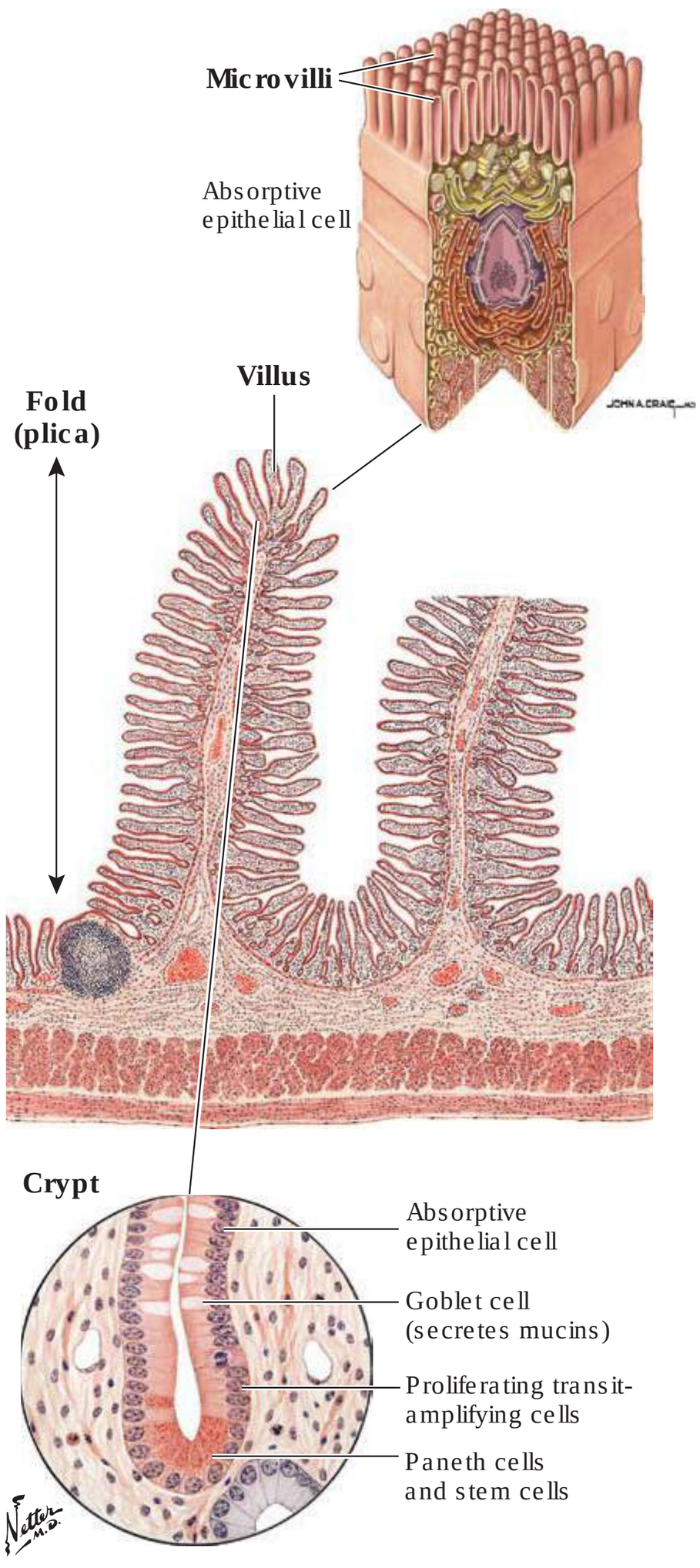


Fig. 18.2 Structure of the jejunum. The structures of the duodenum and ileum are similar, except that there are fewer folds.

2.2. Hydrolysis of Polysaccharides and Disaccharides to Monosaccharides

Carbohydrates account for 30% to 60% of the typical human caloric intake. A good portion of this carbohydrate is starch. An adult thus consumes the equivalent of about 200 to 450 g of glucose per day.

The small intestine contains polysaccharidases and disaccharidases that degrade dietary carbohydrates (Fig. 18.3). In the lumen of the intestine, polysaccharides in food particles are first degraded by **amylase**. Some of the amylase is derived from saliva (where it degrades amylose on teeth, the tongue, and the rest of the mouth, but plays only a minor role in the overall digestion of starch). Most of the amylase is secreted by the pancreas. Amylase degrades amylose and the linear portions of amylopectin mostly to **maltose** and **maltotriose**. In this way, amylopectin gives rise to **limit dextrins**, which are glucose polymers with linear portions of at most 10 monosaccharides. **Maltase-glucoamylase** and **sucrase-isomaltase** hydrolyze glucose residues near the branch points of limit dextrins, and isomaltase then hydrolyzes α(1→6)-linked residues. Mono-, di-, and oligosaccharides diffuse through the layer of mucus that covers the intestinal epithelium. In this way, they reach disaccharidases that are anchored to the luminal surface of intestinal epithelial cells. Some of the starch and glycogen escapes complete digestion in the small intestine and is degraded by **bacteria** in the ileum and colon (see Section 4 below). Normally, few bacteria colonize the stomach, duodenum, and jejunum.

Intestinal microvilli contain four different **disaccharidase complexes** (Fig. 18.4), which are encoded by four different genes and contain seven different hydrolytic enzyme activities, as described below. Each complex is derived from a single protein, protrudes into the intestinal lumen, and is anchored in the membrane of a microvillus (most often, the anchor consists of a membrane-spanning stretch of hydrophobic amino acids). Proteases, secreted from the pancreas into the lumen of the intestine, gradually degrade the disaccharidases. As a result, the disaccharidases have a mean lifetime of approximately 7 hours, and their activity changes up to two fold over the course of a day. Data on disaccharidase activities in biopsy material therefore must be interpreted accordingly.

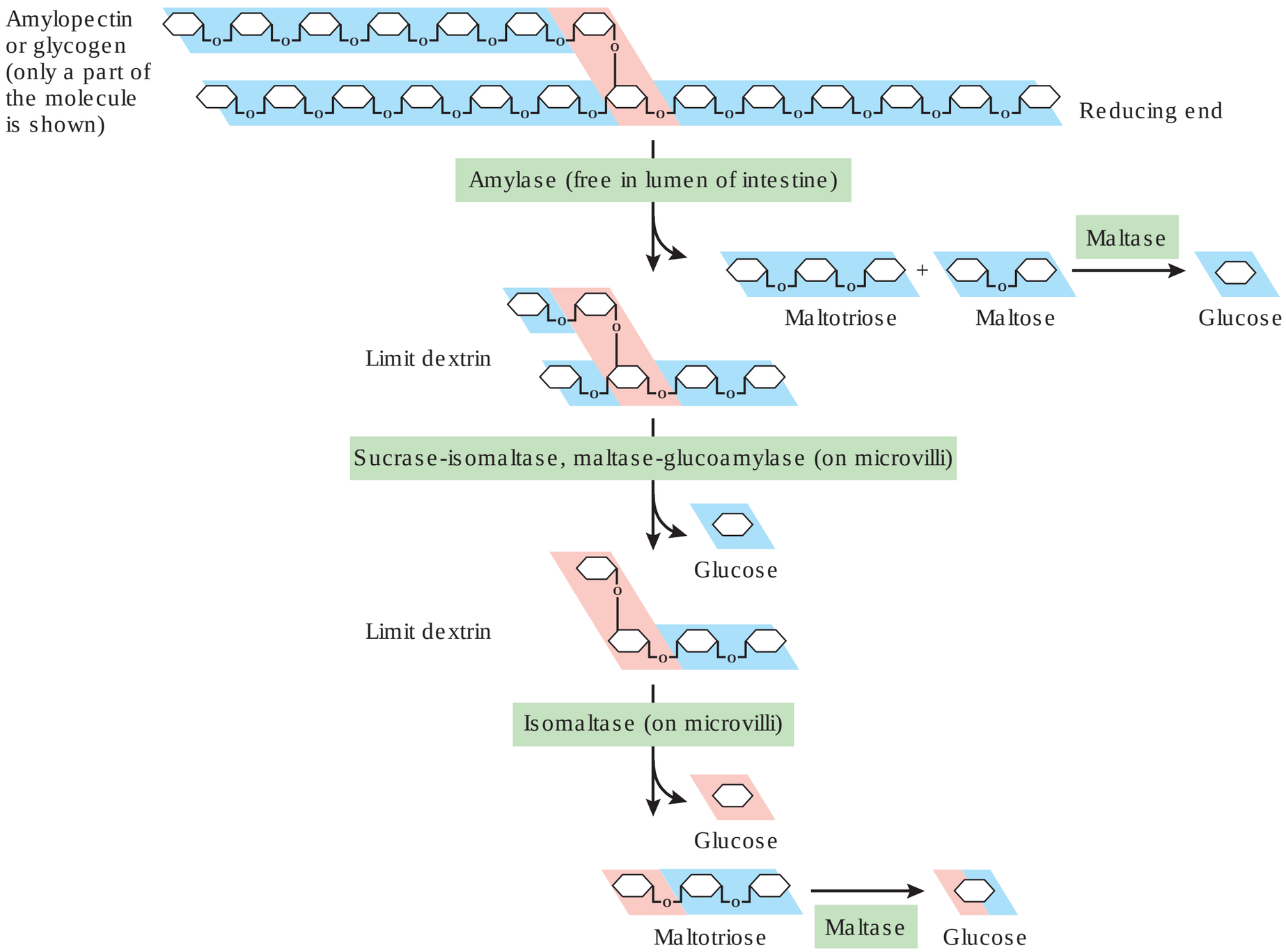


Fig. 18.3 **Hydrolysis in the intestine of amylopectin and glycogen to glucose.**

T e **sucrase** subunit of sucrase-isomaltase (see Fig. 18.4) hydrolyzes **sucrose** (see Fig. 18.1) into glucose and fructose; the isomaltase subunit hydrolyzes α(1→6)-glycosidic bonds that form the branch points in **limit dextrins**, which arise from the degradation of the branched polysaccharides amylopectin and glycogen (see Fig. 18.3). Sucrase-isomaltase also accounts for about 80% of the **maltose**-hydrolyzing activity in the intestine.

T e **maltase** subunit of maltase-glucoamylase (see Fig. 18.4) hydrolyzes maltose (see Fig. 18.1) to glucose, while the **glucoamylase** subunit hydrolyzes α(1→4)-glycosidic bonds in **starch** to glucose. Maltase accounts for about 20% of the maltose-hydrolyzing activity in the intestine. Compared with amylase, glucoamylase prefers shorter polyglucose chains; furthermore, while amylase produces only maltose and maltotriose, glucoamylase produces only glucose. In addition, glucoamylase works at the end of a polyglucose chain, whereas amylase functions better at internal sites.

T e **lactase** domain of lactase-glycosylceramidase (see Fig. 18.4) catalyzes the hydrolysis of **lactose** (see Fig. 18.1) to galactose and glucose, while the glycosylceramidase domain catalyzes the hydrolysis of a **glucosylceramide** to glucose and ceramide (ceramides contain two fatty acids; see Chapter 11). Lactase-glycosylceramidase is chiefly expressed in infancy (see Section 5 below).

Trehalase (see Fig. 18.4) hydrolyzes trehalose to glucose. **Trehalose** is the disaccharide glucose-α(1→α1)-glucose, which is found chiefly in mushrooms.

3. TRANSPORT OF MONOSACCHARIDES

Sodium-driven transporters *f*or glucose and galactose pump glucose and galactose into intestinal epithelial cells and cells o*f* the kidney tubules. Other transport proteins then *f*acilitate equilibration o*f* sugars between these epithelial cells and the blood. All other cells contain monosaccharide transporters that only *f*acilitate equilibration (not pumping) across the plasma membrane. Some cells insert these transporters into the plasma membrane only in the presence o*f* insulin or upon exercise.

3.1. Intestinal Monosaccharide Transport

T e microvilli of the small intestine contain a sodium-driven transporter for glucose and galactose (Fig. 18.5). Glucose and galactose are chiefly derived from the hydrolysis of di- and polysaccharides (see Section 2.2). Intestinal epithelial cells, like other cells in the body, contain a relatively low concentration of Na^+, while the intestinal lumen contains a much higher

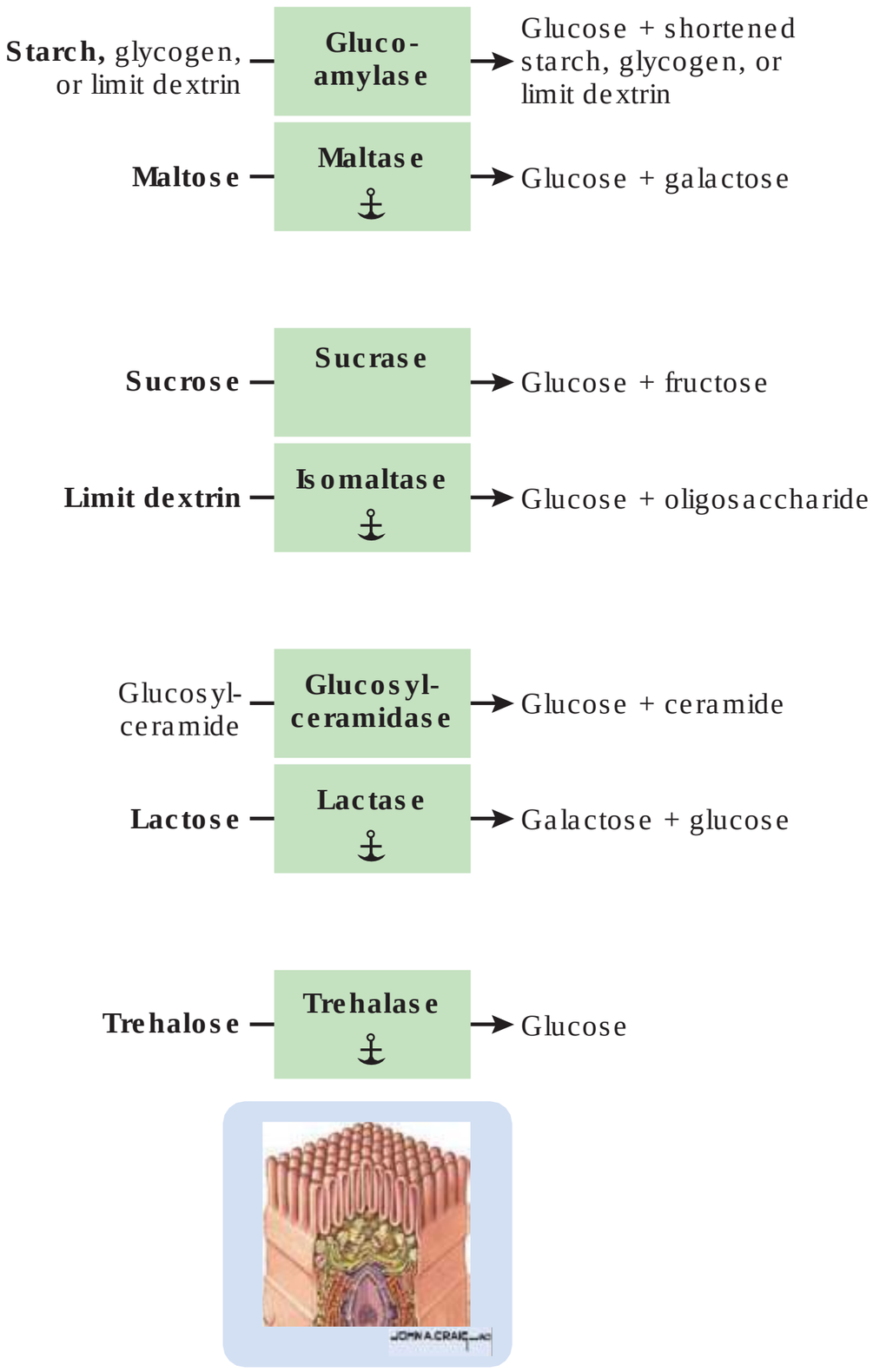


Fig. 18.4 Disaccharidases of the small intestine are anchored to the microvilli. Close stacking of enzyme names indicates tight binding of two enzymes; the anchoring unit is labeled.

concentration of Na^+ (the Na^+ in the intestinal lumen derives mostly from the fluid that is secreted by the exocrine pancreas). The inward-directed concentration gradient for Na^+ drives the uptake of glucose and galactose through the **Na:glucose cotransporter** (**SGLT1**). This transporter has a 2 Na^+:1 monosaccharide stoichiometry, which allows virtually complete uptake of glucose and galactose from the intestinal lumen. The Na:glucose cotransporter transports either glucose or galactose.

The transport of glucose into the intestine tends to collapse the transmembrane Na^+ gradient; however, the basolateral membrane contains a **Na^+, K^+-ATPase**, which pumps Na^+ out of the cell into the extracellular space, from where it enters the bloodstream (Na^+ returns to the intestinal lumen via the pancreas; K^+ leaves the cell via K^+-channels).

The basolateral membrane of small-intestinal epithelial cells contains a transporter (**GLUT-2**) that facilitates the equilibration of **glucose** and **galactose** between the epithelial cells and the blood. Thus, whenever the concentration of glucose or galactose is higher in the intestinal epithelial cells than in the blood, glucose or galactose show a net movement from the absorptive cells into the blood. Conversely, when the blood contains a higher concentration of glucose or galactose, these monosaccharides move from the blood into intestinal epithelial cells.

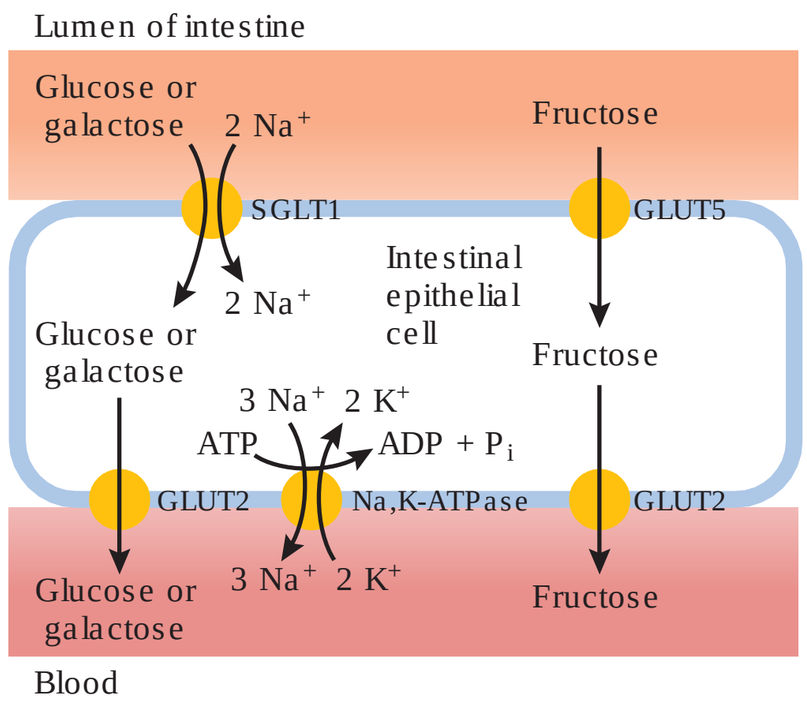


Fig. 18.5 Transport of glucose, galactose, and fructose from the intestinal lumen to the bloodstream.

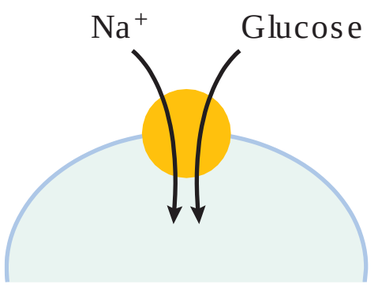
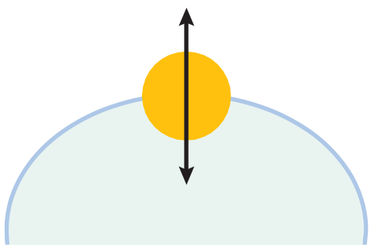
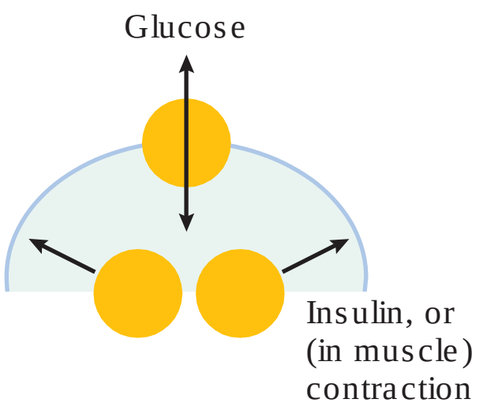
The small-intestinal epithelial cells also contain a transporter (**GLUT-5**) that facilitates the flow of **fructose** from the intestinal lumen into epithelial cells. Efflux of fructose from intestinal epithelial cells and uptake into other cells largely occurs via GLUT-2 transporters. The liver removes fructose from the blood and phosphorylates it, thereby acting as a sink for fructose (see Chapter 20).

3.2. Monosaccharide Transport in Tissues Other Than the Intestine

Different glucose transporters move glucose and galactose between the blood and peripheral cells (Table 18.1). Some of these transporters are in the cell membrane at all times, as in red blood cells or hepatocytes. Others are stored intracellularly and move into the plasma membrane only when the concentration of **insulin** is elevated; this is the case in adipose tissue and skeletal muscle. In skeletal muscle, contraction also causes plasma membrane insertion of these glucose transporters (for more details, see Chapters 19, 24, 25, and 26). In a variety of tissues, **hypoxia** can induce the expression, plasma membrane insertion, and activity of yet other passive glucose transporters.

The **GLUT glucose transporters** bind glucose on one side of the membrane, undergo a **conformational change**, and then release glucose on the other side of the membrane (Fig. 18.6). Thus, the substrate-binding site is alternately accessible from the inside and outside of a cell. Since these are passive transporters that facilitate diffusion, transport occurs from the side with the higher to the side with the lower glucose concentration. Section 2.2 of Chapter 11 contains a general discussion and classification of transport proteins in membranes.

Table 18.1 Glucose Transporters in Human Tissues

Category of Transporter	Tissue Location	Specific Transporter, Comments
Na+-driven pump 	Intestinal epithelium (luminal side)	SGLT1 accepts glucose and galactose and is a 2 Na^+:1 monosaccharide cotransporter.
	Kidney tubules	SGLT2 accepts glucose (not galactose) and is a 1 Na^+:1 monosaccharide cotransporter; it moves the bulk of filtered glucose. SGLT1 moves the remainder of the filtered glucose.
Facilitated diffusion, not insulin-regulated 	Erythrocytes	GLUT-1 facilitates uptake of glucose into erythrocytes for energy generation.
	Liver	GLUT-2 allows the liver to take up glucose in the fed state and release it in the fasting state.
	Intestinal epithelium	GLUT-2 equilibrates glucose between the basolateral side of epithelial cells and the blood.
	Kidneys	GLUT-1 and GLUT-2 equilibrate glucose with the blood.
	Islet β-cells	GLUT-2 equilibrates extracellular and intracellular glucose, which facilitates intracellular glucose sensing.
	Brain	GLUT-1 and GLUT-3 allow neurons and glial cells to take up glucose for energy generation.
Facilitated diffusion, insulin-sensitive 	Adipocytes	GLUT-4 is active postprandially to transport glucose for deposition of fatty acids as triglycerides.
	Muscle (skeletal and cardiac)	GLUT-4 is active during muscle contraction and also postprandially to provide glucose for contraction and glycogen synthesis.

The glucose transporters **GLUT-1**, **GLUT-3**, and **GLUT-4** also transport **dehydroascorbic acid**. Many animals, but not humans, can derive **L-ascorbic acid** (**vitamin C**) from glucose. L-ascorbate is an important redox cofactor with a variety of functions. It plays a role in reducing Fe^{3+} in the lumen of the intestine to Fe^{2+} that is then taken up (see Section 2 in Chapter 15). It is a required cofactor for the hydroxylation of proline residues in collagen to form hydroxyproline (see Section 1.2 in Chapter 12) and in the synthesis of norepinephrine in the nervous system (see Fig. 35.14). L-ascorbate also functions as an antioxidant throughout the body (see Section 2.3 in Chapter 21), but it is present at an especially high concentration in the brain. When L-ascorbate donates two electrons, it becomes dehydroascorbate. While dehydroascorbate is transported by GLUTs, L-ascorbate is mostly transported by the **Na^+-dependent vitamin C transporters 1** and **2**.

4. BACTERIAL METABOLISM OF UNDIGESTED CARBOHYDRATES THAT REACH THE COLON

Bacteria in the distal ileum and the colon degrade some of the unabsorbed carbohydrates. This degradation may give rise to borborygmi, abdominal pain, and flatus. Hydrogen gas in exhaled air is a convenient diagnostic measure of bacterial metabolism following oral carbohydrate intake.

The **intestinal flora** plays important roles in carbohydrate digestion and the conservation of water and electrolytes. Bacteria colonize the colon and distal ileum in large numbers, and adults excrete more than 50 g of microorganisms per day. Bacteria in the colon degrade carbohydrates to diverse products, such as **hydrogen** (H_2), methane, acetic acid, butyric acid, and propionic acid, which are taken up into the bloodstream or leave the intestine with feces. Indeed, oxidation of butyric acid is the principal source of energy in epithelial cells of the colon. When bacteria in the colon have been decimated

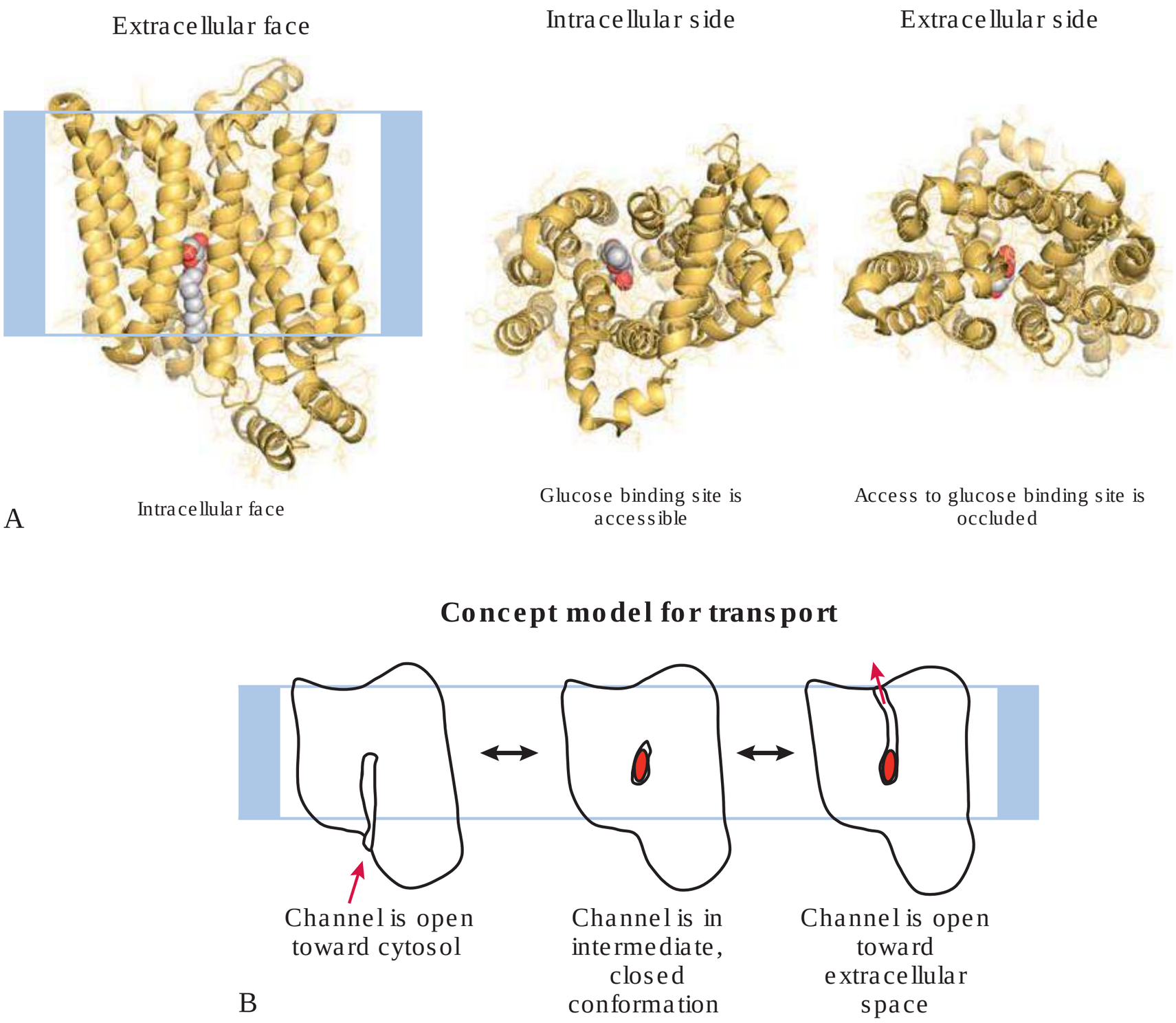


Fig. 18.6 Crystal structure of the human GLUT-1 glucose transporter (open to intracellular side). The structure contains a glucose analog with a 9-carbon alkyl chain. (Data from Protein Data Bank [www.rcsb.org] file 4PYP and Deng D, Xu C, Sun P, et al. Crystal structure of the human glucose transporter GLUT1. *Nature*. 2014;510:121-125.)

(e.g., by **antibiotics**), or when the metabolic capacity of the normal intestinal flora is overwhelmed by unabsorbed carbohydrates, the remaining osmotically active nutrients may attract enough water to cause **diarrhea**, with the attendant loss of water and electrolytes. Per each mole of unabsorbed, osmotically active carbohydrate, the stools contain about 3.5 L of water (for instance, 1 g of lactose in the colon pulls with it about 10 g of water). Therefore, osmotically active, poorly degradable carbohydrates, such as insoluble **fiber** (see Section 1), are useful as **stool softeners**.

A **hydrogen breath test** following a carbohydrate load is often used in the diagnosis of carbohydrate malabsorption (Fig. 18.7). When bacteria digest carbohydrates in the colon, they produce gases, including H_2. H_2 diffuses to the blood, and, upon reaching the lungs, some of it is exhaled. The more carbohydrates the bacteria digest, the more H_2 is found in exhaled air. H_2 in a patient's breath is often measured before and at several time points 0.5 to 8 hours after the administration of a test sugar (e.g., lactose, fructose, xylose; often 25 to 50 g in an otherwise healthy adult). Patients differ in their gut microflora; as a result, up to 20% of patients fail to exhale significant H_2 from undigested carbohydrate. However, such patients can be given **lactulose** (galactose-β(1→4)-fructose), a sugar that is digested only by bacteria, to test for a proper H_2 response. Lactulose is also used as a laxative.

A hydrogen breath test after oral **lactulose** or **glucose** is also used to evaluate patients for **small-intestinal bacterial overgrowth**. An affected patient exhales an abnormally large amount of H_2. Glucose shows lower sensitivity than lactulose because the proximal small intestine absorbs glucose before it reaches the distal portion.

If the **stool pH** of a patient with diarrhea is below 5.6, the patient most likely has pure **carbohydrate malabsorption** (see Section 5). When excessive quantities of carbohydrates (at least about 50 g) reach the colon, the bacteria in the colon produce more acids (e.g., acetic acid, propionic acid, and butyric acid) from these carbohydrates than the colon can absorb, and the acids then lower the pH of feces to an abnormally low value.

Some carbohydrates are normally only partially digested by human enzymes in the small intestine. Thus, normally, 5% to 20% of **starch** (especially amylose) escapes degradation by

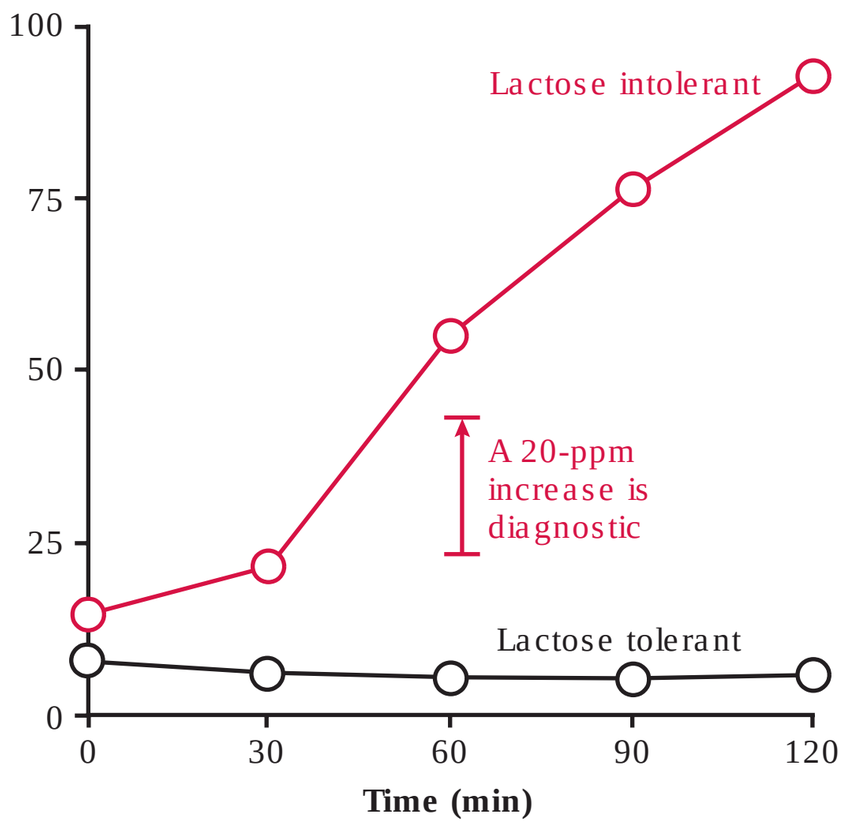
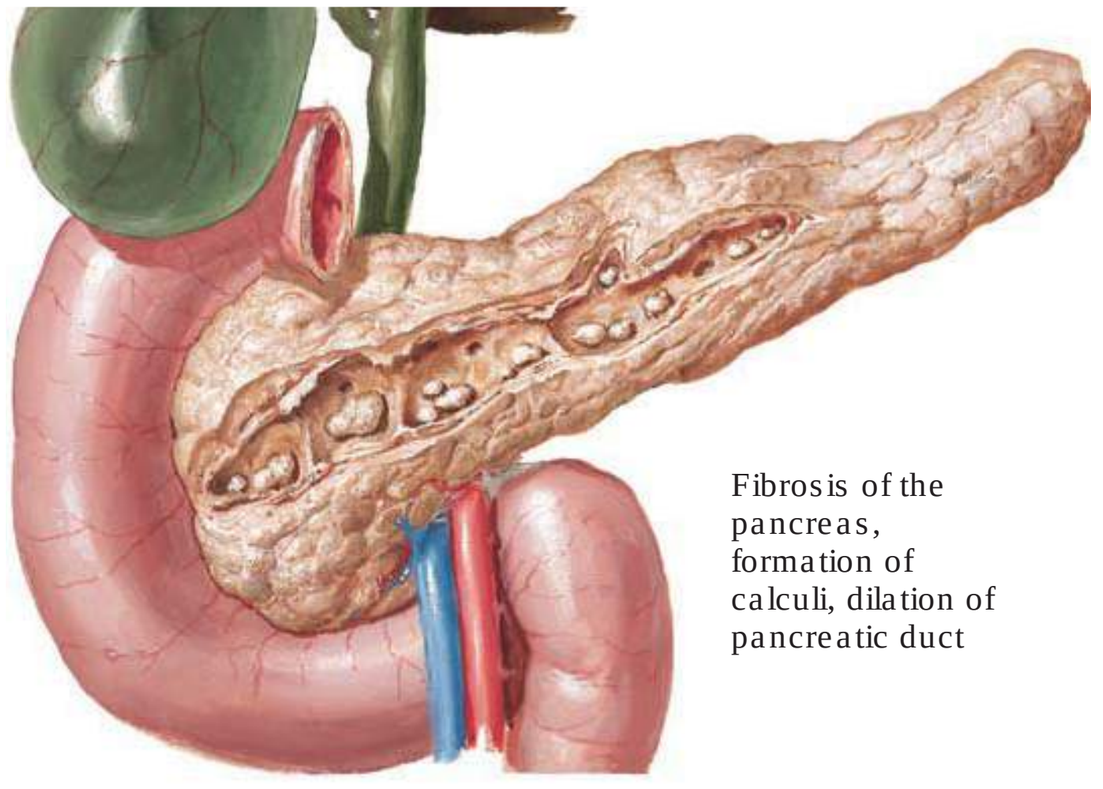


Fig. 18.7 Approximate mean H_2 in breath during a lactose breath hydrogen test. Patients were classified by lactase nonpersistence genotype. (Based on data from Waud JP, Matthews SB, Campbell AK. Measurement of breath hydrogen and methane, together with lactase genotype, defines the current best practice for investigation of lactose sensitivity. *Ann Clin Biochem*. 2008;45:50-58 and Nagy D, Bogacsi-Szabo E, Varkonyi A, et al. Prevalence of adult-type hypolactasia as diagnosed with genetic and lactose hydrogen breath tests in Hungarians. *Eur J Clin Nutr*. 2009;63:909-912.)



Fibrosis of the pancreas, formation of calculi, dilation of pancreatic duct

F. Netter M.D.

Fig. 18.8 Chronic pancreatitis. The disease is commonly due to long-term excessive consumption of alcohol, but it can also be due to a mutation in the chloride transporter CFTR or the protease trypsinogen.

amylase and disaccharidases. Similarly, **fructose**, when present in sufficient quantity, can escape intestinal uptake and lead to abdominal pain and diarrhea. T is frequently happens with children who drink large volumes of apple juice.

Some carbohydrates cannot be digested by human enzymes in the small intestine, whereas bacteria in the ileum and colon can digest them. Since humans do not produce α-galactosidase, they cannot degrade dietary α-galactosides, the major ones being raffinose and stachyose. **Raffinose** (galactose-α(1→6)-glucose-α(1→2)-β-fructose) and **stachyose** ("galactose-elongated raffinose" (i.e., galactose-α(1→6)-galactose-α(1→6)-glucose-α(1→2)-β-fructose) are both found in legumes (e.g., beans) and to a lesser extent in vegetables of the Brassica genus (e.g., cabbage, Brussels sprouts, and broccoli). Bacteria in the colon do possess α-galactosidase and degrade these oligosaccharides. Oral **α-galactosidase** (**Beano**) cleaves dietary raffinose and stachyose into galactose and sucrose while in the small intestine; Beano can thus prevent abdominal discomfort after the ingestion of raffinose or stachyose. Human enzymes also fail to degrade **sorbitol**, a reduced derivative of glucose. Sorbitol is quite abundant in prunes, and somewhat less abundant in pear juice and apple juice. Sorbitol is the most frequently used sugar in "sugarless" chewing gum. Sorbitol can be used as an **excipient**, an inactive substance that acts as a carrier of an active substance in a tablet. Intestinal bacteria do degrade sorbitol, and these bacteria can produce gas.

Most **insoluble fiber** (i.e., cellulose, lignins) cannot be digested by either human enzymes or bacteria in the human intestine. T e nature of insoluble fiber is described in Section 1.

5. CARBOHYDRATE MALABSORPTION

Carbohydrate malabsorption occurs when dietary carbohydrate is not adequately digested either by the patient's pancreatic and small-intestinal enzymes or by bacteria in the colon and distal ileum. Carbohydrate malabsorption is usually due to pancreatic insu*ffi*ciency, diminished *f*unctional capacity o*f* the small intestine to degrade and transport carbohydrates, or an inadequate bacterial *fl*ora.

5.1. General Comments

Inadequate digestion of carbohydrates can be the cause of inadequate growth in young children.

Oral sugar **tolerance tests** provide information about the overall digestive capacity of the intestine. Such tests are less invasive and often more meaningful than measurements of disaccharidase activities in biopsies of the jejunal or duodenal epithelium. T e oral load is usually 2 g/kg of body weight, not to exceed 50 g total. Oral sugar tolerance tests may include measurements of sugar in blood or feces (see Section 1); alternatively, bacterial metabolites may be measured in blood (e.g., acetate), feces (e.g., lactate), or exhaled air (e.g., hydrogen gas; see Section 4).

As a rule, sugars are absent from the stools. If sugars are found in the stools, the patient has carbohydrate malabsorption.

5.2. Pancreatic Insufficiency

Patients with severe pancreatic insufficiency due to **pancreatitis** (Fig. 18.8), pancreatic **cancer**, or **cystic fibrosis** have significantly decreased small-intestinal carbohydrate absorption.

As pancreatic insufficiency develops, digestion of lipids and proteins is impaired before the digestion of starch and glycogen. Oral pancreatic enzymes can make up for the abnormality.

5.3. Diminished Capacity of the Small Intestine to Degrade Carbohydrates

The most common problem of carbohydrate absorption is **lactose intolerance** (also called **lactose restriction**, **lactase deficiency**, and **hypolactasia**), which affects most of the world's **adults**. The exceptions are people from ethnic groups in which the keeping of cattle and the consumption of unfermented milk is or was common (i.e., parts of Africa, Asia, and Europe). These latter adults express sufficient lactase to handle about 50 to 100 g of lactose per day (the amount contained in about 0.8 to 1.7 L of milk). Lactase persistence is inherited in an autosomal dominant fashion. In people with nonpersistence, consumption of as little as 3 g lactose/day may be more than the small-intestinal lactase can handle and may lead to excessive production of gas by bacteria in the colon (see Fig. 18.7). The patient then experiences any of the following: bloating, borborygmi (i.e., rumbling in the intestine from moving gas and fluid), abdominal pain, flatulence, and diarrhea (Fig. 18.9). Patients with poor lactose tolerance should either avoid consuming more lactose than their lactase can handle, or they should take **oral lactase** (drops or pills). Lactose amounts to approximately 6% of the weight of milk (regardless of the fat content), 4% of ice cream, 4% of cheese, and 2% of yogurt. Some milk products are treated with lactase to reduce their lactose content.

The **diagnosis of lactose intolerance** may involve an oral lactose tolerance test (50 g of lactose in water by mouth with repeated measurement of H_2 in exhaled air [Fig. 18.7] or measurement of the concentration of glucose in blood).

Premature babies are often lactose intolerant because cells of the small intestine fully express lactase only late in the third trimester of pregnancy, but **congenital lactase deficiency** is rare. Premature babies and babies with congenital lactase deficiency are both treated with an appropriate low-lactose diet.

Infection of the upper small intestine with the pathogenic protozoan Giardia lamblia is very common. The infection leads to villus atrophy and consequent symptoms of malabsorption.

The decimation of absorptive epithelial enterocytes by cancer **chemotherapy**, protein **malnutrition**, or **bacterial toxins** also leads to carbohydrate malabsorption.

Celiac disease (also called celiac sprue or gluten-sensitive enteropathy) is an autoimmune disease that is characterized by chronic inflammation of the small-intestinal mucosa, atrophy of intestinal villi, and malabsorption. In Europe and North America, celiac disease affects about 1% of the population. In affected patients, inflammation occurs in response to the consumption of **gliadin** proteins (which are part of **gluten**) present in wheat, rye, and barley. The inflammation leads to a loss of epithelial cells and a concomitant loss of digestive and absorptive capacity.

In the treatment of type 2 diabetes, **acarbose**, an analog of polysaccharides, is sometimes used to inhibit amylase and intestinal disaccharidases; acarbose thus induces mild carbohydrate malabsorption. Acarbose inhibits α-glucosidases, such as amylase, maltase, and sucrase; it does not inhibit β-glucosidases (e.g., lactase). Flatulence and diarrhea are side effects of acarbose.

Short bowel syndrome occurs in patients who have less than 50% of the normal length of small bowel. In these

Fig. 18.9 Adverse effects of lactose in lactose-intolerant patients. The same symptoms apply to other components of the diet that elicit extensive gas production by bacteria and/or result in osmotically active compounds in the colon.

patients, absorption of carbohydrates in the small intestine is significantly reduced, while bacterial fermentation is increased.

Trehalase deficiency causes diarrhea after the consumption of mushrooms, but the link between cause and effect is often not recognized. Affected patients should abstain from consuming the offending sugar. Among most populations, trehalase deficiency is probably uncommon.

Congenital sucrase-isomaltase deficiency affects about 0.1% of the population in Europe and the United States and roughly 5% of the Inuit in the arctic regions of Greenland, Canada, and Alaska. The deficiency first manifests in infants when they ingest sucrose with formula or other food (note that many formulas are free of sucrose). The final diagnosis relies on sucrase-isomaltase activity in biopsy material. There are many different sucrase-isomaltase mutations. Almost all patients with sucrase-isomaltase deficiency lack sucrase activity but this can be of limited clinical consequence because maltase-glucoamylase splits α(1→4)-glycosidic bonds. Some patients have no loss in isomaltase activity, whereas others have complete loss of it, which greatly impairs degradation of amylopectins [i.e., α(1→6)-branched starches]. Some 5% of the U.S. population is heterozygous for sucrase-isomaltase deficiency, and some of these patients therefore have a reduced capacity for carbohydrate digestion. Treatment of any sucrase-isomaltase deficiency involves the restriction of starch and/or sucrose intake to a tolerable level.

Glucose-galactose malabsorption is due to a deficiency in the **Na^+-driven glucose transporter** (**SGLT1**) in the intestine. This is rare deficiency becomes apparent shortly after the first feeding. Lactose in milk is hydrolyzed to galactose and glucose, which reach the colon and cause massive diarrhea. Treatment is difficult because patients must not consume appreciable quantities of any carbohydrates that contain glucose or galactose. Patients can absorb fructose, but the consumption of a large amount of fructose is unhealthy (see Chapter 20). The transporter deficiency affects the kidney tubules to a lesser degree because the tubules also express the SGLT2 Na-driven glucose transporter (see Table 18.1).

6. SGLT INHIBITORS: DRUGS THAT INHIBIT Na^+-COUPLED GLUCOSE TRANSPORT

Drugs that inhibit Na^+-coupled glucose uptake in the kidneys are used to lower the concentration of glucose in the blood of patients who have type 2 diabetes.

Without treatment, patients who have **diabetes** have chronically elevated concentrations of glucose in the blood. Long-term, chronic hyperglycemia leads to damage of the retina, kidneys, peripheral nerves, and other systems (see Chapter 39). Treatment of diabetes is aimed at lowering the concentration of glucose in the blood towards the normal value. The treatment should not induce hypoglycemia, which is acutely dangerous.

Inhibition of the Na^+-coupled glucose transporter **SGLT2** by one of the **gliflozin** drugs lowers blood glucose in type 2 diabetic patients by preventing the uptake of some of the filtered glucose in the kidneys. The kidneys use two types of glucose transporter to recover glucose from the glomerular filtrate, SGLT2 and SGLT1. SGLT2 transporters in the proximal convoluted tubules have a 1:1 stoichiometry for Na^+ and glucose and they normally transport most of the filtered glucose. Then, cells in the proximal straight tubule use SGLT1 transporters with a 2 Na^+:1 glucose stoichiometry to take up essentially all remaining glucose. In the kidneys, SGLT1 transporters have a lower transport capacity than the SGLT2 transporters. Near-complete inhibition of SGLT2 by a gliflozin leaves all of the glucose transport to the SGLT1 transporters, which exceeds their capacity and thus results in a ~35% loss of filtered glucose. This is ongoing loss of glucose into the urine leads to a lowering of blood glucose by about 10 mg/dL. Currently, FDA-approved inhibitors are **canagliflozin, dapagliflozin,** and **empagliflozin**.

SUMMARY

- The most common dietary carbohydrates are the monosaccharides glucose, galactose, and fructose; the disaccharides lactose, and sucrose; and the polysaccharides starch and glycogen. Lactose consists of glucose and galactose, while sucrose consists of glucose and fructose. The starches consist of variable mixtures of the linear polysaccharide amylose and the branched polysaccharide amylopectin; the structure of glycogen resembles that of amylopectin. Glucose, galactose, fructose, and lactose are reducing sugars.
- Fiber consists of oligosaccharides, polysaccharides, and lignins that cannot be digested by human enzymes; however, bacteria in the gut can degrade part of this fiber.
- The walls and folds of the small intestine are covered with villi of ~1 mm length. The surface of the villi contains absorptive cells that are continually renewed. The plasma membrane of these absorptive cells forms numerous microvilli, to which disaccharidases are anchored. The microvilli and disaccharidases are covered by mucus, through which di- and oligosaccharides diffuse much faster than do large polysaccharides.
- In the small intestine, amylase degrades the linear polysaccharide amylose to maltotriose and maltose. Sucrase-isomaltase and maltase-glucoamylase hydrolyze maltotriose and maltose to glucose. The branched polysaccharides amylopectin and glycogen are degraded similarly, except that branched oligosaccharides, so-called limit dextrins, are also formed. Sucrase-isomaltase and maltase-glucoamylase hydrolyze limit dextrins to glucose.
- Microvilli of the small intestine contain Na^+-dependent glucose transporters (SGLTs) that pump glucose or galactose into intestinal epithelial cells. Glucose and galactose leave these cells via facilitative glucose transporters of the GLUT family. GLUT transporters also facilitate the entry of glucose and galactose into peripheral cells. SGLT and GLUT transporters also participate in the recovery of glucose from blood filtrate in the kidneys.

- Gliflozin inhibitors of SGLTs induce glucosuria and are used in the treatment of type 2 diabetes.
- Fructose dif uses through facilitative transporters in the absorptive epithelial cells into the bloodstream.
- T e colon and the terminal ileum contain bacteria that degrade unabsorbed carbohydrates to acids and gasses that are either taken up into the bloodstream or leave the intestine via feces or flatus. Since humans do not digest 10% to 25% of dietary starch, bacteria degrade it. Bacteria also degrade some of the dietary fiber (most of it soluble), and most of the dietary sorbitol, lactulose, raffinose, and stachyose, which human enzymes cannot degrade. Saccharides that are osmotically active and at most only partially degraded by bacteria are ef ective as stool softeners.
- Carbohydrate malabsorption occurs as a consequence of inadequate secretion of amylase from the pancreas, inadequate disaccharidase activity, or inadequate uptake of monosaccharides. Laboratory data that are indicative of carbohydrate malabsorption are increased breath hydrogen, blood lactate, or fecal acetate after a carbohydrate load, or a pH of feces lower than 5.6. T e release of pancreatic amylase may be compromised by pancreatitis, pancreatic cancer, or cystic fibrosis. T e absorptive capacity of the intestinal epithelium may be diminished by inflammatory bowel disease, infection, protein malnutrition, or a hereditary deficiency of a disaccharidase or a monosaccharide transporter.

FURTHER READING

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Review Questions

1. A patient has symptoms of lactase deficiency. A hydrogen breath test during an oral 50 g lactose load is negative, even though the patient has symptoms of malabsorption. To determine whether there is a problem with the hydrogen breath test, the test should be repeated with which of the following?

   A. Antibiotics
   B. Fructose
   C. Galactose
   D. Glucose
   E. Lactulose

2. In response to a large mixed meal, the number of glucose transporters in the plasma membrane increases most markedly in which one of the following cell types?

   A. Adipocyte
   B. Hepatocyte
   C. Intestinal epithelial cell
   D. Neuron
   E. Red blood cell

3. A patient who has diabetes routinely takes acarbose, an inhibitor of intestinal α-glucosidases. One day, he accidentally also injects too much insulin and becomes mildly hypoglycemic. Which one of the following carbohydrates should he consume to increase the concentration of glucose in his blood as quickly as possible?

   A. Amylose
   B. Lactose
   C. Maltose
   D. Stachyose
   E. Sucrose

4. Abnormally low lactase activity was found in biopsied material from the small intestine of a 2-month old, ill baby who had persistent severe vomiting and diarrhea. T e baby had been breastfed. T e low lactase activity could be due to which of the following?

   A. Congenital isomaltase deficiency
   B. Deficiency of the intestinal fructose transporter
   C. Insufficient secretion of amylase
   D. Protein malnutrition

Chapter

19 Glycolysis and Its Regulation by Hormones and Hypoxia

SYNOPSIS

- Glycolysis is the process of converting glucose to pyruvate. All cells can perform glycolysis.
- Red blood cells and the glial cells of the brain always depend on glycolysis for adenosine triphosphate (ATP) production; most other cells can produce sufficient ATP from other fuels.
- As you will see in Chapters 22 and 23, mitochondria can metabolize pyruvate to CO_2 and water by the combination of the citric acid cycle and oxidative phosphorylation (Fig. 19.1); this yields a much larger amount of ATP than does glycolysis alone.
- If pyruvate is not metabolized in mitochondria, it is reduced to lactate, which is released into the blood. This happens, for instance, in red blood cells, contracting fast-twitch muscle cells, and hypoxic cells.
- Adenosine monophosphate (AMP) stimulates glycolysis, while ATP feedback inhibits it. As a result, glycolysis tends to maintain a constant, relatively high concentration of ATP. Furthermore, ATP that is produced in the combination of citric acid cycle and oxidative phosphorylation attenuates the flux in glycolysis.
- In some cells, glycolysis is regulated by hormones (e.g., insulin, glucagon, and epinephrine).
- If mitochondrial ATP production is impaired (e.g., due to local hypoxia), glycolysis speeds up and produces lactic acid. The human body cannot handle the acid load that results from persistent anoxia in all organs; the resulting acidosis is fatal.
- Flux in glycolysis is impaired during hypophosphatemia, which is a complication of parenteral nutrition, chronic alcohol abuse, or diabetic ketoacidosis.
- Heritable deficiencies of enzymes of glycolysis are quite rare. In red blood cells, they give rise to hemolytic anemia.

LEARNING OBJECTIVES

For mastery *of* this topic, you should be able to do the *following*:

- Describe the overall purpose of glycolysis, its reactants and products, its cellular location, and its tissue distribution.
- Describe the roles of hexokinase or glucokinase, phosphofructokinase-1 (PFK-1), and pyruvate kinase in glycolysis, thereby paying attention to the compounds that regulate the activity of these enzymes.
- Describe the roles of AMP, ATP, fructose 1,6-bisphosphate, and fructose 2,6-bisphosphate in the regulation of glycolysis.
- Explain how and in which tissues insulin, glucagon, and epinephrine can regulate flux in glycolysis.
- Compare and contrast aerobic and anaerobic glycolysis regarding the tissues and conditions in which they occur. Describe the reactants and products of these two processes.
- Describe the purpose of the reaction catalyzed by lactate dehydrogenase, its reactants, and products, cellular and tissue location. Interpret the findings of an elevated concentration of lactate dehydrogenase in blood plasma.
- Describe the role and fate of cytosolic NADH produced in glycolysis.
- Predict changes in the rate of ATP synthesis and the concentrations of intermediates of glycolysis when there is a deficiency of an enzyme of glycolysis (e.g., diminished activity of glyceraldehyde phosphate dehydrogenase, or pyruvate kinase deficiency).
- Describe the cause, signs, and treatment of pellagra.
- Describe the role and production of 2,3-bisphosphoglycerate in red blood cells, and list the factors that influence its concentration.
- Interpret/explain the reason for a strong positron emission tomography (PET) scan signal from tissue that is exposed to radioactively labeled fluorodeoxyglucose.

1. CHEMICAL REACTIONS OF GLYCOLYSIS

Glycolysis produces two molecules o*f* pyruvate per molecule o*f* glucose.

Glucose transporters move glucose between the extracellular and the intracellular space (see Section 3 in Chapter 18 and Table 18.1). Glycolysis takes place in the cytosol.

T e reactions of glycolysis are shown in Fig. 19.2. T e **net reaction** of glycolysis is:

$$\text{Glucose} + 2\,\text{ADP} + 2\,\text{phosphate} + 2\,\text{NAD}^+ \rightarrow 2\,\text{pyruvate} + 2\,\text{ATP} + 2\,\text{NADH} + 2\,\text{H}^+ + 2\,\text{H}_2\text{O}$$

All cells are capable of glycolysis. As detailed in Section 4, several intermediates and products of glycolysis constitute a starting or end point for other metabolic pathways (see Fig. 19.11).

T ere are no plasma membrane transporters for the phosphorylated intermediates of glycolysis, and the lipid bilayer does not allow them to pass through.

Enzymes that catalyze irreversible reactions are regulated as described in Sections 3 and 5 below. Enzymes that catalyze reversible reactions are not regulated.

Glycolysis by itself yields a modest amount of **ATP** (see Fig. 19.2). Early in the pathway, ATP is consumed, but ATP is later produced such that the return is greater than the initial investment. T e conversion of glucose to pyruvate yields two ATP and 2 NADH. In anaerobic glycolysis, explained in Section 2, the net gain is two ATP, while in aerobic glycolysis, the gain is about 5 to 7 ATP because NADH can also give rise to ATP (see Section 2 and Chapter 23).

In glycolysis, **NAD^+** serves as an electron acceptor (i.e., as an oxidizing agent; see Figs. 19.2 and 19.3). NAD^+ is an abbreviation of nicotinamide adenine dinucleotide. T e pyridine ring of NAD^+ is the electron acceptor. T e nitrogen of the pyridine ring is positively charged, and hence it

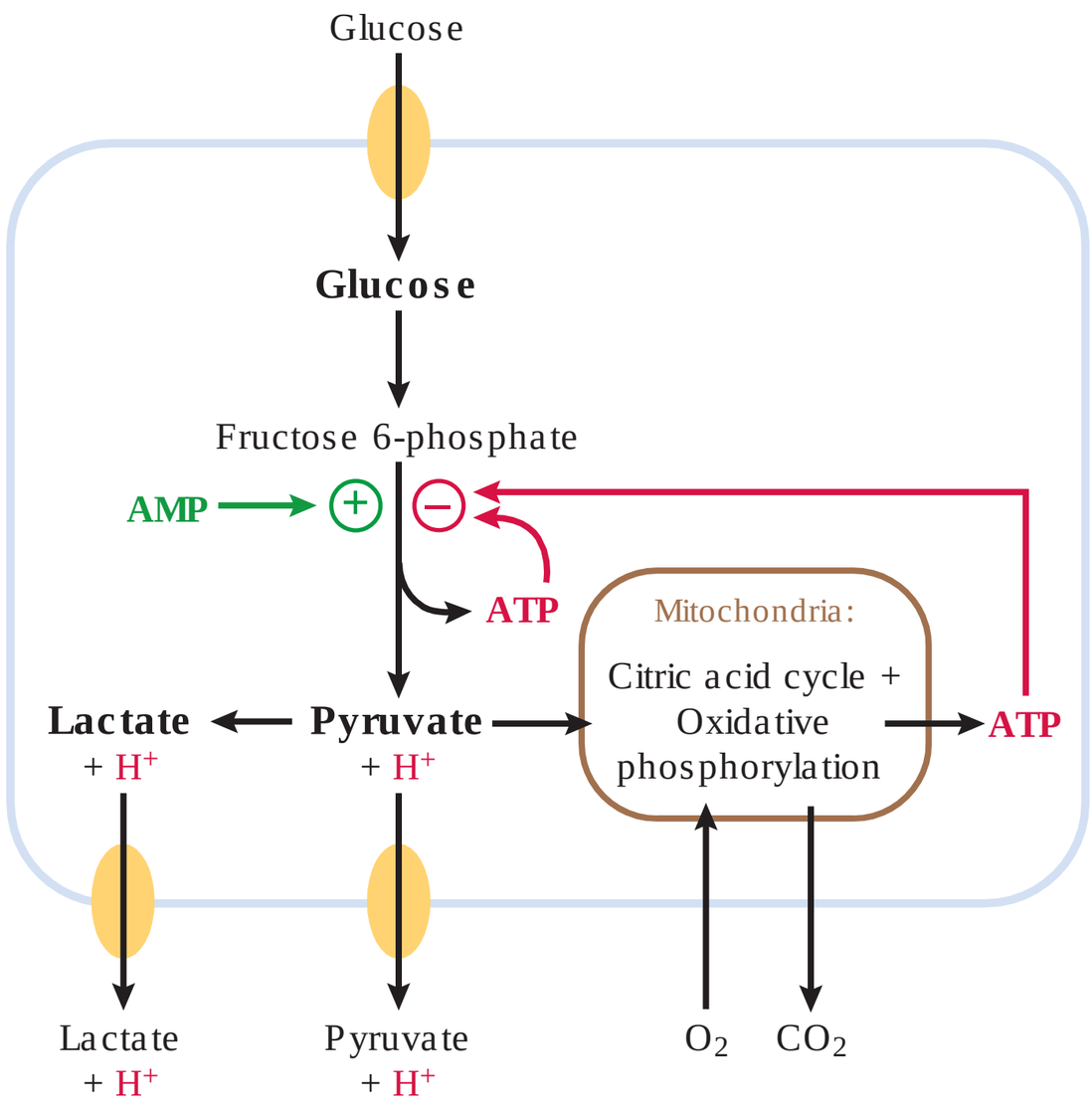

Fig. 19.1 Overview of cellular ATP production and its effect on glycolysis.

is commonplace to write this species as NAD^+; however, at physiologic pH, NAD^+ is in fact negatively charged because it carries two negative charges on its phosphate groups. With the incorporation of one proton and two electrons, NAD^+ is reduced to **NADH**. Many biochemical reactions produce two electrons and two H^+, and the partial redox reaction is then $NAD^+ + 2H^+ + 2e^- \rightarrow NADH + H^+$.

NAD^+ and NADH play a major role in various other metabolic pathways that are linked to ATP generation. Besides glycolysis, NADH is also produced in the citric acid cycle (see Chapter 22), fatty acid oxidation (see Chapter 27), and ketone body oxidation (see Chapter 27). NADH is oxidized to NAD^+ chiefly in oxidative phosphorylation (see Chapter 23), a process that is responsible for most of the ATP production in cells that contain respiring mitochondria.

T e type of ATP synthesis that occurs in glycolysis is called **substrate level phosphorylation** as opposed to oxidative phosphorylation, which occurs in mitochondria (see Chapter 23). In glycolysis, a substrate with a "high-energy" phosphate bond phosphorylates ADP to generate ATP. In oxidative phosphorylation, reduced compounds (e.g., NADH) are oxidized and thereby generate an electrochemical H^+ gradient that drives the phosphorylation of ADP to ATP.

NAD^+ and NADH (as well as $NADP^+$ and NADPH; see Chapter 21) are synthesized from **niacin mononucleotide** (Fig. 19.4). Niacin mononucleotide, in turn, is derived either from **niacin** (**vitamin B_3, nicotinic acid**) in the diet or the degradation of the amino acid **tryptophan** (see Fig. 35.16). Most humans produce about half of their niacin mononucleotide from nicotinic acid in the diet and derive the remainder from the degradation of tryptophan.

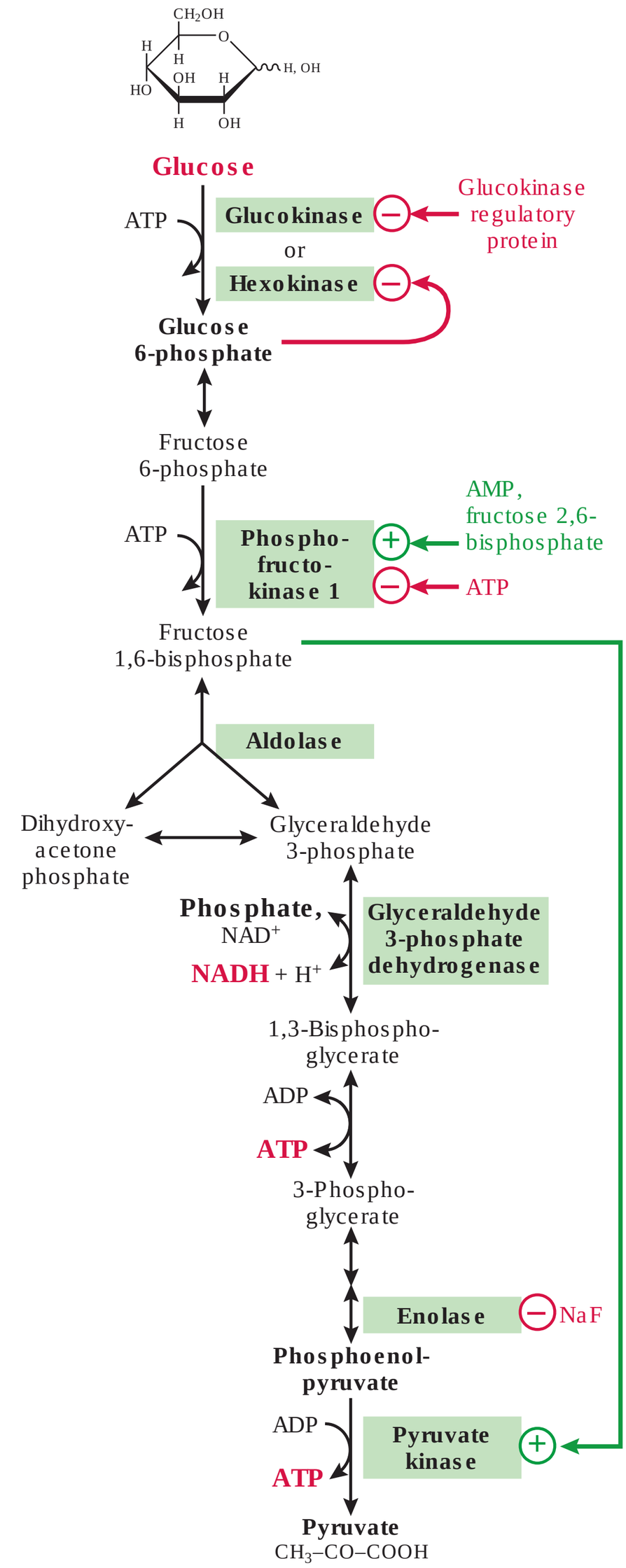

Fig. 19.2 Reactions of glycolysis. NaF is used clinically to inhibit glycolysis in blood samples.

If tryptophan and niacin intake are abnormally low, **pellagra** develops (see Fig. 19.5). Pellagra is associated with dementia, dermatitis, and diarrhea (often called the "three D's"; at times, a fourth "D" is added for death). Pellagra due to inadequate nutrition is endemic in parts of Africa and Asia; in developed nations, it is now seen mostly in patients with an intestinal disease, **Hartnup disease** (which af ects the uptake of tryptophan; see Chapter 34), chronic **alcoholism**, or **anorexia** nervosa.

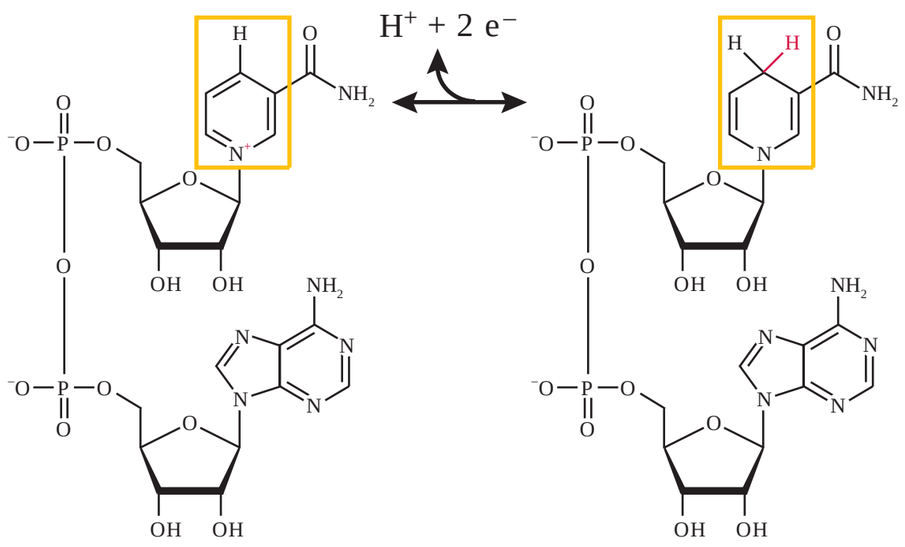

Fig. 19.3 Structures of NAD^+ and NADH. The yellow boxes highlight the pyridine ring, which can gain or lose electrons. As NAD^+ is reduced to NADH, another molecule in a cell is oxidized (e.g., in glycolysis, an aldehyde group, –CHO, is oxidized to a carboxyl group, –COOH).

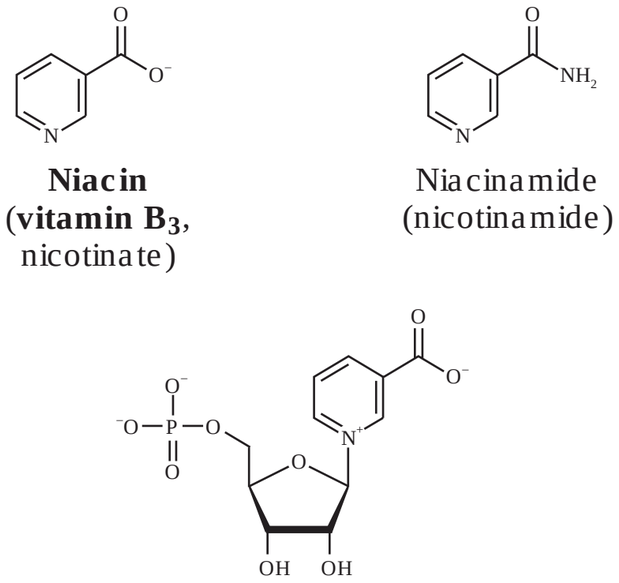

Fig. 19.4 Structures of precursors of NAD^+.

Insufficient conversion of tryptophan to niacin mononucleotide occurs in patients who have a deficiency of **vitamin B_2 (riboflavin**; see Chapter 22) or **vitamin B_6 (pyridoxine**; see Section 2.3 in Chapter 35 and Section 9 in Chapter 36), and in patients taking medications long term, such as **isoniazid**, **5-fluorouracil**, **6-mercaptopurine**, **phenobarbital**, **azathioprine**, or **chloramphenicol**.

Treatment of pellagra involves extra doses of **niacin** or nicotinamide.

Unrelated to pellagra, in patients with dyslipidemia, niacin in very large doses is sometimes used to increase the fraction of HDL particles.

2. AEROBIC VERSUS ANAEROBIC GLYCOLYSIS

Glycolysis can be carried out either in aerobic or anaerobic *f*ashion. In anaerobic glycolysis, reducing power *f*rom NADH is trans*f*erred to pyruvate, producing lactate that is released into the blood as lactic acid. T e advantage o*f* anaerobic glycolysis is that it allows A*T*P to be produced in the absence o*f* oxygen; its disadvantage is that it produces lactic acid, which may cause li*f*e-threatening acidosis. In aerobic glycolysis, the reducing power *f*rom NADH is ultimately trans*f*erred to the O_2 inside mitochondria. T e advantage o*f* aerobic glycolysis (working together with the citric acid cycle and oxidative phosphorylation) is that *f*or each glucose molecule it yields about 15 times more A*T*P than does anaerobic glycolysis.

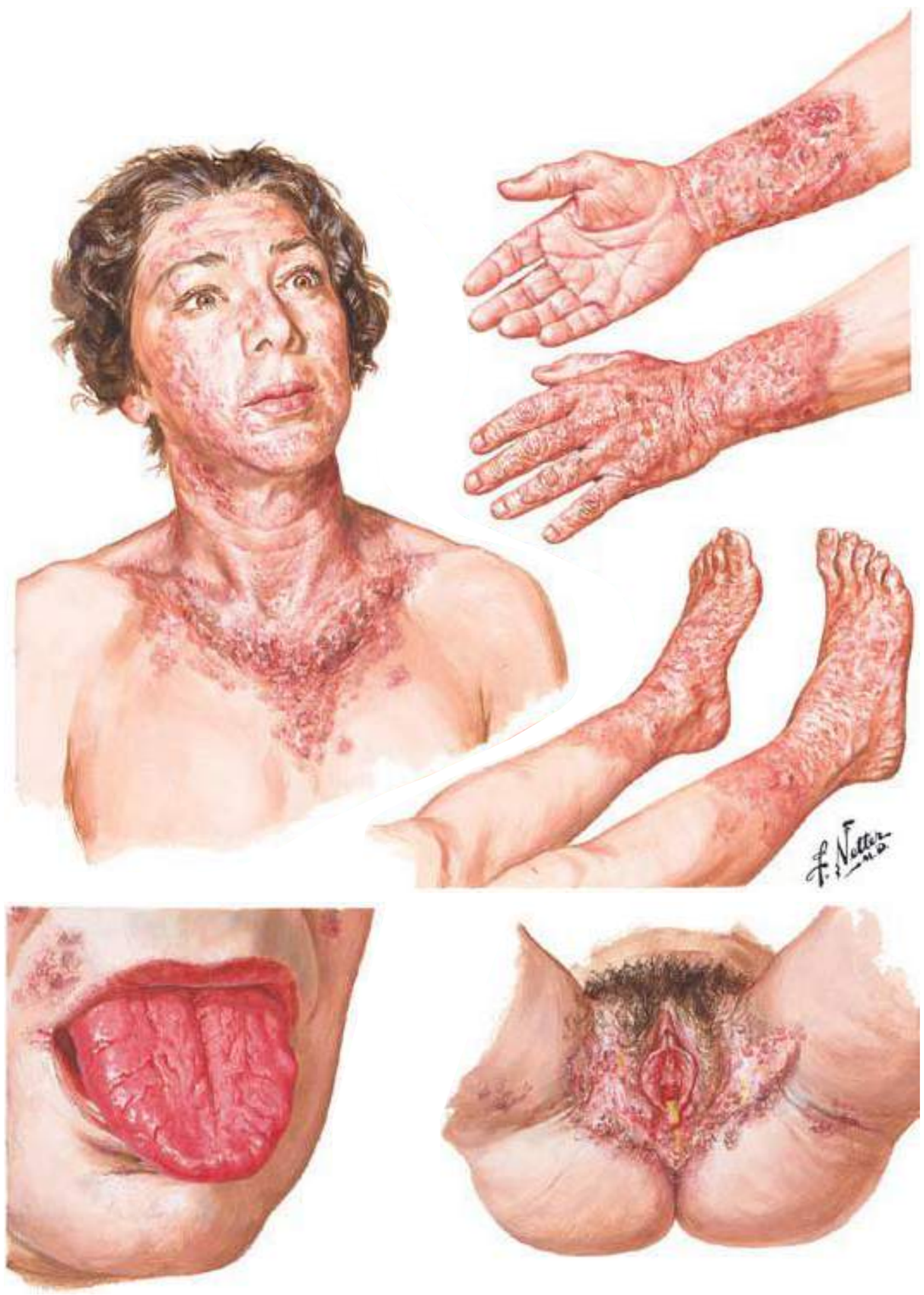

Fig. 19.5 Lesions that are typical of pellagra. Most patients show erythema on the backs of their hands and feet, sometimes in a glove-and-stocking fashion. Lesions on the face and around the neck ("Casal's necklace") are also common. About one-third of patients with pellagra show cheilitis, angular stomatitis, and glossitis. Perineal lesions are common. Women are more readily affected because estrogen inhibits tryptophan degradation to niacin mononucleotide.

2.1. Production of NADH in Glycolysis

In glycolysis, the oxidation of glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate consumes NAD^+ and produces NADH + H^+ (see Fig. 19.2). T e supply of NAD^+ is small compared with the rate of this reaction. Hence, NADH has to be recycled to NAD^+; otherwise, glycolysis rapidly comes to a standstill. NADH can be oxidized to NAD^+ in three dif erent ways (Figs. 19.6 and 19.7): (1) via the reduction of pyruvate to lactate; (2) via the glycerol-phosphate shuttle; or (3) via the malate-aspartate shuttle. Production of lactate works in the

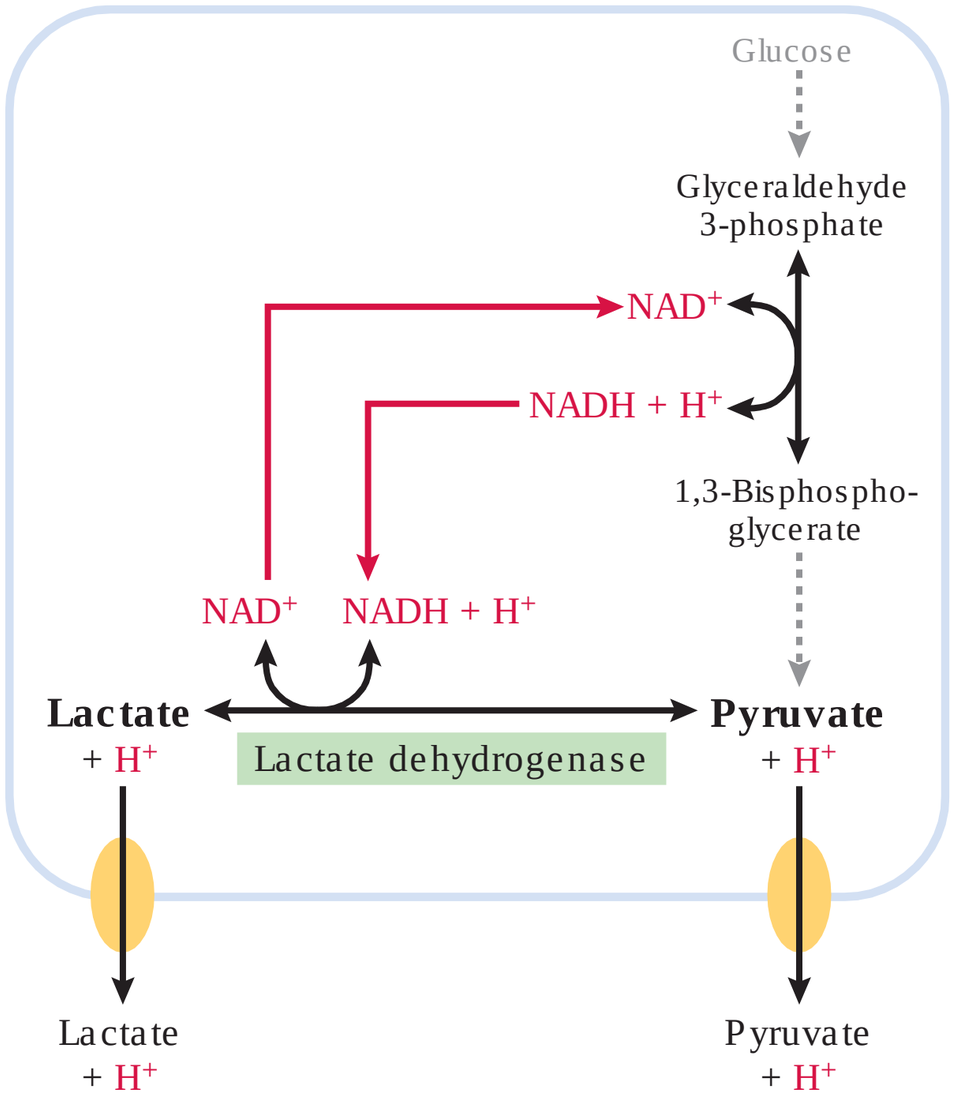

Fig. 19.6 **In anaerobic glycolysis, NADH reduces pyruvate to lactate.**

absence of oxygen and is an integral part of anaerobic glycolysis (see Section 2.2). T e shuttles require the presence of oxygen and functioning mitochondria and permit aerobic glycolysis (see Section 2.3).

2.2. Cells Performing Anaerobic Glycolysis Release Lactic Acid

Anaerobic glycolysis does not require oxygen, and it occurs in cells that have no or few functioning mitochondria (e.g., in red blood cells, contracting fast-twitch muscle fibers, and in all hypoxic cells). NADH reduces pyruvate to lactate, catalyzed by lactate dehydrogenase (LDH; see Fig. 19.6). Lactate leaves the cell through a **monocarboxylic acid transporter**, **MCT**, as lactic acid (MCT transporters also transport pyruvic acid, the ketone bodies acetoacetic acid and β-hydroxybutyric acid [see Section 5 in Chapter 27], and acetic acid).

Anaerobic glycolysis results in the net phosphorylation of 2 moles of ADP to ATP per mole of glucose metabolized. T e cell eventually hydrolyzes the ATP to ADP and phosphate. If ATP hydrolysis and anaerobic glycolysis are combined, the net result is

$$\text{glucose} \rightarrow 2\ \text{lactate}^- + 2\ \text{H}^+ \textit{ or}$$

$$\text{glucose} \rightarrow 2\ \text{lactic acid}$$

Since the pK of lactic acid is about 4, most of the lactic acid in the cytosol and blood plasma dissociates into lactate^- and H^+. From the above equation it is apparent that a cell that performs anaerobic glycolysis releases lactic acid into its environment.

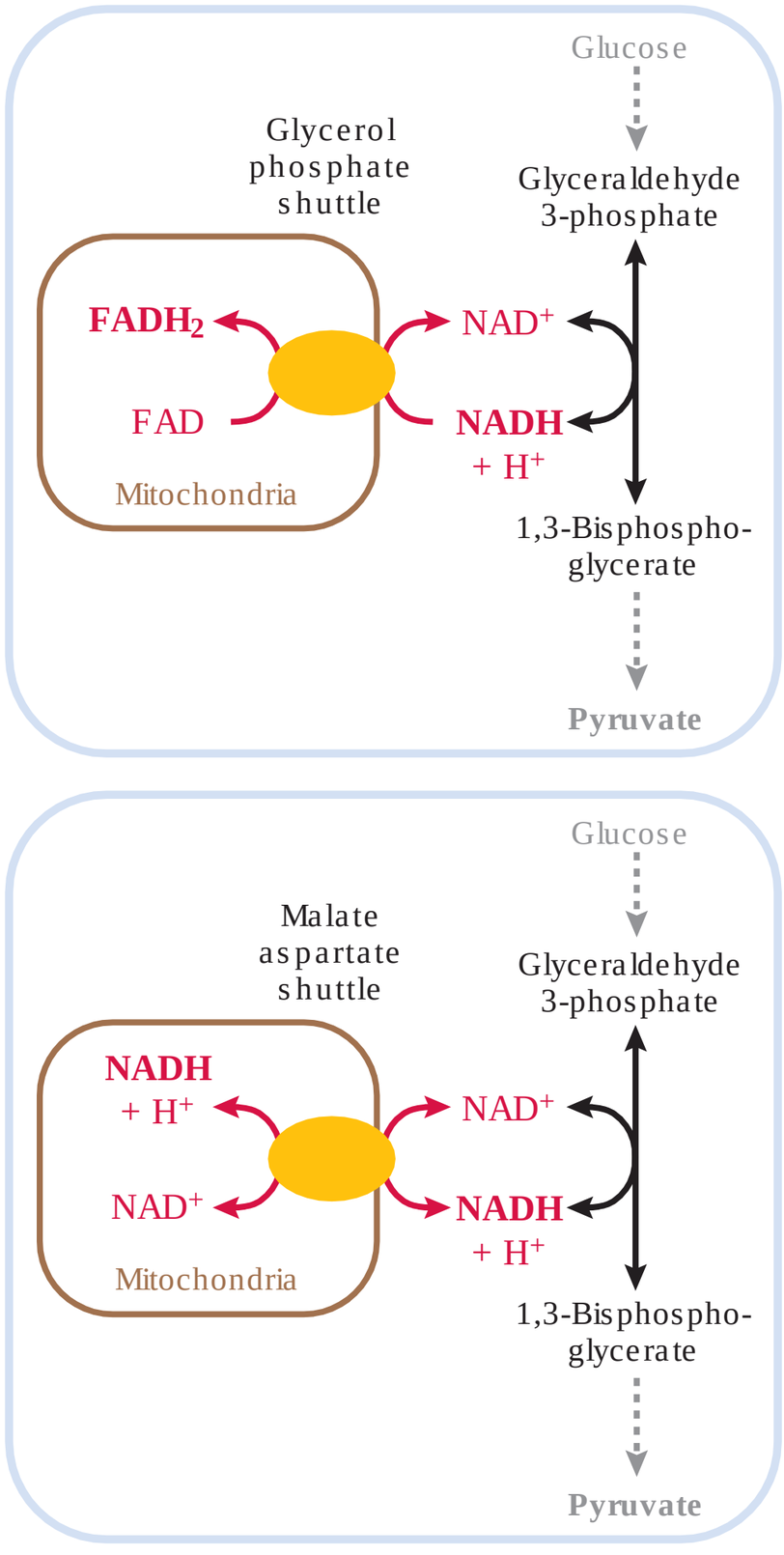

Fig. 19.7 **In aerobic glycolysis, reducing power from NADH is transferred into mitochondria via the glycerolphosphate shuttle (top) or the malate-aspartate shuttle (bottom).** The shuttles are depicted in a simplified manner. There is no transmembrane transport of NAD^+ or NADH.

Tissues that depend on anaerobic glycolysis are red blood cells; the lens of the eye (there are no blood vessels and little ATP is needed; glucose and lactic acid dif use between the lens, the vitreous body, and the aqueous humor); the renal medulla (a tissue that maintains a countercurrent transport gradient, and the cells involved in this are too far from blood vessels to allow proper oxygenation); glial cells of the brain (see Section 5.2) contracting fast-twitch muscles; contracting slow-twitch muscles that exceed their aerobic capacity; and any hypoxic tissue. When these cells release lactate into the bloodstream, other cells (e.g., in the liver, heart, or skeletal muscle) take it up and metabolize it. Cells in the skin epithelium produce lactic acid and release it to the surface as a bacteriostatic agent.

Patients who produce too much lactic acid or do not adequately remove lactic acid from the blood can develop **lactic acidosis**, which may be life-threatening. Lactic acidosis is characterized by an abnormally high concentration of lactic acid (i.e., lactate) and an abnormally low pH in blood.

Causes of lactic acidosis are compromised oxidative phosphorylation (e.g., due to hypoxia), or an inadequate removal of lactate and pyruvate due to severe alcohol intoxication (see Section 3.1 in Chapter 30), pyruvate dehydrogenase deficiency (see Section 5.6 in Chapter 22), or a block in gluconeogenesis (see Section 4.1 in Chapter 25). A low blood pH is neurotoxic.

When lactic acid enters the blood and dissociates into $lactate^-$ and H^+, it acidifies the blood; conversely, when lactic acid leaves the blood, it alkalinizes it. A lactic acid–induced shift to a lower pH is buf ered by bicarbonate, HCO_3^-, which binds H^+ and thereby becomes carbonic acid (H_2CO_3), which in turn dissociates into water and CO_2. Driven by a lowered blood pH, the af ected person exhales extra CO_2 into the environment. T ese processes lead to a low concentration of bicarbonate and CO_2 in the blood plasma (the pH of blood depends on the ratio $[HCO_3^-]/[CO_2]$; see Section 4 in Chapter 16; in acidosis, the ratio is too small). T e kidneys can also neutralize acid (see Chapter 35), but their capacity is much smaller than the CO_2-removing capacity of the lungs.

T e **lactate/pyruvate ratio** in blood plasma is sometimes used to pinpoint the cause of lactic acidosis in a patient. Since the monocarboxylic acid transporters equilibrate both lactic acid and pyruvic acid across the plasma membrane, the lactate/pyruvate ratio in the blood reflects on the ratio of $NADH/NAD^+$ in the cytosol (it is not the same, but proportional to it). T e lactate/pyruvate ratio is increased in patients who have tissue **hypoxia** or a problem in the **electron transport** chain (see Chapter 23). T e lactate/pyruvate ratio is normal in acidotic patients who have a defect in **pyruvate dehydrogenase** or **gluconeogenesis** (see Chapters 22 and 25).

2.3. Aerobic Glycolysis: Cells Produce ATP From the Reducing Power of NADH

In aerobic glycolysis, NADH from glycolysis is oxidized to NAD^+, and the reducing power from NADH is then transferred into the mitochondria by one of the shuttles described below. T e outer mitochondrial membrane is permeable to small molecules, including NAD^+ and NADH. However, NAD^+ and NADH cannot cross the inner mitochondrial membrane (or the plasma membrane) because they are both hydrophilic, charged molecules, and there are no transporters for them.

T e **glycerol phosphate shuttle** transfers the reducing power unidirectionally from the cytosolic NADH to intramitochondrial FAD, thereby generating $FADH_2$ inside the mitochondria (see Fig. 19.7). T e structures of FAD and $FADH_2$ are shown in Fig. 22.5.

T e **malate-aspartate shuttle** transfers reducing power bidirectionally between NADH in the cytosol and NADH in the mitochondria (see Fig. 19.7). T is shuttle can thus generate NADH in the mitochondria or the cytosol. Export of reducing equivalents from the mitochondria through the malate-aspartate shuttle is required for gluconeogenesis from amino acids in the liver and the kidneys (see Chapter 25) and for some of the production of NADPH for fatty acid synthesis (see Chapter 27).

Inside the mitochondria, **oxidative phosphorylation** oxidizes NADH to NAD^+ and $FADH_2$ to FAD, thereby generating ATP (see Chapter 23). In cells with oxygenated mitochondria, pyruvate can be oxidized to CO_2 in the citric acid cycle (see Chapter 22); this yields additional NADH, $FADH_2$, and ATP. In this way, the complete oxidation of glucose to CO_2 yields about 15 times more ATP than does anaerobic glycolysis from glucose to lactate.

3. BASIC MECHANISMS IN THE REGULATION OF GLYCOLYSIS

Glycolysis is regulated such that the concentration of ATP in the cytosol is high (millimolar) and that of free AMP low (nanomolar). In some cells, glycolysis is the principal means of ATP production; in others it has only a backup role. In most normal cells, the activities of hexokinase (or glucokinase), phosphofructokinase 1, and pyruvate kinase are the key determinants of flux through glycolysis. T e activity of these enzymes is regulated by AMP, ATP, fructose phosphates, or the degree of phosphorylation of the enzyme.

3.1. Overview

T e regulation of glucose use varies appreciably from tissue to tissue. In adipose tissue and the hypoglycemic brain, glucose transport limits glucose use. In red blood cells and glycolytic (fast-twitch) muscle, ATP consumption is usually the limiting factor. In the liver, the concentrations of insulin and glucagon are the master controllers. In all cells, hypoxia activates glycolysis via an abnormally high concentration of AMP. In chronically hypoxic cells, there is also increased synthesis of enzymes of glycolysis.

3.2. Introduction to Insulin, Glucagon, Epinephrine, and Norepinephrine

Insulin, glucagon, epinephrine, and norepinephrine are among the many hormones that af ect the rate of glycolysis in a tissue-specific manner. T e release of these hormones into the bloodstream is covered in Chapter 26. A brief overview of this material is provided here, sufficient to understand the regulation of glycolysis. Pancreatic islet β-cells secrete **insulin** into the blood in response to an elevated concentration of glucose (e.g., after a carbohydrate meal). Islet α-cells secrete **glucagon** when the concentration of glucose is low. Chromaffin cells in the medulla of the adrenal glands secrete **epinephrine** and **norepinephrine** in response to sympathetic input from splanchnic nerves. T ese nerves in turn fire in response to exercise or hypoglycemia.

Insulin, glucagon, epinephrine, and norepinephrine are water-soluble hormones that cannot cross plasma membranes;

they bind to plasma membrane receptors that transmit a signal to the cytosol (see Chapters 26 and 33). Insulin stimulates glucose use and storage, whereas glucagon promotes endogenous glucose production. Epinephrine and norepinephrine promote glucose use by the heart.

3.3. Introduction to AMP-Dependent Protein Kinase

AMP-dependent protein kinase (**AMPK**) is an important energy sensor inside cells. **AMP** and **ADP** activate AMPK, whereas ATP inhibits it. Several kinases and phosphatases further modulate AMPK activity.

When AMPK is active, it phosphorylates enzymes so as to modify cell growth, proliferation, the production of new mitochondria, and flux in metabolism. In metabolism, high AMPK activity leads to increased ATP production via glycolysis and fatty acid oxidation, and to reduced use of ATP due to inhibition of the synthesis of fatty acids, triglycerides, cholesterol, and proteins. (Further details are provided in Chapters 8, 25, 27, and 39.) Indirect activation of AMPK activity with **metformin** has proven very successful in the treatment of **diabetes** (see Chapter 39). Metformin is sometimes also used to treat **polycystic ovary syndrome** (see Chapter 26).

3.4. Regulation of Glucose Transport

T ere are several types of glucose transporters (see Table 18.1 and Section 3.2 in Chapter 18). After synthesis, glucose transporters of most types are inserted into the plasma membrane and remain there. Transporters of the **GLUT-4** subtype, however, frequently move between intracellular storage vesicles and the plasma membrane. When the concentration of **insulin** is low, the GLUT-4 transporters are mostly stored intracellularly. When the concentration of insulin is high, the storage vesicles fuse with the plasma membrane and the GLUT-4 transporters are incorporated into the plasma membrane; this increases the capacity for glucose uptake. (Details of the signal transduction mechanism for insulin are provided in Chapter 26.) GLUT-4 transporters are predominantly found in adipose tissue and muscle. In muscle, besides insulin, exercise also leads to the insertion of GLUT-4 transporters into the plasma membrane. T is ef ect is at least in part mediated by AMPK. Muscle can thus store glucose as glycogen after exercise and after a meal, and it can use glucose from the blood during exercise.

3.5. Regulation of Hexokinase and Glucokinase Activities

Humans have three hexokinases with a relatively low K_m for glucose (i.e., they are half-maximally saturated by <0.2 mM glucose; Fig. 19.8). Humans also produce **glucokinase** (also called type IV hexokinase), which has a comparatively high $S_{0.5}$ for glucose (it is half-maximally active at ~5 mM glucose).

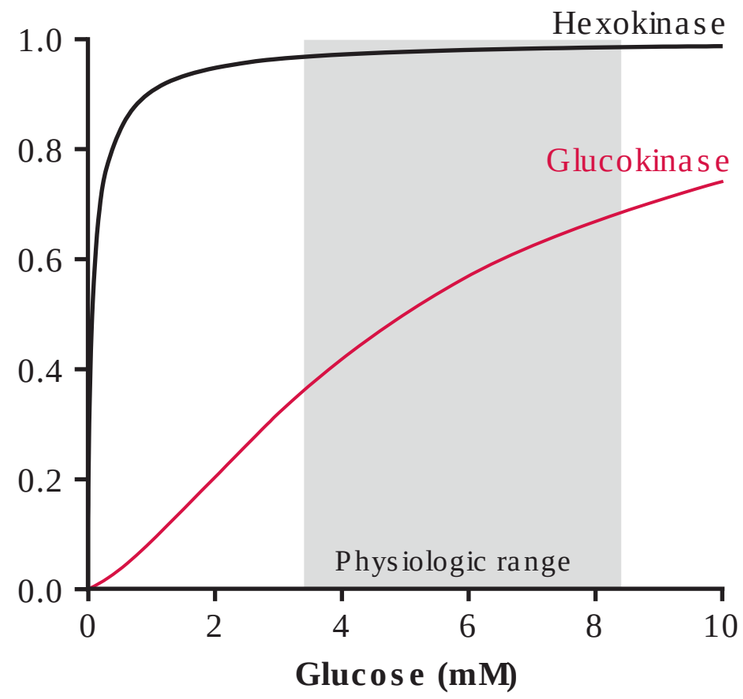

Fig. 19.8 Difference in the kinetic behavior between hexokinases (types I to III) and glucokinase (hexokinase type IV). Five mM glucose corresponds to 90 mg glucose/dL.

Almost all cells contain a low-K_m **hexokinase**, and unless glucose transport is limiting, these enzymes are nearly saturated with glucose even during hypoglycemia (see Fig. 19.8). Glucose 6-phosphate, the product of the hexokinase-catalyzed reaction, inhibits hexokinase; this is variably called product inhibition or *f*eedback inhibition. By itself, the product inhibition ensures that the concentration of glucose 6-phosphate is held constant.

Glucokinase is found only in the liver and in glucose-sensing cells of the pancreas, jejunal enterocytes, and hypothalamus. T e activity of glucokinase changes markedly between hypo- and hyperglycemia because glucokinase has a low affinity for glucose, shows cooperativity toward glucose, and is not inhibited by glucose 6-phosphate. In the liver, the activity of glucokinase is further regulated by a **glucokinase regulatory protein** (GKRP) that responds to dietary fructose; details are provided in Section 5.6. T e role of glucokinase in glucose-sensing inside pancreatic β-cells is described in Chapter 26.

3.6. Regulation of Phosphofructokinase 1

Glucose-6-phosphate is not only an intermediate of glycolysis; it is also an intermediate in the synthesis and degradation of glycogen (see Chapter 24) and one of the starting points for the pentose phosphate shunt pathway (see Chapter 21). T e phosphorylation of fructose 6-phosphate to fructose 1,6-bisphosphate is thus the first irreversible reaction that commits a metabolite to glycolysis. T is reaction is catalyzed by **phosphofructokinase 1** (**PFK1**); this enzyme usually exerts the greatest control over glycolysis (pancreatic islet β-cells are an exception; see Chapter 26).

T ere are several isoenzymes for PFK1; **AMP** stimulates all of them, and **ATP** inhibits them. Since **adenylate kinase** maintains the equilibrium 2 ADP ↔ AMP + ATP, an elevated concentration of AMP is an indicator of poor phosphorylation of ADP and AMP. In some tissues, **citrate** (from the citric acid cycle; see Chapter 22) also inhibits PFK1.

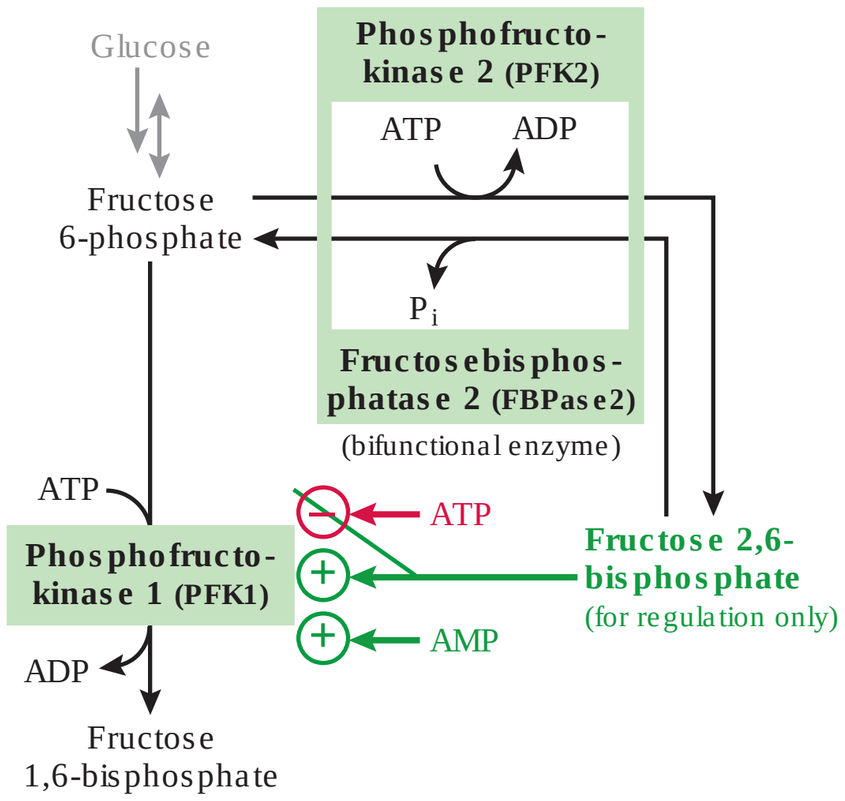

Fig. 19.9 Regulation of phosphofructokinase 1 by fructose 2,6-bisphosphate.

T e regulatory sugar phosphate **fructose 2,6-bisphosphate** markedly increases the activity of PFK1 and thereby abrogates the inhibitory ef ect of ATP. Fructose 2,6-bisphosphate is synthesized from fructose 6-phosphate by the bifunctional enzyme **phosphofructokinase 2/fructose bisphosphatase 2** (=**6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase**, **PFK2/FBPase 2**; Fig. 19.9). T ere are four isoenzymes, the activity of which is regulated by signaling pathways that respond, for example, to cellular stress, hypoxia, AMP, insulin, glucagon, or epinephrine. Fructose 2,6-bisphosphate can stimulate glycolysis beyond the flux that would be needed to produce ATP from glucose. Intermediates and products of glycolysis can thus be used for other metabolic pathways.

3.7. Regulation of Pyruvate Kinase

In all cells, the rate of the pyruvate kinase–catalyzed reaction is regulated allosterically by the concentration of **fructose 1,6-bisphosphate** so that the concentration of intermediates in glycolysis stays approximately constant, regardless of flux (Figs. 19.2 and 19.10). T is ensures that intermediates are available for other pathways (Fig. 19.11). T e activation of pyruvate kinase by fructose 1,6-bisphosphate is an example of feed-forward activation. All reactions between fructose 1,6-bisphosphate and phosphoenolpyruvate are reversible (i.e., they proceed according to the concentrations of reactants and products). If one reactant piles up, all reactants of upstream reversible reactions also pile up. T erefore, if the pyruvate kinase–catalyzed reaction proceeds at too low a rate, the concentration of fructose 1,6-bisphosphate rises, which in turn increases the activity of pyruvate kinase, thus forming a regulatory loop.

When certain cells in the liver and kidneys carry out **gluconeogenesis** (see Chapter 25), hormone-induced phosphorylation inactivates pyruvate kinase.

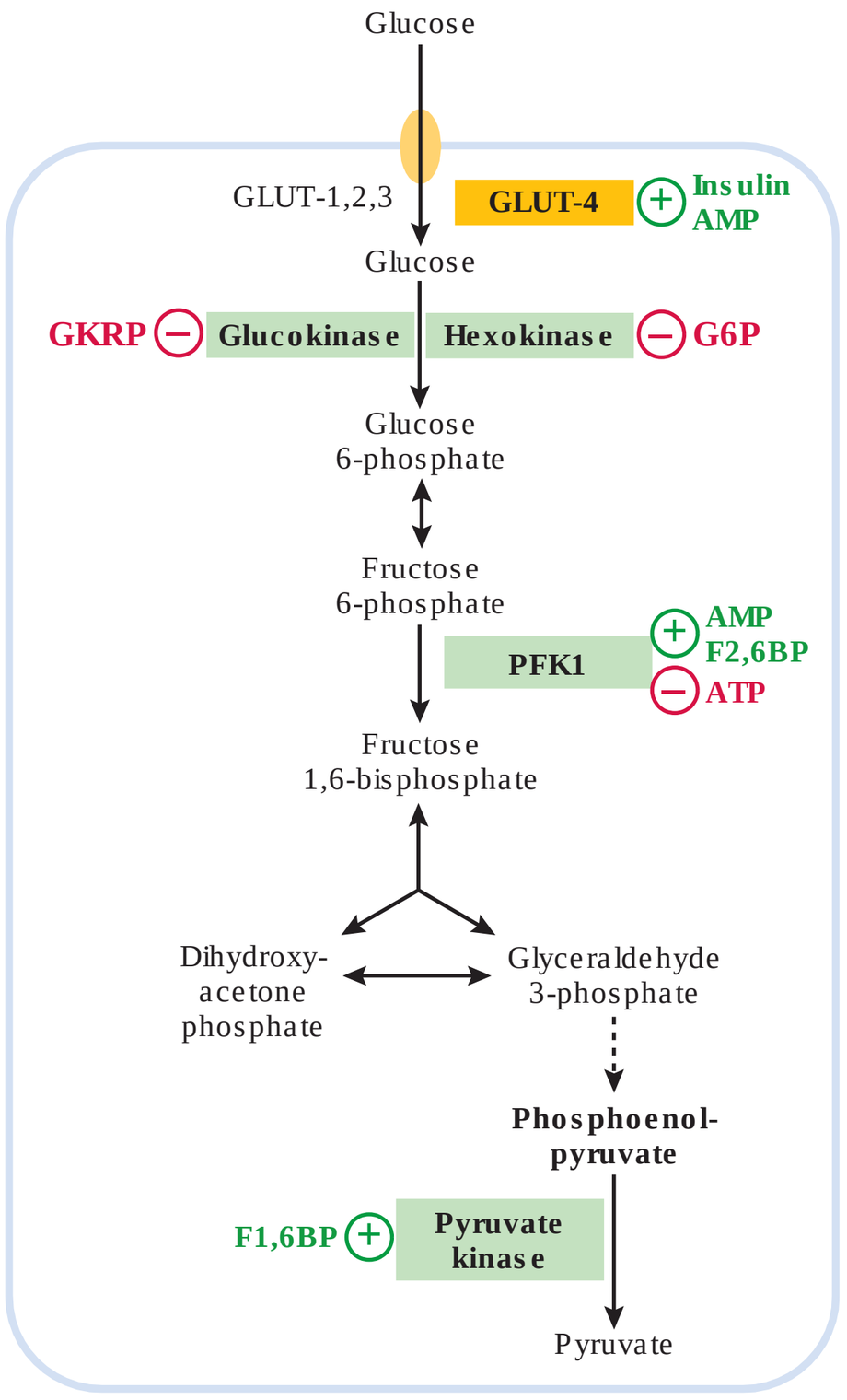

Fig. 19.10 Summary of mechanisms that can regulate flux through glycolysis.

3.8. Summary of the Regulation of Flux in Glycolysis

Fig. 19.10 summarizes mechanisms that can regulate flux through glycolysis.

4. INTERACTIONS OF GLYCOLYSIS WITH OTHER PATHWAYS

Intermediates o*f* glycolysis *f*eed into multiple other pathways or derive metabolites *f*rom them. T e main pathways are glycogen metabolism, the pentose phosphate shunt pathway, the citric acid cycle, and amino acid metabolism.

Fig. 19.11 shows the connections between glycolysis and other metabolic pathways. **Glycogen** synthesis and degradation are described in Chapter 24. T e degradation of **galactose** and **fructose** is described in Chapter 20. T e **pentose phosphate shunt** pathway is explained in Chapter 21. T e production of **2,3-bisphosphoglycerate** is detailed in Section 5.1 below. Chapters 22 and 23, respectively, are devoted to the **citric acid cycle** and **oxidative phosphorylation**. **Fatty acid synthesis** from **citrate** is described in Chapter 27, while **triglyceride synthesis** is presented in Chapter 28. T e conversion of lactate or alanine to pyruvate and the use of pyruvate and glycerol for

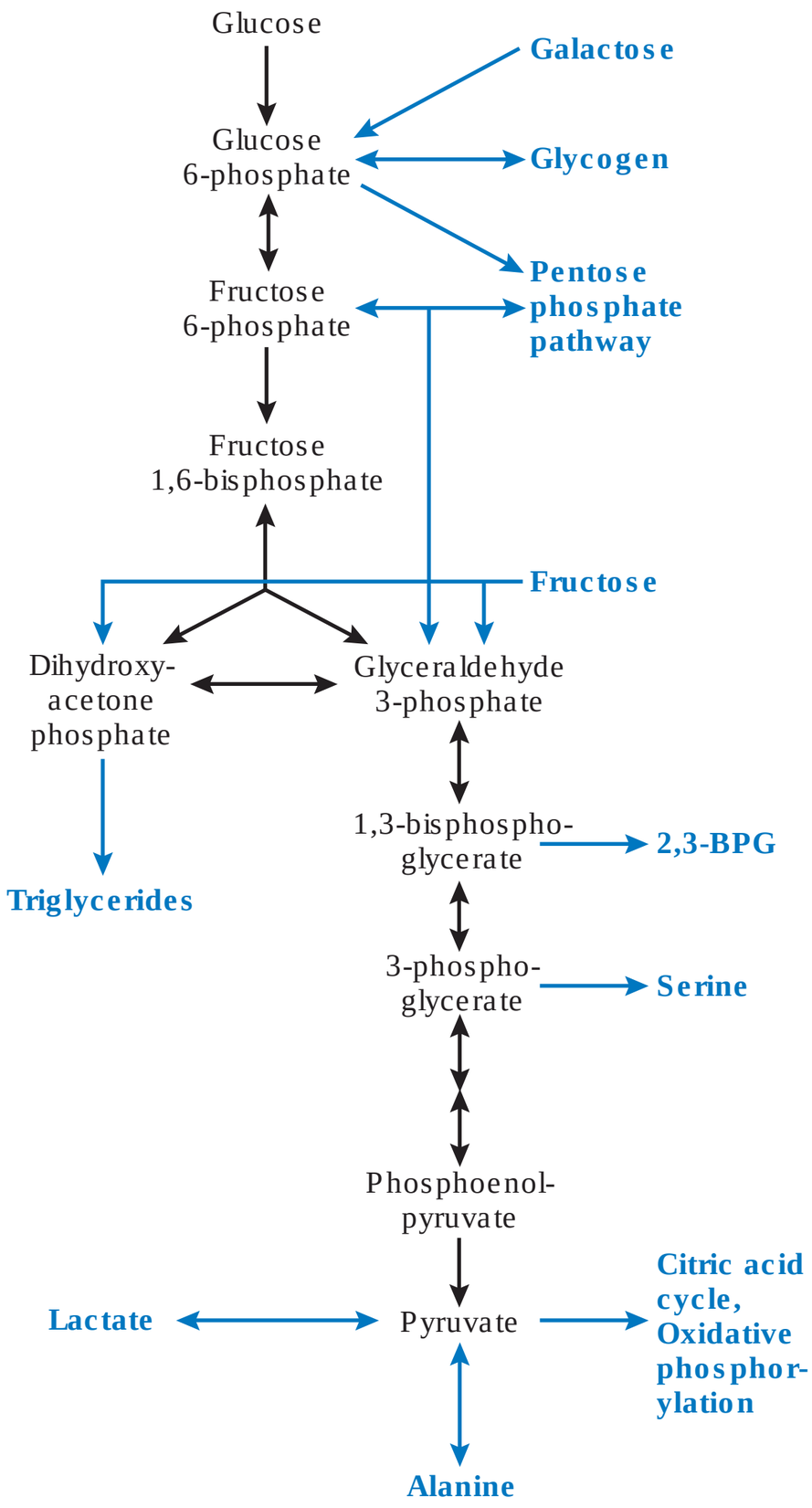

Fig. 19.11 **Overview of glycolysis and its links with other pathways.**

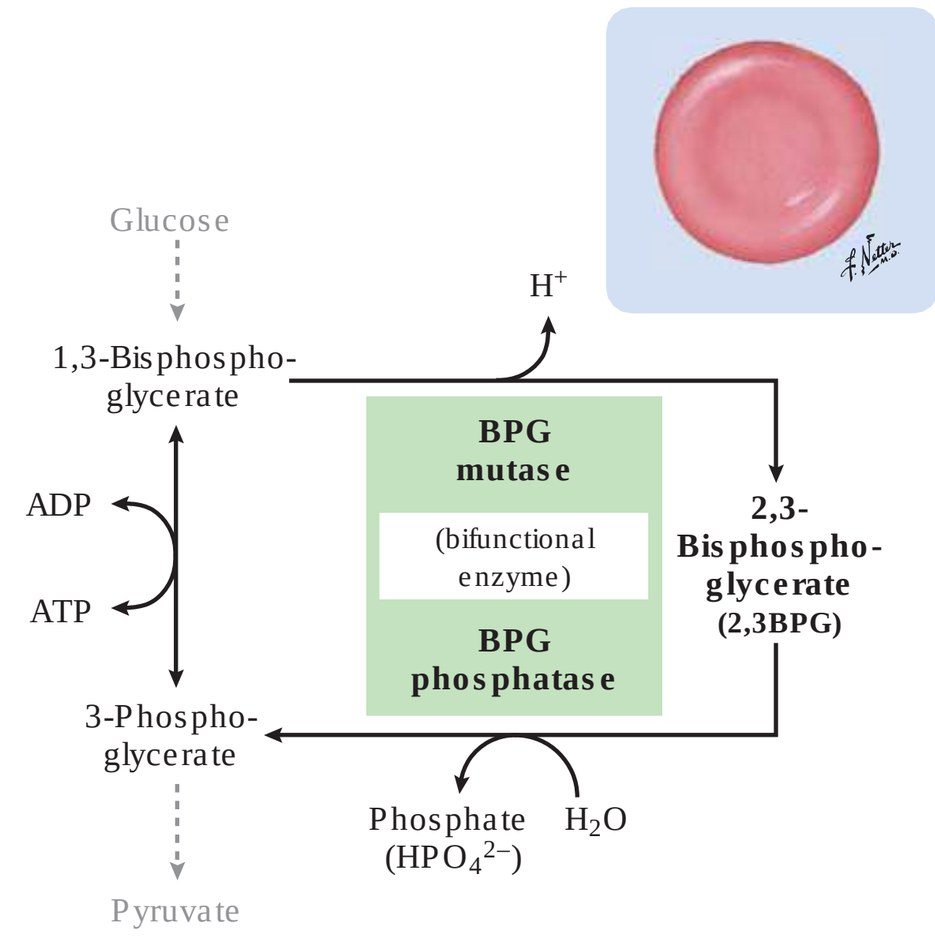

Fig. 19.12 **Production of 2,3-bisphosphoglycerate in red blood cells.**

the synthesis of glucose (i.e., gluconeogenesis) are described in Chapter 25.

5. TISSUE-SPECIFIC REGULATION OF GLYCOLYSIS

Glycolysis is a central pathway of metabolism and crucial for a cell's survival. Accordingly, regulation of glycolysis is extraordinarily complex; however, it is yet incompletely understood. This section provides an introduction to the regulation of glycolysis in the context of key metabolic processes in red blood cells, brain, adipose tissue, heart, skeletal muscle, and the liver.

5.1. Regulation of Glycolysis in Red Blood Cells

In red blood cells, glycolysis is the only pathway that generates ATP. GLUT-1 glucose transporters are always in the plasma membrane and equilibrate intracellular and extracellular glucose. Hexokinase produces a near-constant concentration of glucose 6-phosphate, which is needed both for glycolysis and for the production of NADPH (used to repair oxidative damage; see Chapter 21). PFK1 regulates flux through glycolysis based on the concentrations of AMP and ATP. Fructose 1,6-bisphosphate feed-forward activates pyruvate kinase. This activation ensures that the concentrations of the intermediates between fructose 1,6-bisphosphate and phosphoenolpyruvate are near constant, which is important for the production of 2,3-bisphosphoglycerate, a regulator of the oxygen affinity of hemoglobin (see below). In mature red blood cells, which do not contain mitochondria, lactate is the end product of glycolysis. In contrast, **reticulocytes** (which still synthesize hemoglobin; see Chapter 16) contain mitochondria that can oxidize some of the pyruvate to CO_2.

Red blood cells use **2,3-bisphosphoglycerate** (2,3-BPG) to adjust the affinity of hemoglobin for O_2 (see Chapter 16). 2,3-BPG is produced from 1,3-bisphosphoglycerate (1,3-BPG) and later metabolized to 3-phosphoglycerate by a single, bifunctional enzyme, bisphosphoglycerate mutase/bisphosphoglycerate phosphatase (Fig. 19.12). The balance of the mutase and the phosphatase activities of this enzyme determines the concentration of 2,3-BPG. During hypoxia or at higher altitude, the concentration of 2,3-BPG is increased. However, at a molecular level, the regulation of the BPG mutase/BPG phosphatase is not understood. In erythrocytes, about one of every four 1,3-BPG molecules takes the 2,3-BPG "detour"; hence, these cells gain only about 1.5 mol ATP per mol glucose metabolized. When the concentration of 1,3-BPG is abnormal due to a problem in glycolysis, the concentration of 2,3-BPG is typically also abnormal. **Hereditary, deficient BPG mutase activity** (a very rare disease) is associated with high hematocrit, a fine illustration of the need for 2,3-BPG to optimize the oxygen affinity of hemoglobin.

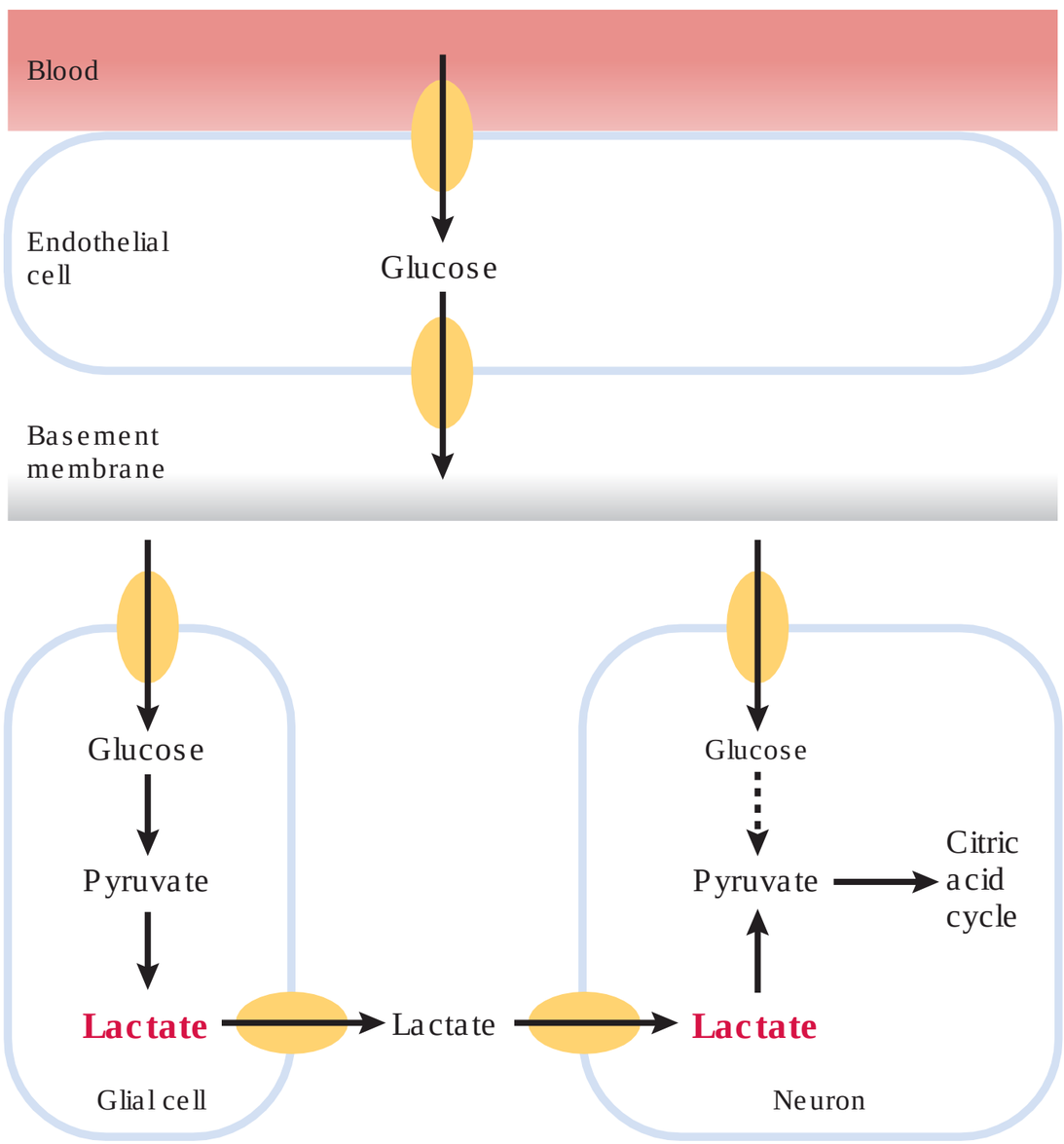

Fig. 19.13 **Supply of fuel to the brain.** Endothelial cells form the walls of blood vessels. The blood vessel walls are enveloped by a basement membrane that consists largely of extracellular matrix proteins (see Chapter 12). The basement membrane forms a filter, through which small molecules can pass. Glial cells convert glucose to lactate and "feed" the lactate to neurons. Neurons oxidize lactate via the citric acid cycle (see Chapter 22) to CO_2.

5.2. Regulation of Glycolysis in the Brain

The concentration of glucose in brain tissue is considerably lower than in the cerebrospinal fluid or in the blood. Endothelial cells that line the walls of blood vessels in the brain contain glucose transporters that transport glucose from the blood to the extracellular space (Fig. 19.13). From there, glucose diffuses through the basement membrane (a meshwork of extracellular matrix proteins that envelopes the blood vessels) to the extracellular space near glial cells and neurons. The concentration of glucose in cerebrospinal fluid (obtained by lumbar puncture) is ~55% that in serum. However, at a plasma glucose concentration of 90 mg/dL or more, the concentration of glucose in the brain tissue (measured by magnetic resonance imaging [MRI]) is only about 30% of that in blood plasma. Furthermore, when the plasma glucose concentration is only about 40 mg/dL, there is essentially no more free glucose in brain tissue.

Glial cells take up glucose from the extracellular space and convert it to lactate, which they release for the benefit of **neurons** (see Fig. 19.13). Glial cells tend to neurons by regulating their growth and properties, feeding them, insulating their axons, or recycling their neurotransmitters. Neurons take up lactate, oxidize it in the citric acid cycle (see Chapter 22), and obtain energy from oxidative phosphorylation (see Chapter 23). In addition, neurons also directly take up a small amount of glucose, which then enters into the pathway sequence glycolysis–citric acid cycle/oxidative phosphorylation to produce CO_2 and H_2O.

To a limited extent, the brain can use **lactate** and **ketone bodies** in place of glucose. Lactate uptake from the blood can replace only a small fraction of the glucose that the brain normally uses. However, the brain of an adult who fasts for 8 days can cover about 75% of its energy needs with ketone bodies. In adults, ketone body production is normally low and increases substantially only after 1 to 2 days of fasting. Thus, after an overnight fast, the brain covers only about 5% of its energy needs with the oxidation of ketone bodies. Newborns and young children produce ketone bodies in response to fasting much sooner than adults. For instance, both children who are a few months old and fast for ~8 hours and newborns produce and consume ketone bodies at a rate (relative to body weight) that adults achieve only after more than 48 hours of fasting.

In a normoglycemic or hyperglycemic person, **hexokinase** provides a near-constant concentration of glucose 6-phosphate. A very small amount of glucose 6-phosphate is used for the synthesis of glycogen as an emergency fuel reserve (see Chapter 24). PFK1 activity is the chief determinant of glycolytic flux; the cytosolic concentrations of **AMP**, ATP, and **fructose 2,6-bisphosphate** (much more so in glial cells than in neurons) determine the activity of PFK1. Fructose 2,6-bisphosphate, produced by phosphofructokinase 2, provides glial cells with an override of the ATP inhibition of glycolysis so that these cells can release lactate for the benefit of neurons.

During **hypoglycemia** (an abnormally low concentration of glucose in the blood), the brain can no longer take up an adequate amount of glucose and therefore loses function. Hypoglycemia most frequently occurs after the accidental administration of excess **insulin** or oral **hypoglycemic agents** that stimulate insulin secretion despite the hypoglycemia (see Chapter 39); less frequently it is due to an inherited abnormality of carbohydrate or lipid metabolism that comes to the fore in the fasting state (see Chapters 24, 25, and 27). If the concentration of glucose in the blood falls to 20 to 35 mg/dL, stupor and coma set in, and eventually brain electrical activity disappears. With severe hypoglycemia of less than 25 mg glucose/dL in the blood plasma, glial cells cannot produce enough lactate for neurons (see Fig. 19.13), the concentration of ATP inside neurons drops precipitously, neurotransmitters flood the extracellular space, dendrites swell, and necrosis sets in.

5.3. Regulation of Glycolysis in Adipocytes

When the concentration of insulin is elevated after a mixed meal, adipocytes increase their glucose uptake and deposit

fatty acids as triglycerides (see Chapter 28). An elevated concentration of **insulin** leads to translocation of GLUT-4 glucose transporters from intracellular storage vesicles to the plasma membrane; at the same time, the lipoprotein lipase activity in the blood vessels of the adipose tissue increases, which brings about an influx of fatty acids (see Chapter 28). Via glycolysis and a side reaction to it, glucose is converted to **glycerol 3-phosphate**, which is esterified with fatty acids to form triglycerides (see Figs. 19.11, 28.2, and 28.5).

In the fasting state, adipocytes produce most of their energy from sources other than glucose (i.e., endogenous and exogenous triglycerides, as well as exogenous ketone bodies).

5.4. Regulation of Glycolysis in Heart Muscle

ATP use of the heart is very high. The heart can use fatty acids, glucose, lactate, and ketone bodies for ATP production.

At rest, in the fasting state, or after a high-fat/low-carbohydrate meal, the heart uses almost no glucose and produces its ATP mostly from the β-oxidation of fatty acids (see Chapter 27). Under these conditions, the heart also uses some lactate and ketone bodies.

The heart substantially increases its use of glucose after a high-carbohydrate meal, during exercise or a high workload, and during hypoxia, whereby insulin, epinephrine, AMP, and AMP-dependent protein kinase are key regulators. **Insulin** and active **AMP-dependent protein kinase** (**AMPK**) can each induce the insertion of **GLUT4 glucose transporters** into the cardiomyocyte plasma membrane. **AMP** directly activates PFK1, the main rate-limiting enzyme of glycolysis. AMP also activates AMPK. Insulin, **epinephrine**, and AMPK each increase **phosphofructokinase 2** activity, which leads to an increase in the concentration of fructose 2,6-bisphosphate, which in turn activates PFK1.

While **insulin** increases glucose use by the heart, it decreases **fatty acid** use. The mechanisms for increased glucose use are described above. Fatty acid use is decreased by an insulin-induced increase in the concentration of malonyl-CoA, which inhibits the transport of fatty acids from the cytosol into the mitochondria (see Chapter 27).

Hypoxia increases glucose use in the heart, as it does in all tissues. Hypoxia slows ATP production via oxidative phosphorylation, which leads to an increased concentration of AMP, which in turn increases the uptake and use of glucose, in part via AMPK (see above).

During a **high workload**, the heart increases its glucose use and decreases its fatty acid use because ATP production from glucose requires less **oxygen** than ATP production from fatty acids. The complete oxidation of glucose via glycolysis, conversion of all pyruvate to acetyl-CoA, the oxidation of acetyl-CoA in the citric acid cycle, and oxidation of all NADH and $FADH_2$ in oxidative phosphorylation with concomitant production of ATP yields about 2.7 ATP per single O-atom used. In contrast, the activation of a C-16 fatty acid and complete oxidation via β-oxidation, the citric acid cycle, and oxidative phosphorylation yields only about 2.4 ATP per O-atom used.

5.5. Regulation of Glycolysis in Skeletal Muscle

Muscle consists of fibers of several types that differ in their use of oxygen for glucose metabolism. Some fibers perform mostly anaerobic glycolysis, others mostly aerobic glycolysis with oxidation of most substrates to CO_2. The gastrocnemius muscle (Fig. 19.14), for instance, contains mostly fibers that perform anaerobic glycolysis. The soleus muscle, on the other hand, contains mostly fibers that oxidize substrates to CO_2. Both muscles extend the foot; the gastrocnemius is used mainly for high-force, short-duration activities, and the soleus is used mainly for maintaining posture.

Glycolytic fibers are fast-twitch fibers that contain only a few mitochondria; they can contract and deliver full force in

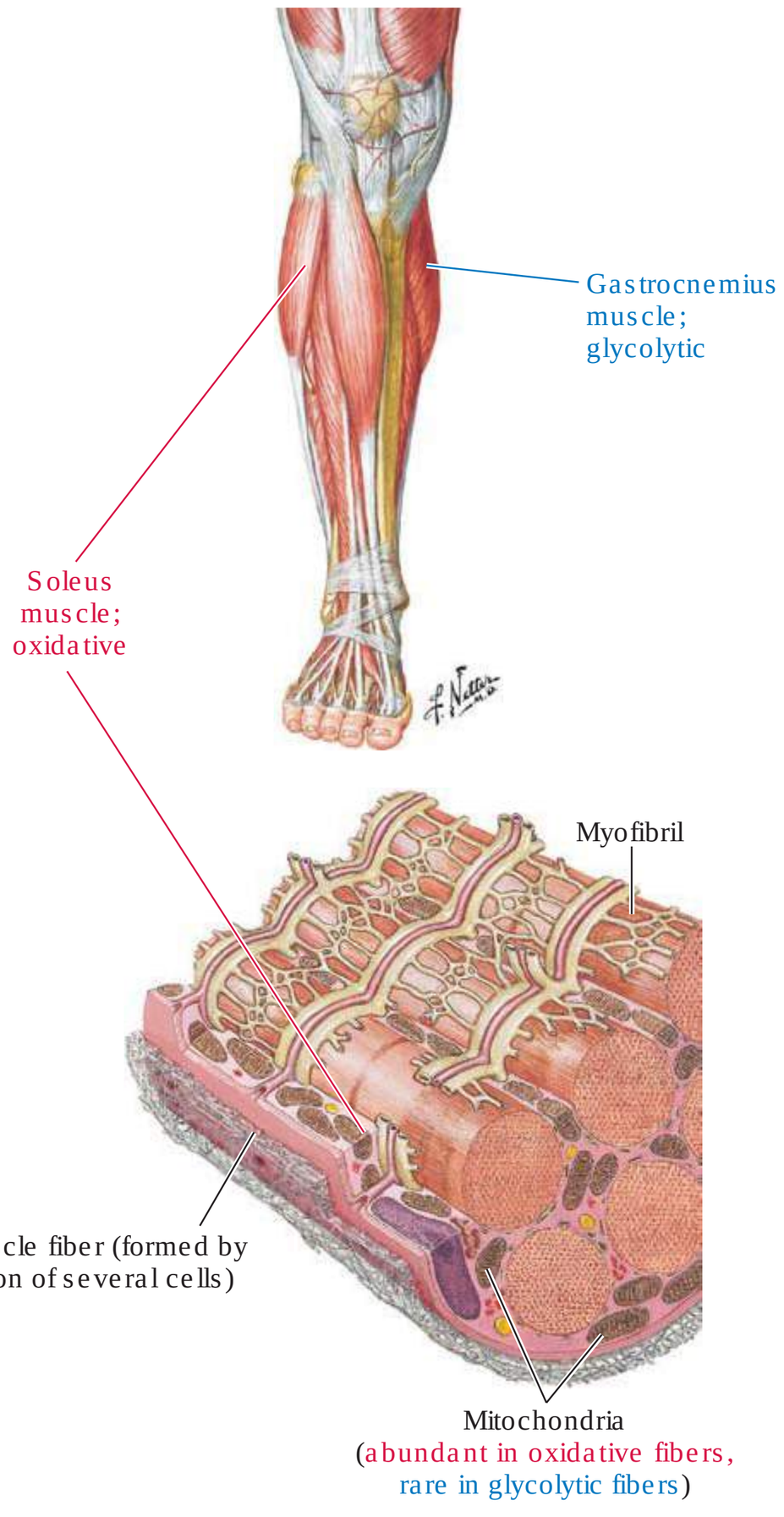

Fig. 19.14 **Anterior view of the muscles of the left leg (superficial dissection).**

less than 0.1 second because all components for anaerobic glycolysis are present (if insufficient glucose is available, endogenous glycogen can be degraded to glucose 6-phosphate; see Chapter 24). The drawback of glycolytic fibers is that they produce and release lactic acid; if all muscle fibers performed only anaerobic glycolysis during exercise, the human body could not remove enough lactic acid from the blood to prevent life-threatening acidosis.

Oxidative fibers (the majority of fibers in the soleus muscle) are slow-twitch fibers that have abundant mitochondria and continuously need to import oxygen; however, increased oxygen delivery depends on an increase in blood flow. Even though these fibers can contract within a fraction of a second, blood flow typically takes a few minutes to become maximal. This is a reason for athletes to warm up before a competition. Besides glucose, oxidative fibers also use fatty acids and ketone bodies for energy generation. When oxygen is limiting, oxidative fibers can also perform anaerobic glycolysis.

Successful **sprinters** typically rely on muscles that perform appreciable anaerobic glycolysis, while **marathon runners** rely on muscle fibers that oxidize substrates to CO_2 and produce very little lactic acid.

During **contraction**, glycolytic and oxidative skeletal muscle fibers use glucose from the blood and also glucose 6-phosphate from the degradation of **glycogen** (see Chapter 24). Glucose and glucose 6-phosphate undergo glycolysis to pyruvate. When most of the glycogen is used up, or when the **intracellular pH** is low, **muscle fatigue** sets in.

In response to exercise or insulin, the main glucose transporter in skeletal muscle, GLUT-4, moves from intracellular vesicles to the plasma membrane. Exercise increases the concentration of AMP, which in turn activates PFK1 and **AMPK** (see Section 3). AMPK favors the translocation of GLUT-4 to the plasma membrane.

In the **recovery** phase, the glycogen stores are built up again; this happens both after exercise has ended and even more so after a meal when an elevated concentration of insulin causes the translocation of GLUT-4 to the plasma membrane and activates glycogen synthase (see Chapter 24).

5.6. Regulation of Glycolysis in the Liver

After a meal, the liver oxidizes glucose (see Chapters 22 and 23), stores glucose as glycogen (see Chapter 24), and converts a small amount of glucose into triglycerides (see Chapters 27 and 28). In the fasting state, the liver degrades glycogen to glucose (see Chapter 24); converts lactate, many amino acids, and glycerol into glucose (see Chapter 25); and turns fatty acids into ketone bodies (see Chapter 27), whereby it releases glucose and ketone bodies for the benefit of other tissues.

The plasma membrane of hepatocytes always contains **glucose transporters** (GLUT-2). The liver takes up glucose after a carbohydrate-containing meal and releases glucose in the fasting state. Although hormones do not regulate the membrane insertion of glucose transporters in the liver, they regulate glucose metabolism, which in turn affects the direction and rate of glucose transport across the hepatocyte plasma membrane.

In contrast to other tissues, the liver expresses **glucokinase** and a **GKRP**. Glucose 6-phosphate does not inhibit glucokinase. However, GKRP binds to glucokinase, inhibits it, and induces its translocation to the nucleus. In the fasting state, glucokinase is largely sequestered in the nucleus; this process is modestly enhanced by the high concentration of fructose 6-phosphate that is typical of the glucose-producing liver in the fasting state (see also Chapters 24 and 25). Conversely, **glucose** influx from the diet into hepatocytes leads to a partial release of glucokinase from GKRP in the nucleus, followed by transport into the cytosol. As a result, glucose-phosphorylating activity in intact hepatocytes shows an even greater dependence on blood glucose than does glucokinase alone (see Fig. 19.8). Fructose 1-phosphate, which is present only when dietary **fructose** or **sorbitol** are absorbed (see Chapter 20), further enhances the release of glucokinase from GKRP.

Since glycolysis and gluconeogenesis have essentially the same intermediates and use the same enzymes for the reversible steps, it is important that enzymes that catalyze the irreversible steps in glycolysis are inactivated during **gluconeogenesis**. Similarly, enzymes that catalyze irreversible steps in gluconeogenesis need to be inactivated during glycolysis. To this end, glucokinase activity is regulated as described above by GKRP, PFK1 activity is regulated by fructose 2,6-bisphosphate, and pyruvate kinase is regulated by phosphorylation/dephosphorylation (see Chapter 25).

AMP, ATP, and **fructose 2,6-bisphosphate** allosterically control the activity of PFK1. The regulation of PFK1 (and thus glycolysis) by AMP and ATP ensures adequate ATP production. *After* a meal, when the concentration of **insulin** is relatively high, protein phosphatase 2C, which is part of the insulin signaling network (see Chapter 26), dephosphorylates the bifunctional enzyme PFK2/FBPase 2 and thereby activates its phosphofructokinase 2 domain (see Fig. 19.9). This leads to an increase in the concentration of fructose 2,6-bisphosphate, which in turn activates PFK1. Activation of PFK1 by fructose 2,6-bisphosphate overrides the inhibition of PFK1 by ATP. As a result, although the concentration of ATP is high, glycolysis can proceed at a high rate and its intermediates and products can be used for the synthesis of fatty acids and triglycerides (see Chapters 27 and 28). In the *f*asting state or during vigorous, prolonged exercise, **glucagon** and **epinephrine** activate protein kinase A (see Chapters 26 and 33), which phosphorylates PFK2/FBPase 2 and thereby activates its fructosebisphosphatase 2 domain (see Fig. 19.9). This leads to the degradation of the regulatory sugar fructose 2,6-bisphosphate. Without fructose 2,6-bisphosphate bound to it, PFK1 is readily inhibited by ATP. This ATP stems from mitochondria that oxidize fatty acids. The resulting low PFK1 activity means that glucose enters glycolysis only sparingly.

The activity of **pyruvate kinase** is regulated not only by a feed-forward activation from **fructose 1,6-bisphosphate** but

also by inhibition from **glucagon-** or **epinephrine-**induced phosphorylation. T is signaling pathway is described in detail in Chapters 24 and 26. When gluconeogenesis is active, the inactivation of pyruvate kinase prevents a vicious cycle between phosphoenolpyruvate and pyruvate. In the fed state, **insulin** antagonizes the ef ects of glucagon and epinephrine.

6. COMMON LABORATORY METHODS AND ASSAYS

6.1. Preservation of Metabolites in Blood Samples

For lab assays (e.g., of blood glucose and lactate), **blood collection tubes** often contain sodium **fluoride** (NaF) to inhibit **enolase**, the enzyme that converts 2-phosphoglycerate to phosphoenolpyruvate (see Fig. 19.2). Inhibition of any step in glycolysis leads to the inhibition of the entire pathway (see Chapter 10).

6.2. Plasma or Serum Lactate Dehydrogenase

It is common to measure the activity of **LDH** in blood plasma of patients. On an ongoing basis, damaged cells leak this enzyme into the blood. All tissues have the same concentration of LDH, and cells normally contain a greater than 500-fold higher concentration of LDH than blood plasma. T e liver eliminates LDH from blood plasma. T ere are several isozymes of LDH in plasma, and they have plasma half-lives of several hours to several days. Most of the LDH in blood plasma derives from red blood cells and platelets. Even a slight amount of hemolysis increases the concentration of LDH in the plasma.

An elevated LDH by itself does not provide any information on which tissue is damaged. It is uncommon for liver disease to be the cause of an elevated LDH. If blood tests of aspartate aminotransferase, alanine aminotransferase, creatine kinase, hemoglobin, bilirubin, or reticulocyte count are not sufficiently diagnostic, electrophoresis for LDH isoenzyme distribution can help narrow down the tissue source of LDH.

6.3. 2-Fluoro-Deoxyglucose Positron Emission Tomographic Scans

Radioactive 2-^{18}F-deoxyglucose (FDG) together with positron emission tomography (PET) scanning is used to assess glucose metabolism in tissues. FDG contains a fluorine atom in place of the hydroxyl group found in glucose. FDG enters cells through glucose transporters. Inside cells, FDG mixes with glucose. Hexokinase phosphorylates glucose in proportion to the rate of glycolysis since hexokinase is designed to maintain a constant concentration of glucose 6-phosphate. Hexokinase can phosphorylate FDG to FDG 6-phosphate, and it does so in proportion to glucose. In most tissues FDG 6-phosphate is not appreciably metabolized further or degraded. Hence, FDG (used at an appropriately small dose) accumulates at a rate that is proportional to the rate of glycolysis.

FDG-PET scans are primarily used for imaging tumors and the brain. Tumors and active regions of the brain take up FDG at an especially high rate (see Fig. 8.19). FDG-PET scans help locate metastases and help diagnose certain dementias.

7. DISEASES THAT INVOLVE AN ABNORMAL FLUX IN GLYCOLYSIS

An elevated concentration of lactate in the blood is most often the result of tissue hypoxia that increases the rate of anaerobic glycolysis. Hypophosphatemia impairs glycolysis (and with it ATP production) in a wide variety of tissues. Hypophosphatemia is most commonly seen in patients who get too little phosphate or waste too much of it in the kidneys. Hereditary def ciencies of enzymes of glycolysis are uncommon and usually affect predominantly the red blood cells, leading to premature hemolysis. T e most common disease is pyruvate kinase def ciency.

7.1. Lactate Accumulation

T e concentration of lactate in the blood is a function of both tissue lactate production and lactate consumption. Compared with daily lactate production, the amount of lactate that circulates in the blood is very small (<1/1000). Hence, even a relatively small change in production or consumption can have a sizable ef ect on the plasma concentration of lactate.

Hyperlactatemia is a condition of an abnormally high concentration of lactate in the blood, which is typically defined as greater than 2 mM lactate (normal reference value: 0.5 to 1.5 mM).

Lactic acidosis develops when lactate production exceeds consumption such that the pH in the blood drops and the concentrations of both bicarbonate and CO_2 become abnormally low (due to buf ering and increased ventilation; see Chapter 16). Lactic acidosis is typically seen when the blood lactate concentration is 4 mM or greater.

T e term **anion gap** refers to the dif erence between measured serum or plasma concentrations of the cations Na^+ and K^+ (K^+ is often omitted) and the anions Cl^- and HCO_3^-. Blood contains other cations and anions that are not usually measured. Blood must always have the same number of positive and negative ions. T e anion gap is therefore an indicator of the concentration of "unmeasured" ions, which often means anions such as lactate or formate (see Chapter 36) or acetoacetate and β-hydroxybutyrate (see Chapter 27). A patient who has a lactic acidosis may thus have an increased anion gap.

Lactate production increases when a tissue experiences **hypoxia** that forces it to decrease ATP production from oxidative phosphorylation and increase ATP production via anaerobic glycolysis (see Section 2.2).

Shock is a condition of tissue hypoperfusion that leads to tissue hypoxia and hyperlactatemia. Early on, the shock is reversible, but later it leads to multi-organ failure and death. Shock can have a variety of causes. **Hypovolemic (hemorrhagic) shock**, for instance, is a consequence of massive blood loss. It is accompanied by vasoconstriction and the redistribution of blood flow away from the skin, kidneys, and gastrointestinal tract**. Septic shock** is due to severe sepsis from a severe infection that leads to vasodilation, an increase in microvascular permeability, and leukocyte accumulation in tissues that are remote from the primary site of infection. Patients with septic shock may have tissue hypoperfusion with consequent injuries to their lungs and kidneys, and sometimes also their liver.

Besides the abovementioned disorders, hyperlactatemia may also be caused by near-maximal **exercise**, a grand mal **seizure**, metabolism of **ethanol** (see Chapter 30), or any impairment of **mitochondrial ATP production**.

7.2. Effect of Hypophosphatemia on Glycolysis

In health care, standard laboratory methods report the concentration of **phosphorus** (P) in the serum. Phosphorus is not present in the blood in elemental form, but mostly as free phosphate (mostly as HPO_4^{2-}). The reported phosphorus is normal at about 3.0 to 4.5 mg P/dL (1.0 to 1.5 mM phosphate). The term **hypophosphatemia** refers to an abnormally low concentration of phosphate in the blood, and this is evident from an abnormally low reported serum phosphorus.

Hypophosphatemia impairs flux in glycolysis by reducing the activity of **glyceraldehyde 3-phosphate dehydrogenase**, which converts glyceraldehyde 3-phosphate to 1,3-bisphosphoglycerate (see Fig. 19.2). In tissues with mitochondria, hypophosphatemia also impairs ATP production by **oxidative phosphorylation** (see Chapter 23). **Tissue oxygenation** suffers because red blood cells contain less **2,3-bisphosphoglycerate** to facilitate the dissociation of O_2 from hemoglobin.

Hypophosphatemia progressively impairs the function of red blood cells, white blood cells, glial cells, skeletal muscle, and heart muscle. Phosphate-deficient red blood cells hemolyze prematurely. Diminished leukocyte function can lead to sepsis. When serum phosphorus falls below about 1 mg P/dL, the patient may stop breathing, have seizures, and show cardiac arrhythmias.

To make up for losses, humans need to take in about 0.7 g of phosphorus per day. Even though total body stores amount to 500 g or more of "phosphorus," phosphate cannot be sufficiently mobilized to cover a deficit in intake of a few days.

Hypophosphatemia is most commonly observed in connection with parenteral nutrition, malnourishment, chronic alcohol abuse, diabetic ketoacidosis, sepsis, respiratory alkalosis, or primary hyperparathyroidism (Fig. 19.15). The need for phosphate was first recognized when **parenteral nutrition** was developed. Patients who receive parenteral nutrition after a period of severe **fasting** (e.g., due to **anorexia**) or **malnutrition** need extra phosphate in the infusate to avoid developing hypophosphatemia as part of **refeeding syndrome**, which also includes other electrolyte abnormalities. Patients who chronically abuse **alcohol** take in little phosphate, and their kidneys lose an excessive amount of phosphate. Prolonged **acidosis** (e.g., **diabetic ketoacidosis**) leads to the loss of phosphate from tissues and the body. Normalization of metabolism is often accompanied by increased tissue uptake of phosphate,

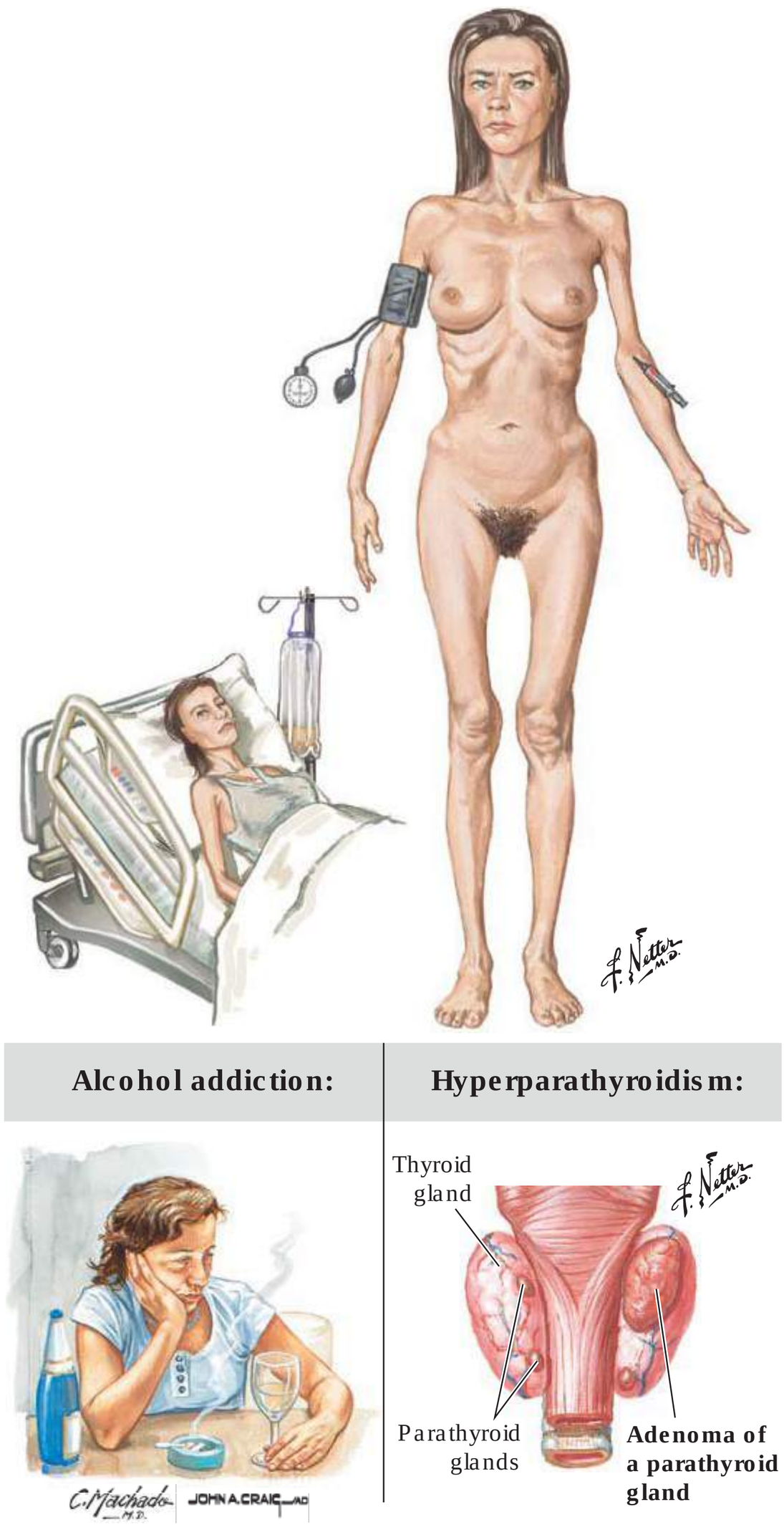

Fig. 19.15 **Some causes of hypophosphatemia.**

which can lead to hypophosphatemia. In **sepsis**, hypophosphatemia develops early and is a predictor of patient survival. Though persistent hypophosphatemia itself can lead to sepsis, most patients develop sepsis for other reasons. Patients who have **respiratory alkalosis** due to mechanical hyperventilation shift phosphate from the blood into the tissues. **Parathyroid hormone** inhibits the recovery of filtered phosphate in the kidneys. Patients with **hyperparathyroidism** become hypophosphatemic because they tend to lose too much phosphate. Hyperparathyroidism is most often due to an adenoma of a parathyroid gland.

Patients with significant symptoms and severe hypophosphatemia are treated with an intravenous infusion of a solution containing phosphate. Moderate hypophosphatemia is often treated with an oral phosphate salt.

7.3. Hemolytic Anemias Due to Hereditary Deficiencies of Enzymes of Glycolysis

Hemolytic anemia can have many causes, such as a problem with hemoglobin, as in sickle cell anemia or thalassemia (see Chapter 17), a problem with handling oxidative stress, as in G6PD deficiency (see Chapter 21), or a problem with an enzyme in glycolysis, as discussed below. Since mature red blood cells cannot synthesize enzymes, red blood cells are more readily affected by unstable mutant proteins than other cells that can still synthesize proteins.

Pyruvate kinase deficiency is the most common deficiency of a red blood cell enzyme of glycolysis. It has a worldwide distribution and shows a recessive pattern of inheritance. About 1% of all people are carriers, and about 1:10,000 are affected by the disease. In the United States, the disease is particularly common among the Amish. Red blood cell pyruvate kinase deficiency (with <30% of the normal activity remaining) is accompanied by nonspherocytic hemolytic anemia, usually with persistent hyperbilirubinemia, high reticulocyte count, a tendency to accumulate excessive amounts of iron (presumably due to ineffective erythropoiesis and hypoxia; see Chapter 15), and an increased incidence of gallstones (due to the increased excretion of bilirubin glucuronides; see Chapter 14). The pyruvate kinase deficiency is commonly due to a decreased affinity of the enzyme for the activator fructose 1,6-bisphosphate, and it always reduces ATP production in red blood cells so that processes such as ion pumping and maintenance of cell shape are compromised. The pyruvate kinase deficiency also causes an accumulation of all intermediates between fructose 1,6-bisphosphate and phosphoenolpyruvate (including 1,3-bisphosphoglycerate); as a result, the concentration of 2,3-bisphosphoglycerate also rises; this, in turn, lowers the oxygen affinity of hemoglobin inside red blood cells (see Chapter 16). At low altitude, this latter effect is beneficial because it improves oxygen delivery in these anemic patients; the drawback is a smaller O_2 reserve in circulating red blood cells.

Pyruvate kinase in red blood cells and liver derives from the same gene, though each tissue uses a different promoter and with that a different first exon. Even if the mutation is in a common exon (i.e., exons ≥3), a red blood cell pyruvate kinase deficiency does not affect glucose metabolism in the liver in a clinically relevant fashion.

For a detailed history of a patient with pyruvate kinase deficiency, see Bowman and Procopio in the Further Reading section.

Deficiencies of **other enzymes of glycolysis** are known, but they are very rare.

SUMMARY

- Glycolysis produces pyruvate from glucose. Tissues without mitochondria or cells without adequate oxygen perform anaerobic glycolysis. In anaerobic glycolysis, pyruvate is converted to lactic acid, which is released into the bloodstream.
- Cells that have well-oxygenated mitochondria can perform aerobic glycolysis; thereby pyruvate enters the citric acid cycle.
- Pellagra is characterized by diarrhea, dermatitis, and dementia. The disease is due to a deficiency of the vitamin niacin, which is needed for the synthesis of NAD^+, NADH, $NADP^+$, and NADPH. Some niacin can be made in the body by degradation of excess tryptophan. Pellagra is due to inadequate nutrition or drugs that interfere with NAD^+ production from tryptophan.
- Hypoglycemia primarily leads to an impairment of the central nervous system. Glial cells normally produce lactate for the benefit of neurons; during hypoglycemia, glial cell lactate production is insufficient, and neurons become short of ATP. Severe hypoglycemia (plasma glucose <20 mg/dL in an adult) leads to permanent damage of neurons and is often lethal.
- Hypophosphatemia is a common complication of parenteral nutrition, long-standing acidosis, ketoacidosis, chronic alcohol abuse, sepsis, or renal failure. Hypophosphatemia inhibits the glyceraldehyde 3-phosphate dehydrogenase–catalyzed reaction in glycolysis. This leads primarily to the hemolysis of red blood cells, to decreased production of 2,3-bisphosphoglycerate and thus increased oxygen affinity of hemoglobin, and to impaired brain function due to decreased glycolysis and ATP production; the latter is also due to decreased oxygen delivery from red blood cells. Severe hypophosphatemia can be fatal.
- Hereditary deficiencies of enzymes of glycolysis are uncommon. Among them, a deficiency of the pyruvate kinase that is expressed in red blood cells (and also in the liver) is the most common; it leads to chronic hemolytic anemia with hyperbilirubinemia, and patients are at an increased risk of hemochromatosis and gallstones. However, glycolysis in the liver is not affected in a clinically recognizable manner.

FURTHER READING

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Ann Intern Med. 1963;567-591. T is paper contains detailed descriptions of five individual cases.
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Review Questions

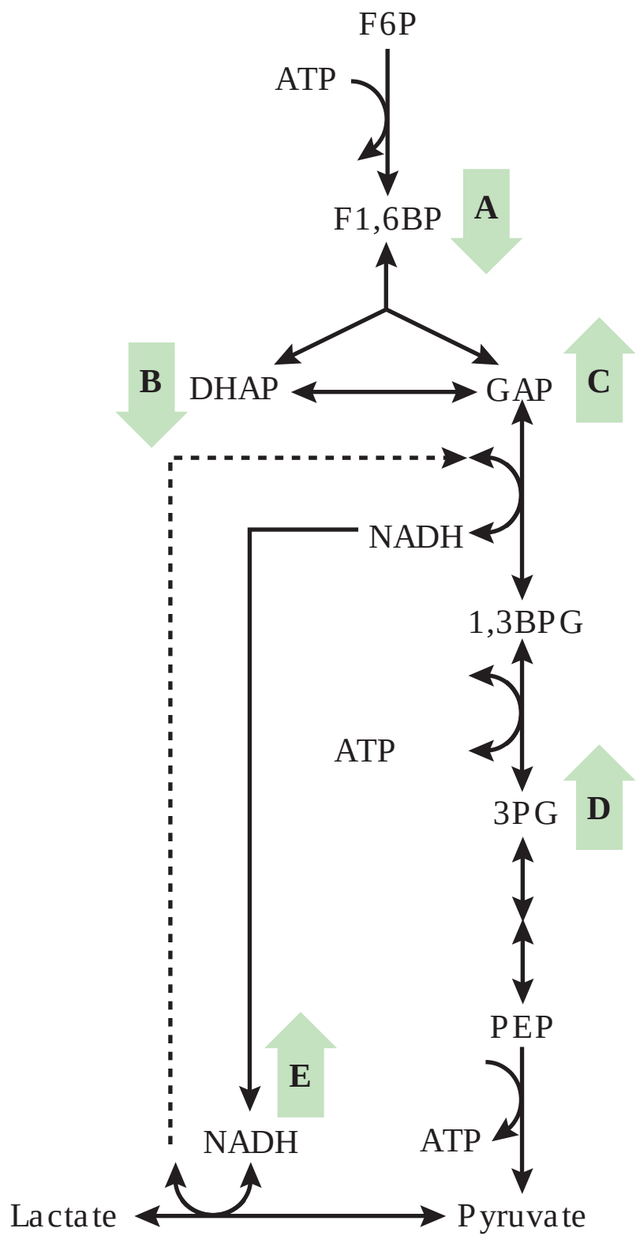
1. In the fed state (but not in the fasting state), fat cells have to make dihydroxyacetone phosphate to esterify fatty acids and deposit them as triglycerides in an intracellular droplet. Insulin-dependent activation of which one of the following enzymes allows fat cells to produce dihydroxyacetone phosphate from glucose even at a low concentration of AMP?

   A. 2,3-bisphosphoglycerate (2,3-BPG) mutase
   B. Aldolase
   C. Enolase
   D. Glyceraldehyde 3-phosphate dehydrogenase
   E. Lactate dehydrogenase
   F. Phosphofructokinase 1 (PFK1)
   G. Pyruvate kinase

2. A 2-year-old Amish girl has a hereditary pyruvate kinase deficiency, such that her red blood cells show only 5% of the normal pyruvate kinase activity. T e patient's erythrocytes have a decreased lifespan, and her skin chronically has a yellow tinge. Which one of the following ef ects on red blood cells is also characteristic of this disorder?

   A. In glycolysis, about 20 times more ATP is generated in the step 3-phosphoglycerate → 2-phosphoglycerate than in the step phosphoenolpyruvate → pyruvate.
   B. Instead of lactate, erythrocytes release phosphoenolpyruvate into the blood.
   C. T e concentration of 2,3-bisphosphoglycerate (2,3-BPG) is abnormally high.
   D. T e concentration of all intermediates between fructose 1,6-bisphosphate and phosphoenolpyruvate is abnormally low.

3. Below is a schematic diagram of a part of glycolysis. Which arrow correctly identifies a hypophosphatemia-induced change in the concentration of an intermediate of glycolysis?



   A. A
   B. B
   C. C
   D. D
   E. E

Chapter

20

Fructose and Galactose Metabolism: Hereditary Fructose Intolerance and Galactosemia

SYNOPSIS

- Fructose is a monosaccharide. The main sources of dietary fructose are table sugar, industrially prepared high-fructose corn syrup, and fruits. In the Western world, the daily per capita consumption of fructose is about 50 g/day; without sweeteners, it would be about 8 g/day.
- Dietary fructose enters the bloodstream via fructose transporters in intestinal epithelial cells. The liver and the kidneys phosphorylate fructose and thus act as a fructose sink. Phosphorylated fructose is cleaved, and the products enter glycolysis in the liver and kidneys.
- Hereditary fructose intolerance is due to a deficiency of aldolase B, the enzyme that cleaves phosphorylated fructose. The prevalence of hereditary fructose intolerance is about 1 in 20,000. Many affected patients have not been diagnosed. Hence, patients should not be infused with fructose or sorbitol (which is converted to fructose). If a patient with this deficiency regularly consumes fructose, the function of the liver and kidneys becomes severely compromised.
- Galactose is also a monosaccharide. Lactose (milk sugar) is the main dietary source of galactose. Galactose is absorbed via glucose transporters and is degraded in most tissues. The degradation products of galactose enter glycolysis.
- Galactosemia is due to a hereditary inadequate degradation of galactose. Most often, galactosemia is due to a deficiency of galactose 1-phosphate uridyltransferase. In affected infants, the consumption of milk leads to an acutely toxic state with jaundice, hepatomegaly, and vomiting. Treatment involves the elimination of most galactose from the diet.

LEARNING OBJECTIVES

For mastery of this topic, you should be able to do the following:

- List the foods that are rich in fructose.
- Outline the normal metabolism of dietary fructose.
- Describe the molecular basis of hereditary fructose intolerance.
- Explain the pathogenesis of organ damage in patients who have hereditary fructose intolerance and who consume an amount of fructose in their diet that is typical of the unaffected population.
- Explain renal Fanconi syndrome and describe its effects on kidney function.
- Describe the polyol pathway, paying attention to conditions that activate it.
- List the foods that are rich in galactose.
- Identify the molecular cause of classic galactosemia.
- Explain the pathogenesis of organ damage in patients who have classic galactosemia.
- Compare and contrast hereditary fructose intolerance and classic galactosemia with regard to cause, pattern of inheritance, pathogenesis, age of onset of symptoms, and treatment.

1. NORMAL METABOLISM OF FRUCTOSE

Fructose is a constituent of sweeteners, fruits, and vegetables. It is metabolized chiefly in the liver and kidneys. Fructokinase and aldolase B catalyze the first two steps of fructose degradation.

1.1. Sources of Fructose

Fructose is a 6-carbon sugar that is a natural part of the human diet. Fructose (Fig. 20.1) is also called **fruit sugar**. Like glucose, fructose is a reducing sugar. The largest amounts of fructose are usually consumed with sweeteners, such as high-fructose corn syrup, sucrose (table sugar; see Fig. 18.1), honey, and maple syrup. Table 20.1 provides a list of the fructose content of various foods and beverages. The main calorie-containing sweeteners—sucrose, high-fructose corn syrup, and honey—consist of roughly equal amounts of glucose and fructose.

In developed nations, the per capita fructose consumption is about 50 g/day (total carbohydrate consumption is about 400 to 500 g/day); without added sweeteners, fructose consumption would only be near 8 g/day (equivalent to <2% of all calories). Adverse effects of a high fructose intake are discussed in Section 3.4. The World Health Organization recommends that less than 10% of all calories come from honey, syrups, fruit juices, or mono- and disaccharides that are added to foods. At a total intake of 2000 kcal/day, less than 50 g of added sweeteners should be consumed; this translates to less than 25 g of fructose.

1.2. Uptake of Fructose

The microvilli of small-intestinal epithelial cells contain a **fructose transporter** (GLUT-5) that facilitates the movement of fructose down a concentration gradient and into the cells (see Fig. 18.5). GLUT-2 glucose transporters in the basolateral membrane of intestinal epithelial cells allow further equilibration of fructose with the extracellular space and blood. From there, fructose enters the liver and, to a lesser degree, the kidneys and the intestine via the same transporters. These organs act as sinks for fructose because they trap fructose by phosphorylation (see Section 1.3 and Fig. 20.2).

1.3. Metabolism of Dietary Fructose

Fructose metabolism is normally limited by aldolase B activity, and fructose metabolites enter glycolysis. Fig. 20.2 shows

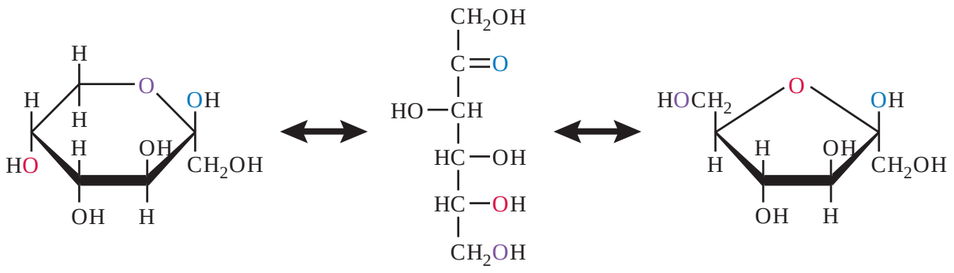


Fig. 20.1 **Structure of D-fructose.**

Table 20.1 **Sugar Content of Various Foods**

	Content in g/100 g Food		
	Total Carbohydrate	**Fructose**	**Galactose**
White sugar	100	51	0
Honey	82	43	0
High-fructose corn syrup, HFCS-42	71	30	0
High-fructose corn syrup, HFCS-55	77	42	0
Cola (with HFCS)	10	5	0
Apple	13	8	0
Cooked carrot	7	2	0
Boiled white potato	18	0	0
Cooked white rice	28	0	0
Whole cow's milk	5	0	3
Plain yogurt	6	0	3

the metabolism of fructose in detail. **Fructokinase** (also called **ketohexokinase**) has a much higher rate of flux than **aldolase B**; as a consequence, fructose 1-phosphate transiently accumulates. In the liver, fructose 1-phosphate serves as a signal for the influx of dietary carbohydrate, and it activates glucokinase (see Sections 3.5 and 5.6 in Chapter 19); as a result, dietary *f*ructose helps clear dietary glucose from the blood.

Humans have three aldolase isoenzymes (A, B, and C); all of them function in glycolysis and gluconeogenesis (see Chapter 25), but only aldolase B can cleave fructose 1-phosphate. Fructokinase and aldolase B are expressed mainly in the liver, kidneys, and intestine.

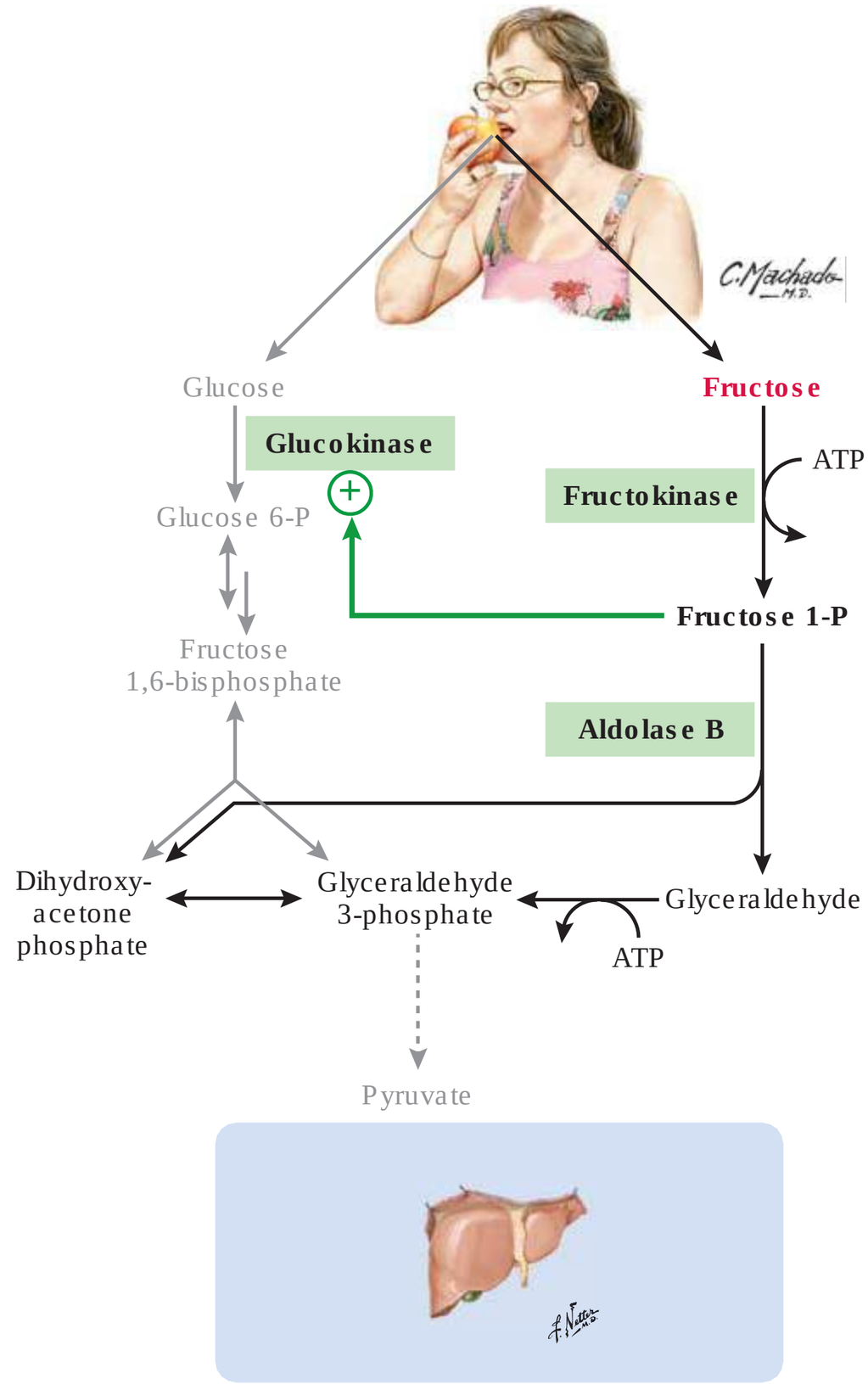


Fig. 20.2 **Metabolism of fructose in the liver.**

2. POLYOL PATHWAY AND ITS ROLE IN DISEASE

In the polyol pathway, glucose is reduced to sorbitol, which in turn is oxidized to *f*ructose. In patients with chronic hyperglycemia, increased production o*f* sorbitol and *f*ructose *f*rom glucose contributes to cataract *f*ormation and small-vessel disease that brings about retinopathy, nephropathy, and peripheral neuropathy.

T e **polyol pathway** consists of the two steps: glucose → sorbitol → fructose (Fig. 20.3), and it is present in a wide variety of cells. Sorbitol is a sugar alcohol. Aldose reductase, the rate-limiting enzyme of the polyol pathway, has a very low affinity for glucose ($K_m \geq 50$ mM). T us, the flux in the pathway is markedly increased during hyperglycemia.

In the medulla of the **kidney**, hypertonicity stimulates the synthesis of aldose reductase, and sorbitol helps to maintain the intracellular osmotic pressure. In other cells, aldose reductase may detoxify various aldehydes. In the male reproductive tract, the polyol pathway produces fructose as a fuel for sperm (see below).

A large amount of natural **sorbitol** is found in dried prunes (15 g/100 g); decreased amounts are found in other fruits (0.1 to 2.5 g/100 g). Sorbitol is also found in some pills, chewing gums (sorbitol does not promote caries), jams, baked goods, and ice creams. T e liver converts dietary sorbitol to fructose, which it degrades.

On U.S. food labels, sorbitol is considered to be a carbohydrate, not a sugar. Consequently, foods with added sorbitol may carry the label "no sugar added."

In diabetic patients who have chronic **hyperglycemia**, activity in the polyol pathway is thought to contribute to lens **cataracts**, peripheral **neuropathy**, **retinopathy**, and **nephropathy** (Fig. 20.4). Increased aldose reductase activity lowers the concentration of NADPH and thus also lowers the concentration of reduced glutathione (Fig. 20.5). T is in turn may permit the presence of increased amounts of reactive oxygen species, which then inflict damage to various cell components (see Chapter 21). In addition, sorbitol may accumulate in tissues that have low sorbitol dehydrogenase activity. T is may damage cells through osmotic ef ects and contribute to neuropathy, retinopathy, and the formation of cataracts. Diabetes-related complications are discussed further in Chapter 39.

In the evaluation of **infertility**, **fructose** is often measured in the ejaculate. About 90% of the fructose in the ejaculate is derived from the **seminal vesicles** (Fig. 20.6), which synthesize fructose via the polyol pathway. T e concentration of fructose in semen (normally more than about 7 mM when measured 1 hour after ejaculation) thus serves as an indicator of the function of the seminal vesicles. In the absence of fructose, sperm have a much shortened lifespan.

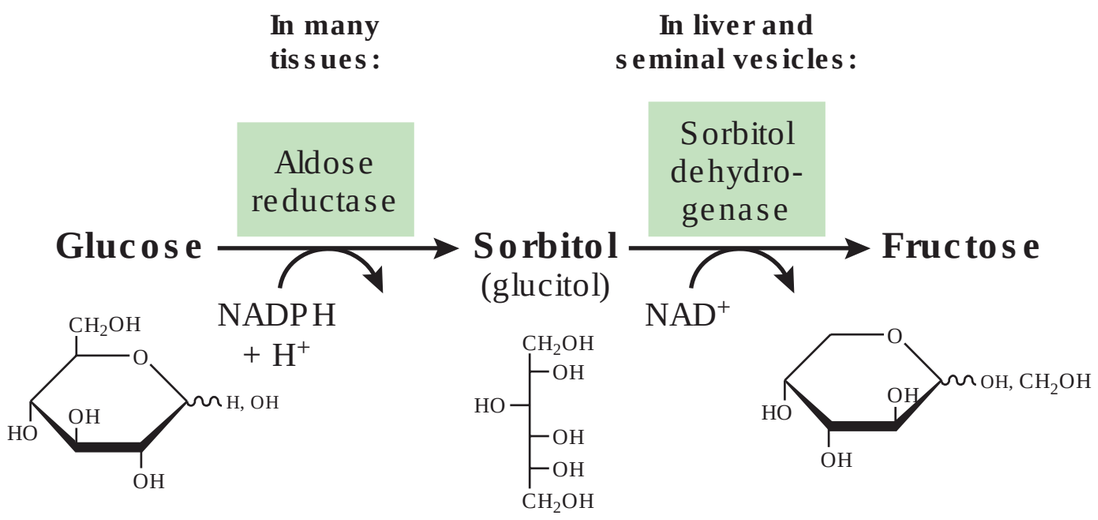


Fig. 20.3 The polyol pathway. Aldose reductase also reduces galactose, producing galactitol (see Fig. 20-9).

3. ABNORMAL FRUCTOSE ABSORPTION AND METABOLISM

T e most signif cant heritable disease o*f f*ructose metabolism is a def ciency o*f* aldolase B, which causes hereditary

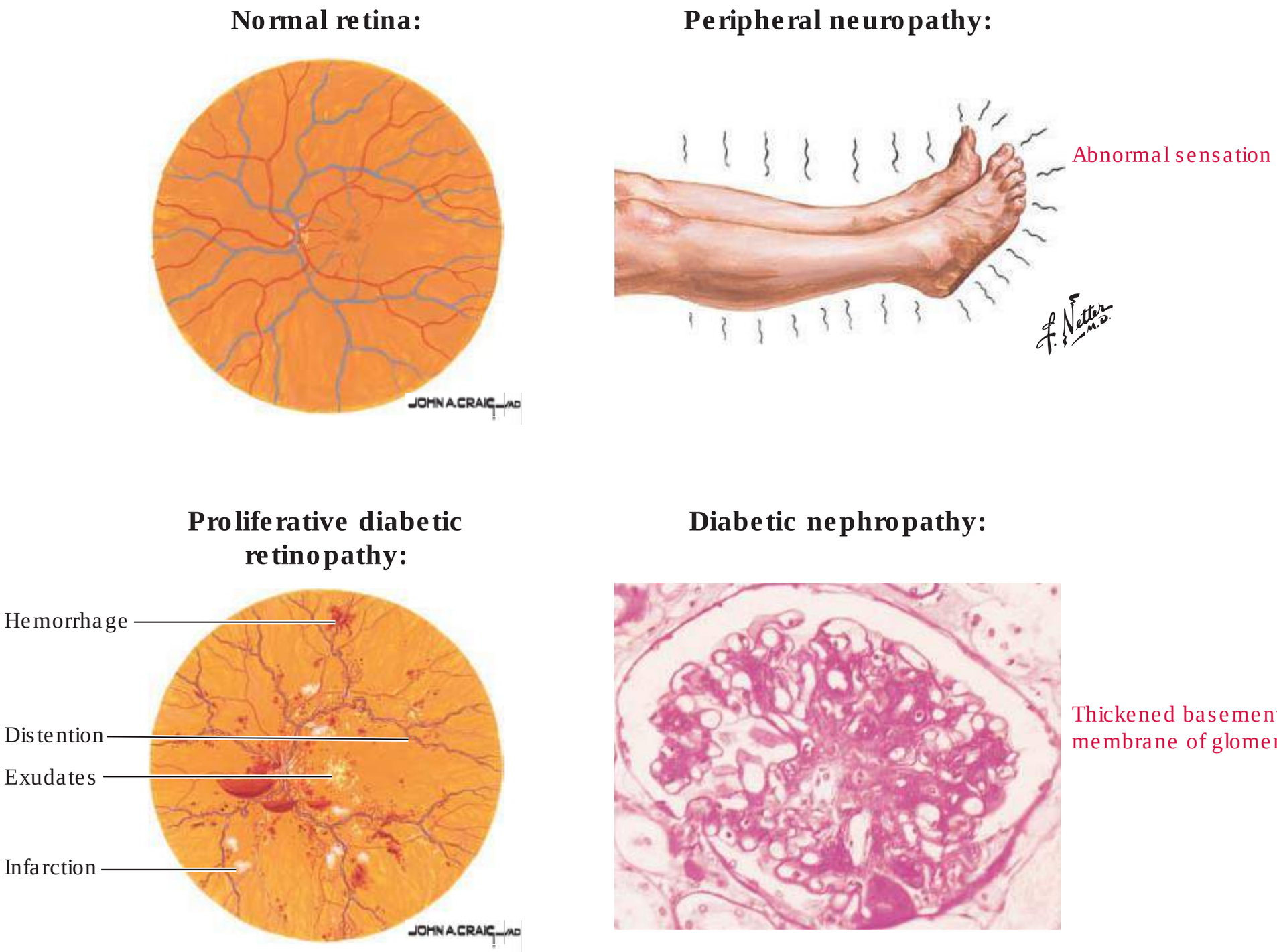


Fig. 20.4 Complications of diabetes due in part to increased flux through the polyol pathway.

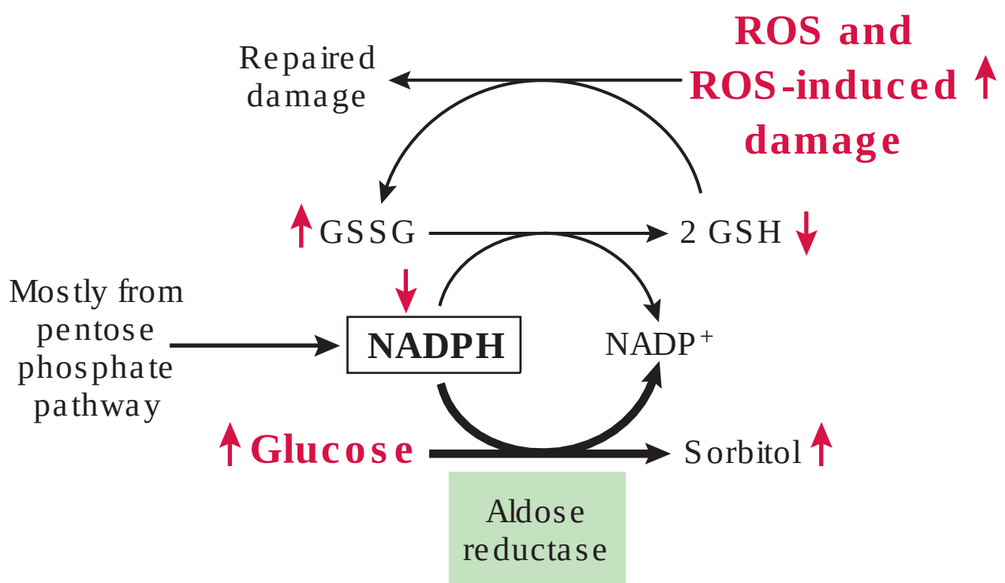


Fig. 20.5 **Hyperglycemia-induced increase in flux through the polyol pathway causes increased reactive oxygen species (ROS) and ROS-induced damage. GSH, glutathione; GSSG, oxidized glutathione.**

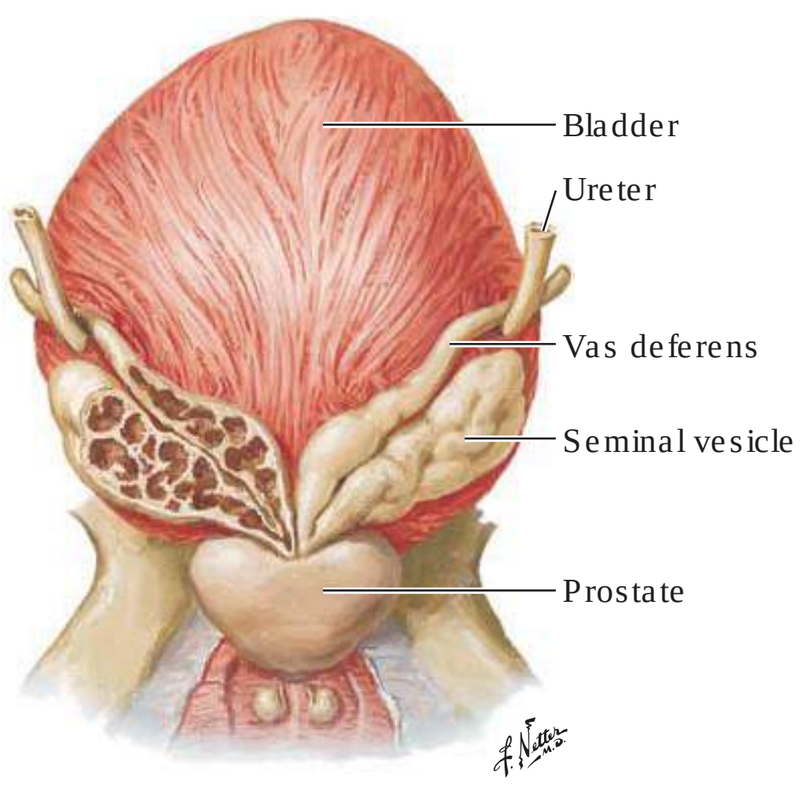


Fig. 20.6 **Most of the fructose in semen stems from fructose secreted by the bilateral seminal vesicles.**

*f*ructose intolerance (HFI). I*f* a*ff*ected patients consume *f*ructose, their liver and kidneys su*ff*er *f*rom a depletion o*f* intracellular adenosine triphosphate (A*T*P) and phosphate and thus *f*ail to per*f*orm their regular *f*unctions; this can be lethal.

3.1. Fructose Malabsorption

All humans suf er from intestinal discomfort if they consume more fructose than they can absorb. Normally, the small intestine can absorb ~15 g or more of fructose from a pure fructose solution. If fructose reaches the colon, it may give rise to abdominal discomfort (from gas produced by bacteria) and diarrhea (from osmosis; see Chapter 18).

Patients who have fructose malabsorption (Fig. 20.7) have intestinal discomfort after a relatively low dose of fructose. Malabsorption is often determined by a hydrogen breath test (see Section 4 in Chapter 18). T ere is no agreed-upon definition for fructose malabsorption, and it is not clear why certain patients can tolerate only a small amount of fructose.

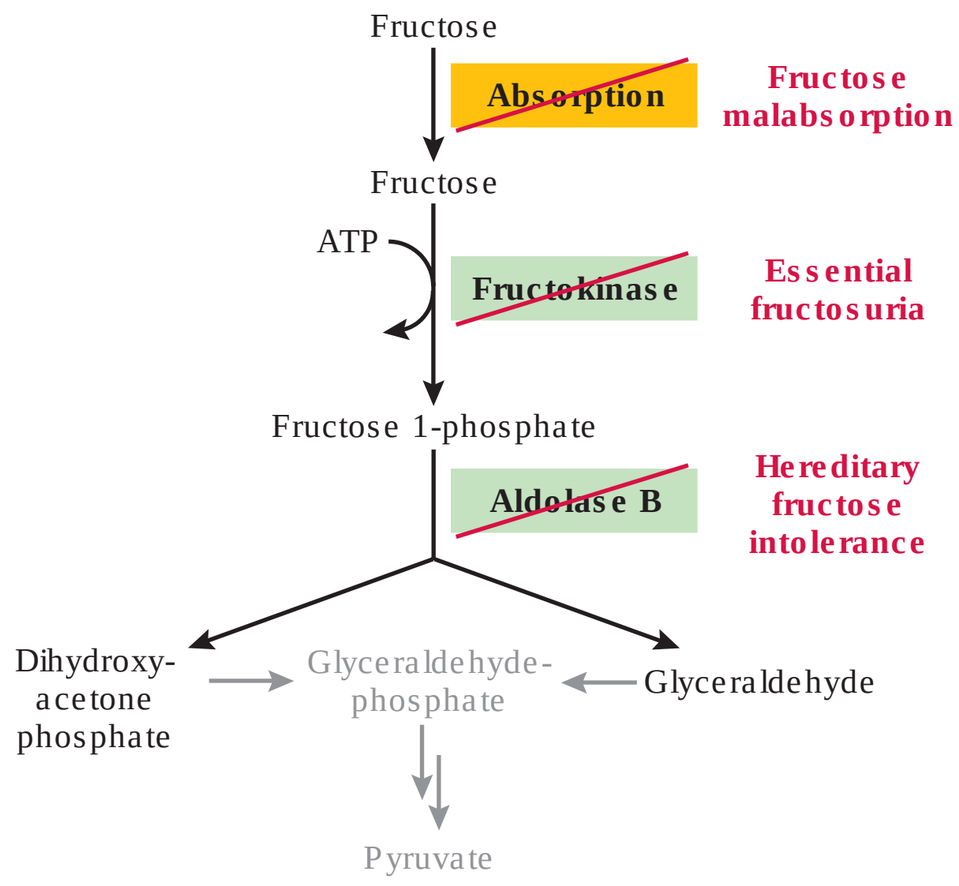


Fig. 20.7 **Sites of problems with fructose metabolism.**

3.2. Fructosuria

Fructosuria is a very rare and harmless disease that is due to the absence of **fructokinase** (see Fig. 20.7). Af ected patients do not efficiently phosphorylate fructose; as a result, dietary fructose remains in the bloodstream for several hours. Fructose is lost from the bloodstream by filtration in the kidneys and excretion with urine (hence the name fructosuria), as well as by metabolism (details are poorly known).

3.3. Hereditary Fructose Intolerance

Patients with HFI have an **aldolase B deficiency** and become extremely ill when they consume even an average amount of fructose. Aldolase B cleaves fructose 1-phosphate (see Fig. 20.7). HFI shows autosomal recessive inheritance and af ects about 1 in 20,000 people. More than 60 mutations in the aldolase B gene are known, but in Europe and North America, about 75% of af ected patients are homozygous or compound heterozygous for the mutations A149P and A174D. T e first symptoms usually appear in infants with the introduction of fructose-containing foods (see Table 20.1). Some soy-based infant formulas contain fructose in the form of sucrose. Most babies start consuming fructose when they start eating solid foods. Af ected babies experience abdominal pain within a few minutes of fructose consumption, which is accompanied by nausea, vomiting, and hypoglycemia. Af ected children who chronically ingest fructose have retarded growth and may incur irreversible or even lethal damage to their liver and kidneys. Since there is associated discomfort, fructose-intolerant children learn to avoid the most of ending foods; however, for good health, they must knowingly exclude additional fructose-containing foods so that the daily fructose consumption is below 1.5 g. (See Baerlocher et al. in the Further Reading section for individual histories of several patients with HFI.)

If a patient with HFI consumes fructose, the liver, kidneys, and intestine accumulate fructose 1-phosphate to a high concentration (up to about 10 mM; Fig. 20.8). In the process, cytosolic ATP is used. Furthermore, phosphate is temporarily locked up in fructose 1-phosphate, leading to a severe deficit in cytoplasmic phosphate. The release of phosphate from hydroxyapatite in the mitochondria and the uptake of phosphate from the blood are too slow to maintain a near-normal cytoplasmic concentration of phosphate. As a result of the cell-wide phosphate deficiency, insufficient ADP is phosphorylated to ATP. Under these circumstances, ADP and AMP are degraded to urate, which is released into the blood (see Chapter 38). Marked depletion of ATP in the kidneys and liver causes failure of these organs. In the kidneys, the proximal tubules fail to take up electrolytes from the glomerular filtrate, a condition called renal **Fanconi syndrome**. The liver no longer secretes adequate amounts of clotting factors, and patients may show petechiae. Patients also have fructose-induced hypoglycemia, which is incompletely understood. The hypoglycemia may be due to excessive activation of glucokinase in the liver (see Chapter 19) and the inhibition of glycogenolysis and gluconeogenesis (see Chapters 24 and 25). Glycogenolysis is impaired by the low concentration of phosphate and gluconeogenesis by the low concentration of ATP.

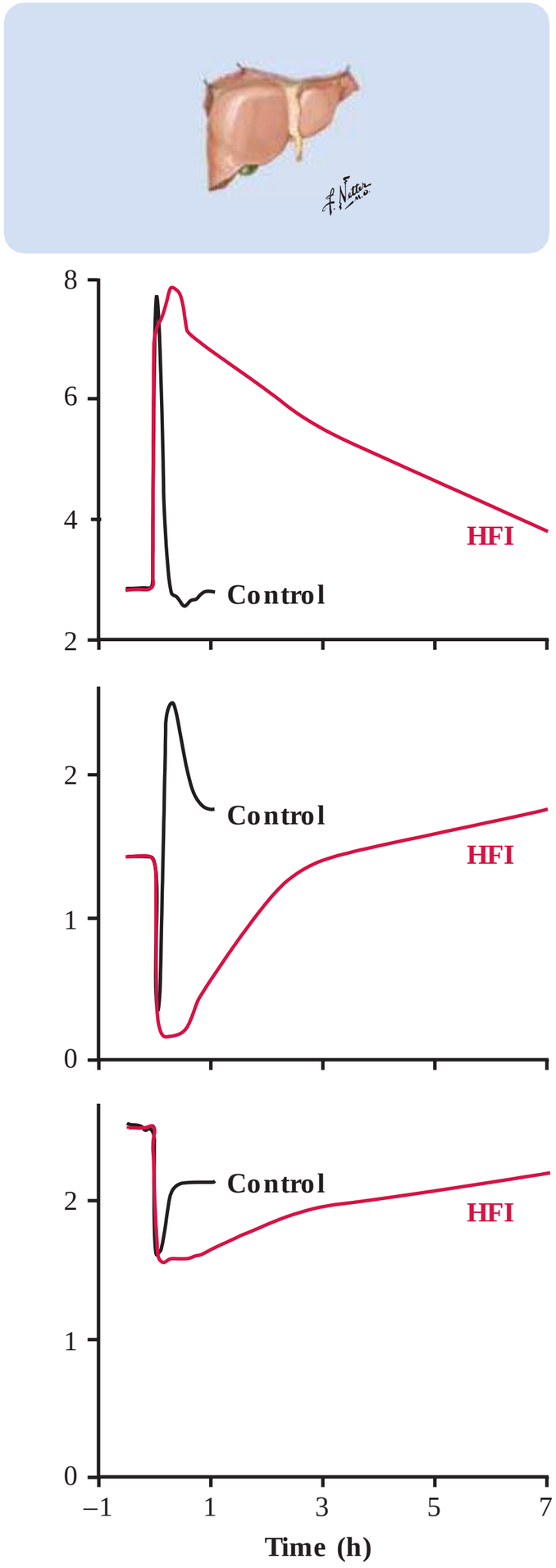


Fig. 20.8 Effect of intravenous fructose on phosphate-containing compounds in the liver. Magnetic resonance imaging studies of three healthy volunteers and one volunteer with hereditary fructose intolerance were performed. After an overnight fast, patients were infused with 0.2 g of fructose/kg body weight (~30% of average daily Western consumption). The change in the phosphomonoester concentration is mostly due to fructose 1-phosphate. Note that even in the controls, the concentration of adenosine triphosphate (ATP) did not recover to its original value. (Modified from Boesiger P, Buchli R, Meier D, Steinmann B, Gitzelmann R. Changes of liver metabolite concentrations in adults with disorders of fructose metabolism after intravenous fructose by 31P magnetic resonance spectroscopy. *Pediatr Res*. 1994;36:436-440.)

In patients with HFI who do not consume fructose, glycolysis and gluconeogenesis in the liver and kidneys are not significantly impaired because these tissues also express aldolase A and because aldolase B has residual activity toward its substrates in glycolysis and gluconeogenesis.

3.4. Concerns About High Fructose Consumption in the General Population

A small amount of fructose is part of the traditional human diet, appears to serve as an indicator of the influx of carbohydrate from the diet (see Chapter 19), and improves glucose tolerance. However, a high consumption of fructose, typically from fructose-containing sweeteners, may have adverse effects. Fructose consumption of 100 g/day or more is associated with **hypertriglyceridemia** and often with **hypercholesterolemia**. The following mechanism has been suggested as a cause of the hypertriglyceridemia (see Figs. 19.11 and 20.2). Given the normal abundance of enzymes of fructose metabolism and glycolysis, and because of a difference in control, fructose metabolism can feed trioses into glycolysis at a greater rate than can occur with glucose. Then, pyruvate and citrate are also produced at a higher rate, fatty acyl-CoA are synthesized at a higher rate, and fatty acyl-CoA are readily esterified with glycerol 3-phosphate, thereby giving rise to triglycerides.

High fructose intake is also associated with **gout** (see Section 4 in Chapter 38). A high intake of **fructose** from sucrose, high-fructose corn syrup, fruit juices, or fruit can lead to an accumulation of AMP in the liver and kidneys. AMP is then degraded into urate, which can lead to hyperuricemia, a precondition for gout.

Approximately 1 in 70 persons is a carrier for HFI and has decreased aldolase B activity and thus an impaired capacity to metabolize fructose 1-phosphate. Whether an average rate of fructose consumption is particularly harmful to this population remains to be determined. The data on ATP shown in

Fig. 20.8 raise the possibility that the current high fructose consumption is detrimental even to the general population.

For healthy persons, the benefits of consuming fruit are thought to outweigh the potential adverse effects of fructose in the fruit.

3.5. Problems With the Use of Fructose or Sorbitol in Medicine

In most countries the intravenous administration of fructose or sorbitol is restricted. As is evident from Fig. 20.8 and Sections 3.3 and 3.4, infusion of fructose can pose serious threats to the liver of patients with normal or compromised aldolase B activity. Sorbitol is metabolized to fructose (see Section 2) and is therefore equally troublesome. Many patients with HFI do not know that they are fructose intolerant; a significant infusion of fructose could be lethal to such a patient.

4. NORMAL METABOLISM OF GALACTOSE

A large number of tissues degrade galactose to glucose 6-phosphate.

The principal dietary source of galactose is the disaccharide **lactose** (see Fig. 18.1) from milk products (see Table 20.1). The structure of galactose is shown in Fig. 20.9. Galactose is a reducing sugar.

In the small intestine, **lactase** cleaves lactose into glucose and galactose (see Chapter 18). Galactose moves from the lumen of the intestine into absorptive epithelial cells via the same Na^+-driven glucose transporters (SGLT1) as does dietary glucose (see Fig. 18.5). Then, via glucose transporters, galactose is released into the bloodstream and taken up from the blood by other tissues.

All cells can metabolize galactose and feed the products into glycolysis, glycogen synthesis, or gluconeogenesis (see Fig. 20.9). The pathway from galactose to glucose 1-phosphate is also called the **Leloir pathway**. Glycogen synthesis is discussed in Chapter 24. Gluconeogenesis is active in the fasting state and decreases in the fed state (see Chapter 25).

5. GALACTOSEMIA

Classic galactosemia is due to a deficiency of galactose 1-phosphate uridyltransferase. Affected infants show cataracts and hepatomegaly. Patients with galactosemia need to follow a lifelong diet that is largely free of galactose.

5.1. Classical Galactosemia

Classical galactosemia is due to a deficiency of **galactose 1-phosphate uridyltransferase** (see Fig. 20.9), which leads to increased concentrations of galactose 1-phosphate, galactose, and galactitol. Since most tissues (including red blood cells) normally metabolize galactose, damage in patients with galactosemia affects many tissues. Galactose 1-phosphate accumulates inside cells and temporarily traps **phosphate** (this is comparable to the accumulation of fructose 1-phosphate in patients with HFI; see Fig. 20.8). As a result, phosphorylation of ADP to ATP is impaired, which leads to cell damage. Furthermore, since the phosphorylation of galactose to galactose 1-phosphate is a reversible reaction, a galactose 1-phosphate uridyltransferase deficiency also leads to an elevated concentration of galactose. Galactose then activates aldose reductase from the polyol pathway, which generates **galactitol** (the K_m of aldose reductase for galactose is ~15 mM; see Section 2). Galactitol is not a substrate of sorbitol dehydrogenase, the second enzyme of the polyol pathway. Galactitol, once formed, cannot be degraded; it is lost into the blood, filtered in the glomeruli, and not recovered from kidney tubules; thus it is excreted in the urine. Tissue damage may be due to a combination of osmotic effects of galactitol that alter intracellular signaling and free radical damage due to depletion of NADPH from the formation of galactitol (see Fig. 20.9). Finally, a

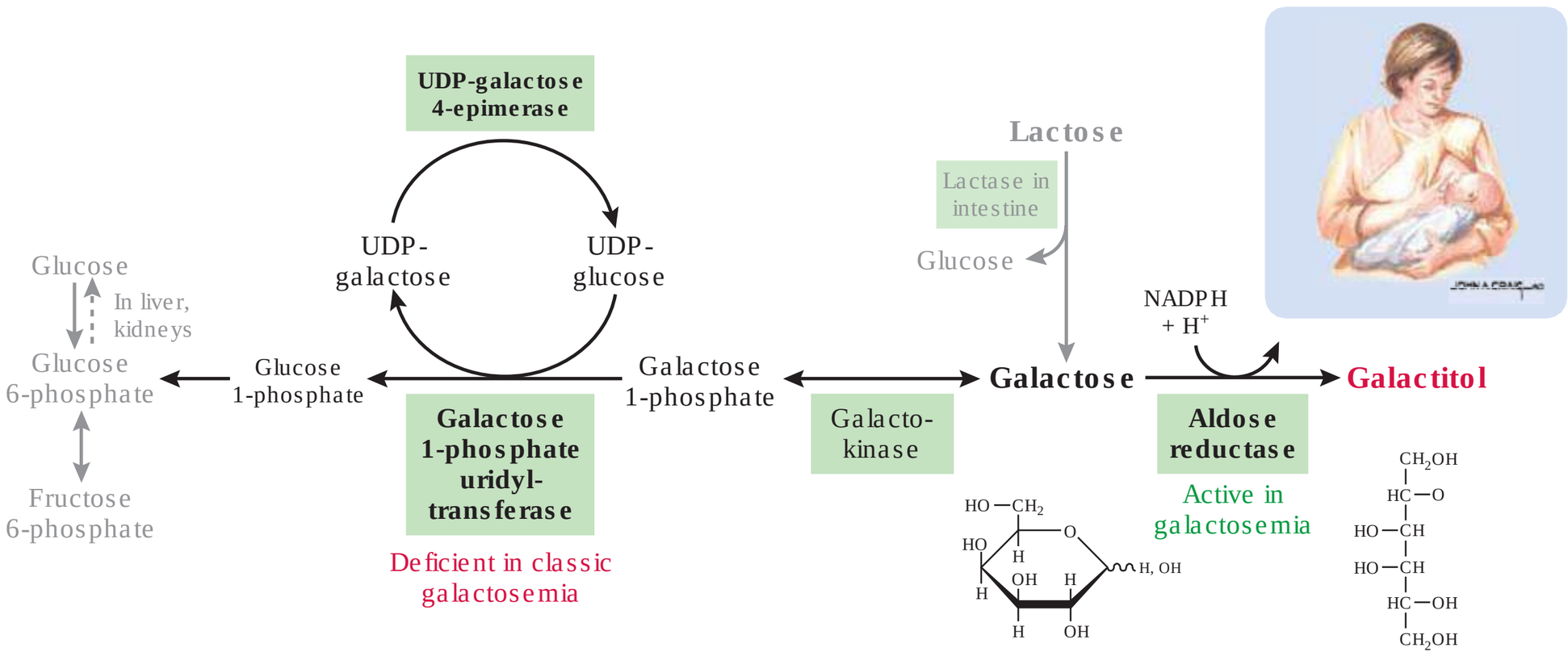


Fig. 20.9 Metabolism of galactose.

deficiency of galactose 1-phosphate uridyltransferase might also alter glycosylation of proteins (see Chapter 7).

In Europe and North America, the **incidence** of classical galactosemia is on the order of 1 in 35,000, with appreciable variation between countries. The disease is inherited in an autosomal **recessive** fashion.

Although well over 200 **mutations** in galactose 1-phosphate uridyltransferase are known, only a handful of mutations are common among affected patients. The mutation S135L is common among individuals with African ancestry, while Q188R is common among individuals with European ancestry. The severity of the disease can be correlated with certain mutations. DNA-based prenatal diagnosis is possible.

Classical galactosemia is usually evident after a few days of milk ingestion by a newborn, who may then exhibit symptoms of jaundice, hepatomegaly, vomiting, diarrhea, and Fanconi syndrome (Fig. 20.10). Some newborns also develop sepsis or coagulation defects. The urine of affected newborns tests positive for a reducing substance (galactose; see Section 1 in Chapter 18) and tests negative for glucose. Fanconi syndrome is due to a low concentration of ATP in the kidneys, as in patients with HFI after fructose ingestion (see Section 3.3). Galactosemia may also be detected by a newborn screening test.

Treatment of galactosemia involves greatly reducing dietary galactose intake by stopping breastfeeding or milk-based formula and feeding newborns a soy-based formula that has a low galactose content. Without treatment, patients become severely intellectually impaired. Dairy products (containing the disaccharide lactose or the monosaccharide galactose) are excluded from the diet. The degradation of glycoproteins and glycolipids in the body itself generates a significant amount of galactose, which is tolerated so that the complete exclusion of dietary galactose is unnecessary. Hence, patients who have classic galactosemia can consume fruits and vegetables, even if they contain small amounts of galactose (up to ~0.3 g galactose/100 g food). The success of treatment can be followed with measurements of the concentration of galactose 1-phosphate in red blood cells.

Treatment with a galactose-restricted diet is lifesaving and prevents severe mental impairment, but treated patients still face major problems with **mentation**, reproduction, and eyesight. About 80% of children with galactosemia have a full-scale IQ score of less than 90 (the test is designed for a maximum of 160 and a mean of 100 in the population at large). Affected children are likely to have problems with speech. Virtually all females develop **hypergonadotrophic hypogonadism** in their teens (i.e., an impaired response of the gonads to gonadotropins with subsequent hypertrophy of the gonadotropin-secreting anterior pituitary gland). Some of these women are sterile due to atrophy of the ovaries. About 10% of patients who have galactosemia develop **cataracts** at an early age; this is treatable with surgery. Some of the long-term problems seen in patients with galactosemia may be a consequence of in utero exposure to galactose.

5.2. Nonclassical Galactosemia

Patients with nonclassical galactosemia have a deficiency of **galactokinase** or **uridine diphosphate (UDP)-galactose/UDP-glucose 4-epimerase**. Patients with a galactokinase deficiency primarily develop cataracts due to a persistently elevated concentration of galactose in the blood and the consequent formation of galactitol in the lenses. These patients do not show damage to liver, kidneys, and brain that is typical of classical galactosemia. Patients with a very rare, generalized epimerase deficiency have symptoms that are similar to those of patients with classical galactosemia.

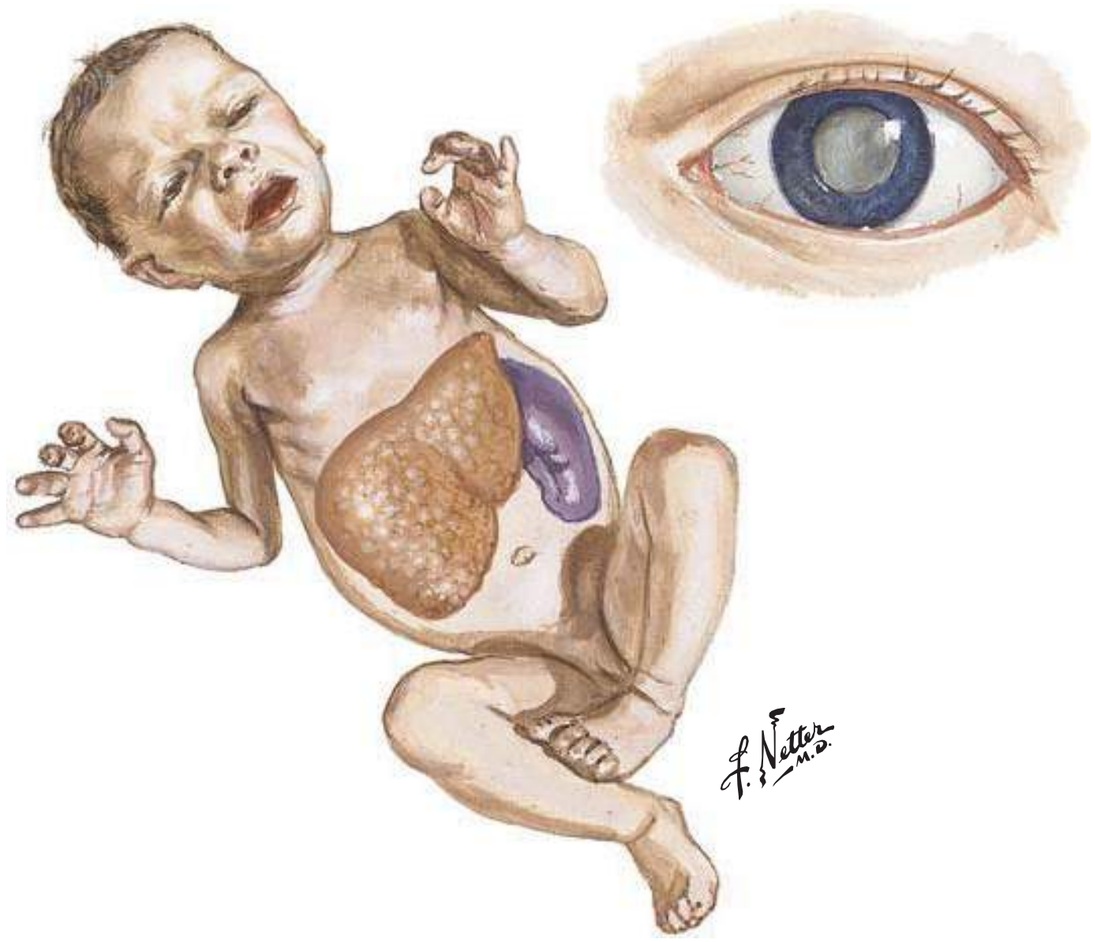


Fig. 20.10 Galactosemia often manifests itself within the first few days of life. Cataracts are occasionally seen at birth but generally develop only later.

6. LACTOSE SYNTHESIS IN THE LACTATING BREAST

The lactating breast synthesizes lactose *from* glucose and galactose. Most *of* the lactose is derived *from* glucose in the blood. Lactose is the principal osmolyte *of* milk and thus plays a role in determining milk volume.

Fig. 20.11 shows the pathway of lactose synthesis in the lactating mammary gland.

The synthesis of lactose depends on the presence of **lactose synthase**, which is a complex of soluble **α-lactalbumin** and a membrane-anchored **galactosyl transferase** in the Golgi apparatus. Most cells express a small amount of galactosyl transferase for the purpose of protein glycosylation in which the enzyme transfers galactose to glucose that is covalently linked to the side chain amino group of a protein (see Chapter 7). Several weeks before pregnancy term and throughout lactation, the mammary gland expresses vastly increased amounts of galactosyl transferase. Late in pregnancy, the mammary glands also express α-lactalbumin, and there is a further

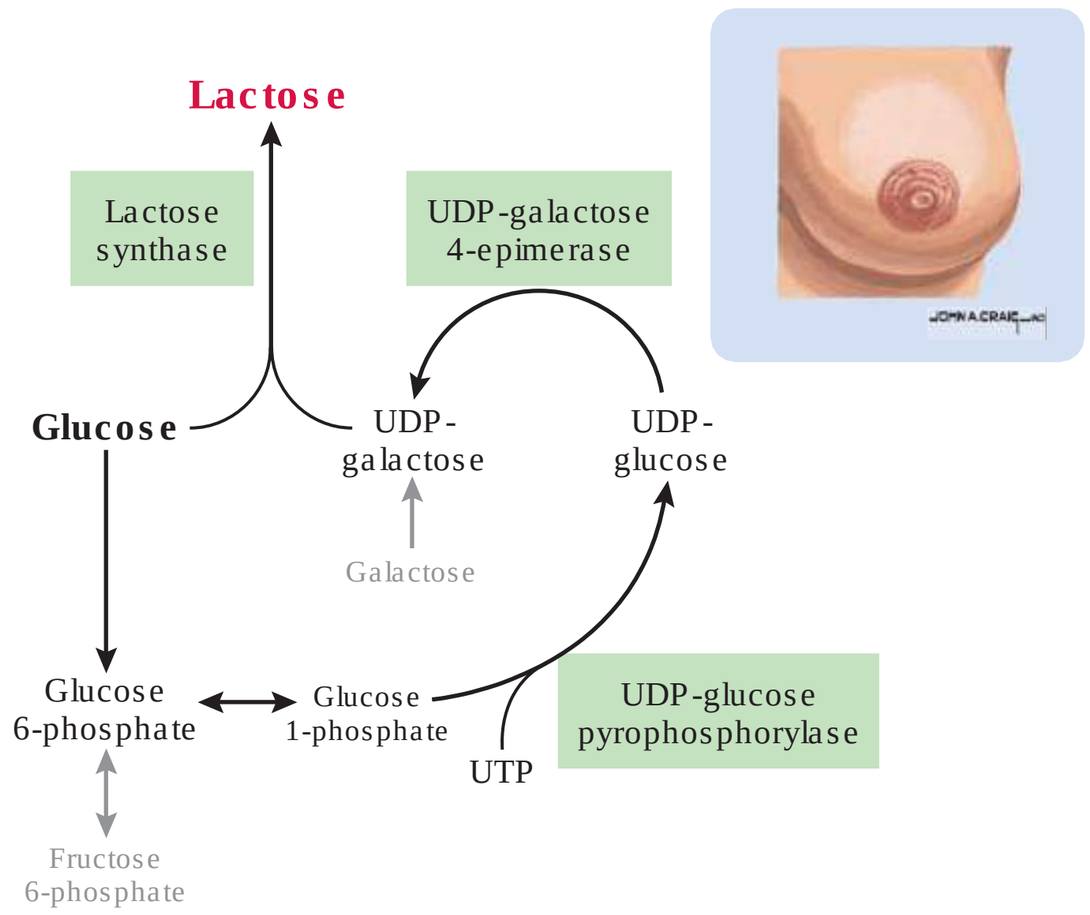


Fig. 20.11 Synthesis of lactose from glucose in the lactating breast. All cells can carry out the above reactions except the lactose synthase–catalyzed reaction. Only the mammary gland produces lactose synthase, and it does so only during the third trimester of pregnancy and during lactation. Cells use uridine diphosphate (UDP)-glucose for the synthesis of glycogen (see Chapter 24) and UDP-galactose for glycosylation of proteins (see Chapter 7). UTP, uridine triphosphate.

marked increase around the time of delivery. α-Lactalbumin binds glucose and forms a complex with galactosyl transferase such that glucose (instead of protein-linked glucose) becomes the favored acceptor of galactose, thus forming lactose.

In the lactating breast, in the *f*ed state, all of the glucose and about 70% of the galactose in lactose are derived from glucose in the blood. After a 1-day *f*ast, about 70% of the glucose and about 50% of the galactose are derived from glucose in the blood. It is unclear which processes contribute the remaining glucose and galactose. Gluconeogenesis, the process of glucose synthesis from nonglucose precursors that normally occurs in the liver and kidneys (see Chapter 25), might also be active in the lactating mammary gland. Galactose can serve as a precursor for lactose synthesis if the lactating woman consumes a large amount of galactose (typically as lactose from dairy products; see Figs. 20.9 and 20.11).

α-Lactalbumin and lactose are both secreted into milk. Milk contains about 7 g of lactose/dL (i.e., about 70 times more carbohydrate on a weight basis than is found in blood plasma). Milk also contains about 1 to 2 g of protein/dL, of which about 0.2 to 0.3 g/dL is α-lactalbumin. Since lactose is the main osmolyte of milk, it determines milk volume.

SUMMARY

- Fructose is present in many sweeteners as well as fruits and vegetables. T e liver, kidneys, and intestinal mucosa degrade fructose to intermediates of glycolysis (or gluconeogenesis). In the liver, one intermediate, fructose 1-phosphate, activates glucokinase and thus serves as an indicator of the influx of carbohydrate from the diet.
- T e polyol pathway converts glucose to sorbitol and fructose, thereby consuming NADPH. In hyperglycemic patients, increased flux through this pathway is responsible for some of the damage to the lenses and small blood vessels in the retina, glomeruli, and peripheral nerves. In patients with classic galactosemia and a consequently elevated concentration of galactose in tissues, the production of galactitol by the polyol pathway gives rise to lens cataracts.
- Hereditary fructose intolerance (HFI) has an incidence of about 1 in 20,000. T e disease is due to a deficiency of aldolase B. Upon consumption of fructose, this leads to an accumulation of fructose 1-phosphate in the liver and the kidneys. T is, in turn, is accompanied by a severe drop in intracellular phosphate, as well as a drop in intracellular ATP, that impairs the function of the liver and kidneys. Af ected patients must exclude fructose-containing sweeteners, fruits, and many vegetables from their diet.
- Many patients have undiagnosed HFI, and carriers for the disease (about 1% of the population) also show a mild impairment of fructose metabolism. Most countries restrict the intravenous infusion of fructose or its precursor, sorbitol.
- Due to the extensive use of fructose-containing sweeteners, per capita fructose consumption in developed nations is several times higher than it used to be in antiquity. High fructose consumption is associated with hyperlipidemia and gout.
- Galactose is present predominantly in lactose in milk products. Most tissues degrade galactose to glucose 6-phosphate, which enters glycolysis or gluconeogenesis.
- Patients who have classical galactosemia are deficient in galactose 1-phosphate uridyltransferase. After the consumption of galactose, the deficiency leads to an accumulation of galactose 1-phosphate and a drop in intracellular phosphate and ATP. After consuming milk, af ected newborns vomit, and they develop hepatomegaly and Fanconi syndrome. Treatment involves the lifelong exclusion of most dietary galactose.

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Review Questions

1. Newborns who have galactosemia due to a galactose 1-phosphate uridyltransferase deficiency and who are breastfed during their first three weeks of life usually develop liver dysfunction, bleeding, hyperbilirubinemia (too much bilirubin in the blood), abnormalities of ion transport in the kidney tubules, diarrhea, and vomiting. T ey also tend to become septic. T ese pathologic events are most likely due to which of the following?

   A. Bacterial metabolism of lactose to various gases and acids
   B. Impaired ATP production in various tissues
   C. Impaired uptake of glucose and galactose in the intestine
   D. Protein malnutrition, which leads to decreased synthesis of clotting factors

2. Common to HFI and galactosemia is that consumption of the of ending sugar:

   A. is followed by metabolism of the sugar in liver, kidneys, muscle, heart and brain.
   B. leads to an increase in the concentration of HCO_3^- in blood plasma.
   C. lowers the concentration of phosphate inside hepatocytes.
   D. normally occurs during the first few days of life.

3. A patient with a deficiency of aldolase B is expected to suf er from nausea the most after consuming 20 g of which of the following carbohydrates?

   A. Amylopectin
   B. Galactose
   C. Glucose
   D. Lactose
   E. Sucrose

Chapter

21 Pentose Phosphate Pathway, Oxidative Stress, and Glucose 6-Phosphate Dehydrogenase Deficiency

SYNOPSIS

- The pentose phosphate pathway branches off glycolysis and can feed back into glycolysis, albeit at a different point of the pathway. All cells are capable of the pentose phosphate pathway.
- The pentose phosphate pathway provides 5-carbon sugars for biosyntheses and reducing power (in the form of reduced nicotinamide adenine dinucleotide phosphate [NADPH]) for repair processes and biosyntheses.
- Cells with mitochondria can also make NADPH via another pathway.
- Reactive oxygen species (ROS) damage DNA, lipids, and proteins. Antioxidants (e.g., vitamin E, vitamin C, and glutathione) react with ROS or play a role in repairing ROS-induced damage.
- Some of the patients whose ancestors are from areas in which malaria has been endemic have a decreased activity of glucose 6-phosphate dehydrogenase (G6PD); their pentose phosphate pathway can therefore produce NADPH only at a decreased rate. Reduced NADPH production impairs the repair of oxidative damage in red blood cells. As a result, red blood cells may lyse in the bloodstream. Hence, affected patients must avoid oxidizing drugs.

LEARNING OBJECTIVES

For mastery *of* this topic, you should be able to do the *f*ollowing:

- Describe the overall purpose of the pentose phosphate pathway as well as its reactants, products, and cellular location.
- Describe the role of reduced glutathione (GSH) in the body and the contribution of NADPH to its formation.
- Compare and contrast the production and removal of radicals, taking into account the roles of vitamin E, vitamin C, and glutathione.
- Describe an episode of drug-induced hemolytic anemia in a patient who has G6PD deficiency, and list key drugs that might be contraindicated in this patient.
- Select laboratory tests to diagnose G6PD deficiency.
- Predict the results of a complete blood count in a person who has a G6PD deficiency and just passed a hemolytic crisis.

1. STEPS OF THE PENTOSE PHOSPHATE PATHWAY

T e pentose phosphate pathway can be divided into an oxidative branch and a nonoxidative branch. T e oxidative branch produces NADPH. T e nonoxidative branch produces predominantly ribose 5-phosphate, which is used *f*or the synthesis o*f* nucleotides.

1.1. General Comments

T e pentose phosphate pathway is also called **pentose phosphate shunt**, **hexose monophosphate shunt**, or **6-phosphogluconate pathway**.

Fig. 21.1 shows a condensed version of the pentose phosphate pathway with its connections to glycolysis. Detailed versions of the two major branches of the pathway are shown in Figs. 21.3 and 21.4. T e oxidative branch can proceed in only one direction, whereas the nonoxidative branch can proceed in two directions.

T e pentose phosphate pathway is found in the cytosol of all cells, and it produces NADPH and ribose 5-phosphate. **NADPH** is used to maintain a reducing environment, synthesize fatty acids and steroids, and eliminate highly damaging oxidative radicals and peroxides. **Ribose 5-phosphate** is used in the de novo synthesis of purine and pyrimidine nucleotides as well as in the salvage of purine nucleotides (see Chapters 37 and 38).

Flux in the pentose phosphate pathway dif ers among tissues and varies over time. In red blood cells and brain, about 5% to 7% of all glucose 6-phosphate metabolism occurs via the pentose phosphate shunt. In the brain, the pentose phosphate pathway is about three times more active in patients who have a traumatic brain injury than in healthy persons. In the normal heart, the flux of glucose 6-phosphate through the pentose phosphate pathway is only about 1% of the flux through glycolysis.

1.2. Oxidative Branch

T e oxidative branch of the pentose phosphate pathway produces **NADPH**, the structure of which is shown in Fig. 21.2. T e oxidized form of NADPH is written as $NADP^+$ to indicate that a nitrogen at the business end of the molecule carries a positive charge, analogous to conventions about NAD^+ (see Fig. 19.3 and Section 1 of Chapter 19). NADPH dif ers from NADH in a phosphate group. Most enzymes distinguish between NADPH and NADH; in metabolism, these reducing agents function essentially independent of each other. In most cells, the ratio of NADPH/$NADP^+$ is about 100, whereas the ratio of NADH/NAD^+ is only about 0.01. T is makes NADPH a stronger reducing agent than NADH.

In the oxidative branch of the pentose phosphate pathway, **glucose 6-phosphate dehydrogenase** (**G6PD**) and 6-phosphogluconate dehydrogenase each give rise to NADPH (Fig. 21.3). T e reactions in the oxidative branch are

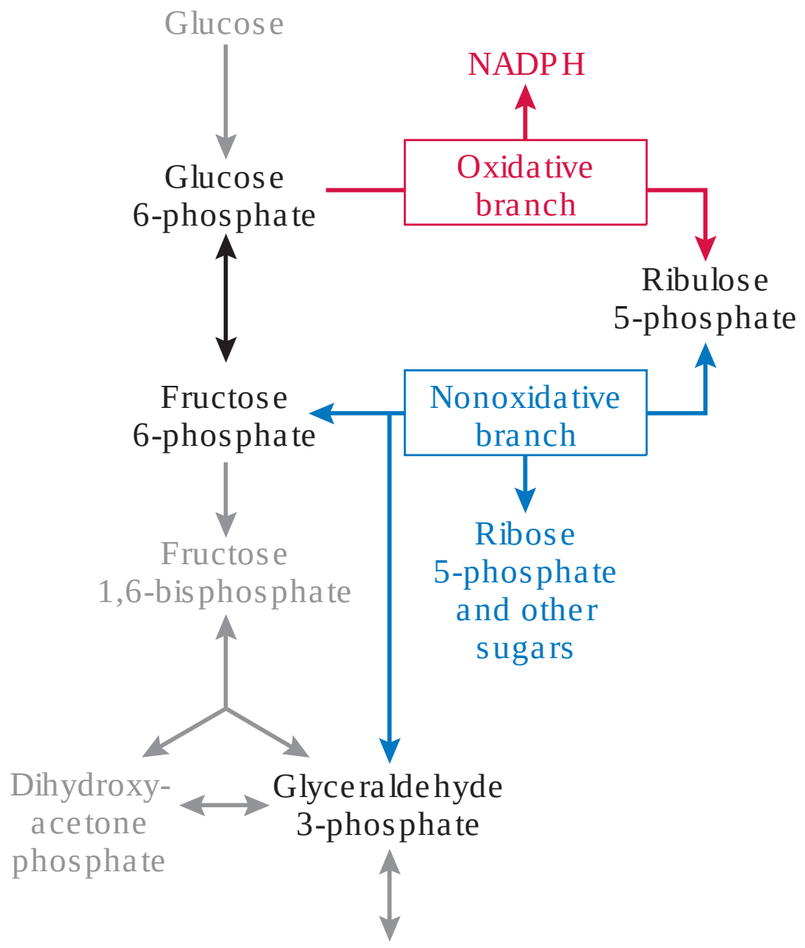


Fig. 21.1 The core elements of the pentose phosphate pathway and its interface with glycolysis.

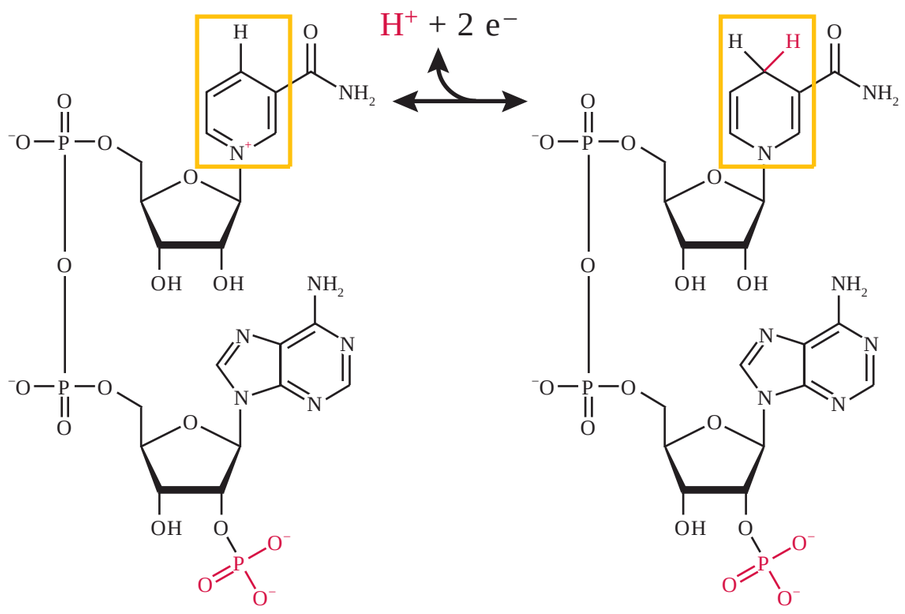


Fig. 21.2 NADP⁺ and its reduced form, NADPH. Compared with NADH and NAD⁺, NADPH and NADP⁺ have a phosphate group (bottom, red) in place of a hydroxyl group.

irreversible and start with glucose 6-phosphate from the glycolytic pathway. T e first enzyme, G6PD, is the rate-limiting enzyme. It is controlled by product inhibition from NADPH and by the depletion of the substrate $NADP^+$. In the liver, enzyme expression is also enhanced after a carbohydrate-rich meal (this helps with NADPH production when turning carbohydrates into fatty acids; see Chapter 27). T e second enzyme, 6-phosphogluconate dehydrogenase, gives rise to the sugar end product of the oxidative branch, ribulose 5-phosphate. T e activity of 6-phosphogluconate dehydrogenase is not regulated. T e net reaction of the oxidative branch is:

$$\text{Glucose 6-phosphate} + H_2O + 2\,NADP^+ \rightarrow \text{ribulose 5-phosphate} + CO_2 + 2\,NADPH + 2\,H^+$$

As described in Section 3, a hereditary deficiency of G6PD can lead to hemolytic anemia.

1.3. Nonoxidative Branch

T e nonoxidative branch of the pentose phosphate pathway produces sugar phosphates, principally ribose 5-phosphate, which is used for the production of nucleotides. A simplified scheme is shown in Fig. 21.4. All reactions are reversible, and there is no regulation of the activity of the enzymes in this branch. Hence, the concentrations of sugar phosphates in the nonoxidative branch of the pentose phosphate pathway depend on the concentrations of fructose 6-phosphate and glyceraldehyde 3-phosphate in glycolysis.

T e net reaction for the conversion of ribulose 5-phosphate to intermediates of glycolysis via the pentose phosphate pathway is:

$$3\,\text{ribulose 5-phosphate} \rightarrow 2\,\text{fructose 6-phosphate} + \text{glyceraldehyde 3-phosphate}$$

Transketolase activity inside red blood cells is used to estimate the **thiamine** reserves in the body. Patients who have thiamine deficiency have abnormally low transketolase

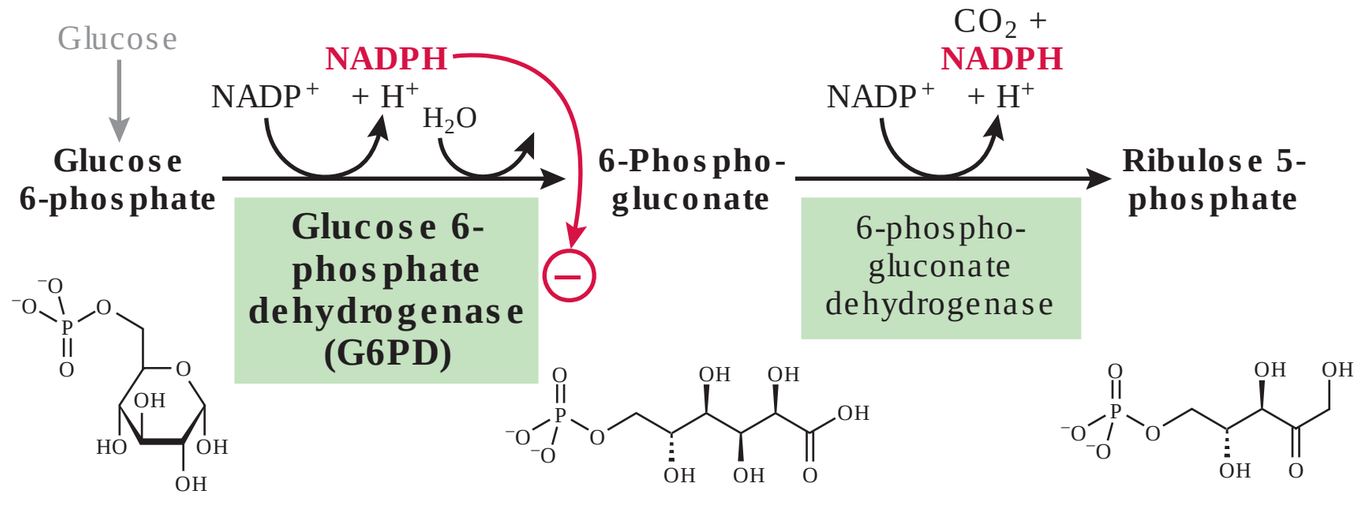


Fig. 21.3 The oxidative branch of the pentose phosphate pathway produces NADPH. When there is little need for the production of NADPH, G6PD activity is limited by a low concentration of $NADP^+$ and by product inhibition from NADPH.

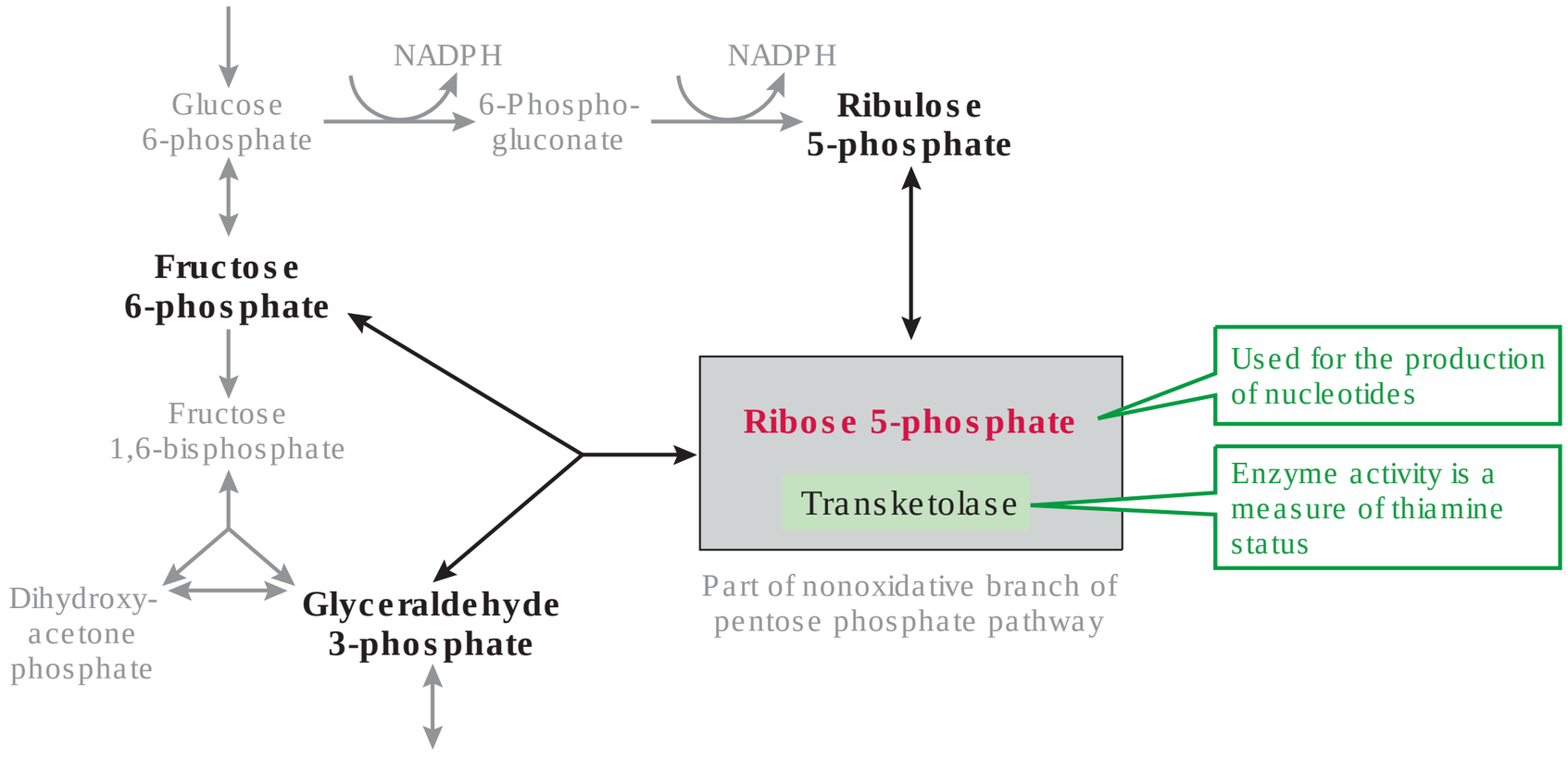


Fig. 21.4 The nonoxidative branch of the pentose phosphate pathway produces mainly ribose 5-phosphate. The reactions and stoichiometry of the nonoxidative branch are very complex and are thus depicted in a simplified version as a gray box and two connecting reactions. The nonoxidative branch includes several enzymes, including transketolase.

activity. However, low transketolase activity plays no role in the pathologic effects of thiamine deficiency. Thiamine deficiency is described in Section 5.2.2 of Chapter 22.

1.4. Independent Versus Joint Operation of the Branches

The oxidative and the nonoxidative branches of the pentose pathway can operate independently or jointly. The oxidative branch is unidirectional, and the nonoxidative pathway is bidirectional. When a cell needs only NADPH, it runs the oxidative branch and the nonoxidative branch from ribulose 5-phosphate to intermediates of glycolysis. When a cell needs only ribose 5-phosphate, it uses only the nonoxidative branch, starting with fructose 6-phosphate and glyceraldehyde 3-phosphate in glycolysis. When a cell needs both NADPH and ribose 5-phosphate, it uses the oxidative branch, followed by the conversion of ribulose 5-phosphate to ribose 5-phosphate via the nonoxidative branch.

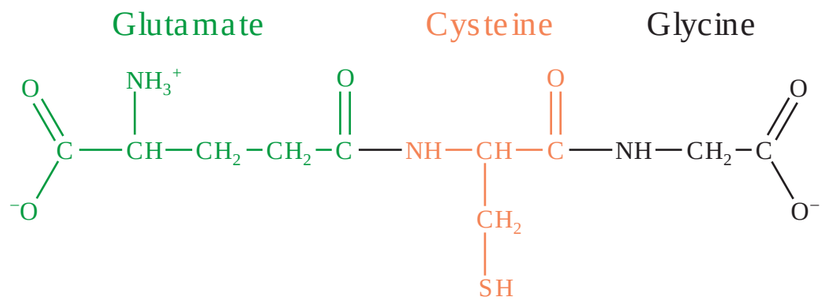
2. PROCESSES THAT USE NADPH INSIDE CELLS

NADPH is used in biosyntheses and the defense against ROS and radicals inside cells. NADPH, together with antioxidants (e.g., vitamins C and E) and enzymes, helps reduce the amount of free radicals and repair oxidative damage.

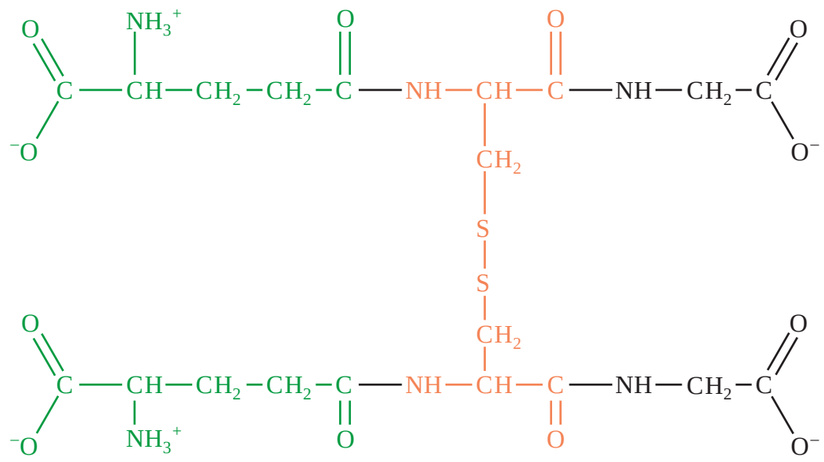
2.1. Use of NADPH in Biosynthetic Pathways

The liver, and to a lesser degree, the adipose tissue, uses NADPH to synthesize fatty acids from carbohydrates. Besides NADPH from the pentose phosphate pathway, the liver also uses NADPH produced by malate dehydrogenase (decarboxylating). This and the de novo synthesis of fatty acids are detailed in Chapter 27.

Cytochrome P450 enzymes use NADPH to produce steroids (see Chapter 31) or to detoxify various drugs and other xenobiotic compounds.



Glutathione (GSH, reduced glutathione)
γ-glutamyl-cysteinyl-glycine



Oxidized glutathione (GSSG, glutathione disulfide)
Bis(γ-glutamyl-cysteinyl-glycine) disulfide

Fig. 21.5 Reduced and oxidized forms of glutathione.

2.2. NADPH Reduces Oxidized Glutathione

Glutathione is a reducing agent inside cells. The structure of glutathione is shown in Fig. 21.5. Glutathione consists of the

tripeptide γGlu-Cys-Gly. Glutathione is synthesized by two enzymes (i.e., not by the mechanism of mRNA translation). T e side chain of the Cys is the business end of glutathione. Reduced glutathione is commonly written as **GSH** (the SH stands for the thiol group in the Cys side chain). Two molecules of GSH can become oxidized to the homodimer oxidized glutathione (**GSSG)** by forming a disulfide bridge between the Cys side chains of two GSH molecules (see Fig. 21.5).

Glutathione reductase uses NADPH to reduce GSSG to GSH. Inside healthy cells, the concentration of GSH far exceeds that of GSSG. T us, cells maintain large reservoirs of both NADPH and GSH.

2.3. Removal of ROS and Repair of ROS-Induced Damage

ROS are highly reactive oxygen-containing molecules, such as the **superoxide anion radical** ($\bullet O_2^-$), the **hydroxyl radical** (•OH), **organic peroxides** (ROOH), and **hydrogen peroxide** (HOOH, H_2O_2). T e extreme reactivity of radicals is due to the presence of an unpaired electron (denoted by the dot).

ROS-induced damage to DNA, proteins, and lipids contributes significantly to pathologic processes. Damage to DNA that is not properly repaired (see Chapter 2) can lead to genetic changes that result in **cancer**. Damage to proteins, particularly those with long half-lives, such as collagen, elastin, and crystallin (a protein of the eye lens), leads to changes associated with **aging**. Damage to lipids is a significant contributor to **atherosclerosis**.

Some ROS are formed intentionally and are important to the function of peroxisomes and the normal immune response, whereas other ROS are produced in undesired side reactions. About 1% of the oxygen consumed by the body is converted to ROS, mostly as an unintentional byproduct of **oxidative phosphorylation** (Fig. 21.6; see Chapter 23). Free **iron** considerably enhances the undesired formation of ROS via the Haber-Weiss and Fenton reactions (see Chapter 15). Neutrophils generate hypochlorous acid (HOCl) to kill pathogens.

Superoxide anions ($\bullet O_2^-$) derive chiefly from oxidative phosphorylation and cytochrome P450 enzymes (see Fig. 21.6). T e **superoxide anion** is a weak radical, but in the presence of iron it gives rise to highly reactive **hydroxyl radicals** (•OH). •OH is one of the most aggressive radicals; it dif uses only 5 to 10 times its diameter until it reacts. •OH can react with guanine in DNA (forming 8-oxo-guanine; see Section 1 in Chapter 2), hydroxylate phenylalanine and tyrosine in proteins, and give rise to lipid radicals. Oxidative damage to lipids leads to a self-propagating cycle of damage (Fig. 21.7).

Glutathione peroxidase, which removes H_2O_2 and lipid peroxides, contains **selenium** (Se) in **selenocysteine** that is part of the catalytic site (see Chapter 9). Adequate Se in the diet has a cancer-preventive ef ect, perhaps through reduced free radical damage to DNA.

We acquire **Se** mainly from cereals and animals. T e amount of Se in cereal foods depends on the amount of Se in the soil. For instance, wheat from the United States is high in Se, whereas wheat from unfortified soils in the United Kingdom and Finland is low in Se.

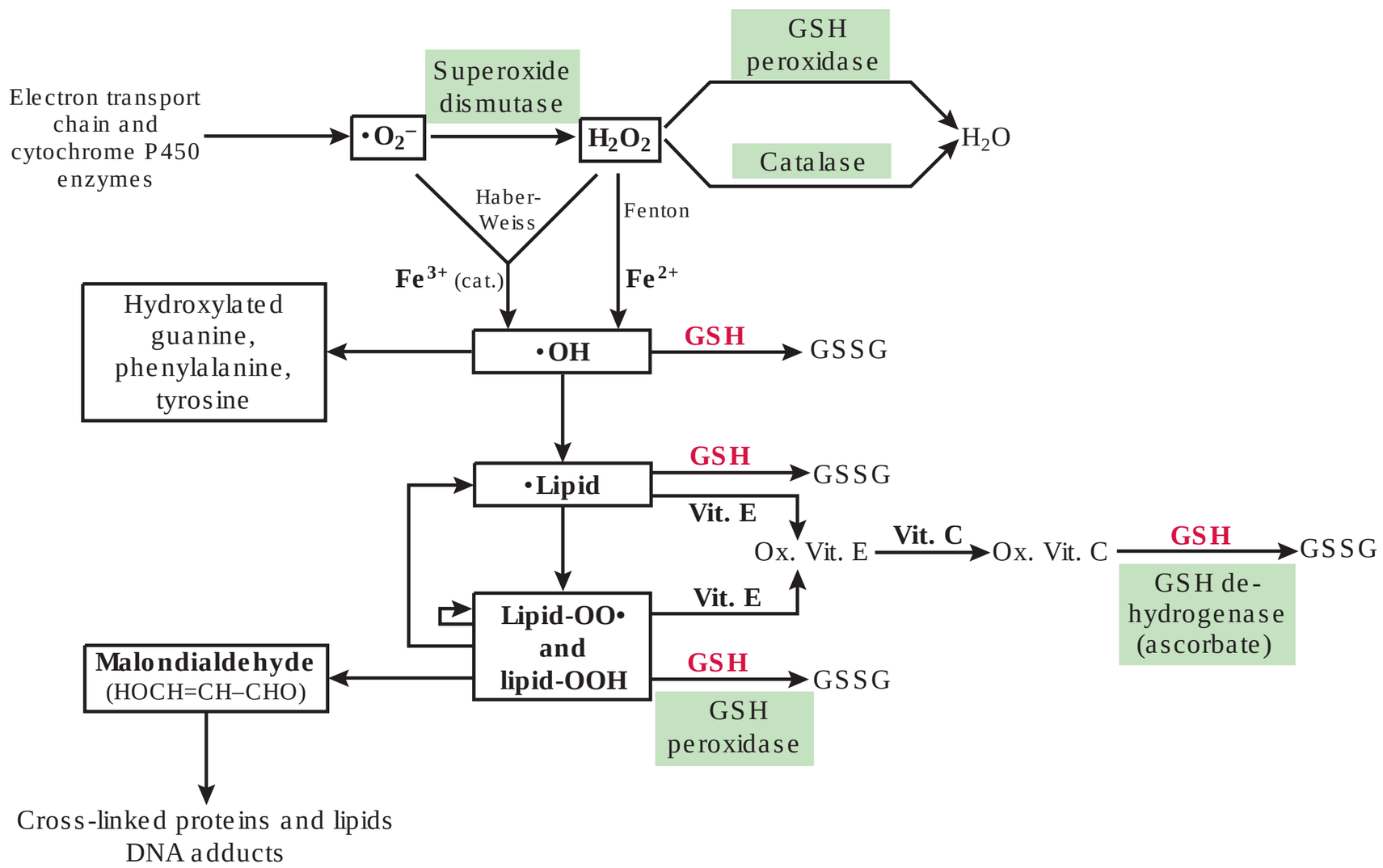


Fig. 21.6 Overview of reactive oxygen species, associated damage, and repair. Glutathione (GSH), vitamin E, and vitamin C are involved in removing radicals. Maintenance of an adequate concentration of GSH requires an adequate supply of NADPH from the pentose phosphate shunt.

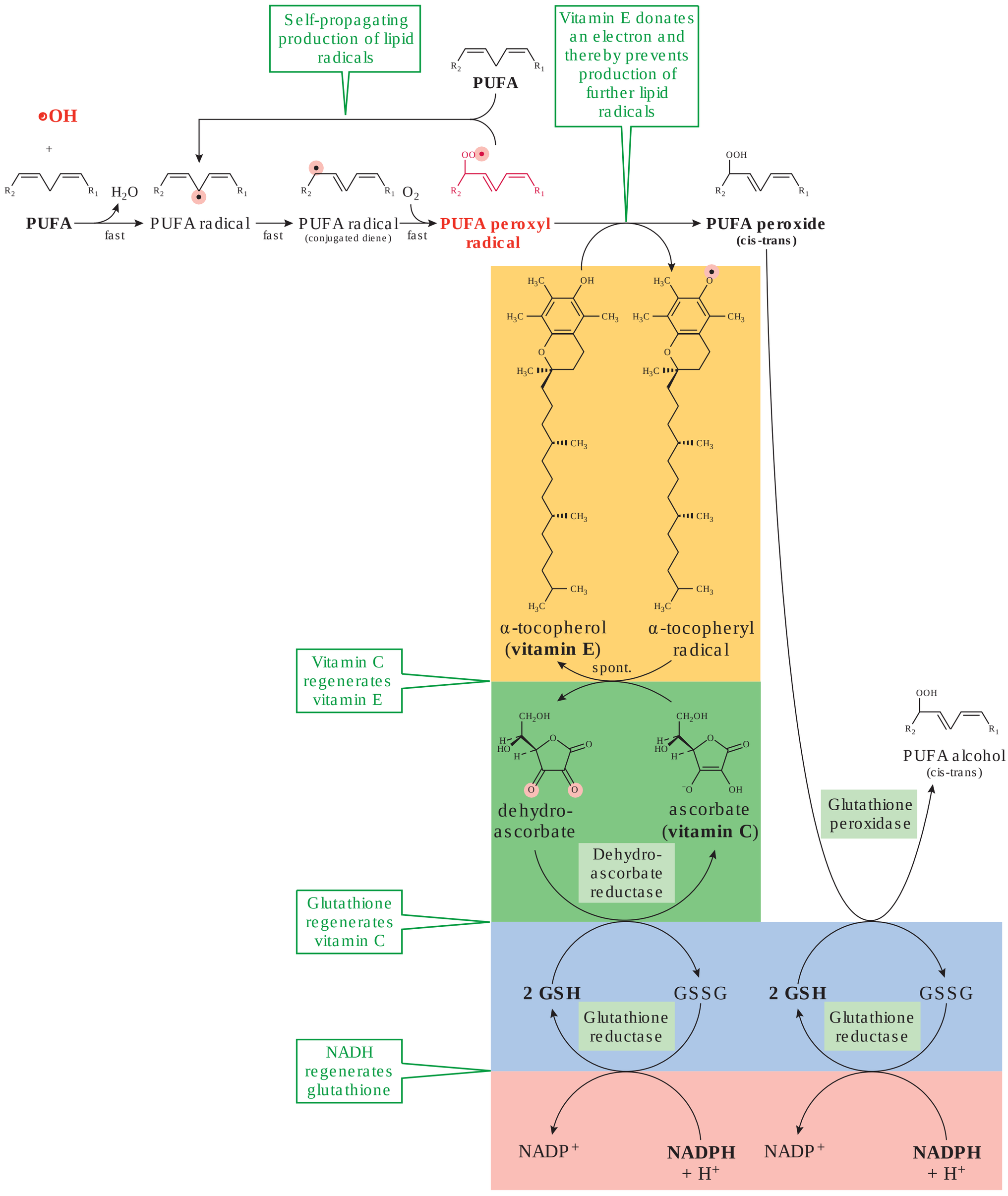


Fig. 21.7 Removal of lipid peroxyl radicals and lipid peroxides with vitamin E, vitamin C, glutathione, and NADPH. *PUFA*, polyunsaturated fatty acid (see Chapter 27).

When **SE deficiency** develops, glutathione peroxidase activity is first to dwindle. SE-deficient patients have decreased antioxidant defenses and increased susceptibility to infection, cancer, myopathy, and cardiomyopathy. Susceptibility to infection increases further with vitamin E deficiency.

The body's defense against oxidative damage rests in part on removing ROS (see Fig. 21.6). **Superoxide dismutase** converts $\bullet\mathbf{O_2^-}$ to H_2O_2 ($2\ \bullet O_2^- + 2\ H^+ \rightarrow H_2O_2 + O_2$), and both **glutathione peroxidase** and **catalase** remove $\mathbf{H_2O_2}$ ($2\ GSH + H_2O_2 \rightarrow GSSG + 2\ H_2O$; $2\ H_2O_2 \rightarrow 2\ H_2O + O_2$). Together with the maintenance of an environment of a low concentration of

free **iron**, this minimizes the production of •OH. •OH cannot be ef ectively removed by an enzyme because it reacts before it could dif use to an enzyme. •OH and the lipid radicals it gives rise to are removed to some degree by **glutathione**, which is ubiquitous. Glutathione also removes lipid radicals (2 GSH + 2 •lipid → GSSG + 2 lipid). Fig. 21.7 shows how the fat-soluble **vitamin E**, ascorbate, glutathione, and NADPH work together in a chain to react with various lipid radicals.

Degradation of cyclized lipid peroxyl radicals yields **malondialdehyde**, which in turn **crosslinks proteins** and/or **lipids** (see Fig. 21.6). In clinical research, the concentration of circulating malondialdehyde is sometimes used as a measure of oxidative damage.

T e terms **antioxidant** and **free radical scavengers** are used synonymously for compounds that react with oxidants by donating at least one H with its one electron. Antioxidants are frequently divided into fat-soluble and water-soluble compounds. T e major **fat-soluble antioxidants** in the human body are:

- **Vitamin E.**
- **Carotenes,** including **vitamin A, β-carotene** (in carrots, pumpkins), and **lycopene** (in tomatoes).
- **Coenzyme QH2 (ubiquinol)** is found in all membranes, reacts with ROS, and thereby protects unsaturated fatty acids in membranes. Ubiquinol is also part of the electron transport chain (see Chapter 23) in the inner mitochondrial membrane.
- **Dihydrolipoic acid** is active both in the cytosol and in membranes. Lipoic acid (see Fig. 22.5) is sometimes used in the treatment of painful diabetic neuropathy. Cells reduce exogenous lipoic acid to the antioxidant dihydrolipoic acid. T e R(+) enantiomer of lipoic acid also serves as a reversibly reduced prosthetic group of pyruvate dehydrogenase, α-ketoglutarate dehydrogenase, and branched-chain ketoacid dehydrogenase.
- **Bilirubin** is a degradation product of heme that circulates in blood as part of its transport from the spleen to the liver (see Chapter 14).

T e major **water-soluble** antioxidants include:

- **Ascorbate**
- **Glutathione**
- **Uric acid,** which preferentially reacts with peroxynitrite, the product of a reaction between superoxide anion and nitric oxide

Finally, the body's defense against oxidative damage also includes the repair of damage to DNA and proteins. DNA **base excision repair** repairs 8-oxo-guanine in DNA (see Section 1 in Chapter 2), which is the predominant DNA base damage inflicted by ROS (from •OH, reacting with guanine). T e concentration of 8-oxo-guanine can be used as a measure of a cell's stress. Damage to proteins from ROS and hypochlorous acid af ects mostly cysteine and methionine residues; this can lead to the formation of cysteine disulfides and methionine sulfoxide, for example. T ioredoxin- or glutaredoxin-dependent enzymes reduce disulfide bonds (–S–S–). Methionine sulfoxide reductase reduces methionine sulfoxide. Other types of damage often remain unrepaired and are dealt with by the ongoing degradation of proteins and concurrent synthesis of new proteins.

3. GLUCOSE 6-PHOSPHATE DEHYDROGENASE DEFICIENCY

A def ciency o*f* G6PD is a common X-linked disorder that is prevalent in populations originating *f*rom certain regions where malaria has been, or still is, endemic. It is marked by an increased incidence o*f* neonatal jaundice and then, throughout li*f*e, by susceptibility to hemolysis a*ft*er oxidative stress.

Deficiency of G6PD (see Section 1) is very common in populations originating from parts of the world where malaria has been or still is endemic (i.e., equatorial Africa, Middle East, Mediterranean, and Southeast Asia; see Fig. 17.1). T e reason for this finding is that G6PD-deficient persons are less likely to have severe malaria. About 5% to 25% of people from the above populations have one of the variant alleles with low G6PD activity. T e gene is located on the X chromosome; males are more frequently symptomatic than females. Low G6PD activity limits production of NADPH and maintenance of GSH (glutathione), thereby impairing the defense against oxidative stress (see Section 2). A total lack of the enzyme is lethal.

When G6PD-deficient erythrocytes experience excessive oxidative damage due to impaired defense against ROS, they hemolyze in the bloodstream (**intravascular hemolysis**) and give rise to **hematuria**. In unaf ected persons, only a small number of red blood cells normally lyse in the bloodstream, and most erythrocytes are instead cleared by the spleen and liver (see Chapter 14). In G6PD-deficient persons, the hemolysis is more extensive than normal and can lead to anemia and jaundice. Some patients with severe disease require a transfusion of fresh red blood cells. Often, **Heinz bodies** (aggregates of damaged globin) accumulate inside red blood cells of G6PD-deficient individuals who experience increased oxidative stress. Heinz bodies are detected by microscopic observation of a stained blood smear. T e hemolysis resolves once the oxidizing substance that triggered the attack is removed.

About 140 mutations in the G6PD gene are known, which explains in part the variation in patient response to oxidative stress. A variant called **G6PD A–** is common in people whose origin is in Africa. T e variant entails two point mutations that lead to the amino acid substitutions Val68Met and Asn126Asp. **G6PD Mediterranean** is common in people from the Mediterranean, the Middle East, and India; it entails a point mutation that leads to a Ser188Phe substitution. T e large majority of G6PD variants af ects the **stability** of the G6PD protein. T ese variants have minimal ef ect on cells that continuously synthesize new G6PD. However, the instability of G6PD

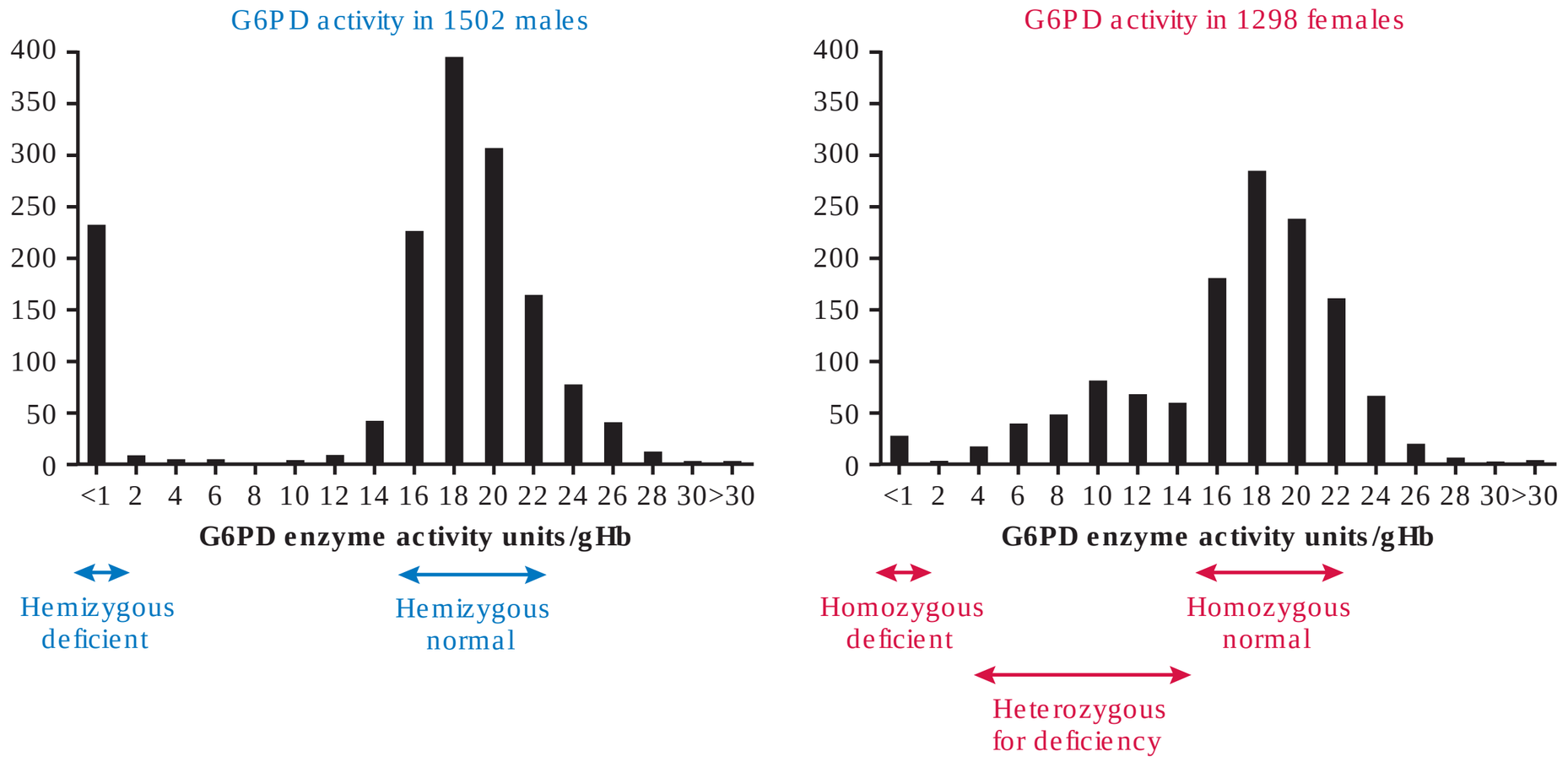


Fig. 21.8 G6PD activity in male and female newborns. G6PD activity was measured in newborns of Sephardic Jewish ethnicity, a group in which G6PD deficiency is common. Blue and red arrows indicate approximate ranges. (Modified from Algur N, Avraham I, Hammerman C, Kaplan M. Quantitative neonatal glucose-6-phosphate dehydrogenase screening: distribution, reference values, and classification by phenotype. *J Pediatr.* 2012;161:197-200.)

af ects red blood cells, which cannot synthesize new G6PD molecules (mature erythrocytes do not have DNA, RNA, or ribosomes). Severe G6PD deficiency can also impair the function of peripheral nerves, perhaps due to loss of G6PD activity during transport in axons.

Since **females** show patchy, random **X-inactivation**, heterozygous females show a broad range of approximately intermediate G6PD activity that eludes easy classification (Fig. 21.8).

G6PD-deficient newborns are at increased risk for **neonatal jaundice** (see Chapter 14) and its complications. Severe hyperbilirubinemia leads to brain damage. Many cases of severe neonatal hyperbilirubinemia are due to a G6PD deficiency. Hence, all newborns who are at risk of G6PD deficiency due to parental carrier status or ethnic background should have their bilirubin levels monitored closely.

Erythrocytes of G6PD-deficient individuals may hemolyze during an **infection**, such as during pneumonia, hepatitis B, or symptomatic infection with cytomegalovirus (a herpes virus).

After ingestion of **fava beans** (also called broad beans), erythrocytes of G6PD-deficient persons may hemolyze. Fava beans (from the plant Vicia *f*aba) contain vicine, which is an oxidizing agent. Hemolysis precipitated by fava beans has been known since antiquity and has been referred to as **favism**.

Oxidative drugs that predictably cause hemolysis in patients with at least mild G6PD deficiency are **primaquine** and **dapsone** (used as antimalarials), **methylene blue** (used to treat methemoglobinemia), **phenazopyridine** (used as an analgesic and antipyretic), and the H_2O_2-producing **uricases,** such as rasburicase and pegloticase (used mostly in the treatment of tumor lysis syndrome and in gout that cannot be treated with other drugs). Severe hemolysis can lead to acute kidney injury. In a setting of mild hemolysis, an oxidative drug is sometimes continued because the number of susceptible older erythrocytes abates with time. Hence, some oxidizing drugs are given only to at-risk patients who have been screened for a G6PD deficiency, whereas others are given without prescreening but are discontinued at the first sign of significant hemolysis.

Table 21.1 WHO Classification of G6PD Deficiency

Class	Hemolysis	G6PD Activity, % of Normal
I	Chronic	<10
II	Only with infection or drugs	<10
III	Only with infection or drugs	10–60

Information on drug sensitivity often refers to the World Health Organization's **classification** (Table 21.1).

Testing for G6PD deficiency involves measuring the activity of G6PD in red blood cells or assessing the presence of variant G6PD alleles. A semiquantitative **fluorescence spot test** measures NADPH fluorescence in red blood cells. It is positive for patients who have less than about 20% of the normal G6PD activity. A **quantitative test of G6PD activity** measures NADPH production by G6PD from NADP and glucose 6-phosphate, using a red blood cell hemolysate. G6PD activity is reported relative to the number of red blood cells (i.e., the red blood cell count) or the hemoglobin concentration in blood. T e presence of a significant amount of white blood cells can increase measured G6PD activity and thereby mask a G6PD deficiency in red blood cells. Lab results can

also be misinterpreted if there is an unusual fraction of red blood cells with good G6PD activity, such as after old red blood cells have undergone hemolysis, after transfusion of normal red blood cells, or after the entry of a wave of new reticulocytes into the blood. For this reason, the most reliable measurements of G6PD activity are made well after hemolytic crises and transfusions. **DNA-based methods** currently often detect only a limited number of frequently occurring mutations. DNA-based assays can be performed regardless of hemolytic crisis. DNA-based methods can also identify heterozygous females much more reliably than a quantitative test of G6PD activity would (see Fig. 21.8).

A very small proportion of G6PD-deficient individuals have a severe deficiency that causes **chronic nonspherocytic hemolytic anemia** and may eventually also give rise to damage to peripheral nerves.

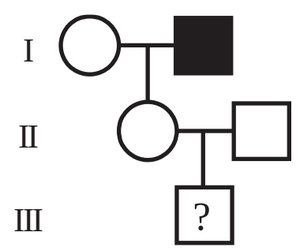
SUMMARY

- T e pentose phosphate pathway provides pentoses and/or reducing power in the form of NADPH.
- In erythrocytes, the pentose phosphate pathway is the only pathway that generates NADPH.
- In the presence of iron, superoxide anions and H_2O_2 give rise to highly reactive hydroxyl radicals that attack DNA, proteins, and lipids. Resulting lipid radicals can self-propagate and thus give rise to an avalanche of lipid radicals.
- Enzymes remove superoxide anions and H_2O_2. GSH reacts with hydroxyl radicals and thus removes them. Vitamin E, vitamin C, and reduced glutathione play a role in removing lipid and lipid peroxyl radicals. NADPH reduces oxidized glutathione.
- G6PD-deficient patients produce NADPH at a reduced rate and are prone to hemolytic attacks due to infections and/or oxidizing agents (e.g., drugs or fava beans).
- G6PD deficiency is X-linked. T e deficiency is most common among people whose ancestors are from areas in which malaria is or was endemic.

FURTHER READING

- Dahl J-U, Gray MJ, Jakob U. Protein quality control under oxidative stress conditions. J Mol Biol. 2015;427:1549-1563.
- Luzzatto L, Seneca E. G6PD deficiency: a classic example of pharmacogenetics with on-going clinical implications. Br J Haematol. 2014;164:469-480.

Review Questions

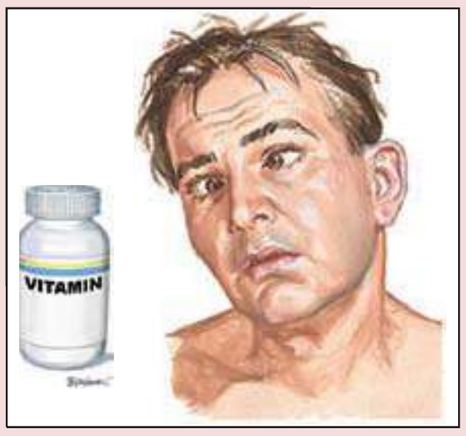


1. In the above pedigree of G6PD deficiency, I-2 has a moderate G6PD deficiency. I-1 and II-2 have no family history of G6PD deficiency, and G6PD deficiency is generally rare in people of their ethnic background. What is the likelihood that III-1 will have a G6PD deficiency?

   A. 0%
   B. 12.5%
   C. 25%
   D. 50%
   E. 100%

2. A 25-year-old man returning from a country with endemic malaria was given primaquine for prevention of malaria. Shortly after starting the drug, he presented with dark urine. Which of the following ef ects did primaquine have on his red blood cells?

   A. It damaged the cells by acting as a free radical scavenger or antioxidant.
   B. It exerted more oxidative stress on his red blood cells than the cells could handle.
   C. It inhibited G6PD and therefore led to an increase in the concentration of oxidized glutathione.
   D. It irreversibly reduced oxidized glutathione to GSH.
   E. It required more flux in the sugar phosphate branch of the pentose phosphate shunt pathway than the maximal flux that was available.



Chapter

22 Citric Acid Cycle and Thiamine Deficiency

SYNOPSIS

- The citric acid cycle occurs in the mitochondria. It chiefly oxidizes acetyl-coenzyme A (acetyl-CoA) to CO_2, thereby generating reduced nicotinamide adenine dinucleotide (NADH), reduced flavin adenine dinucleotide ($FADH_2$), and guanosine triphosphate (GTP) (Fig. 22.1). Acetyl-CoA is derived from either the oxidative decarboxylation of pyruvate or the degradation of fatty acids or ketone bodies. Other compounds that enter the citric acid cycle are derived primarily from amino acids (see Chapter 25).
- Intermediates of the citric acid cycle serve as precursors for gluconeogenesis and the biosynthesis of fatty acids, heme, and various amino acids (see Chapters 14, 25, 27, 34, and 35).
- Pyruvate not only generates acetyl-CoA for the citric acid cycle, it also gives rise to oxaloacetate, which helps maintain the concentration of all other intermediates of the citric acid cycle.
- NADH and $FADH_2$ generated in the citric acid cycle are fed into oxidative phosphorylation for the production of adenosine triphosphate (ATP; see Chapter 23). When the use of NADH by oxidative phosphorylation slows, the citric acid cycle also slows, because an elevated concentration of NADH inhibits the citric acid cycle.
- The enzymes and reactions that link pyruvate to the citric acid cycle, and some of the enzymes and reactions of the citric acid cycle itself, require vitamins. Deficiencies of thiamine, riboflavin, niacin, and biotin are clinically important.
- A portion of gliomas (brain tumors), acute myelogenous leukemias, tumors in the cavity or on the surface of bone, and melanomas contain a mutant isocitrate dehydrogenase that generates an abnormal, tumorigenic metabolite.
- Patients who are heterozygous for a deficiency of succinate dehydrogenase or fumarase (two enzymes of the citric acid cycle) are at an increased risk of developing certain tumors, such as pheochromocytomas, paragangliomas, renal cell carcinoma, and fibroids.

LEARNING OBJECTIVES

For mastery of this topic, you should be able to do the following:

- Describe the overall purpose of the pyruvate dehydrogenase (PDH) complex as well as its reactants and products, cellular location, and tissue distribution.
- Describe the regulation of PDH and the conditions that must be met for pyruvate to be oxidized to acetyl-CoA and for acetyl-CoA to be fed into the citric acid cycle.
- Describe the overall purpose of the citric acid cycle as well as its reactants and products, cellular location, and tissue distribution.
- Explain how metabolite concentrations in the citric acid cycle are maintained.
- Describe in broad strokes how reducing equivalents produced in the oxidative decarboxylation of pyruvate and in the citric acid cycle give rise to ATP.
- Describe the regulation of flux through the citric acid cycle vis-à-vis the flux through glycolysis, the electron transport chain, and oxidative phosphorylation.
- Describe the clinical result of severe thiamine deficiency, and connect the symptoms to the biochemical role of thiamine in the PDH complex and the citric acid cycle.
- Explain the rationale for providing thiamine along with glucose to patients who have hypoglycemia and/or alcohol intoxication.
- Describe the role of biotin in mitochondrial metabolism.
- Explain what a biotinidase deficiency is and how it is treated.
- Provide a credible scenario for tumorigenesis in cells that contain no or minimal activity of succinate dehydrogenase (SDH) or fumarase (i.e., fumarate hydratase [FH]). Do the same for cells that express a mutant isocitrate dehydrogenase (IDH) that produces 2-hydroxo-glutarate.

1. MITOCHONDRIA CONVERT PYRUVATE TO ACETYL-COA

Transport proteins move pyruvate into mitochondria. There, the PDH complex (a large multienzyme complex) converts pyruvate to acetyl-CoA. The conversion of pyruvate to acetyl-CoA requires thiamine (vitamin B_1) and riboflavin (vitamin B_2).

Pyruvate stems from glycolysis, lactate, or alanine (see Chapters 19, 25, 34, and 35). Mitochondria take up pyruvate via a transporter in the inner mitochondrial membrane (the outer membrane of the mitochondria is permeable to small molecules such as pyruvate; Fig. 22.2).

The **PDH complex** oxidatively and irreversibly decarboxylates pyruvate and thereby forms acetyl-CoA (see Fig. 22.2). CoA is an abbreviation for **coenzyme A**, the structure of which is shown in Fig. 22.3. The synthesis of coenzyme A requires **pantothenic acid** (**vitamin B_5**). Pantothenic acid deficiency in humans is not well described.

The PDH complex is a huge complex of proteins. It consists of many copies of E1 (PDH), E2 (dihydrolipoyl acetyltransferase), and E3 (dihydrolipoyl dehydrogenase). The complex also contains an E3-binding protein (protein X, dihydrolipoyl dehydrogenase-binding protein) that binds to both E2 and E3, and two types of regulatory enzymes, PDH kinase, and PDH phosphatase.

The E1, E2, and E3 subunits of the PDH complex contain prosthetic groups. Thus, the E1 subunits contain **thiamine pyrophosphate**, which is derived from **thiamine** (**vitamin B_1**; Fig. 22.4). We consume thiamine mainly with cereals, and the consequences of its deficiency are well known and serious (see Section 5). The E2 subunits contain **lipoic acid**. Lipoic acid is

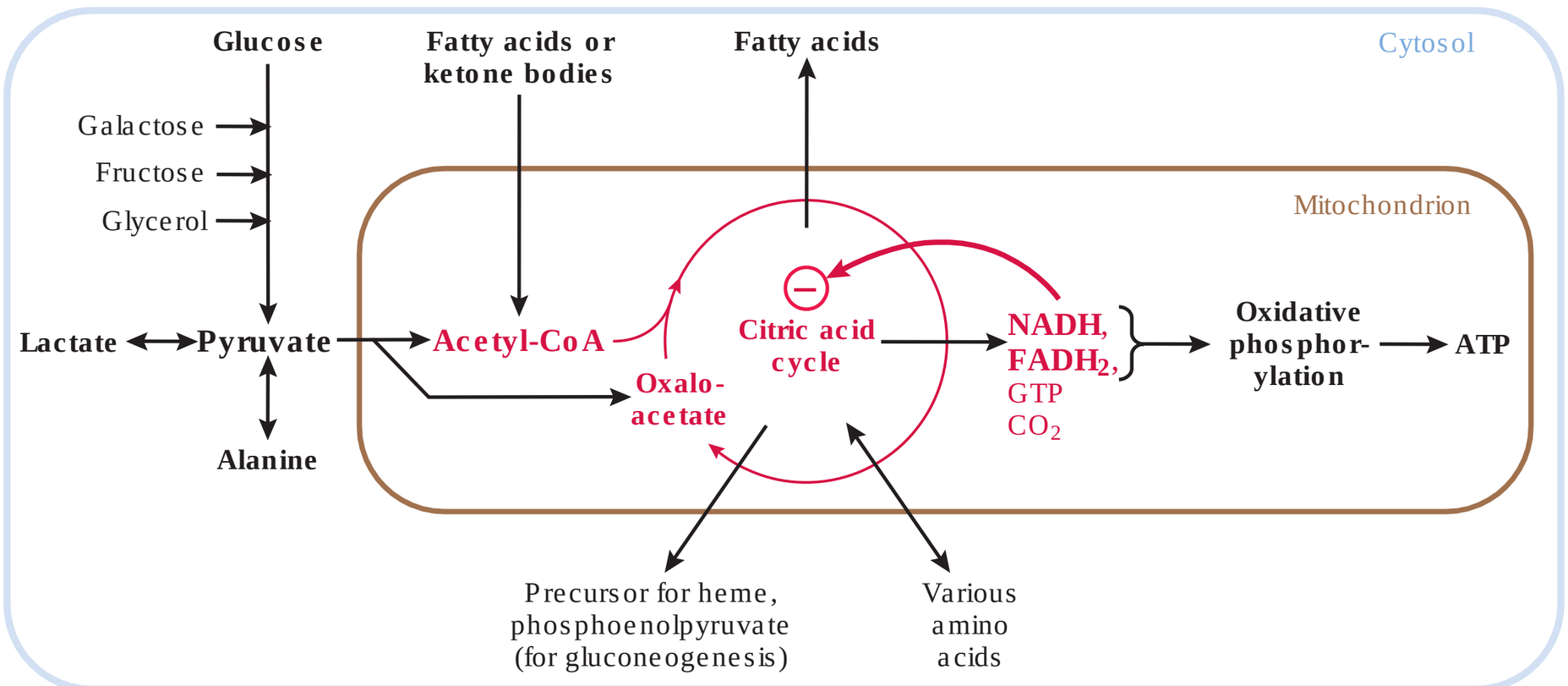


Fig. 22.1 Overview of the metabolites that feed into the citric acid cycle or are derived from it.

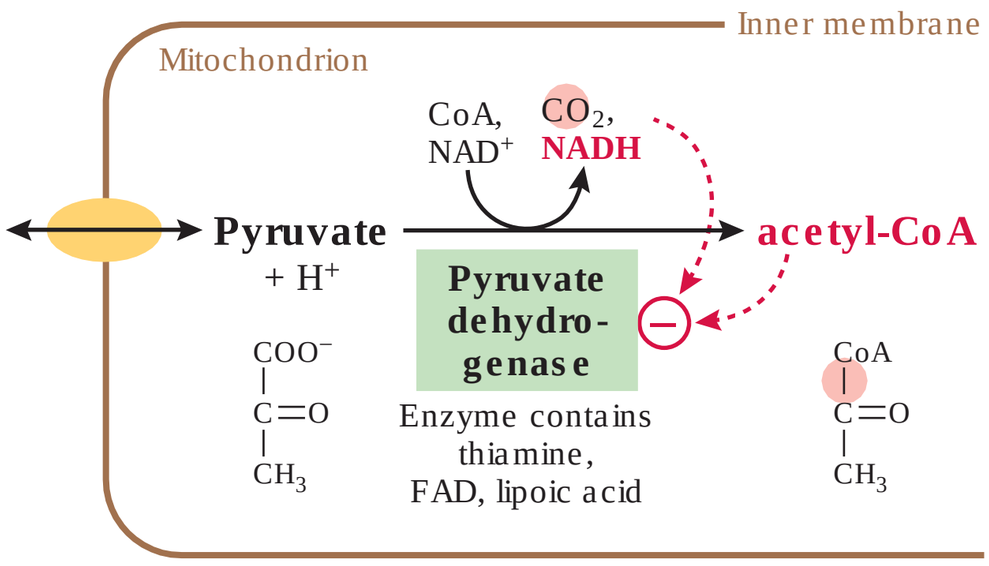
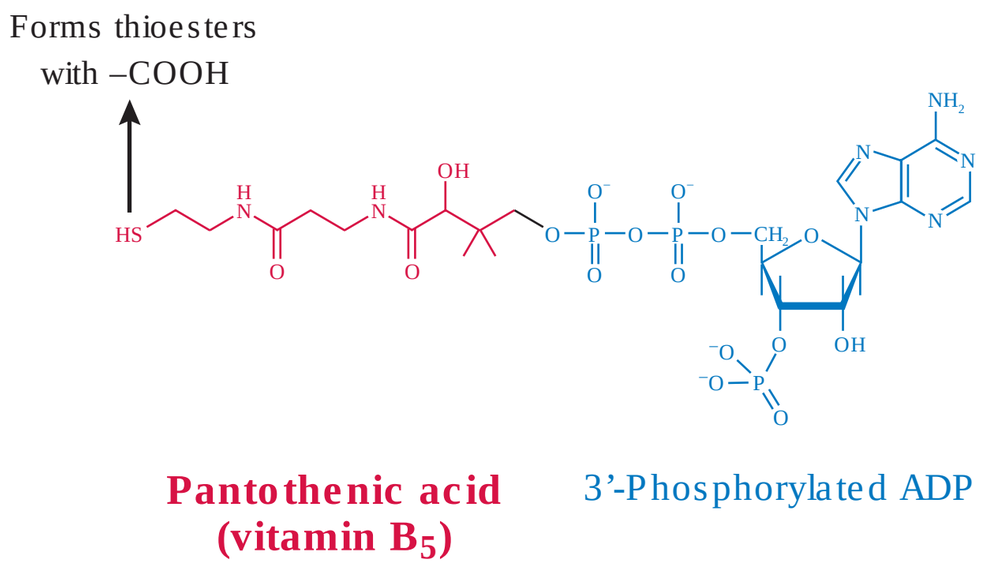


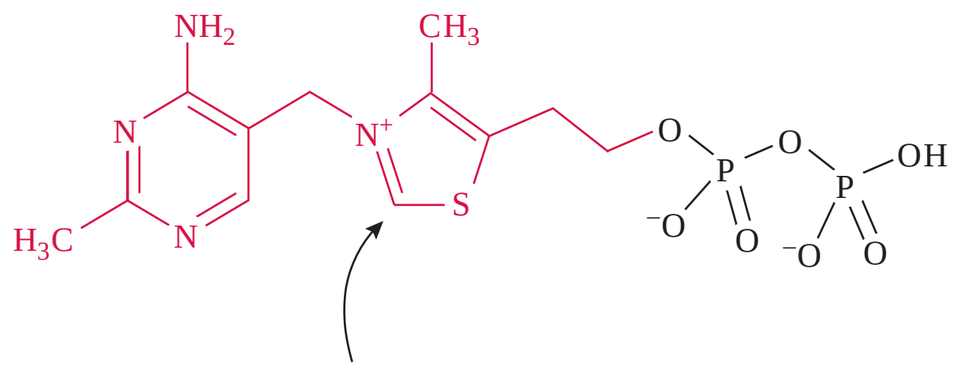
Fig. 22.2 Uptake of pyruvate into mitochondria and oxidative decarboxylation of pyruvate to acetyl-coenzyme A (CoA). The structure of CoA is shown in Fig. 22.3. FAD, flavin adenine dinucleotide.



Pantothenic acid (vitamin B_5)

Fig. 22.3 Structure of coenzyme A. Coenzyme A, a thiol, forms thioesters with carboxylic acids such as acetate, propionate, acetoacetic acid (a ketone body), and fatty acids; thus, coenzyme A "carries" acyl groups (i.e., it carries R–CO–). Coenzyme A also "activates" acyl groups because these thioesters release a large amount of energy when they are hydrolyzed, resulting in an energetically favorable transfer of the acyl group to a new acceptor.

Thiamine pyrophosphate



Reacts with C of keto-group of R-CO-COO⁻, then facilitates removal of -COO⁻ to liberate CO_2

Lipoic acid

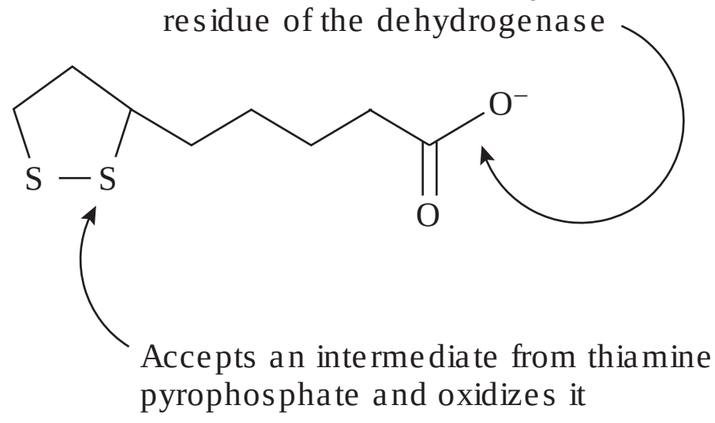


Fig. 22.4 Structures of thiamine pyrophosphate and lipoic acid. Thiamine pyrophosphate is produced from thiamine (vitamin B_1) with the help of thiamine pyrophosphate synthetase and ATP. Both thiamine pyrophosphate and lipoic acid are tightly bound to the pyruvate dehydrogenase complex, the α-ketoglutarate dehydrogenase complex (see Section 2), and the branched-chain ketoacid dehydrogenase complex (see Chapter 35).

not a vitamin. Lipoic acid accepts and oxidizes an intermediate from thiamine pyrophosphate, whereby lipoic acid is reduced to dihydrolipoic acid. **Arsenite** (O=As–O–), a potent poison, prevents lipoic acid from participating in this reaction. The E3 subunits contain bound flavin adenine dinucleotide (**FAD**; Fig. 22.5), which helps oxidize dihydrolipoic acid back to lipoic acid. FAD is derived from **riboflavin (vitamin B_2)**. Riboflavin is mainly found in milk, dairy products, meat, fish, and dark-green vegetables. Riboflavin deficiency is described in Section 5.

FAD (see Fig. 22.5) is typically covalently bound to an enzyme, where it often plays a role in forming C=C double bonds and disulfide bonds. Thus, FAD is also required for the activity of succinate dehydrogenase in the citric acid cycle and the electron transport chain of oxidative phosphorylation (see Chapter 23); glycerol phosphate dehydrogenase in the glycerol phosphate shuttle (see Chapter 19); acyl-CoA dehydrogenases in fatty acid β-oxidation both in mitochondria and peroxisomes (see Chapter 27); and in methylenetetrahydrofolate reductase in one-carbon metabolism (see Chapter 36).

Flavin mononucleotide (FMN), a moiety of FAD, is a prosthetic group of the NADH dehydrogenase complex in the electron transport chain of oxidative phosphorylation (see Chapter 23), and of NADPH-cytochrome P450 reductase, which plays a role in steroid synthesis.

The regulation of the activity of the PDH complex is described in Section 4.

In patients who have **primary biliary cirrhosis** due to an autoimmune disease, the **E2** component of the **pyruvate dehydrogenase complex** (**PDC** or **PDC-E2**) is the dominant antigen. Primary biliary cirrhosis primarily affects middle-aged women, and the measurement of autoantibodies against the E2 component of PDC is often part of the diagnosis. In this disease, the epithelial cells that line the small bile ducts inside the liver are selectively destroyed. Eventually, this leads to fibrosis and secondary liver damage.

α-Ketoglutarate dehydrogenase (an enzyme of the citric acid cycle, see Section 2) and **branched-chain ketoacid dehydrogenase** (see Chapter 35) resemble the PDH complex in that they too require coenzyme A, thiamine pyrophosphate, lipoic acid, and FAD.

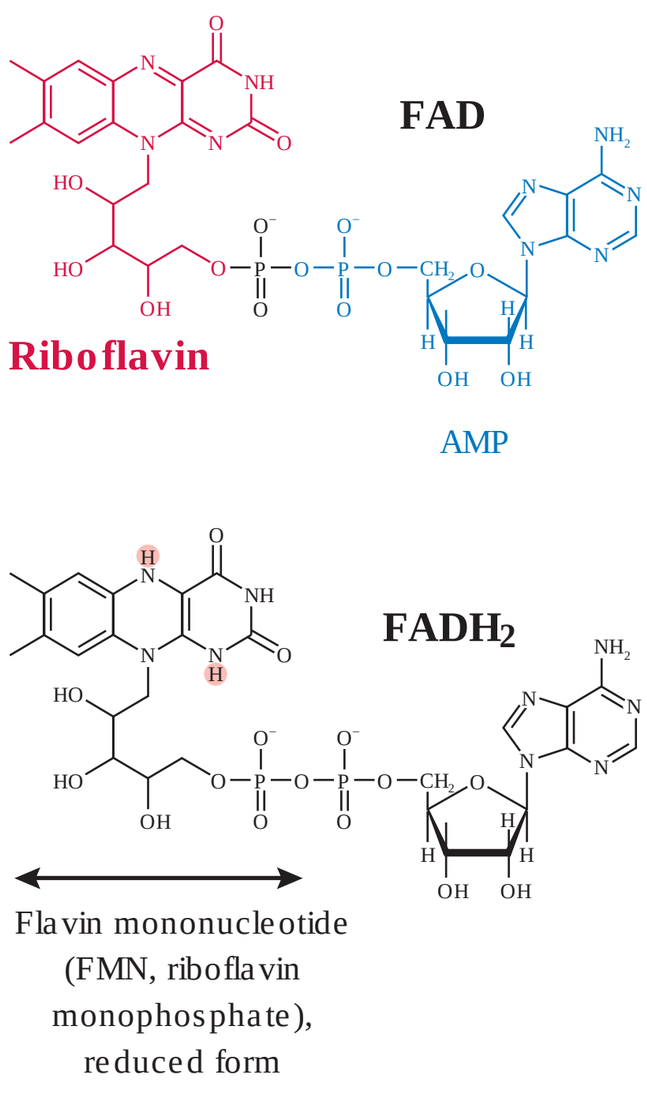


Fig. 22.5 Structure of flavin adenine dinucleotide (FAD) and related compounds.

2. REACTIONS OF THE CITRIC ACID CYCLE

The citric acid cycle can oxidize acetyl-CoA to CO_2 and thereby produce GTP, NADH, and $FADH_2$. Enzymes of the citric acid cycle use derivatives of the vitamins thiamine, niacin, riboflavin, and pantothenic acid.

The citric acid cycle is also referred to as the **tricarboxylic acid cycle** (TCA cycle) or **Krebs cycle** (named after Dr. Hans Krebs, who realized the cyclic nature of the pathway).

The citric acid cycle takes place inside **mitochondria**.

The citric acid cycle can oxidize the acetyl group of acetyl-CoA to CO_2. Depending on the tissue and the feeding/fasting state, acetyl-CoA stems from pyruvate, fatty acids, ketone bodies, or ketogenic amino acids (see Chapters 19, 27, and 35). During the oxidation of the acetyl group, the citric acid cycle produces GTP, which can phosphorylate ADP to ATP. Furthermore, the citric acid cycle produces NADH and $FADH_2$, which can transfer their reducing power to the pathway of **oxidative phosphorylation** for the production of additional ATP (see Chapter 23). Fig. 22.6 shows the reactions of the citric acid cycle in detail.

Several pathways feed the citric acid cycle with intermediates, while others drain intermediates from it. This is the topic of Section 3.

Flux through the citric acid cycle depends substantially on flux through oxidative phosphorylation. This is the topic of Section 4.

The citric acid cycle requires the same vitamins as the PDH complex. NAD^+ and NADH derive from **niacin** (see Chapter 19). The **α-ketoglutarate dehydrogenase complex** in the citric acid cycle resembles the PDH complex and thus requires **pantothenic acid**, **thiamine**, and **riboflavin** as precursors of prosthetic groups (see Figs. 22.3 to 22.5). Like the α-ketoglutarate dehydrogenase complex, succinate dehydrogenase has a covalently bound prosthetic group of FAD, which is derived from riboflavin. Deficiencies of these vitamins are discussed in Section 5.

3. OXALOACETATE HELPS REPLENISH CITRIC ACID CYCLE INTERMEDIATES

Intermediates of the citric acid cycle are used for gluconeogenesis and the biosynthesis of fatty acids, amino acids, and heme. To this end, carboxylation of pyruvate supplies the citric acid cycle with oxaloacetate.

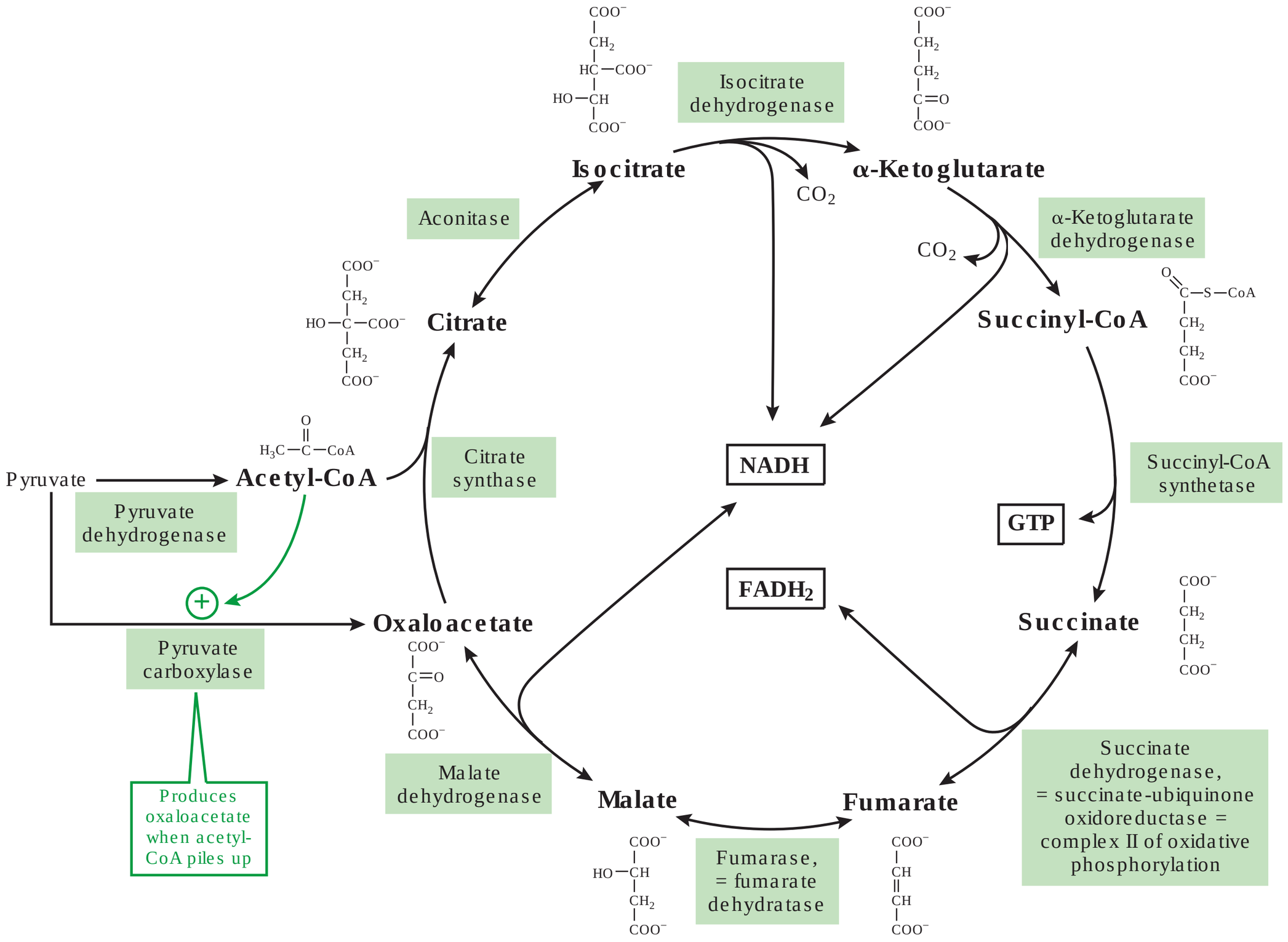


Fig. 22.6 **Reactions of the citric acid cycle.** NADH is free to diffuse through the matrix of the mitochondria. In contrast, $FADH_2$ is covalently bound to succinate dehydrogenase.

While the full citric acid cycle is used for energy generation, parts of it also play a role in other metabolic pathways. As shown in Fig. 22.7, intermediates of the citric acid cycle serve as a starting point for the synthesis of fatty acids (see Chapter 27), amino acids (see Chapter 34), heme (see Chapter 14), or glucose (see Chapter 25). Conversely, the degradation of some amino acids yields intermediates of the citric acid cycle (α-ketoglutarate, succinyl-CoA, fumarate, and oxaloacetate).

Pyruvate carboxylase converts pyruvate to **oxaloacetate** and thereby replenishes the citric acid cycle (see Fig. 22.7). Such replenishment is called **anaplerosis**. A rising concentration of **acetyl-CoA** increases the activity of pyruvate carboxylase, which then forms more oxaloacetate. Pyruvate carboxylase activity is not only important for sustained energy production by the citric acid cycle, but also for the synthesis of **cholesterol** and the elongation of **fatty acids** from exported citrate, for **gluconeogenesis** from exported oxaloacetate and malate, for cytosolic NADPH production from exported malate, and for nitrogen elimination by the **urea cycle**, which depends on the amination of oxaloacetate.

Pyruvate carboxylase requires the vitamin **biotin** as a cofactor (Fig. 22.8). Biotin is present in many dif erent foods. Intestinal microorganisms synthesize biotin as well, but it is unclear whether this becomes available to humans.

Biotin (Fig. 22.8) is covalently bound to a lysine residue near the active site of several dif erent carboxylases. T e reaction between biotin and the carboxylase is catalyzed by **holocarboxylase synthetase**. In addition to pyruvate carboxylase, other carboxylases that use biotin as a prosthetic group are **acetyl-CoA carboxylase** (the rate-limiting enzyme in fatty acid synthesis; see Chapter 27), **propionyl-CoA carboxylase** (involved in the degradation of branched-chain amino acids, methionine, fatty acids with an odd number of carbons, and propionic acid that bacteria in the intestine produce from undigested carbohydrate; see Chapter 36), and **3-methylcrotonyl-CoA carboxylase** (involved in the degradation of leucine; see Chapter 35).

When biotin-containing enzymes are degraded, biotin is recovered and recycled. Protein degradation yields **biocytin** (biotinyl-lysine). **Biotinidase** then cleaves biocytin into biotin and lysine; this enzyme also liberates biotin from short, biotin-containing peptides.

Biotin deficiency and its causes are described in Section 5.2.5.

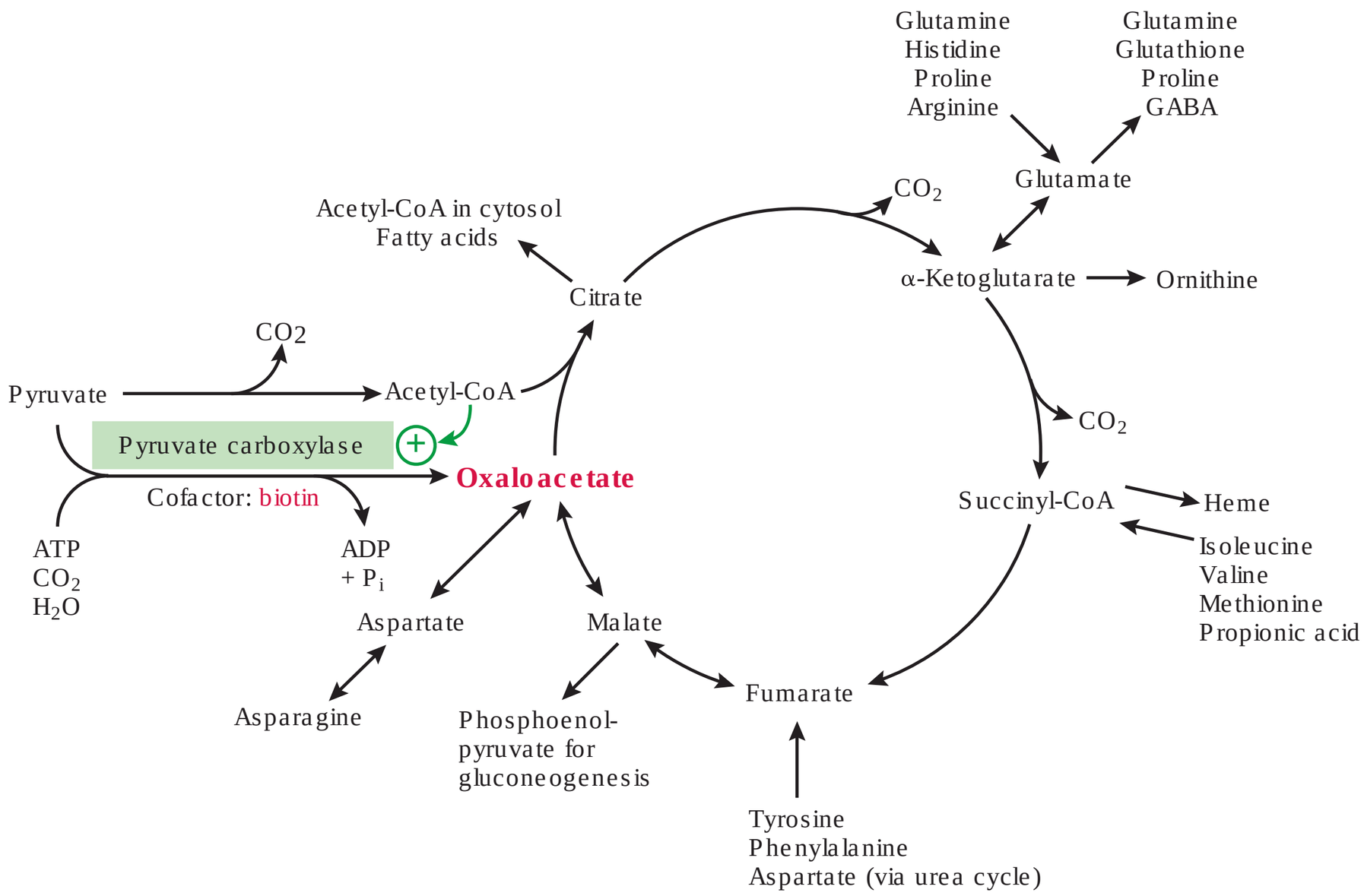


Fig. 22.7 Connections of citric acid cycle intermediates to metabolism, and the supply of oxaloacetate to the cycle. GABA (γ-aminobutyric acid) is a neurotransmitter.

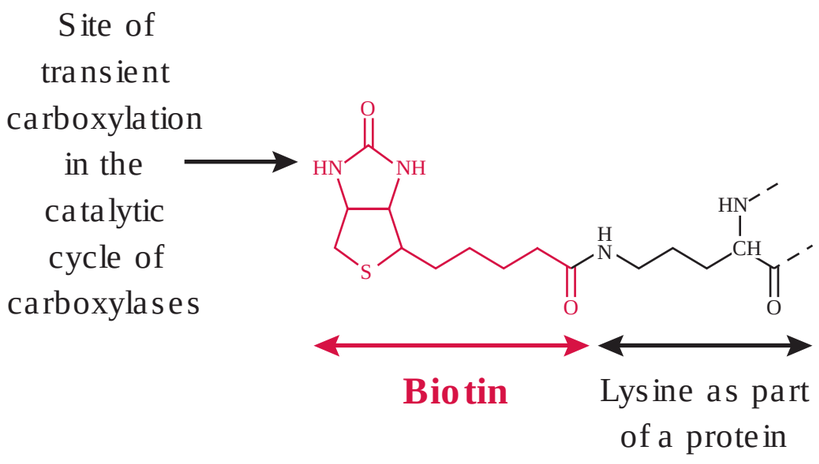


Fig. 22.8 Structure of biotin.

4. REGULATION OF THE CITRIC ACID CYCLE AND THE USE OF PYRUVATE

PDH activity determines the rate of pyruvate use for the production of acetyl-CoA. In muscle, the activity of PDH is highest during intense exercise, when acetyl-CoA is used for energy production. In the liver, the activity of PDH is highest in the fed state, when acetyl-CoA is used to produce citrate for fatty acid synthesis.

An elevated concentration of NADH inhibits PDH and the citric acid cycle. The concentration of NADH is highest when the rate of NADH consumption by oxidative phosphorylation is very low (i.e., when the cell uses little ATP). Hence, flux through the citric acid cycle normally depends on a cell's use of ATP. Flux through PDH depends not only on ATP consumption, but also on the consumption of intermediates of the citric acid cycle, and in some tissues on the concentrations of insulin and glucagon.

The citric acid cycle is almost exclusively regulated on a short-term basis. The amount of enzymes in the citric acid cycle does not appreciably change between the fed and the fasting state.

In untrained individuals, aerobic **exercise training** increases the volume of mitochondria in skeletal muscle, thus enabling cells to produce ATP at a higher rate.

The citric acid cycle is chiefly inhibited by an increasing concentration of **NADH** (Fig. 22.10). NADH product inhibits PDH (see Fig. 22.2), as well as several enzymes of the citric acid cycle.

The citric acid cycle depends on the rate of **oxidative phosphorylation**, which in turn depends on the rate of **ATP consumption**. As shown in Section 2, the citric acid cycle produces NADH and $FADH_2$. As detailed in Chapter 23, oxidative phosphorylation uses the reducing power of NADH and $FADH_2$ to drive the phosphorylation of ADP to ATP. In cells with mitochondria, this process generates the majority of ATP. When a cell hydrolyzes ATP at a high rate and therefore produces ADP at a high rate, oxidative phosphorylation uses a large amount of NADH and $FADH_2$, and the citric acid cycle is very active. On the other hand, when a cell uses little ATP and therefore produces little ADP, oxidative phosphorylation also uses little NADH and $FADH_2$, the concentration of NADH rises, and the citric acid cycle activity declines.

Acetyl-CoA, the main carbon input to the citric acid cycle, can be derived from pyruvate (which in turn derives from glucose, glycogen, lactate, or alanine; see Section 1), from fatty acid β-oxidation or, in nonhepatic tissues, from the oxidation of ketone bodies (see Chapter 27). In most cells, controls are

such that fatty acids and ketone bodies from the blood are preferred over pyruvate as a source of acetyl-CoA. In the fasting state, this is critical for preserving glucose because the body has large stores to produce fatty acids and ketone bodies but comparatively small stores from which it can produce glucose.

Acetyl-CoA product inhibits the PDH complex (Fig. 22.11). T is prevents the conversion of pyruvate to acetyl-CoA when acetyl-CoA is abundant. It also enables cells to produce acetyl-CoA preferentially from fatty acids and ketone bodies rather than pyruvate (see Chapter 27). T is ef ect allows pyruvate to be used for other purposes and thus helps prevent hypoglycemia in the fasting state (see Chapter 25).

Acetyl-CoA activates **pyruvate carboxylase** (see Fig. 22.9). Pyruvate carboxylase is important to the proper function of several metabolic pathways (see Section 3). T e concentration of acetyl-CoA determines how much pyruvate is converted to acetyl-CoA and how much to oxaloacetate.

Two intermediates of the citric acid cycle, **citrate,** and **succinyl-CoA,** inhibit the enzymes that generate them. T is inhibition prevents the buildup of high concentrations of intermediates in the citric acid cycle.

In the **liver**, acting via PDH phosphatases and a PDH kinase, **insulin**, **epinephrine**, and **ADP** all increase PDH activity. In the fed state, PDH tends to be high, while in the fasting state it tends to be low to preserve pyruvate for gluconeogenesis (see Chapter 25).

In contracting **skeletal muscle**, the elevated concentration of **Ca^{2+}**, acting via a PDH kinase and a PDH phosphatase, leads to increased PDH activity.

5. PROBLEMS ASSOCIATED WITH THE CITRIC ACID CYCLE

T e citric acid cycle plays a crucial role in energy generation by mitochondria. I*f* *fl*ux through the citric acid cycle is impaired, organs with high A*T*P needs (e.g., the central nervous system, the heart, and skeletal muscles) su*ff*er damage. Clinically, the most commonly encountered diseases that have a relationship to the citric acid cycle are hypoxia, thiamine def ciency, ribo*fl*avin def ciency, niacin def ciency, paragangliomas, and pheochromocytomas.

5.1. Inhibition of the Citric Acid Cycle Secondary to Impaired Oxidative Phosphorylation

An impairment of oxidative phosphorylation leads to a marked shift in energy production from the citric acid cycle plus oxidative phosphorylation toward anaerobic glycolysis. If a substantial amount of cells in the body switch to anaerobic glycolysis, production of lactic acid surpasses consumption, leading to lactic acidosis and then lactic acidemia (see Chapter 19). Inhibition of oxidative phosphorylation is seen in patients with acute or chronic **hypoxia**. Acute hypoxia may be due to stroke, heart attack, asphyxia, or drowning; chronic hypoxia may be due to cardiac, pulmonary, renal, or hemolytic disease. Oxidative phosphorylation can also be impaired by a poison, such as cyanide or uncouplers (see Chapter 23).

5.2. Clinically Significant Vitamin Deficiencies

5.2.1. Overview

Fig. 22.10 provides an overview of the vitamins that are required for reactions of the citric acid cycle, the PDH complex, and pyruvate carboxylase.

5.2.2. De*fi*ciency of Thiamine (Vitamin B_1)

T e most significant amounts of thiamine are found in whole grains, most vegetables, pork (not in beef, poultry, or fish), and milk. In developed nations, food supplementation is common. In North America and Europe, approximately half of the daily thiamine intake stems from the consumption of grains, and about a quarter each from the consumption of vegetables and animal products. T iamine uptake in the small

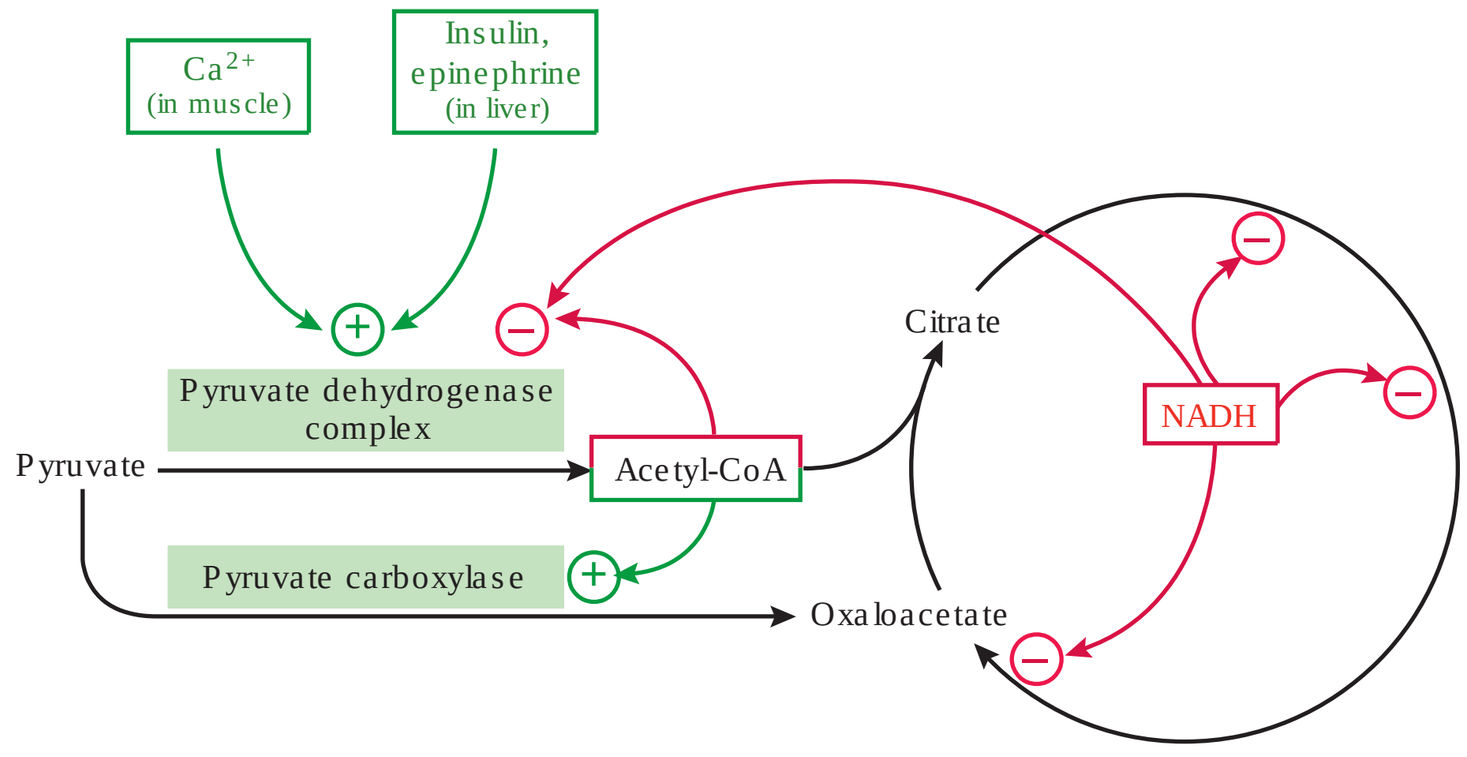


Fig. 22.9 Overview of the vitamins that are cofactors of pyruvate dehydrogenase, pyruvate carboxylase, or enzymes of the citric acid cycle.

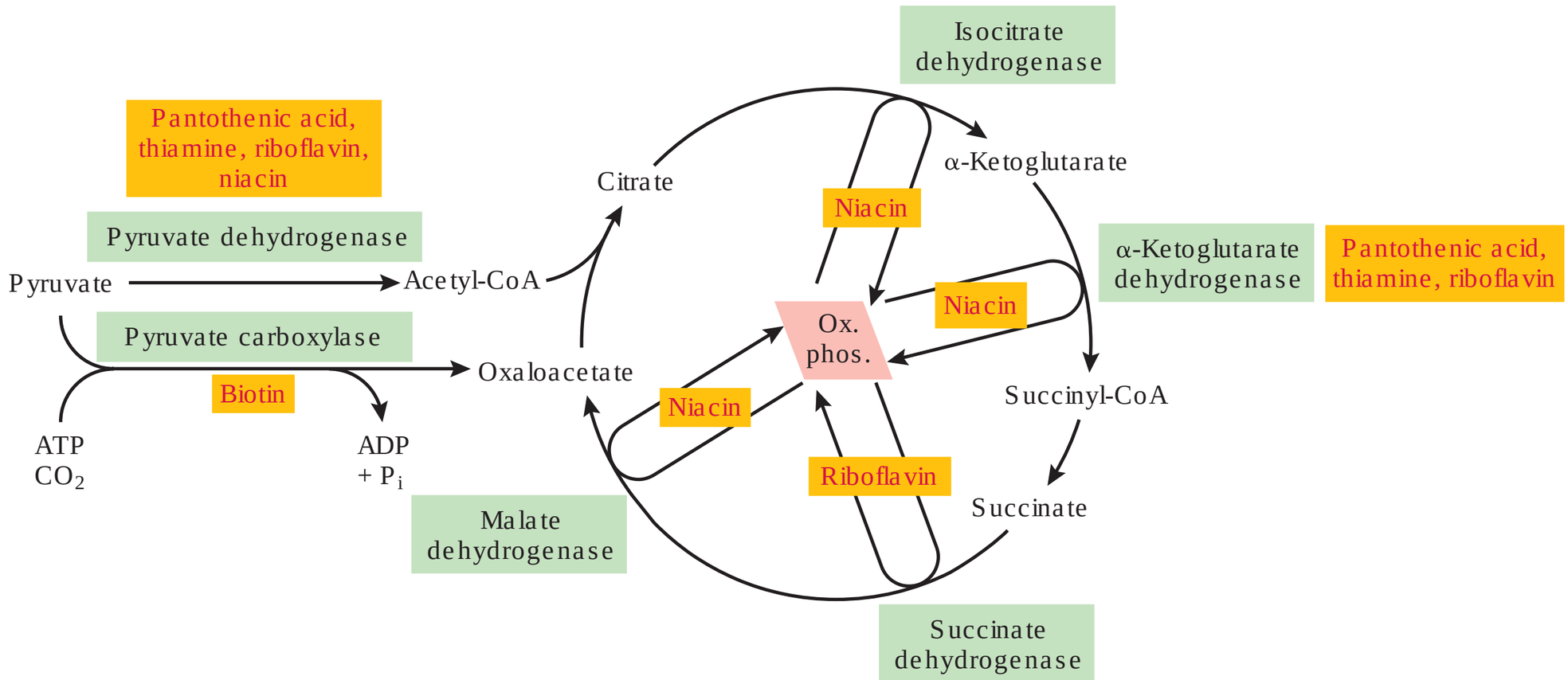


Fig. 22.10 Regulation of the activity of pyruvate dehydrogenase and the citric acid cycle. When oxidative phosphorylation does not use NADH, the citric acid cycle comes to a virtual standstill. Ox. phos., oxidative phosphorylation.

intestine is reduced in the presence of **alcohol**. It is also reduced in patients of advanced **age**. Furthermore, **diuretics** increase the loss of thiamine into the urine.

In the developed world, thiamine deficiency is most commonly seen in patients who regularly abuse **alcohol**. T e diet of these patients does not contain an adequate amount of thiamine, and thiamine absorption by the intestines is impaired. T e treatment of these patients is outlined below.

Patients who undergo frequent **hemodialysis** and patients who have advanced **cancer** are also susceptible to thiamine deficiency.

In **refugee** populations, thiamine deficiency due to inadequate dietary intake is quite common. Af ected people often consume mainly only milled white grains.

For the diagnosis of a thiamine deficiency, one commonly compares the activities of red blood cell **transketolase** (an enzyme that requires thiamine pyrophosphate; see Chapter 21) with and without extra thiamine pyrophosphate (added to a sample in the laboratory).

A chronic thiamine deficiency is usually accompanied by Wernicke cardiomyopathy, which is characterized by an enlarged heart, shortness of breath, and fatigue. In alcoholics, chronic thiamine deficiency typically also leads to **peripheral neuropathy** (see Fig. 22.11).

Wernicke encephalopathy (see Fig. 22.11) is typically provoked by administration of a large amount of carbohydrates to a thiamine-deficient person (i.e., a person who may already have peripheral neuropathy or Wernicke cardiomyopathy; see above). Wernicke encephalopathy is accompanied by petechial hemorrhage in the brain. If initially conscious, af ected patients become agitated, confused, and ataxic. Wernicke encephalopathy is a particularly well-known complication of infusing an **alcohol**-dependent (and therefore often thiamine-deficient) patient with glucose in the hospital. About 5% of the adults in North America and Australia regularly abuse alcohol.

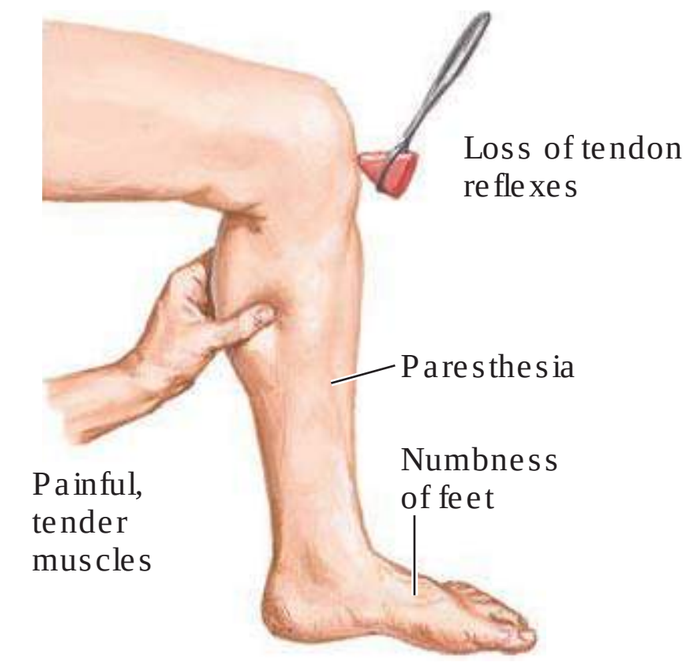


Fig. 22.11 Thiamine deficiency can lead to peripheral neuropathy and Wernicke encephalopathy.

Autopsies reveal that 20% or more of these individuals have had at least one episode of Wernicke encephalopathy.

Wernicke-Korsakof syndrome refers to a combination of Wernicke encephalopathy (initiated by thiamine deficiency) and **Korsakof psychosis.** T e psychosis is a result of the encephalopathy and becomes apparent while the patient

recovers. Korsakof psychosis is characterized by greatly impaired short-term memory. When patients in an **alcoholic coma** or with **alcohol withdrawal syndrome** are infused with glucose, they are routinely also given thiamine to prevent Wernicke-Korsakof syndrome.

Emergency personnel responding to comatose patients often administer a "**coma cocktail**" that contains glucose and thiamine (as well as naloxone).

5.2.3. Deficiency of Riboflavin (Vitamin B_2)

A deficiency of riboflavin is also called **ariboflavinosis**.

A deficiency of riboflavin, the precursor of FMN and FAD, is endemic in many regions of the world, particularly in people who have little access to milk, dairy products, and meats, which are especially rich in riboflavin. Accordingly, vegans and vegetarians are also at greater risk of ariboflavinosis than omnivores. Poor riboflavin status is pervasive among children and the elderly even in developed nations. Riboflavin is present in many foods, with almonds, green leafy vegetables, and some legumes containing more riboflavin than other plant foods. In some countries, white flour and cereals are fortified with riboflavin.

Frank riboflavin deficiency is accompanied by lesions of the mouth, lips, skin, and genitalia (Fig. 22.12).

The preferred method of assessing riboflavin status is to compare the activity of red blood cell **glutathione reductase** before and after the addition of extra FAD. This sensitive test shows abnormal readings within only a few days of dietary deprivation.

Since the endogenous production of niacin from tryptophan requires riboflavin, riboflavin deficiency can lead to a **niacin deficiency** (see Chapter 19).

5.2.4. Deficiency of Niacin (Vitamin B_3, Nicotinic Acid)

A deficiency of niacin is called **pellagra** and is described in Section 1 of Chapter 20. Since niacin is a precursor for NAD^+, patients with pellagra presumably have impaired flux through PDH, isocitrate dehydrogenase, and α-ketoglutarate dehydrogenase, which would curtail ATP production by the combination of citric acid cycle and oxidative phosphorylation. This is may contribute to the observed diarrhea, dermatitis, and dementia.

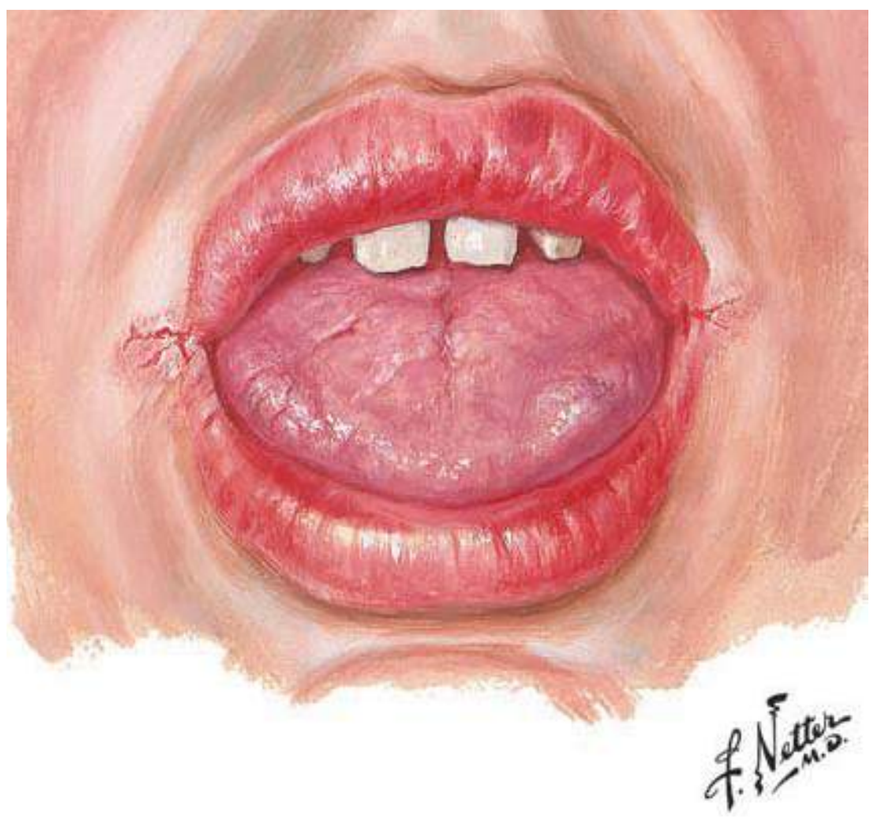


Fig. 22.12 **Ariboflavinosis.**

5.2.5. Deficiency of Biotin

As outlined in Section 3, biotin homeostasis requires both an adequate intake of biotin and adequate recycling of protein-bound biotin; thereby recycling is quantitatively more important. A **nutritional deficiency** of biotin is unusual. Excessive consumption of **raw eggs** can cause a biotin deficiency because egg white contains the protein **avidin**. Avidin binds biotin and prevents its uptake in the intestine. Avidin is largely destroyed when eggs are cooked.

Symptoms of a pronounced biotin deficiency are most often provoked by a severe deficiency of **holocarboxylase synthetase**, the enzyme that conjugates various carboxylases with biotin, or by a deficiency of **biotinidase**, the enzyme that recycles biotin (see Section 3). A severe deficiency of holocarboxylase synthetase manifests itself during the first few days to weeks of life with tachypnea, lethargy, and signs of abnormal central nervous system function. This deficiency affects about 1 in 90,000 persons. Biotinidase deficiency affects about 1 in 60,000 persons. A complete biotinidase deficiency causes symptoms at a few weeks of age or later. Biotinidase deficiency leads to ataxia, seizures, hearing loss, delayed development, and alopecia. Increased protein degradation (e.g., during an illness) exacerbates the biotin deficiency for reasons explained below.

A severe deficiency of biotin, whether caused by excessive consumption of raw eggs, holocarboxylase synthetase deficiency, or biotinidase deficiency, causes an **organic acidemia** and **hyperammonemia**. This acidemia is characterized by elevated concentrations of lactic acid, propionic acid, and ketone bodies (i.e., acetoacetic acid and β-hydroxybutyric acid). The pathogenesis may be explained as follows. The concentration of lactic acid is elevated because the biotin-dependent pyruvate carboxylase does not produce sufficient oxaloacetate; hence, losses of citric acid cycle intermediates are not made up, and acetyl-CoA cannot adequately enter the citric acid cycle. This has two effects: (1) the concentration of acetyl-CoA becomes elevated so that acetyl-CoA inhibits the PDH complex, and (2) the production of ATP by mitochondria decreases. These effects shift ATP production to anaerobic glycolysis in the cytosol, which produces lactate at a high rate (see Chapter 19). The concentration of propionic acid is high because the metabolism of this compound requires biotin (see Chapter 36). At an elevated concentration of propionyl-CoA (the activated form of propionic acid), citrate synthase condenses some oxaloacetate with propionyl-CoA instead of acetyl-CoA; this produces methyl citrate. Methyl citrate inhibits the citric acid cycle; again, this leads to an increase in anaerobic glycolysis. The high concentration of propionic acid also causes hyperammonemia. The increased

concentration of ketone bodies is in part due to a decrease in the rate of their oxidation. The combination of acidosis, decreased energy production in mitochondria, and hyperammonemia foremost impairs the function of neurons.

Screening of newborns often includes tests for deficiencies of holocarboxylase synthetase and biotinidase. Both deficiencies can be treated with high doses of exogenous biotin.

5.3. Pyruvate Carboxylase Deficiency

The pathology of pyruvate carboxylase deficiency attests to the importance of this enzyme and the citric acid cycle in several metabolic processes. Most affected patients have **lactic acidosis** because the lack of oxaloacetate prevents the use of acetyl-CoA, which in turn inhibits PDH, thereby preventing significant entry of pyruvate into mitochondria. The more severely affected patients also have fasting **ketoacidosis**, because a high concentration of acetyl-CoA from fatty acid oxidation in the liver causes overproduction of ketone bodies, whereas a high concentration of acetyl-CoA in other cells inhibits the oxidation of ketone bodies (compare to Figs. 27.14 and 27.16). Patients develop **fasting hypoglycemia** because there is not enough oxaloacetate or malate for gluconeogenesis (see Fig. 25.3). Severely affected patients have **hyperammonemia** because there is not enough oxaloacetate to accept an amino group and form aspartate for nitrogen elimination via the urea cycle (see Fig. 35.7). The low concentration of oxaloacetate also leads to depletion of α-ketoglutarate, from which glutamate and the neurotransmitter γ-aminobutyric acid (**GABA)** are made (see Fig. 35.6). The deficiency in pyruvate carboxylase activity also causes deficient production of **NADPH** via the sequence $malate_{mito} \rightarrow oxaloacetate_{cytosol} \rightarrow pyruvate \rightarrow oxaloacetate_{mito} \rightarrow malate_{mito}$ (see Section 2 in Chapter 27). Without such production of NADPH in the cytosol, there is a diminished defense against **reactive oxygen species** (ROS; see Section 2.3 in Chapter 21) and a reduced elongation of **fatty acids** (see Fig. 27.6) for the production of **myelin**. The increased ROS, impaired myelin production, hyperammonemia, and deficient GABA synthesis give rise to impaired development and maintenance of the **central nervous system**.

5.4. Acute Poisoning With Arsenic

Acute poisoning with arsenic leads to an inhibition of the **PDH** complex, the **α-ketoglutarate dehydrogenase** complex (see Section 1), and **branched-chain ketoacid dehydrogenase** (see Chapter 35). In addition, acute poisoning leads to the inhibition and partial uncoupling of **oxidative phosphorylation**. Affected patients show an impaired function of the gastrointestinal tract, nervous system, heart, and kidneys, possibly due to impaired energy production.

5.5. Tumorigenic Mutations in Isocitrate Dehydrogenase, Succinate Dehydrogenase, or Fumarase

Heterozygous gain-of-function mutations in isocitrate dehydrogenases (IDH) are linked to sporadic cancers, whereas heterozygous inactivating mutations in succinate dehydrogenase (SDH) or fumarase (FH) are linked to hereditary cancer syndromes (Table 22.1). In hereditary cancer syndromes,

Table 22.1 Mutations in Isocitrate Dehydrogenase (IDH), Succinate Dehydrogenase (SDH), or Fumarase Linked to Cancer

Mutated Gene	Enzyme Name	Tumors That May Contain a Mutant Enzyme	Pathway of Tumorigenesis
IDH1	IDH1 (in cytosol and peroxisomes)	Gliomas, acute myeloid leukemias, tumors that arise in the cavity or on the surface of bone, and melanomas	Formation of 2-hydroxy-glutarate, which inhibits dioxygenases
IDH2	IDH2 (in mitochondria)		
SDHA	SDH subunit A	Paragangliomas, pheochromocytomas, and gastrointestinal stromal tumors	Accumulation of succinate, which inhibits dioxygenases
SDHB	SDH subunit B		
SDHC	SDH subunit C		
SDHD, if inherited from the father	SDH subunit D		
SDHAF2	SDH assembly factor 2		
FH	Fumarase = fumarate hydratase (in mitochondria and the cytosol)	Hereditary leiomyomatosis and renal cell cancer, papillary renal cell cancer, Leydig cell tumors	Accumulation of fumarate, which inhibits dioxygenases; fumarate spontaneously reacts with –SH groups of proteins

tumors arise when somatic cells acquire a second mutation that leads to a complete loss of function.

Tumorigenic mutations in IDH, SDH, or FH are similar in that mutant enzymes give rise to metabolites that competitively inhibit **dioxygenases** that use **α-ketoglutarate** (**2-oxoglutarate**) as one of their substrates. These dioxygenases encompass, for example, **HIF prolyl hydroxylase** and the **ten-eleven translocation** (TET) enzymes, which demethylate DNA. As explained in Chapter 16, decreased hydroxylation of hypoxia-inducible factor alpha (HIF-α) leads to increased survival of HIF-α, translocation of HIF-α into the nucleus, and increased transcription of genes, the products of which help the body alleviate hypoxia or cope with it. Many tumor cells exist in a hypoxic environment and therefore benefit from increased HIF-α–induced transcription.

Methylation of promoter regions reduces the rate of transcription of associated tumor suppressor genes and thus favors tumorigenesis. Methylation status is determined by both DNA (cytosine-5-)-methyltransferases (DNMTs) that methylate cytosines in CpG islands of DNA, and by **TET enzymes** that catalyze a multistep demethylation of 5-methyl cytosine by forming 5-hydroxymethyl cytosine, then 5-formyl cytosine, and finally 5-carboxy cytosine. The pathway for the physiologically most relevant re-formation of cytosine from one or more of these intermediates remains to be elucidated. Tumorigenic mutations in IDH, SDH, or FH lead to reduced TET enzyme activity and thus to an increased methylation of DNA near tumor suppressor genes.

Humans have three IDH isoenzymes, but to date only mutations in the genes for IDH 1 and 2 are known to give rise to the oncogenic metabolite **2-hydroxy glutarate** (see Table 22.1). IDH 3 is the main enzyme that is involved in catalyzing a reaction of the citric acid cycle. The normal product of the IDH is 2-oxo-glutarate (α-ketoglutarate). Most pathogenic mutations affect an arginine residue in the active site of the enzyme. These mutations are **acquired** (not inherited) and typically affect only one allele.

Patients who are heterozygous for a germline loss-of-function mutation in one of the five subunits of **SDH** have a high risk of losing the function of the normal allele in certain critical cells; this in turn gives rise to a **paraganglioma** or a **pheochromocytoma** (see Table 22.1). However, only a fraction of patients who have a paraganglioma or a pheochromocytoma has inherited a nonfunctional allele of an SDH subunit. In patients who are heterozygous for an SDH mutation, the loss of the normal allele in some somatic cells is an example of a **loss of heterozygosity** (see Chapters 2 and 8). Pheochromocytomas are derived from the adrenal medulla and are composed of chromaffin cells that release catecholamines (Fig. 22.13). The resulting high concentration of circulating catecholamines causes hypertension, but only about 1 in 500 patients with hypertension has a pheochromocytoma. Paragangliomas are vascular tumors in the head or neck, most frequently at the carotid bifurcation. They too may produce catecholamines, or they may simply be a painless, abnormal mass that can impair normal body function. The expression of pathogenic SDHD mutations is unique in that only a paternal mutation is pathogenic, due to **imprinting** of the maternal allele (imprinting is discussed in Chapter 5). Many patients who have a recognized pheochromocytoma or paraganglioma undergo genetic testing for several known heritable mutations, including mutations in SDH dehydrogenase.

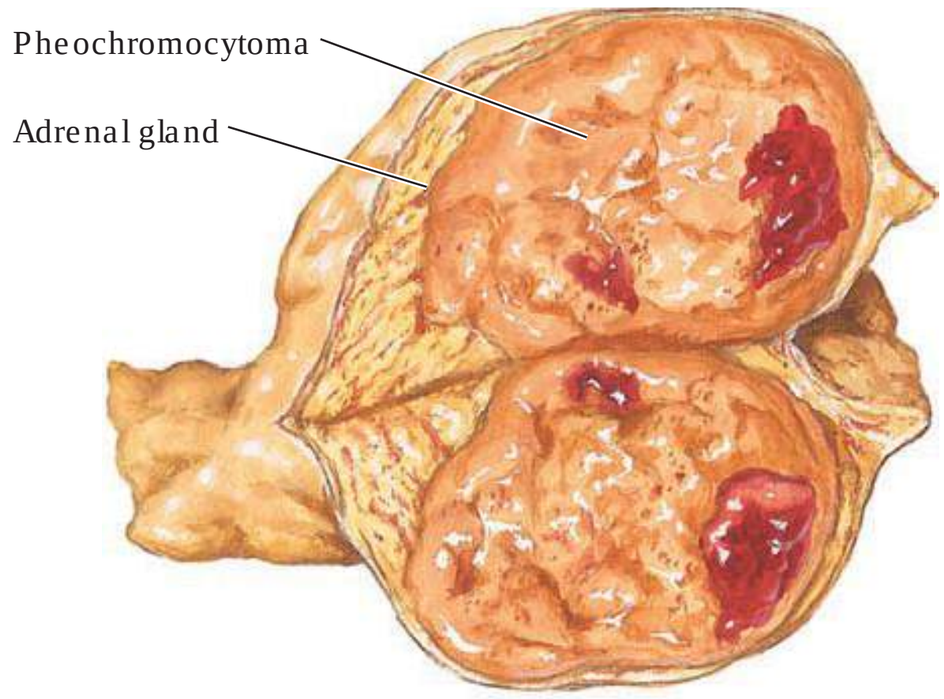


Fig. 22.13 Pheochromocytoma. Pheochromocytomas secrete catecholamines, which cause hypertension, headaches, sweating, and palpitations.

Patients who are homozygous (or compound heterozygous) for mutations in the SDHA gene that result in partial SDH deficiency have **juvenile encephalomyopathy**. This is a very rare disease that appears to be due to impaired energy production in the brain and muscle.

Heterozygous inborn **fumarase** (**FH**) deficiency gives rise to **hereditary leiomyomatosis and renal cell carcinoma** (**HLRCC**; see Table 22.1). Fumarase is required inside mitochondria for the citric acid cycle and in the cytosol of cells that contain the urea cycle (see Chapter 35). Fumarase in the mitochondria and the cytosol is derived from the same gene. When cells lose the function of the remaining normal fumarase allele, the concentration of fumarate increases to a tumorigenic level. With dioxygenase activity inhibited by fumarate, HIF prolyl hydroxylase activity is low so that HIF stimulates the transcription of certain genes (see Fig. 16.5) and thereby promotes angiogenesis. Affected patients develop benign leiomyomas (tumors that derive from smooth muscle) in the **skin**.

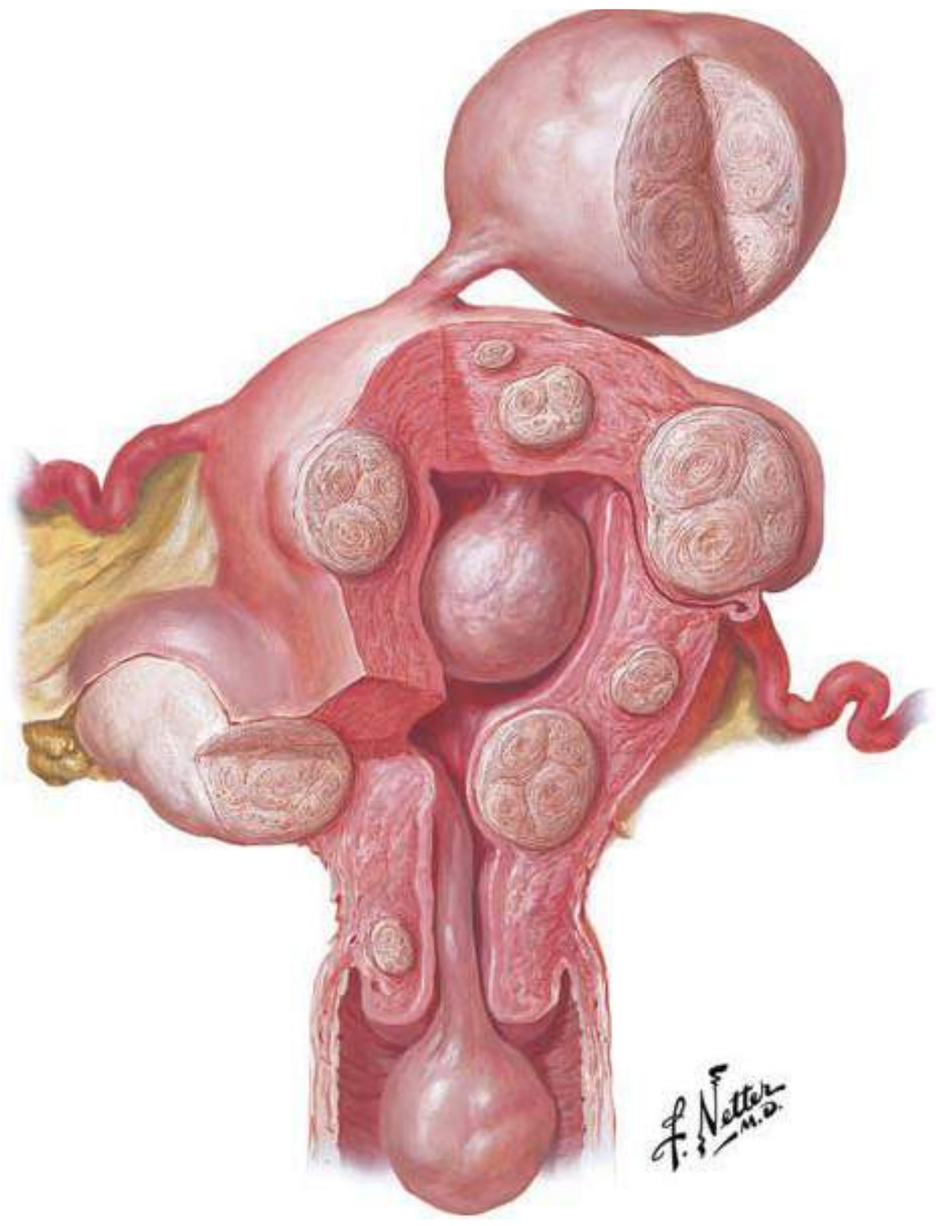


Fig. 22.14 **Leiomyomas in the uterus (fibroids).**

Af ected women also develop benign leiomyomas in the **uterus** (**fibroids**; Fig. 22.14). In addition, some male and female patients develop aggressive, metastatic renal cell cancer. In the absence of skin leiomyomas, most of the commonly occurring uterine leiomyomas and most renal cell carcinomas are not associated with mutations in fumarase.

Homozygous germline mutations that result in **near-complete fumarase deficiency** lead to a severe dysfunction of neurons. T e dysfunction may be due to impaired energy production. Af ected patients often die in early childhood.

5.6. Deficiency of the Pyruvate Dehydrogenase Complex

A deficiency of the PDH complex compromises ATP production, can cause **intellectual disability**, and often leads to **lactic acidosis** and **lactic acidemia**. Most deficiencies of the PDH complex result from mutations in the PDHA1 gene on the X chromosome, the gene that encodes the α-subunit of the E1-component. PDH deficiency is rare, and most mutations occur de novo. In patients with a severe deficiency, all pyruvate must be reduced to lactate, causing massive lactic acidosis at birth and death during the neonatal period. A mild deficiency primarily af ects the function of the brain, which depends on pyruvate for energy production. Ketone bodies (see Chapter 27) can diminish the need for pyruvate; ketogenic diets therefore help some patients.

SUMMARY

- T e pyruvate dehydrogenase (PDH) complex converts pyruvate to acetyl-CoA and thereby produces NADH. Both acetyl-CoA and NADH product inhibit PDH. In the liver, insulin increases PDH activity. In skeletal and cardiac muscle, exercise (acting via Ca^{2+}) increases PDH activity.
- Complete oxidation of acetyl-CoA in the citric acid cycle yields CO_2, GTP, NADH, and $FADH_2$. NADH and $FADH_2$ transfer reducing power to the electron transport chain of oxidative phosphorylation for the production of ATP (see Chapter 23). T e combination of the citric acid cycle and oxidative phosphorylation produces most of the body's ATP. In comparison, ATP production from glycolysis is small.
- T e PDH complex is most active when the concentrations of acetyl-CoA and NADH inside the mitochondria are low.
- Pyruvate carboxylase converts pyruvate to oxaloacetate to help replenish citric acid cycle intermediates that are withdrawn for biosyntheses. Without oxaloacetate, acetyl-CoA cannot enter the citric acid cycle. T e concentration of acetyl-CoA is the main regulator of pyruvate carboxylase activity.
- An elevated concentration of NADH leads to a decrease in flux through the citric acid cycle.
- Flux through the citric acid cycle strongly depends on flux through oxidative phosphorylation. Flux through oxidative phosphorylation in turn strongly depends on the rate of ATP hydrolysis. A low rate of flux through oxidative phosphorylation limits citric acid cycle activity mainly through an increase in the concentration of NADH.
- PDH, pyruvate carboxylase, and some enzymes of the citric acid cycle require vitamins as cofactors. Clinically, the most important vitamin deficiencies are thiamine deficiency, riboflavin deficiency, and biotin deficiency. T iamine deficiency leads to impaired ATP generation and chiefly af ects the heart and the central nervous system. Riboflavin deficiency causes lesions of the mouth, lips, skin, and genitalia. Biotin deficiency af ects pyruvate carboxylase as well as carboxylases in other pathways. It primarily impairs the central nervous system.
- Certain cancers show acquired mutations in the isocitrate dehydrogenase genes IDH1 and IDH2 that lead to the production of 2-hydroxyglutarate, which inhibits 2-oxoglutarate–dependent dioxygenases and thereby leads to DNA hypermethylation and increased transcription of genes that are part of the hypoxia response.
- Heterozygosity for an inactivating mutation in certain subunits of succinate dehydrogenase (SDH) is associated with an increased susceptibility to the formation of pheochromocytomas or paragangliomas.
- Heterozygosity for an inactivating mutation in fumarase is associated with the formation of fibroids and an increased susceptibility to the development of renal cell cancer.
- A congenital deficiency of PDH is rare. Patients in crisis have lactic acidemia.

FURTHER READING

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Review Questions

1. In patients who are at risk of developing Wernicke encephalopathy, a preventive infusion of thiamine ensures adequate activity of which of the following enzymes?

   A. Citrate synthase and malate dehydrogenase
   B. Fumarase and succinate dehydrogenase
   C. α-Ketoglutarate dehydrogenase and pyruvate
   D. Pyruvate carboxylase and succinate dehydrogenase
   E. Transketolase and isocitrate dehydrogenase

2. A patient has severe hypoxia. In this situation, which of the following is the major inhibitor of the citric acid cycle?

   A. α-Ketoglutarate
   B. Acetyl-CoA
   C. ADP
   D. NADH
   E. Pyruvate

Chapter

23 Oxidative Phosphorylation and Mitochondrial Diseases

SYNOPSIS

- Mitochondria are basically stripped-down gram-negative bacteria that specialize in energy production. Human mitochondria consist of an internal compartment (the mitochondrial matrix) that contains the enzymes of the citric acid cycle, fatty acid β-oxidation, ketone body metabolism, and parts of several biosynthetic pathways. The matrix is enclosed by the inner mitochondrial membrane, which contains the proteins for oxidative phosphorylation. The inner mitochondrial membrane is surrounded by an outer mitochondrial membrane that is permeable to small molecules. The region between the two membranes is the intermembrane space.
- Oxidative phosphorylation takes place in the mitochondria and couples the oxidation of reduced nicotinamide adenine dinucleotide (NADH) and other reduced compounds to the production of adenosine triphosphate (ATP; Fig. 23.1). As NADH is oxidized, protons (H^+) are pumped out of the matrix into the intermembrane space as part of a series of oxidation-reduction reactions. An ATP synthase allows protons to flow back into the mitochondrial matrix, and it uses the energy that is freed in this process to phosphorylate adenine diphosphate (ADP) to ATP.
- Mitochondria contain their own DNA. Mitochondria are inherited from only the mother. Some of the proteins needed for oxidative phosphorylation are encoded by the DNA in the mitochondria, but most are derived from the DNA in the nucleus.
- Mitochondrial diseases give rise to deficient oxidative phosphorylation and consequently affect primarily cells and tissues that require a high rate of ATP production, such as the central nervous system, the heart, and skeletal muscle. Pancreatic β-cells are also often affected, since ATP synthesis is required for glucose sensing and insulin secretion.

LEARNING OBJECTIVES

For mastery *of* this topic, you should be able to do the *following*:

- Describe the function, cellular location, and tissue distribution of the electron transport chain and ATP synthase.
- Summarize how components of the electron transport chain undergo oxidation-reduction reactions and how the energy from such reactions is used to pump protons to the intermembrane space.
- Explain the coupling of electron transport and ATP synthase activity.
- Explain the role of creatine kinase, creatine, and phosphocreatine in intracellular energy transport, and list tissues in which these molecules are especially abundant.
- Differentiate the normal regulation and interplay of ATP synthase activity, flux in the electron transport chain, flux in the citric acid cycle, and flux in glycolysis.
- Assess the influence of a limiting concentration of oxygen on oxidative phosphorylation.
- Describe the effects of uncouplers and electron transport chain inhibitors on flux through the electron transport chain and on the rate of oxidative phosphorylation; predict the effects of these agents on flux in glycolysis, in the citric acid cycle, and in the conversion of pyruvate to lactate.
- Describe the role of the supplement coenzyme Q (ubiquinone) in oxidative phosphorylation and in protecting lipid integrity.
- Identify a pattern of mitochondrial inheritance.
- Explain why some mitochondrial diseases are inherited with an X-linked or autosomal recessive pattern, while others show maternal inheritance.
- Explain heteroplasmy and show how it relates to variations in onset, phenotype, and severity of mitochondrial diseases caused by mutations in mitochondrial DNA.

1. OXIDATIVE PHOSPHORYLATION

Oxidative phosphorylation consists *of* an oxygen-requiring electron transport chain and an A*T*P synthase. T e electron transport chain uses the reducing power (electrons and protons) *of* NADH and a *f*ew other reducing agents to reduce O_2 to H_2O. During these reactions, H^+ is pumped out *of* the mitochondrial matrix space into the mitochondrial intermembrane space. T e A*T*P synthase allows H^+ to *fl*ow back into the matrix while using the electrochemical H^+ gradient to synthesize A*T*P *f*rom ADP and phosphate. Inhibitors *of* the electron transport chain and uncouplers *of* oxidative phosphorylation both reduce A*T*P production by oxidative phosphorylation.

1.1. Structure and Function of Mitochondria

Mitochondria are present in most cells. Mature red blood cells do not have mitochondria. Fast white muscle cells have very few mitochondria. In contrast, organs such as the brain and heart contain many mitochondria.

Mitochondria contain an inner and an outer membrane, creating a matrix space and an intermembrane space (Fig. 23.2).

While mitochondria are often drawn in the shape of an elongated bean, they actually form a highly dynamic tubular **reticulum** inside of cells.

T e matrix space contains the enzymes of the citric acid cycle (see Chapter 22); fatty acid β-oxidation, ketone body synthesis, and ketone body oxidation (see Chapter 27); parts of heme synthesis (see Chapter 14); steroid synthesis (see Chapter 31); protein metabolism (see Chapters 34 and 35); and the urea cycle (see Chapter 35). T e inner mitochondrial membrane contains the components of oxidative phosphorylation discussed in this chapter. T e outer membrane is permeable to small molecules.

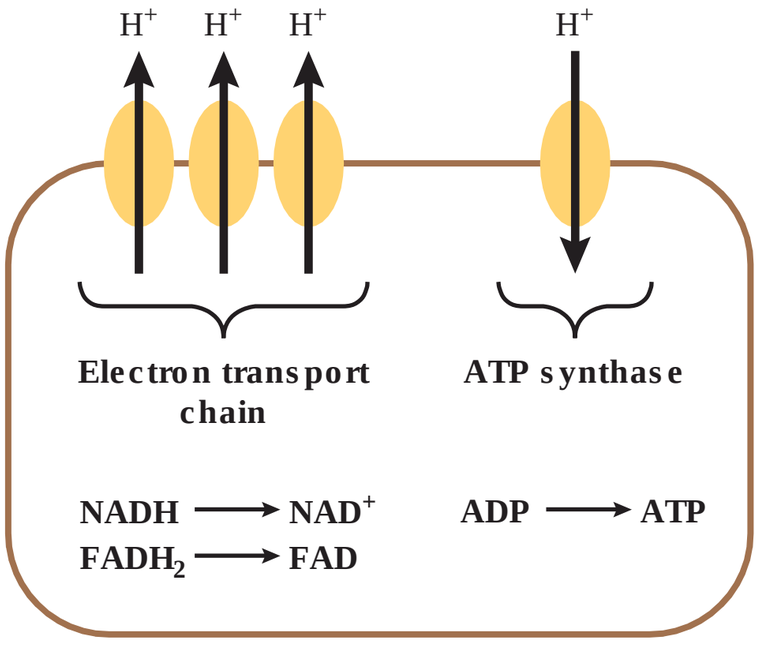


Fig. 23.1 **Formation of ATP via oxidative phosphorylation.**

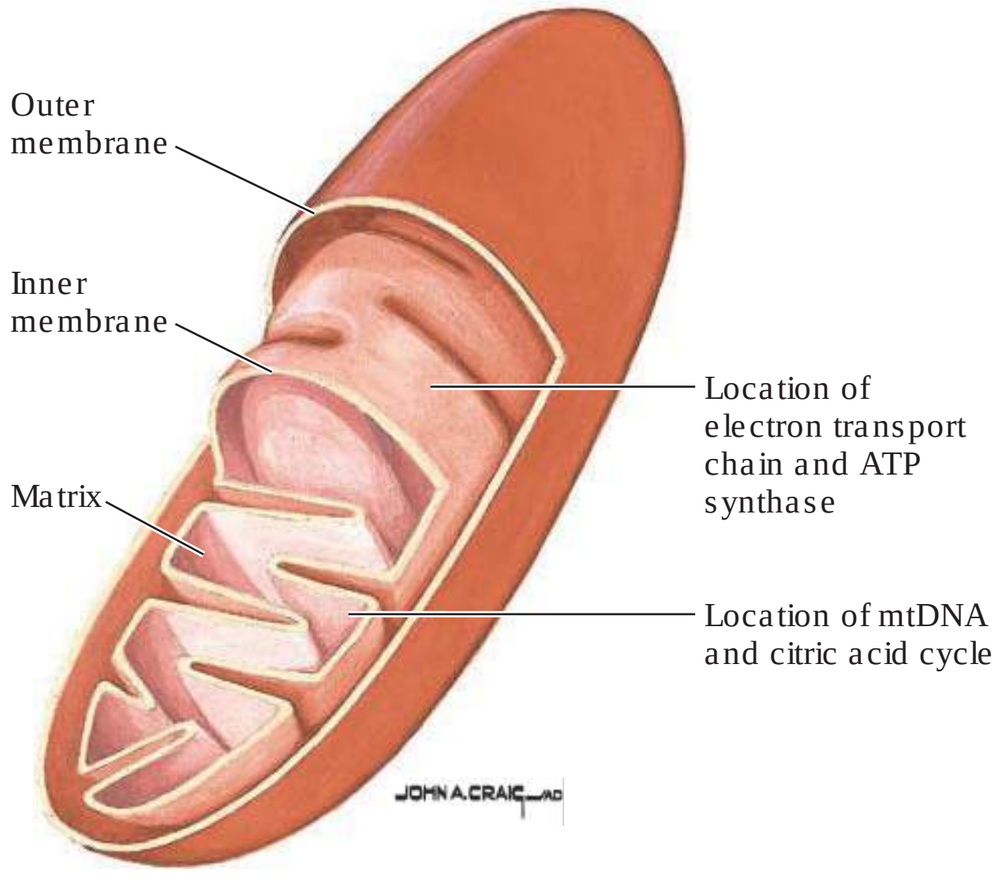


Fig. 23.2 **Structure of mitochondria.**

1.2. Electron Transport Chain

The electron transport chain is sometimes called the **respiratory chain**.

The electron transport chain (Fig. 23.3) has a single endpoint (the reduction of O_2 to water by complex IV), but it has multiple proteins that accept "reducing power" and thereby funnel electrons into the chain. These proteins include complex I (also called NADH dehydrogenase), electron-transferring flavoprotein dehydrogenase, mitochondrial glycerol 3-phosphate dehydrogenase (which is part of the glycerol phosphate shuttle), and complex II (also called succinate dehydrogenase, an enzyme that is part of the citric acid cycle). Complexes III and IV are part of the common and final part of the electron transport chain. Complex III is also called coenzyme Q:cytochrome c oxidoreductase, or cytochrome bc1 complex. Complex IV is also called cytochrome c oxidase.

The electron transport chain contains two electron carriers. Reduced coenzyme Q (QH_2, ubiquinol; see below) is a lipid that freely diffuses in the inner mitochondrial membrane. Every input of the electron transport chain gives rise to QH_2. Catalyzed by complex III, QH_2 then donates its electrons to cytochrome c. Reduced cytochrome c is a protein that is mostly bound to the outside of the inner mitochondrial membrane. Reduced cytochrome c transports electrons from complex III to complex IV.

Only complexes I, III, and IV **pump protons** (H^+) out of the matrix into the intermembrane space. As described in Section 1.4, the energy of the resulting electrochemical gradient is used for the synthesis of ATP.

Coenzyme Q is a lipid-soluble compound (Fig. 23.4) that diffuses within the inner mitochondrial membrane. Coenzyme Q is also called **ubiquinone**. Coenzyme Q can be reduced to **coenzyme QH_2**, which is also called **ubiquinol**. In humans, coenzyme Q has a **polyisoprene** "tail" of 10 units, which gives rise to the designations coenzyme Q10 and CoQ10. Humans synthesize the ring structure of coenzyme Q from tyrosine and derive the polyisoprene tail from the **cholesterol synthesis** pathway (see Chapter 29). Ubiquinol is also present in other membranes and acts as an antioxidant that protects for instance unsaturated fatty acids in phospholipids (see Chapter 21).

Supplemental coenzyme Q10 is used in the treatment of certain disorders of mitochondrial energy production and several rare forms of **heritable deficiencies of coenzyme Q10 synthesis**. CoQ10 supplementation may also have a long-term beneficial effect in **migraine** prophylaxis. In contrast, it is uncertain whether supplementary coenzyme Q10 reduces oxidative damage or is effective in the treatment of **statin-induced myopathy**.

Cytochrome c is a small (104-amino acid) protein in the mitochondrial intermembrane space that is normally bound electrostatically to the outside of the inner mitochondrial membrane. Cytochrome c contains a heme prosthetic group with iron that can be reduced (Fe^{2+}) or oxidized (Fe^{3+}). Cytochrome c is strongly positively charged, and this facilitates its binding to the negatively charged phospholipid **cardiolipin** in the inner mitochondrial membrane. The structure of cardiolipin is shown in Fig. 11.3.

Cytochrome c is not only part of the electron transport chain, but it is also an intracellular signal for **apoptosis**. During apoptosis, cytochrome c can pass through enlarged pores in the mitochondrial outer membrane (see Chapter 8). In the cytosol, cytochrome c binds to apoptotic protease-activating factor 1 (APAF1) and thus gives rise to an apoptosome that favors self-destruction of the cell.

The electron transport chain creates an electrochemical H^+ gradient (i.e., an electrical charge difference and a pH difference). When this gradient equals the chemical driving force for electron transport, electron transport slows and eventually stops (i.e., an equilibrium is reached).

1.3. Clinically Relevant Inhibitors of the Electron Transport Chain

During electron transport by the electron transport chain, some 1% to 4% of electrons do not stay in the chain but are instead accidentally transferred to O_2, giving rise to $\bullet O_2^-$ (i.e.,

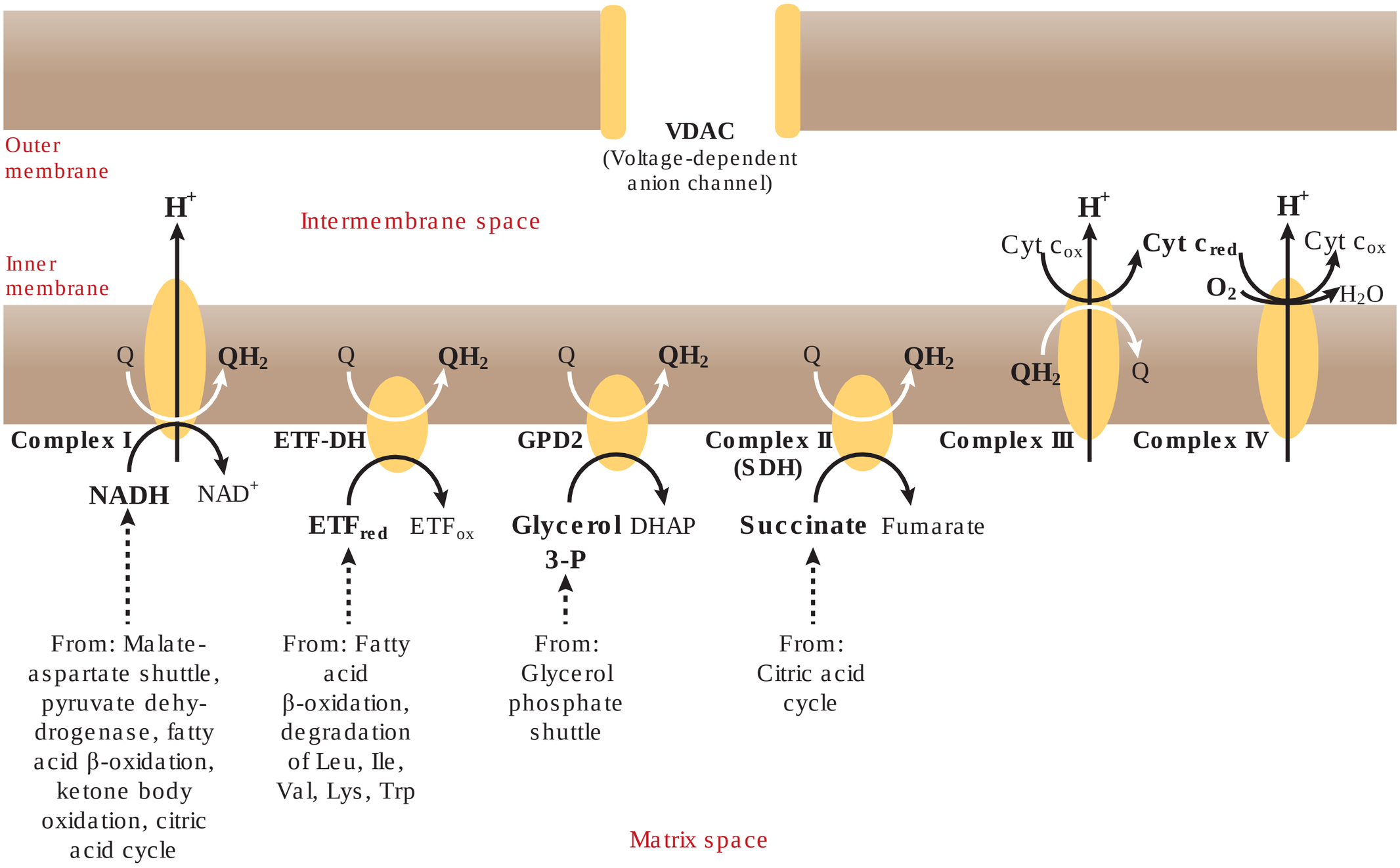


Fig. 23.3 **Key elements of the mitochondrial electron transport chain.** Coenzyme QH_2 transports hydrogen atoms inside the inner membrane. Cytochrome c transports electrons in the intermembrane space. Fatty acid β-oxidation gives rise to both NADH and reduced ETF. Reducing power from NADH that is produced in glycolysis enters the electron transport chain via the malate-aspartate shuttle or the glycerol 3-phosphate shuttle. Q, coenzyme Q (oxidized form); QH_2, coenzyme Q (reduced form); ETF, electron-transferring flavoprotein; ETF-DH, ETF-dehydrogenase; GPD2, mitochondrial glycerol 3-phosphate dehydrogenase; DHAP, dihydroxyacetonephosphate; SDH, succinate dehydrogenase; Cyt c, cytochrome c.

a **superoxide anion**). The superoxide anion is a reactive oxygen species that readily gives rise to a more damaging hydroxyl radical (•OH), which reacts with lipids, proteins, and DNA (see Chapter 21). The main producers of superoxide anions in the electron transport chain are complex I, semiquinol (a radical produced from ubiquinone by the addition of a single H atom), and complex III. An impairment of the electron transport chain increases the production of superoxide anions.

Metformin inhibits complex I, while cyanide, **carbon monoxide**, and **sodium azide** inhibit complex IV. Aggressive **oxygen therapy** is always a part of the treatment of poisoning with cyanide, carbon monoxide, or azide. Sometimes, oxygen therapy is performed in a pressure chamber at up to three times the atmospheric pressure at sea level, a treatment called **hyperbaric oxygen**.

Metformin is used as an antidiabetic agent. It is very effective at suppressing the excessive endogenous glucose production (i.e., chiefly glycogenolysis and gluconeogenesis in the liver) that is seen in type 2 diabetes (see Chapter 39). The mechanism of action of metformin is still debated but is thought to involve the inhibition of complex I that leads to the activation of adenosine monophosphate (AMP)-dependent protein kinase (AMPK), which then inhibits gluconeogenesis.

Cyanide can be produced in building fires, be a part of pesticides, or even be contained in some foods. Cyanide binds predominantly to complex IV (cytochrome c oxidase) and thus blocks the entire electron transport chain, resulting in marked lactic acidemia. Mitochondria contain thiosulfate sulfurtransferase (also called rhodanase), which detoxifies cyanide (CN^-) by converting it to thiocyanate (SCN^-), which is excreted in the urine. The half-life of cyanide in blood plasma is 20 to 60 minutes. Conversion of cyanide to thiocyanate can be enhanced with IV **sodium thiosulfate** ($S_2O_3^{2-}$), a substrate of thiosulfate sulfurtransferase**.** Furthermore, cyanide can be bound to cobalamin, which can be given intravenously as **hydroxocobalamin**. The resulting cyanocobalamin (the traditional form of a vitamin B_{12} supplement) is not toxic. First responders often carry hydroxocobalamin. Cyanide can also be bound to **methemoglobin**. Methemoglobin is formed in the body in response to a therapeutic application of **amyl nitrite** (via inspired air) or **sodium nitrite** (intravenous; see Chapter 16). A common therapeutic goal in adults is to convert about 10% to 30% of hemoglobin to methemoglobin.

Carbon monoxide results from incomplete combustion in many types of fires (including cigarettes). Carbon monoxide binds to both hemoglobin and complex IV, and

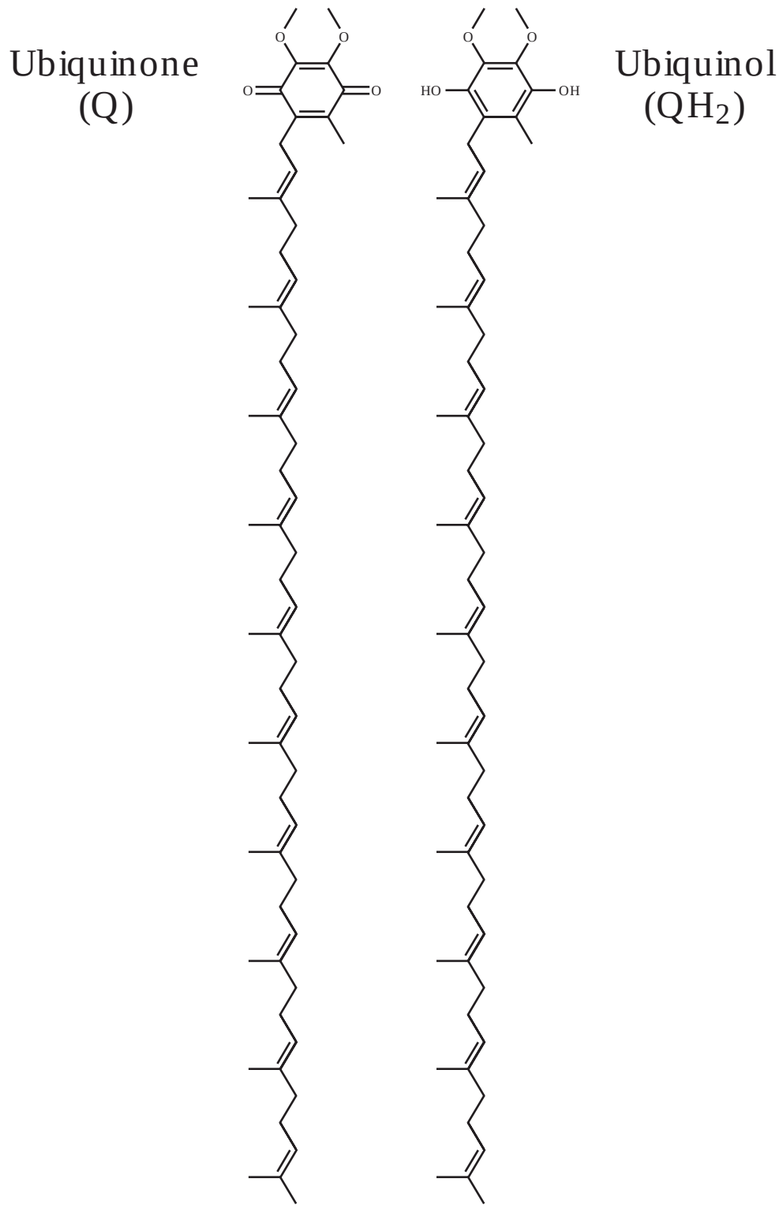


Fig. 23.4 Coenzyme Q10 (ubiquinone) and its reduced form, ubiquinol. Both molecules are dissolved in the membrane.

it impairs both oxygen delivery and oxidative phosphorylation. Oxygen therapy enhances the exchange of CO for O_2 on hemoglobin.

Sodium azide also inhibits complex IV and induces hypotension. Azide is used in explosives (including automobile airbags), as a preservative (often in laboratory settings), and sometimes as a pesticide.

Hydrogen sulfide gas also inhibits complex IV. Hydrogen sulfide is formed in some industrial processes and in places where manure is stored. Poisoned patients are treated with oxygen and can be given sodium nitrite, which gives rise to methemoglobin, which in turn binds sulfide. Furthermore, nitrite gives rise to NO, which can displace sulfide from complex IV.

1.4. ATP Synthase

An **ATP synthase** in the inner mitochondrial membrane allows H^+ to flow from the intermembrane space down the electrochemical gradient into the matrix; it uses the energy of this process to synthesize ATP. Interestingly, the ATP synthase consists in part of subunits that are embedded in the membrane and are essentially static, whereas other subunits form a rotating complex in which H^+ flux powers rotation, the mechanical energy of which causes changes in the conformation of the static complex that drives ATP synthesis.

T e terms **chemiosmotic coupling** and the **Mitchell hypothesis** apply to ATP production by a combination of a proton-pumping electron transport chain and a proton-driven ATP synthase. Peter Mitchell first proposed his theory in 1961, at a time when other investigators looked into other ways of harnessing the reducing power of NADH to produce ATP.

In healthy tissue, oxidative phosphorylation is set up such that the ATP synthase keeps the concentration of **ADP** low. T e ATP synthase becomes more active whenever more ADP becomes available. When the ATP synthase makes ATP and thereby diminishes the electrochemical H^+ gradient, the electron transport chain becomes more active and reestablishes the gradient. T us, the rate of ADP production determines the flux of electrons in the electron transport chain and the rate of oxygen consumption.

Although oxygen consumption is not part of the mitochondrial ATP synthase-catalyzed reaction itself, reduction of O_2 by the electron transport chain is the driving force for this ATP synthesis. Hence, the term oxidative phosphorylation is appropriate. Oxidative phosphorylation is not to be confused with substrate-level phosphorylation, which produces ATP from a high-energy phosphorylated substrate such as phosphoenolpyruvate (see Section 1 in Chapter 19).

It is estimated that feeding 1 NADH into the electron transport chain gives rise to the synthesis of about 2.5 ATP and that the oxidation of 1 QH_2 (ubiquinol) gives rise to about 1.5 ATP.

In most tissues, the vast majority of ATP (typically >90%) is produced by oxidative phosphorylation. In comparison, ATP production from substrate-level phosphorylation in glycolysis is small.

1.5. Transport of Chemical Energy in the Form of ATP and Phosphocreatine

Although ATP is made inside mitochondria, it is mostly consumed outside mitochondria and therefore must be transported across the mitochondrial membranes. T e **adenine nucleotide translocator** exchanges ADP for ATP across the inner mitochondrial membrane. T e outer membrane has large pores through which ADP and ATP can easily pass. A **phosphate carrier** brings phosphate into the mitochondria for ATP synthesis.

Outside the mitochondria, transport of "energy" occurs via two paths (Fig. 23.5): (1) ATP away from mitochondria versus ADP and phosphate toward mitochondria, and (2)

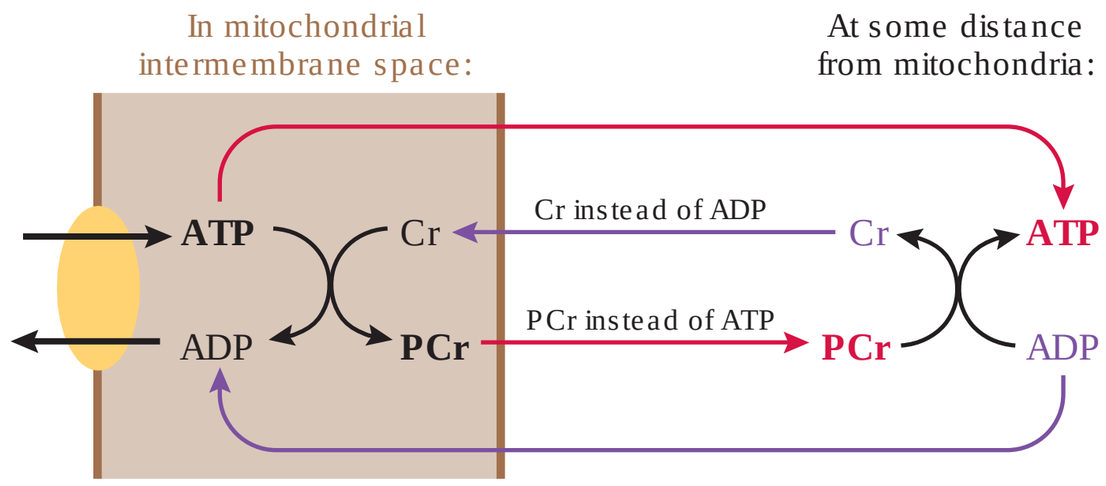


Fig. 23.5 Transport of energy from mitochondria to the cell periphery. Cr, creatine; PCr, phosphocreatine.

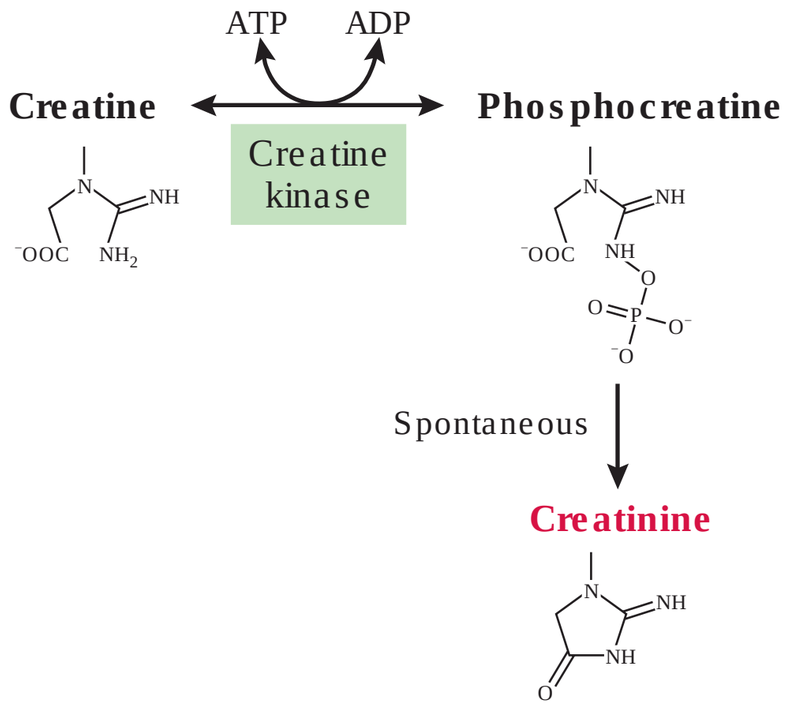


Fig. 23.6 **Formation of creatinine.**

phosphocreatine away from mitochondria versus creatine and phosphate toward mitochondria. The structures of creatine and phosphocreatine are shown in Fig. 23.6. Like ATP, **phosphocreatine** has a high-energy phosphate bond. The concentration of ATP is in the millimolar range, but the free concentration of ADP is usually less than 0.1 mM, which severely curtails transport by diffusion. In contrast, creatine and phosphocreatine can be present in millimolar concentrations. Phosphocreatine and creatine are primarily found in muscle and in the brain, where phosphocreatine is also the primary form of energy storage.

Intake of **exogenous creatine** increases the creatine and phosphocreatine content of various tissues, including muscle. Some athletes take extra creatine to increase their muscle power. Creatine increases power output during repeated short bouts of very intense exercise. Serum **creatinine** levels can rise with creatine supplementation, which complicates the estimation of kidney function that is based on creatinine levels.

Phosphocreatine spontaneously cyclizes to form **creatinine** (see Fig. 23.6), which cannot be remade into creatine and is excreted in the urine. In most people, creatinine is made at a comparable rate; consequently, the amount of creatinine in the blood can be used as a measure of **kidney function** (with significantly decreased filtration, the measured serum concentration of creatinine becomes abnormally high). To make up for the loss of creatinine, the body synthesizes creatine (see Chapter 36).

Creatine kinase catalyzes the phosphorylation of creatine and the dephosphorylation of phosphocreatine (see Fig. 23.6). There are two isoenzymes: one in the intermembrane space of mitochondria and one in the cytosol. Creatine kinase is especially abundant in tissues that have a high concentration of creatine and phosphocreatine (e.g., muscle and the brain).

Measurements of creatine kinase in the serum are used to diagnose and follow various **muscle diseases**. Injury to muscle is accompanied by the release of myocyte contents into the extracellular space and blood. There is a muscle-type (M) and a brain-type (B) creatine kinase in the cytosol. Muscle contains mostly MM dimers; severe exercise or injury may lead to an increased fraction of MB dimers.

1.6. Uncouplers of Oxidative Phosphorylation

Uncouplers are molecules that allow protons to flow from the intermembrane space back into the matrix, bypassing the ATP synthase (i.e., they uncouple electron transport from ATP synthesis). Uncouplers impair ATP synthesis and also stimulate the electron transport chain, which attempts to reestablish a normal electrochemical H^+ gradient. An uncoupler thus increases oxygen consumption.

Brown adipose tissue contains an uncoupling protein, **UCP-1**, that, when active, allows H^+ to flow from the intermembrane space into the matrix space. Active UCP-1 increases thermogenesis because both the electron transport chain itself and the collapse of the electrochemical H^+ gradient generate heat. Brown fat cells are brown or beige because they contain many mitochondria with cytochromes. UCP-1 is activated when norepinephrine activates β-adrenergic receptors on brown fat cells. Uncoupling of the mitochondria in brown fat cells leads to increased oxidation of glucose and fatty acids to CO_2.

Infants have a significant amount of brown fat, but most adults have only relatively small remnants of it, mostly in the neck and above the clavicles. Growing evidence shows that some drugs can induce white fat cells to turn toward a brown phenotype, becoming beige or "brite" adipocytes.

In **positron emission tomography scans**, brown fat often shows up as a tissue that picks up a considerable amount of the radioactive fluorodeoxyglucose tracer. Brown fat oxidizes glucose, and tracer accumulation from labeled fluorodeoxyglucose parallels glucose use (see Section 6.3 in Chapter 19).

2,4-Dinitrophenol is a small-molecule uncoupler that was once tested as a weight-loss drug. It is not currently an approved drug but is available illegally. This drug is dangerous because it can severely impair ATP synthesis and also lead to severe hyperthermia due to stimulation of the respiratory chain.

2. INTERPLAY OF GLYCOLYSIS, CITRIC ACID CYCLE, AND OXIDATIVE PHOSPHORYLATION

As shown above, ATP consumption gives rise to ADP, which in turn stimulates ATP synthase to convert ADP into ATP, thereby consuming a small part of the H^+ gradient. The electron transport chain immediately attempts to reestablish the H^+ electrochemical gradient by oxidizing NADH, electron-transferring flavoprotein, glycerol 3-phosphate, or succinate. Oxidation lowers the concentration of NADH, which in turn increases citric acid cycle activity.

Flux in **glycolysis** is mainly determined by phosphofructokinase activity. As long as oxidative phosphorylation keeps the concentration of ATP high and that of ADP low, flux in glycolysis is small. However, when the concentration of ADP rises, for instance because the citric acid cycle does not get enough acetyl-CoA and thus lowers flux in the electron transport chain and in ATP synthesis, flux in glycolysis increases (Fig. 23.7).

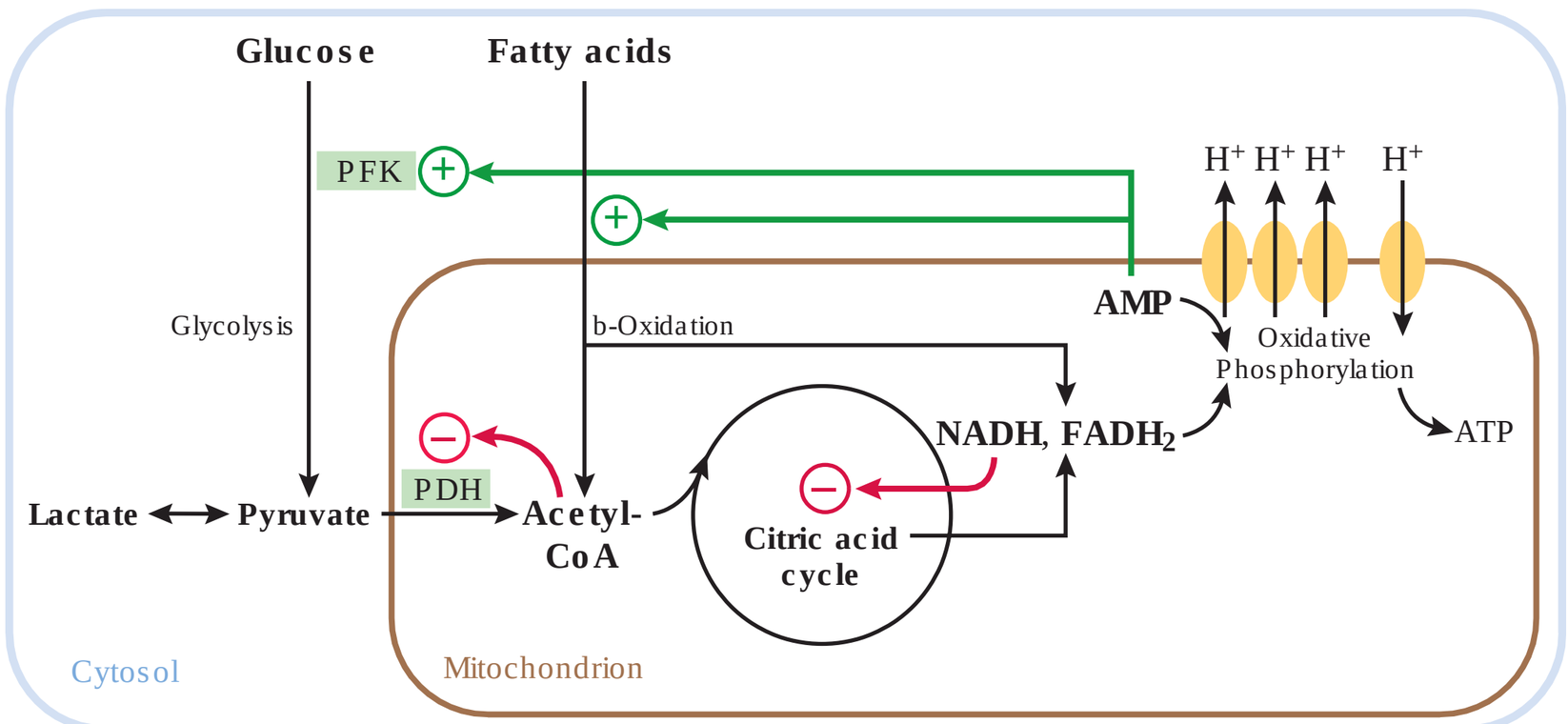


Fig. 23.7 Mutual dependence of glycolysis, fatty acid β-oxidation, citric acid cycle, and oxidative phosphorylation. Fatty acid β-oxidation is also limited by the availability of NAD^+ and FAD (not shown). PFK, phosphofructokinase.

In place of glucose, many cells can use **fatty acids** to produce reducing power for oxidative phosphorylation. In most of these cells, the concentrations of AMP, NAD^+, and flavin adenine dinucleotide (FAD) play a role in regulating the rate of fatty acid β-oxidation (see Chapter 27).

Patients who have impaired oxidative phosphorylation produce more of their ATP via **anaerobic glycolysis**, which may lead to **lactic acidemia** (see Fig. 23.7). Oxidative phosphorylation may be impaired because of **hypoxia** or **anoxia**, or because of an inhibitor of the electron transport chain (e.g., **cyanide**, **carbon monoxide**, or **metformin** overdose). When flux in the electron transport chain decreases, the concentration of NADH increases, and flux in both the citric acid cycle and in pyruvate dehydrogenase decreases. T e impaired electron transport chain leads to a decrease in mitochondrial ATP synthesis, which increases the concentration of free ADP and free AMP. AMP, in turn, activates phosphofructokinase and thus flux in glycolysis. Reducing power from NADH produced in glycolysis can no longer be moved into the mitochondria but must be used to reduce pyruvate to lactate. Appreciable inhibition of the body's capacity for oxidative phosphorylation leads to very marked lactic acidemia. T e acidemia is the cause of death in an anoxic patient.

Although **cancer** cells usually have enough oxygen, they often produce much more pyruvate from glycolysis than they can oxidize via the citric acid cycle and oxidative phosphorylation, a paradox called the **Warburg ef ect**. One of the current hypotheses is that metabolic reprogramming is advantageous to cancer cells because it provides them with more precursors and NADPH for biosynthetic pathways. T ese precursors can be intermediates of glycolysis, intermediates of pathways that interface with glycolysis, or intermediates of the citric acid cycle. T e precursors can then be used for the biosynthesis of amino acids, nucleotides, or lipids. T e metabolic reprogramming is achieved by a mutation or altered expression of genes that play a role in metabolism and signaling.

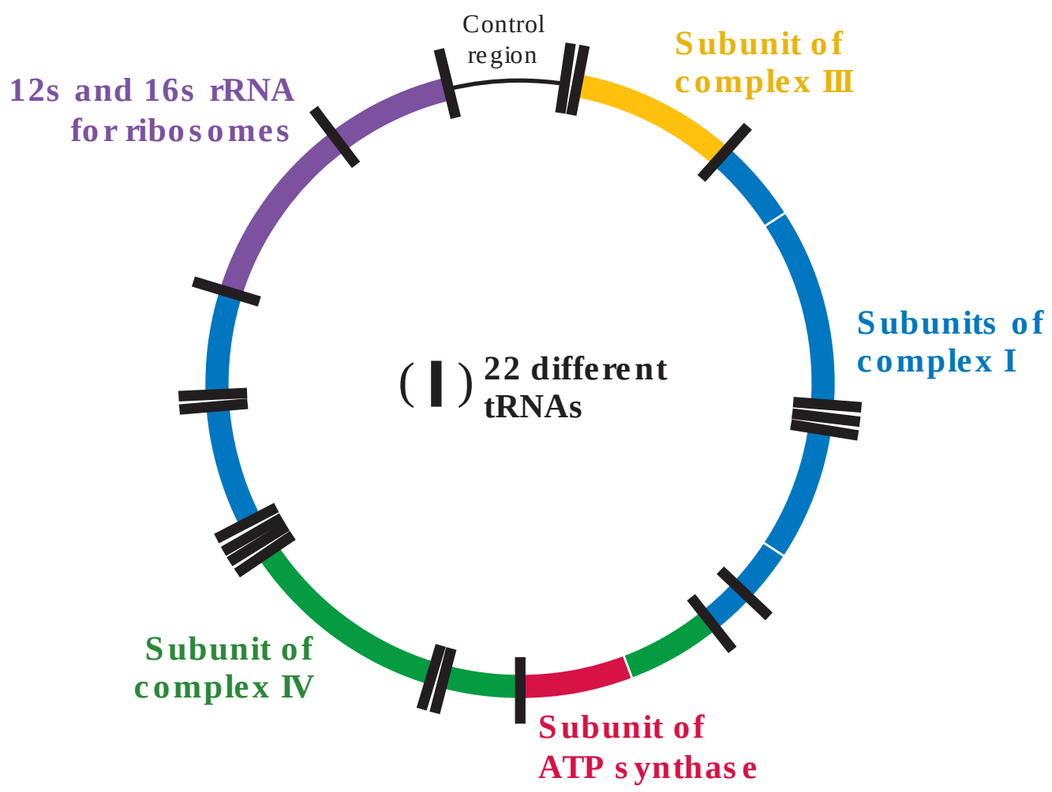


Fig. 23.8 Structure of human mitochondrial DNA (mtDNA). mtDNA consists of two complementary strands. *(Modified from www.mitomap.org.)*

3. MITOCHONDRIAL DNA AND ITS INHERITANCE

Mitochondrial DNA is closed, circular, and contains almost 40 genes that encode mitochondrial tRNAs, rRNAs, and 13 subunits o*f* electron transport complexes and the mitochondrial A*T*P synthase. Mitochondria are passed onto o*ff*spring only via the mother. Most cells have thousands o*f* copies o*f* mitochondrial DNA.

Mitochondria contain their own DNA (mtDNA), which is circular and encodes a few proteins and all of the tRNAs needed for translation (Fig. 23.8). T e genetic codes for translation of mitochondrial- and nucleus-encoded RNAs dif er in two codons. Most proteins in the mitochondria are encoded by genes in the nucleus, synthesized in the cytosol, and then imported into mitochondria. Similarly, most mitochondrial

diseases are due to mutations in nuclear genes and therefore show Mendelian inheritance.

The mitochondrial DNA encodes two rRNAs for its ribosomes, 22 tRNAs for translation, and 13 proteins. The proteins are subunits of the ATP synthase and of complexes I, III, and IV. Other subunits of these protein complexes are encoded in nuclear DNA.

Mitochondria import RNA polymerase, transcription factors, all aminoacyl-tRNA synthetases, initiation factors, and elongation factors (see Section 2 in Chapter 6). The nucleus-encoded DNA polymerase G (or gamma) enters the mitochondria and replicates mtDNA.

Human mtDNA contains about 16,000 nucleotides. On average, unrelated humans differ by about 50 nucleotides. Hence, the mtDNA sequence can serve to identify individuals.

A typical cell contains more than 1000-fold more copies of mtDNA than nuclear DNA.

Mitochondria are **inherited** only from the **mother**. A sperm has fewer copies of mitochondrial DNA than does the egg, few of the mitochondria in sperm enter the egg, and mitochondria from the sperm are rapidly destroyed in the egg. A human egg typically contains more than 100,000 copies of mitochondrial DNA.

The term **homoplasmy** refers to a cell in which all mitochondrial DNA molecules are the same, whereas **heteroplasmy** refers to a cell that contains a mixture of mitochondrial DNA molecules.

During **cell division**, mitochondria and their DNA molecules are divided by chance. Offspring of a mother can therefore have more or less mutant mtDNA than the mother. Furthermore, some cells or tissues in a person may have more or less mutant mtDNA than others. The level of mutant mtDNA in a tissue may even change over time. Clinically, this means that offspring may have greater or lesser severity of disease than the mother. Furthermore, symptoms vary greatly among patients with the same disorder. Due to these chance events described, the terms dominant and recessive inheritance are not used for diseases attributable to mutant mtDNA.

4. DISEASES INVOLVING MITOCHONDRIA

Diseases involving mitochondria are often associated with impaired energy production and affect cells and tissues that use ATP at a high rate. These diseases are acquired or inherited via DNA in the nucleus or mitochondria. Affected patients may benefit from supplements that improve the capacity for oxidative phosphorylation.

4.1. Overview

Mitochondrial diseases are a group of disorders that stem largely from a loss of normal mitochondrial function, particularly oxidative phosphorylation. Major deficiencies of oxidative phosphorylation often impair the nervous system, muscle contraction, insulin secretion from pancreatic β-cells, vision, or hearing (Fig. 23.9). Mitochondria with impaired oxidative phosphorylation may induce **apoptosis** (cell death). Furthermore, such mitochondria can produce **reactive oxygen species** (**ROS**) at an increased rate. The nervous system is particularly sensitive to ROS because it contains an abundance of polyunsaturated fatty acids.

Syndromes of dysfunctional mitochondria are named according to clinical observations rather than cause. This explains why some of these syndromes have more than one cause.

Mitochondria turn over constantly; autophagosomes engulf mitochondria and deliver them to the lysosomes for destruction in a process called **mitophagy**. Impaired function of lysosomes or autophagy appears to impair tissue function.

Mitochondrial disease may arise from **mutations** in mitochondrial or nuclear DNA that affect a wide variety of mitochondrial processes; they can be **acquired** (e.g., by drug

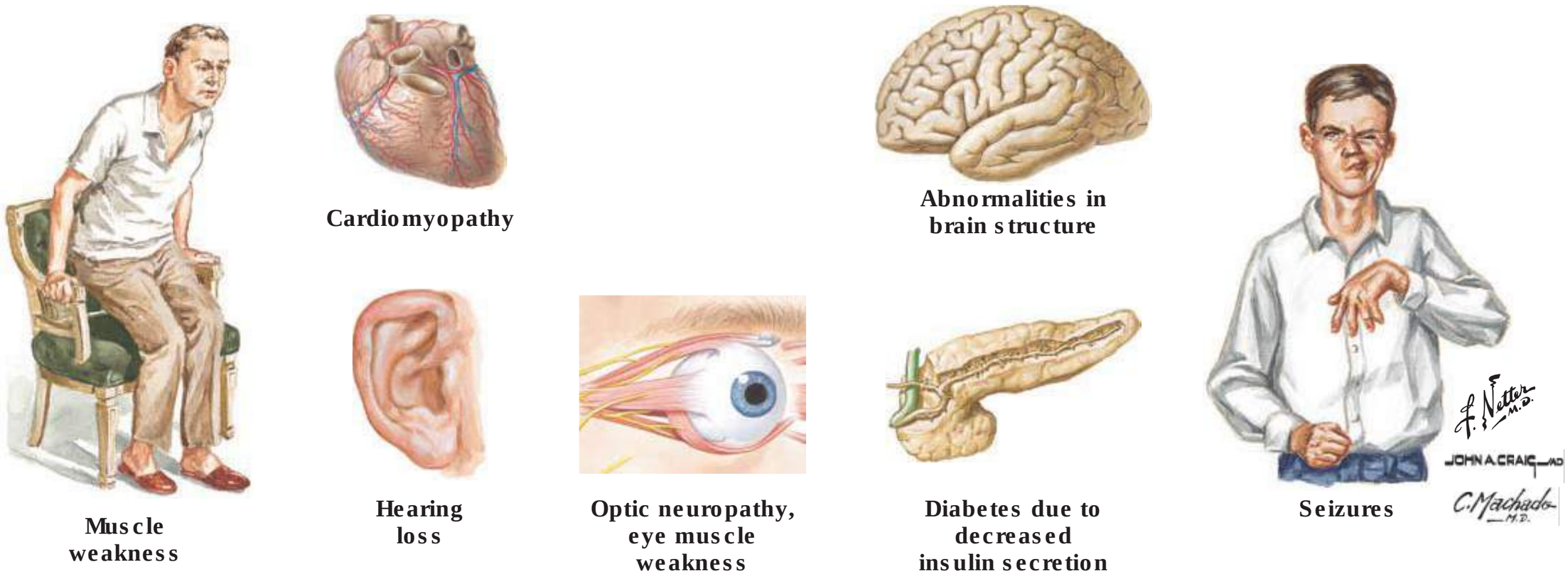


Fig. 23.9 Manifestations of diseases involving mitochondria. Such a disease may affect more than one organ system.

treatment), or they can be of unknown origin. Mutations in **nuclear DNA** show a mendelian pattern of inheritance, whereas mutations in **mtDNA** show a maternal pattern of inheritance. For defects in oxidative phosphorylation to become clinically manifest, there is usually a **threshold** ef ect (i.e., a certain minimal amount of mutant mtDNA must be present). T is threshold depends on the energy needs of a tissue. Hence, the pattern of inheritance of mtDNA mutations may be difficult to interpret because patients with a mutant mtDNA load below the threshold do not exhibit the disease.

Some patients who have a mitochondrial disease benefit from supplements. Supplemental **thiamine** may increase the activity of pyruvate dehydrogenase and α-ketoglutarate dehydrogenase. **Riboflavin** gives rise to flavin mononucleotide (FMN) and FAD, which are used by enzymes that feed into the electron transport chain. Reduced **coenzyme Q10** has a role both as an antioxidant and as an electron transporter. **Ascorbate** works as an antioxidant (see Chapter 21). **Creatine** supplements can markedly increase the creatine content of muscle and brain tissue, which may improve delivery of ATP to peripheral points of cells. **Carnitine** can free up CoA when high concentrations of acyl-CoA are present due to acidemia (see Chapter 27).

4.2. Diseases Associated With mtDNA Mutations

Disease is generally apparent when more than about 60% of the mtDNA is mutant mtDNA, but the thresholds vary by tissue.

Mitochondrial diseases that are symptomatic in the newborn period are often accompanied by lactic acidosis, cardiomyopathy, and hyperammonemia.

T e **diagnosis** of a mitochondrial disease often involves an **analysis of mtDNA**. T e mtDNA can be obtained from kidney epithelial cells in the urine, white blood cells, buccal cells, or muscle cells.

When diseased mitochondria accumulate in myocytes, they give rise to so-called **ragged red fibers** in a trichrome-stained muscle biopsy.

All patients with **Kearns-Sayre syndrome** have a progressive external ophthalmoplegia, show atypical pigment degeneration of the retinae, and experience the onset of symptoms before age 20 years. Many patients have a conduction disorder of the heart or are at high risk of developing one, followed by premature death. Most of these patients have a **large deletion of mtDNA** (often ~5 kb, which is ~30% of the mtDNA) that occurs sporadically (i.e., the disease is not inherited).

T e **A3243G mutation in mtDNA** gives rise to maternally inherited diabetes and deafness (**MIDD**) and sometimes mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (**MELAS**). T e A3243G mutation is in the gene that encodes one of the two mitochondrial $tRNA^{Leu}$. T e mutation is found in 1 in 500 to 15,000 people, depending on the population, with many patients remaining undiagnosed. T e mutation leads to diminished synthesis of all proteins of oxidative phosphorylation that are encoded by mtDNA. T e milder deficiency in oxidative phosphorylation manifests itself with MIDD in adulthood, whereas more severe deficiencies are associated with MELAS and onset during childhood or young adulthood.

Leigh syndrome is a progressive neurodegenerative disorder. T ere are many dif erent genetic causes of Leigh syndrome. Mutations can be in the mtDNA or nuclear DNA, and they af ect a gene that encodes a protein of the electron transport chain, the ATP synthase, or the pyruvate dehydrogenase complex. In many patients, the genetic cause of the disease is unknown. Disease onset is typically before age 2 years. T ere is a wide spectrum of disease manifestations, of which the more common are regression of development, seizures, impaired control of muscles, and lactic acidosis. T e diagnosis rests in part on magnetic resonance imaging showing symmetric necrotic lesions in the brain.

4.3. Diseases Associated With Dysfunctional Mitochondria Due to Mutation in the Nucleus

Mutations in genes in the nucleus can af ect one of the many components of the electron transport chain, ATP synthase, proteins that play a role in the transport and assembly of proteins in mitochondria, or anything else that af ects the function of mitochondria.

Huntington disease (Fig. 23.10) is an autosomal dominantly inherited disorder that is due to an expanded trinucleotide repeat in an exon of the huntingtin gene, which leads to an aggregation of huntingtin and severe defects in the neurons of the striatum. It af ects about 1 in 15,000 people. T e disease often becomes evident when patients are in their 40s. Patients lose control of their movements and some

Fig. 23.10 Huntington disease. Affected patients lose control over motor movements.

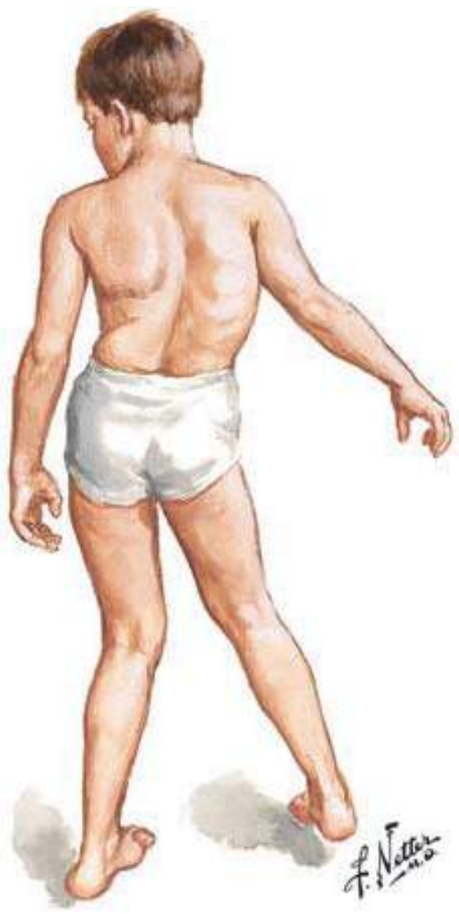


Fig. 23.11 Friedreich ataxia. The disease presents with progressive ataxia, a wide gait, and scoliosis.

Fig. 23.12 Parkinson disease. Patients have tremors and gait disturbances.

cognitive functions. Mitochondria most likely play a role in the neurodegeneration. There is a reduced capacity for oxidative phosphorylation, but the role of this deficit in the overall disease process is unclear.

Friedreich ataxia (Fig. 23.11) is an autosomal recessively inherited disease that is due to a trinucleotide repeat expansion in the FXN gene that leads to a **frataxin** deficiency in mitochondria. The prevalence is about 1 in 50,000. Frataxin likely plays a role in the insertion of iron into proteins that contain iron-sulfur clusters, such as complexes I, II, and III of the electron transport chain, and aconitase of the citric acid cycle (see Chapter 22). Frataxin deficiency also leads to iron overload of the mitochondria, which may increase oxidative stress. Friedreich ataxia is associated with the degeneration of the peripheral nervous system, central nervous system, heart, and pancreatic β-cells.

4.4. Idiopathic or Acquired Diseases of Mitochondria

In **Parkinson disease** (Fig. 23.12) the membrane potential of mitochondria is reduced (suggesting impaired ATP production via oxidative phosphorylation), and there is evidence that an inadequate turnover of mitochondria (mitophagy), impaired Ca^{2+} homeostasis by mitochondria, an increased load of mutant mtDNA, and mitochondria-induced increased apoptosis contribute to the pathology.

Turnover of mitochondria can be impaired, for example, by certain lysosomal storage diseases (e.g., Gaucher disease, due to the deficient degradation of glucocerebroside to glucose and ceramide) or by mutations in proteins that regulate turnover of mitochondria (e.g., parkin or PINK1, both of which are associated with hereditary, early-onset forms of Parkinson disease).

Some **drugs** are known to impair the function of mitochondria. Mitochondria evolved from bacteria. **Aminoglycosides** (e.g., streptomycin, kanamycin, neomycin, gentamicin, tobramycin, and amikacin) inhibit the function of mitochondrial ribosomes and can impair hearing when used systemically; they are also neurotoxic and nephrotoxic. **Chloramphenicol** affects mitochondria such that hematopoiesis may be impaired. **Linezolid** decreases protein synthesis in mitochondria and may lead to lactic acidemia or even peripheral and optic neuropathy. Of the **antiretroviral drugs** that have been developed for the treatment of HIV, those with the highest affinity for DNA-polymerase gamma (the DNA polymerase for replication of mtDNA inside mitochondria) showed considerable toxicity to mitochondria, such that their use is no longer recommended.

SUMMARY

- Oxidative phosphorylation takes place in the mitochondria and provides most of the body's ATP. The electron transport chain reduces oxygen to water and thereby pumps protons into the intermembrane space. The ATP synthase uses the proton electrochemical gradient for the synthesis of ATP.
- The electron transport chain receives input chiefly from NADH, reduced electron-transferring flavoprotein, glyceraldehyde 3-phosphate, and succinate.
- The electron transport chain consists of four multisubunit complexes (three of which pump protons), and the two electron carriers ubiquinol and reduced cytochrome c.
- An adenine nucleotide translocator transports ADP into and ATP out of mitochondria. Chiefly in muscle and the brain, creatine and phosphocreatine facilitate the transport

of chemical energy from the mitochondria to sites of consumption in the cytosol; phosphocreatine is also an energy reserve.

- Hypoxia, uncouplers, and inhibitors of oxidative phosphorylation reduce ATP production in mitochondria, which leads to a compensatory activation of anaerobic glycolysis that may lead to lactic acidemia. Inhibitors of oxidative phosphorylation decrease oxygen consumption, and uncouplers increase it. Clinically relevant inhibitors of oxidative phosphorylation are metformin, cyanide, carbon monoxide, sodium azide, and hydrogen sulfide. The uncoupling protein UCP-1 serves the purpose of heat production in brown fat.
- Mitochondria contain their own DNA, which encodes subunits of complexes I, III, and IV as well as the ATP synthase. In addition, mtDNA encodes the rRNAs and tRNAs needed for translation inside mitochondria. Each cell typically contains thousands of copies of mtDNA. mtDNA is passed to offspring by their mothers.
- Impaired oxidative phosphorylation plays a role in the pathogenesis of most mitochondrial diseases. However, an impaired turnover of mitochondria, impaired control of Ca^{2+} in the cytosol, acquired mutations in mtDNA, excessive apoptosis, and increased production of reactive oxygen species (ROS) often also participate.
- Mitochondrial diseases preferentially involve tissues that have high demands for energy and depend on mitochondria for proper function. Affected patients often present with dysfunction of the nervous system, musculature, auditory perception, or pancreatic β-cells.
- Antimicrobial drugs such as aminoglycosides, chloramphenicol, and linezolid impair the function of mitochondria and must be administered with appropriate precautions.
- A mutation in a mitochondrial $tRNA^{Leu}$ gives rise to maternally inherited diabetes and deafness (MIDD) or mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS). A large deletion of mtDNA gives rise to Kearns-Sayre syndrome.
- Leigh syndrome is characterized by symmetrical necrotic lesions in the brain and has many different causes, either in nuclear or mitochondrial DNA.
- Friedreich ataxia is due to defective iron metabolism in mitochondria caused by mutant nuclear-encoded frataxin.
- Huntington disease is due to mutant, nuclear-encoded huntingtin, and impaired oxidative phosphorylation plays a role in the loss of motor control.
- Parkinson disease is most often an idiopathic or acquired disease with multifaceted dysfunction of mitochondria.

FURTHER READING

- Borron SW, Bebarta VS. Asphyxiants. Emerg Med Clin North Am. 2015;33:89-115.
- DiMauro S, Schon EA, Carelli V, Hirano M. The clinical maze of mitochondrial neurology. Nat Rev Neurol. 2013;9:429-444.
- Perier C, Vila M. Mitochondrial biology and Parkinson's disease. Cold Spring Harb Perspect Med. 2012;4:a009332.

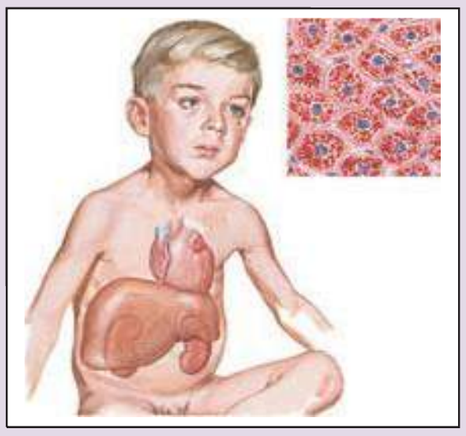
Review Questions

1. A patient with carbon monoxide poisoning is best treated with which one of the following?

   A. Hydroxocobalamin
   B. O_2
   C. Sodium nitrite
   D. Sodium thiosulfate

2. A 5-month-old infant with a selective deficiency in one of the subunits of complex I most likely presents with which of the following?

   A. Leigh syndrome
   B. Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS)
   C. Maternally inherited diabetes and deafness (MIDD)



Chapter

24

Glycogen Metabolism and Glycogen Storage Diseases

SYNOPSIS

- Glycogen is a branched polymer of glucose that is present in a granular form in the cytosol of virtually every cell. The largest glycogen stores are in muscle and the liver.
- Fig. 24.1 shows the basic reactions of glycogen metabolism along with connections to other metabolic pathways in the liver.
- After a meal, muscle and liver synthesize glycogen. During exercise, muscle degrades its glycogen for its own use, and the liver degrades some of its glycogen to provide muscle with glucose. During an overnight fast, the liver degrades some of its glycogen and releases glucose into the blood for the benefit of other tissues.
- Lysosomes continually degrade glycogen particles at a low rate.
- Glycogen storage diseases (glycogenoses) are quite rare; their combined incidence is about 1:20,000. Affected patients may be glucose intolerant (and thus at an increased risk of developing diabetes), have fasting hypoglycemia, develop a myopathy, or have seizures.

LEARNING OBJECTIVES

For mastery *of* this topic, you should be able to do the *f*ollowing:

- Describe the reactants, products, and tissue distribution of glycogen synthesis and glycogenolysis.
- Compare and contrast how feeding, fasting, and exercise influence glycogen synthesis and glycogenolysis in the liver and skeletal muscle.
- Explain the contribution of glycogenesis and glycogenolysis to blood glucose homeostasis during the fed state, the fasting state, and exercise.
- Explain the role of muscle glycogen in exercise.
- Explain the pathogenesis of hypoglycemia in patients who have glucose 6-phosphatase deficiency.
- List the enzyme deficiencies that give rise to the most common hereditary glycogenoses and predict their effects on blood glucose concentration, the amount of tissue glycogen, and damage to tissues.
- Compare and contrast the pathogenesis and pathology of Pompe disease (lysosomal acid maltase deficiency) and Lafora disease.

1. SYNTHESIS OF GLYCOGEN (GLYCOGENESIS)

A glycogen particle consists o*f* glycogenin and a branched polymer o*f* glucose. Under most circumstances o*f* glycogen synthesis, an existing glycogen particle is enlarged. Less o*ft*en, a new particle is started *f*rom glycogenin. Glycogen is synthesized in the liver and muscle a*ft*er a carbohydrate meal and in muscle also a*ft*er exercise. Glycogen synthase adds glucose *f*rom an activated *f*orm, uridine diphosphate (UDP)-glucose. Glycogen branching enzyme creates a branch *f*rom a linear glucose polymer chain.

1.1. Structure and Role of Glycogen

Glycogen consists of a branched polymer of glucose that is formed on a tyrosine side chain of the **glycogenin** protein (Fig. 24.2). T e glucose residues are mostly linked in α(1→4) fashion, and occasionally in α(1→6) fashion to create branch points. Branching increases the solubility of glycogen.

During glycogen synthesis and degradation, there are limits for particle size. T e smallest particles contain about 2,000 glucosyl residues, the largest about 60,000. Small glycogen particles are also called **proglycogen**, large ones **macroglycogen**.

Glycogen particles are visible by electron microscopy after staining with a heavy metal, or by light microscopy after treatment with **periodic acid–Schif (PAS) stain**, which generates a colored complex (Fig. 24.3). T e iodine binds into the left-handed helices of glucose moieties in the linear portions of glycogen. Normal muscle glycogen particles have a diameter of up to 0.04 µm. In the liver, rosettes form that contain 20 to 40 such particles.

Muscle and liver store the largest amounts of glycogen; most other cells store only a small amount of glycogen. T e liver of a typical, healthy, postprandial adult contains up to about 100 g of glycogen (i.e., about 7% of the wet weight of the liver); in the absence of exercise, the skeletal muscles contain up to about 400 g of glycogen (or about 2% of the wet weight of muscle). If a person exercises to exhaustion and then consumes a meal very rich in carbohydrates, the exercised muscles can contain as much as 5% of their wet weight as glycogen.

Glycogen synthesis and breakdown help even out the concentration of glucose in the blood in the course of a day. Glycogen in liver and muscles is synthesized chiefly during the first few hours after a meal (Fig. 24.4). In the subsequent fasting period, when glucose use exceeds glucose influx from the intestine, liver glycogen is degraded to glucose, which is released into the blood; this helps maintain a normal fasting concentration of glucose in the blood. Muscles degrade their glycogen during exercise to provide energy for contraction. Muscles do not release glucose into the blood, but the degradation of intracellular glycogen reduces the need for glucose uptake from the blood into muscle.

1.2. Reactions of Glycogen Synthesis

Glucose is activated to UDP-glucose, from which an additional glucose residue can be added to glycogen (Fig. 24.5). Glycogen synthesis takes place in the cytosol. Glycogen synthesis requires a modest amount of energy in the form of UTP,

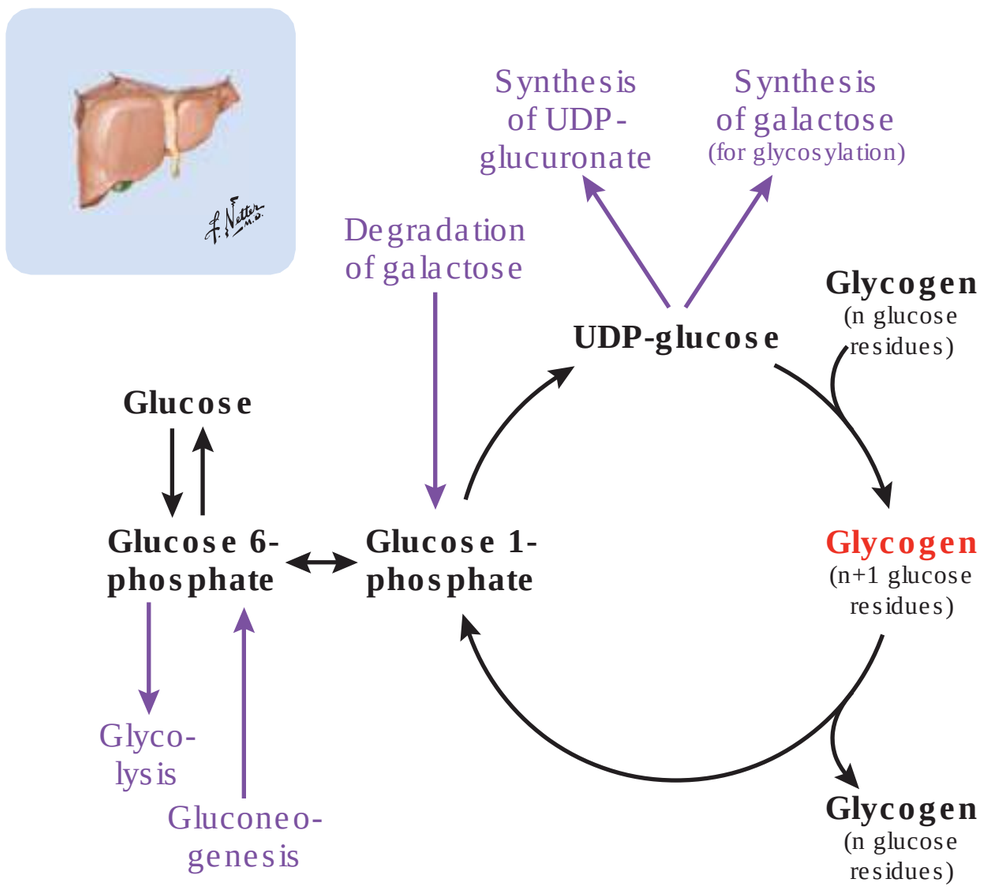


Fig. 24.1 Position of glycogen metabolism in overall metabolism in the liver. UDP, uridine diphosphate.

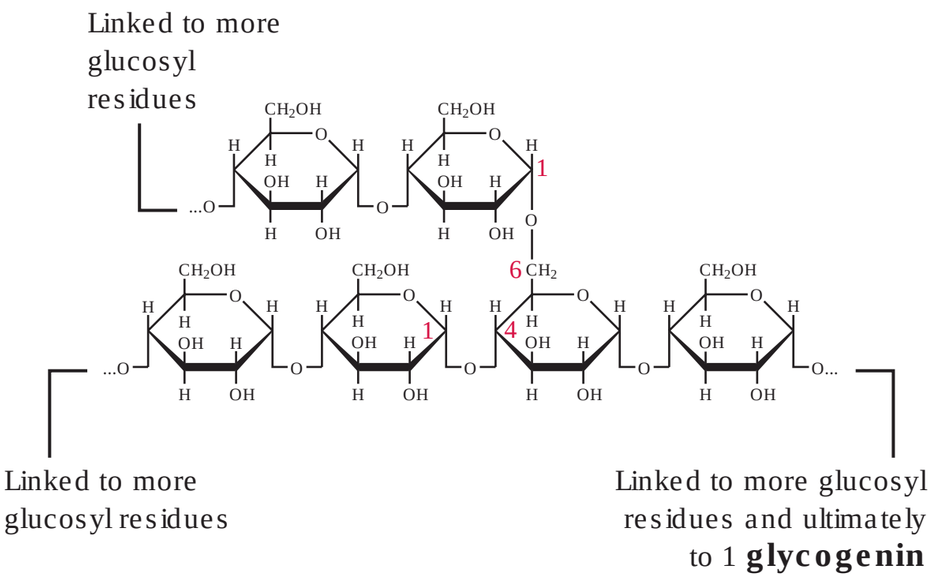


Fig. 24.2 Partial structure of glycogen. Red numbers reflect the standard nomenclature for numbering carbons in sugars.

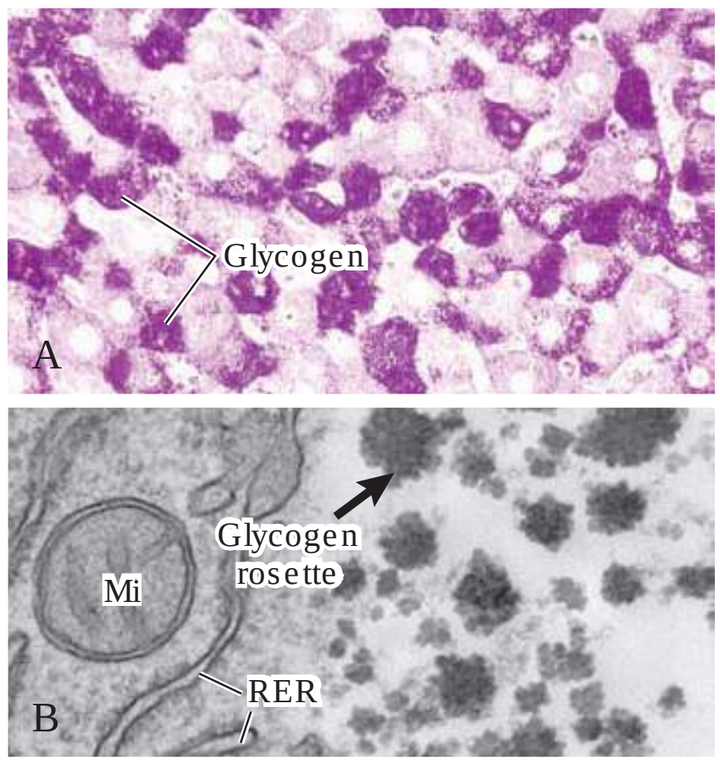


Fig. 24.3 Glycogen in the liver. (A) Light microscope image of PAS (periodic acid–Schiff)-stained tissue. (B) Transmission electron micrograph. Mi, mitochondrion; RER, rough endoplasmic reticulum.

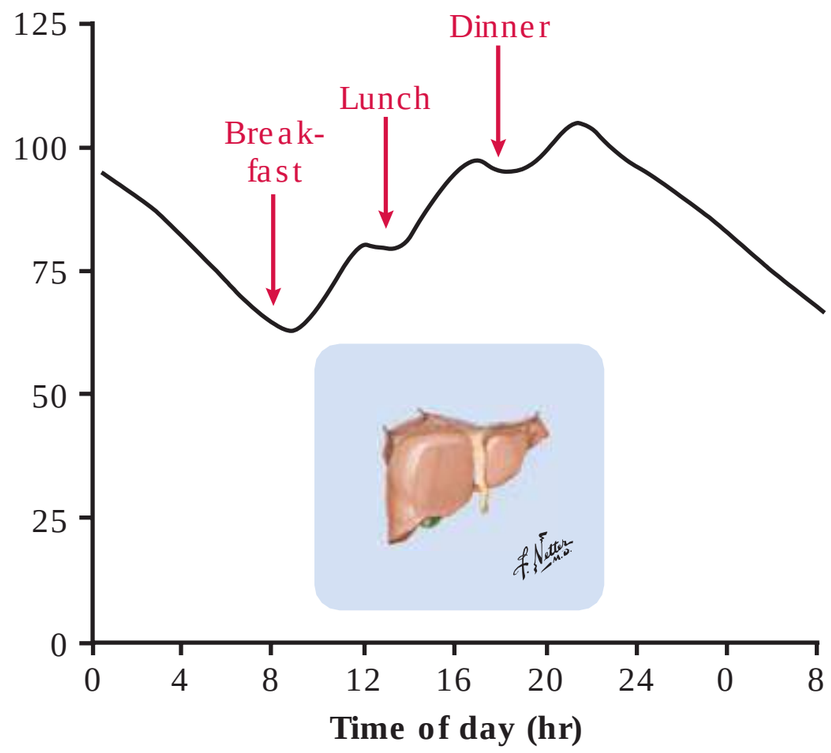


Fig. 24.4 Approximate daily time course of the amount of glycogen in the liver of resting volunteers. Data are based on 13C magnetic resonance spectroscopic measurements. Volunteers consumed weight-maintaining mixed meals. (Data from Hwang J-H, Perseghin G, Rothman DL, et al. Impaired net hepatic glycogen synthesis in insulin-dependent diabetic subjects during mixed meal ingestion; a 13C nuclear magnetic resonance spectroscopy study. *J Clin Invest*. 1995;95:783-787; Taylor R, Magnusson I, Rothman DL, et al. Direct assessment of liver glycogen storage by 13C nuclear magnetic resonance spectroscopy and regulation of glucose homeostasis after a mixed meal in normal subjects. *J Clin Invest*. 1996;97:126-132; and Krssak M, Brehm A, Bernroider E, et al. Alterations in postprandial hepatic glycogen metabolism in type 2 diabetes. *Diabetes*. 2004;53:3048-3056.)

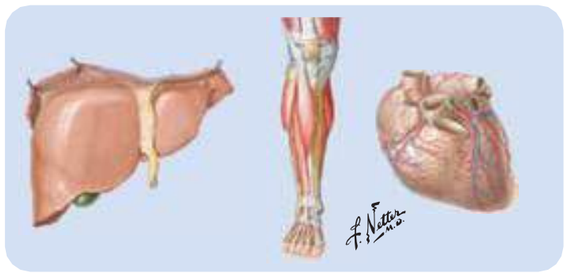
which in turn is made with the help of ATP. Significant glycogen synthesis occurs in muscle and the liver.

The **glycogen branching enzyme** (recommended name: **1,4-α-glucan branching enzyme**) introduces α(1→6) branches (Fig. 24.6). The branching enzyme cuts a stretch of linear, α(1→4)-linked terminal glucose residues and links carbon-1 of this stretch to carbon-6 of an upstream glucose residue, thus generating an α(1→6) glucosidic linkage that starts a new branch. Such branching increases the solubility of glycogen. A deficiency in branching is associated with cell damage (see Section 3.3).

In a healthy person, the center of a glycogen particle is more highly branched than the periphery, and the peripheral half of the weight of a glycogen particle consists of linear branches.

1.3. Regulation of Glycogen Synthesis

Skeletal muscle synthesizes glycogen in response to depleted glycogen stores; this synthesis is strongly enhanced by insulin. Antecedent exercise and an elevated concentration of insulin each increase both the number of **glucose transporters** in the plasma membrane and the activity of **glycogen synthase** in the cytosol (see Fig. 24.5). As a result of these control mechanisms, postexercise glycogen synthesis proceeds at a relatively low rate in the fasting state, and at a markedly higher rate after a carbohydrate-containing meal. During extended exercise, an elevated concentration of epinephrine prevents glycogen synthesis.



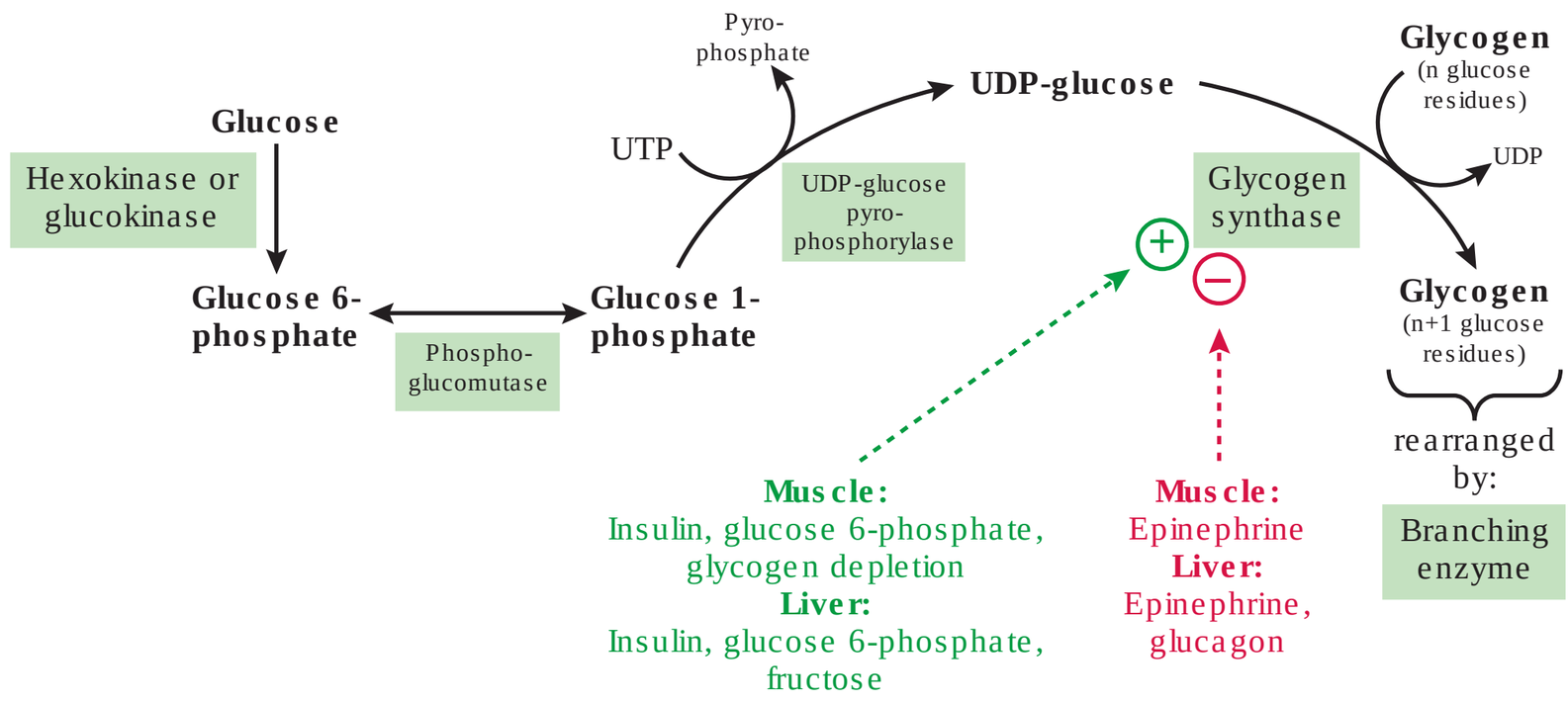


Fig. 24.5 **Glycogen synthesis.** For details of the branching enzyme, see Fig. 24.6. UDP, uridine diphosphate; UTP, uridine triphosphate.

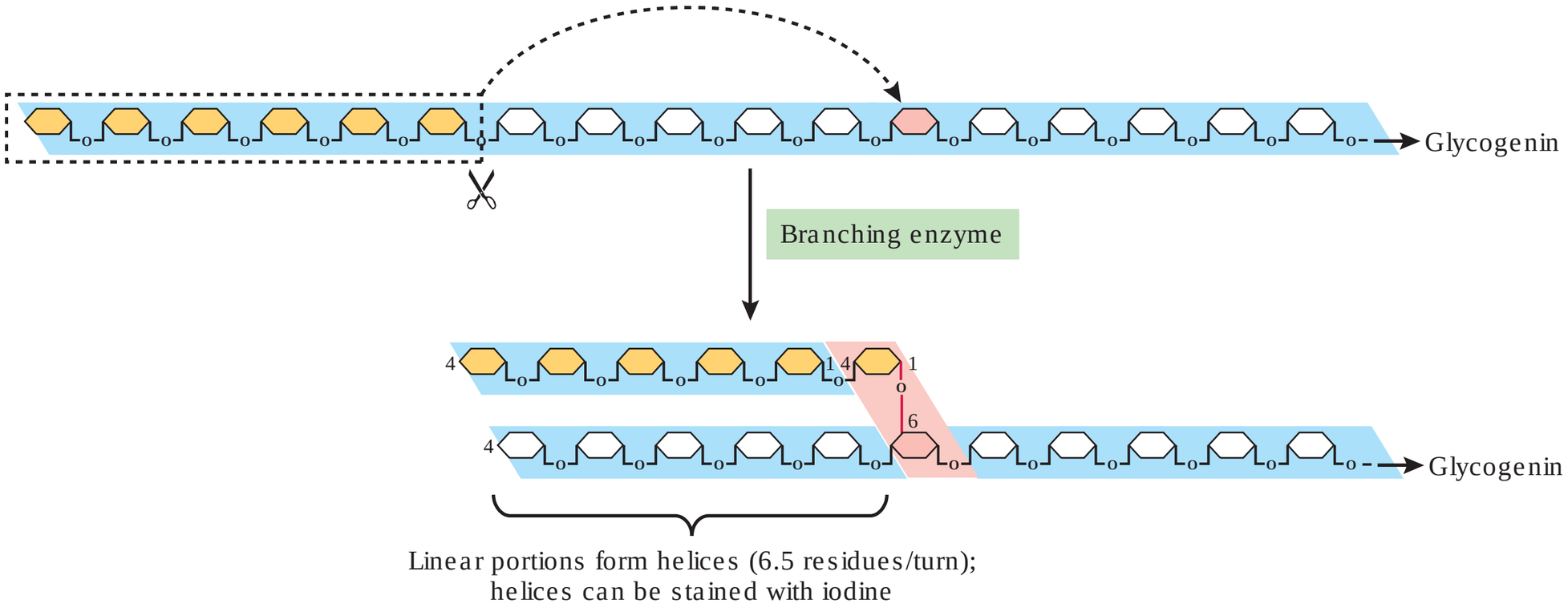


Fig. 24.6 **Introduction of branch points into glycogen by the glycogen branching enzyme.**

T e higher the carbohydrate content of the diet, the higher the muscle glycogen stores. In the short term, a **diet** with greater than 90% of calories from carbohydrate can lead to three- to fourfold greater muscle glycogen stores than a diet of less than 10% carbohydrate. In the long term, dif erences between low- and high-carbohydrate diets are smaller.

Athletes can maximize their endurance by maximizing their muscle glycogen stores. To this end, they can deplete muscles of glycogen through intense exercise, followed by 2 to 3 days of rest during which they consume a high-carbohydrate diet. Such a regimen leads to approximately double the normal glycogen stores, a phenomenon called **supercompensation**. To make glycogen stores peak near the start of a competition, athletes often consume a high-carbohydrate meal a few hours before exercise starts.

In the **heart**, glycogen depletion due to an acute increase in workload or due to ischemia subsequently stimulates glycogen synthesis. Filled glycogen stores have a favorable ef ect on maximal power output and hypoxia tolerance.

In the **liver** (see Fig. 24.5), glucose, fructose, and insulin are the main stimuli for glycogen synthesis. Dietary fructose, glucose, and insulin receptor signaling activate glucokinase, which leads to an increased concentration of glucose 6-phosphate. Glucose 6-phosphate and insulin signaling enhance glycogen synthase activity, which is the main determinant of the rate of glycogen synthesis.

2. DEGRADATION OF GLYCOGEN (GLYCOGENOLYSIS)

Glycogen phosphorylase degrades the linear portions of glycogen to glucose 1-phosphate, which is in equilibrium with glucose 6-phosphate. Glycogen phosphorylase ends its activity a few residues before a branch point. The glycogen debranching enzyme then moves the remaining short, linear chain of glucosyl residues to the end of another linear chain and produces glucose from the glucosyl residue at the branch point. In the liver, glucose 6-phosphatase dephosphorylates glucose 6-phosphate to glucose for export into the blood. Muscle does not have glucose 6-phosphatase and does not export glucose.

As part of the turnover of cell components, lysosomes occasionally engulf glycogen particles. Inside lysosomes, acid α-glucosidase degrades glycogen particles to glucose.

2.1. Degradation of Glycogen to Glucose 6-Phosphate and Glucose

Glycogen degradation takes place in muscle during exercise and in the liver during the first day of fasting.

Glycogen phosphorylase catalyzes the phosphorolysis of glycogen to form glucose 1-phosphate, which is then isomerized to glucose 6-phosphate (Fig. 24.7). Glycogen phosphorylase activity is rate limiting. The isomerization of glucose 6-phosphate and glucose 1-phosphate (a reversible reaction) is part of both glycogen synthesis and glycogen degradation (as well as the degradation of galactose; see Chapter 20).

The degradation of glycogen near α(1→6) branch points requires glycogen debranching enzyme activity. Once a linear branch of glycogen is only four glucosyl residues long, glycogen phosphorylase can no longer shorten it. Glycogen that has all of its linear branches shortened maximally by glycogen phosphorylase is called a **limit dextrin**. The **debranching enzyme** (Fig. 24.8) cleaves the remaining stretch of three linearly linked glucosyl residues (i.e., a maltotriose unit) and transfers it to the C-4 end of another linear portion of glycogen. Next, the debranching enzyme produces glucose from the remaining glucosyl residue that forms the branch point. The degradation of glycogen by the combined actions of glycogen phosphorylase and debranching enzyme thus yields mostly glucose 1-phosphate and some glucose.

A glucose 6-phosphatase in the endoplasmic reticulum hydrolyzes glucose 6-phosphate to produce glucose (Fig. 24.9). The hydrolysis requires three different activities: (1) a **glucose 6-phosphate/phosphate antiporter** (encoded by the SLC37A4 gene) in the membrane of the endoplasmic reticulum, (2) **glucose 6-phosphatase** activity that hydrolyzes glucose 6-phosphate to glucose + phosphate, and (3) a **glucose transporter** that releases glucose from the endoplasmic reticulum into the cytosol.

Glucose 6-phosphatase activity is somewhat increased by glucagon and epinephrine, whereas insulin decreases it.

Major hydrolysis of glucose 6-phosphate to glucose is seen in the liver (for glycogenolysis and gluconeogenesis) and the kidneys (for gluconeogenesis; see Chapter 25).

2.2. Regulation of Glycogenolysis

Glycogenolysis is mostly regulated by intracellular signals in muscle and extracellular signals in the liver.

In muscle, the three main types of **muscle fibers** (Table 24.1) differ in their metabolism. Most muscles contain several types of fibers, whereby the proportions depend on the function of the muscle (see Section 5.5 in Chapter 19). Exercise typically involves the use of several muscles, which together derive energy from intracellular glycogen and triglycerides, as well as from blood-derived glucose and fatty acids.

Blood flow to muscle becomes maximal only several minutes after the start of exercise; in the meantime, muscle glycogen provides the necessary extra fuel for adenosine triphosphate (ATP) generation, in part via anaerobic

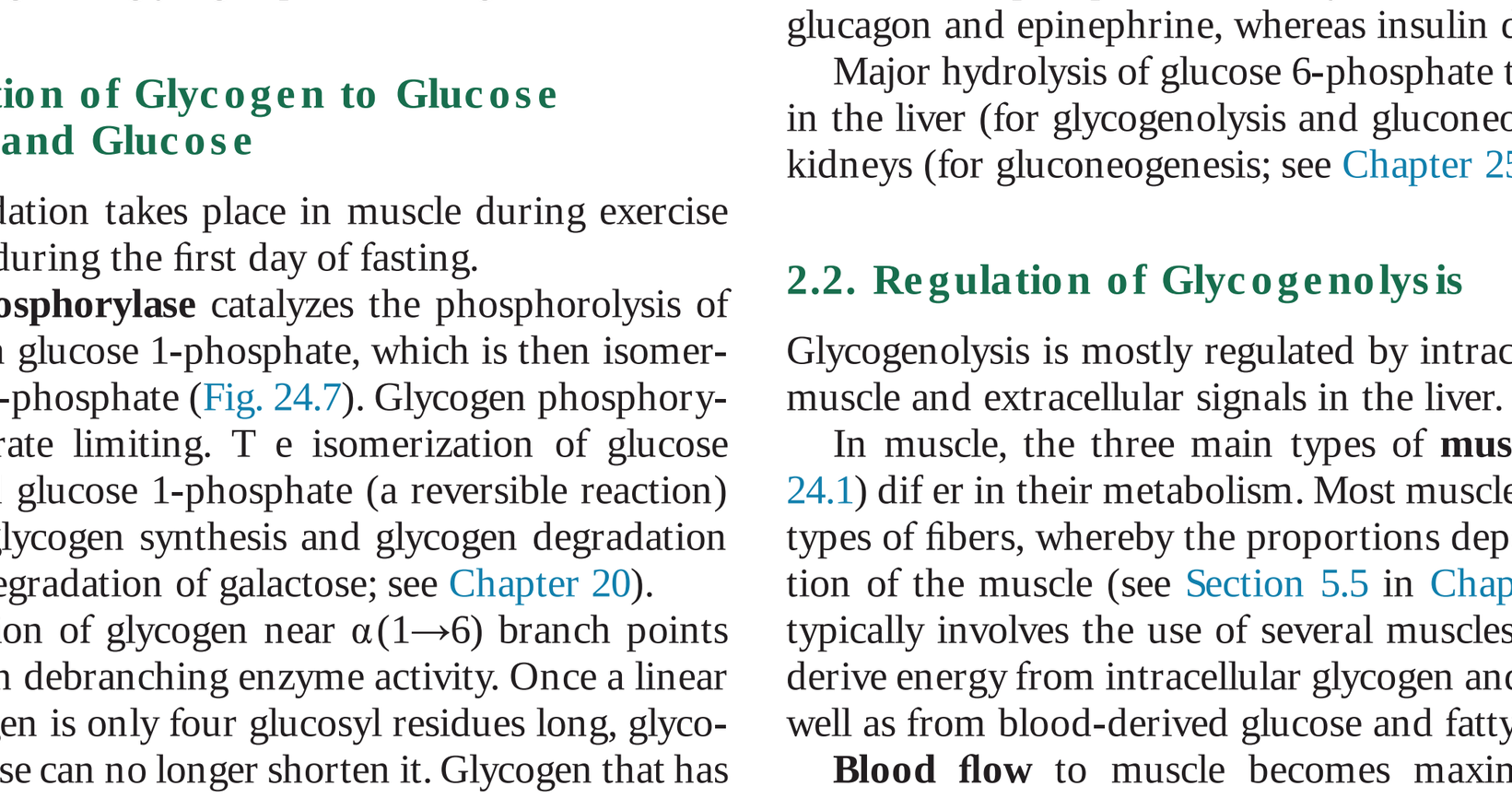


Fig. 24.7 Degradation of glycogen (glycogenolysis). For details of the debranching enzyme, see Fig. 24.8. AMP, adenosine monophosphate; ATP, adenosine triphosphate.

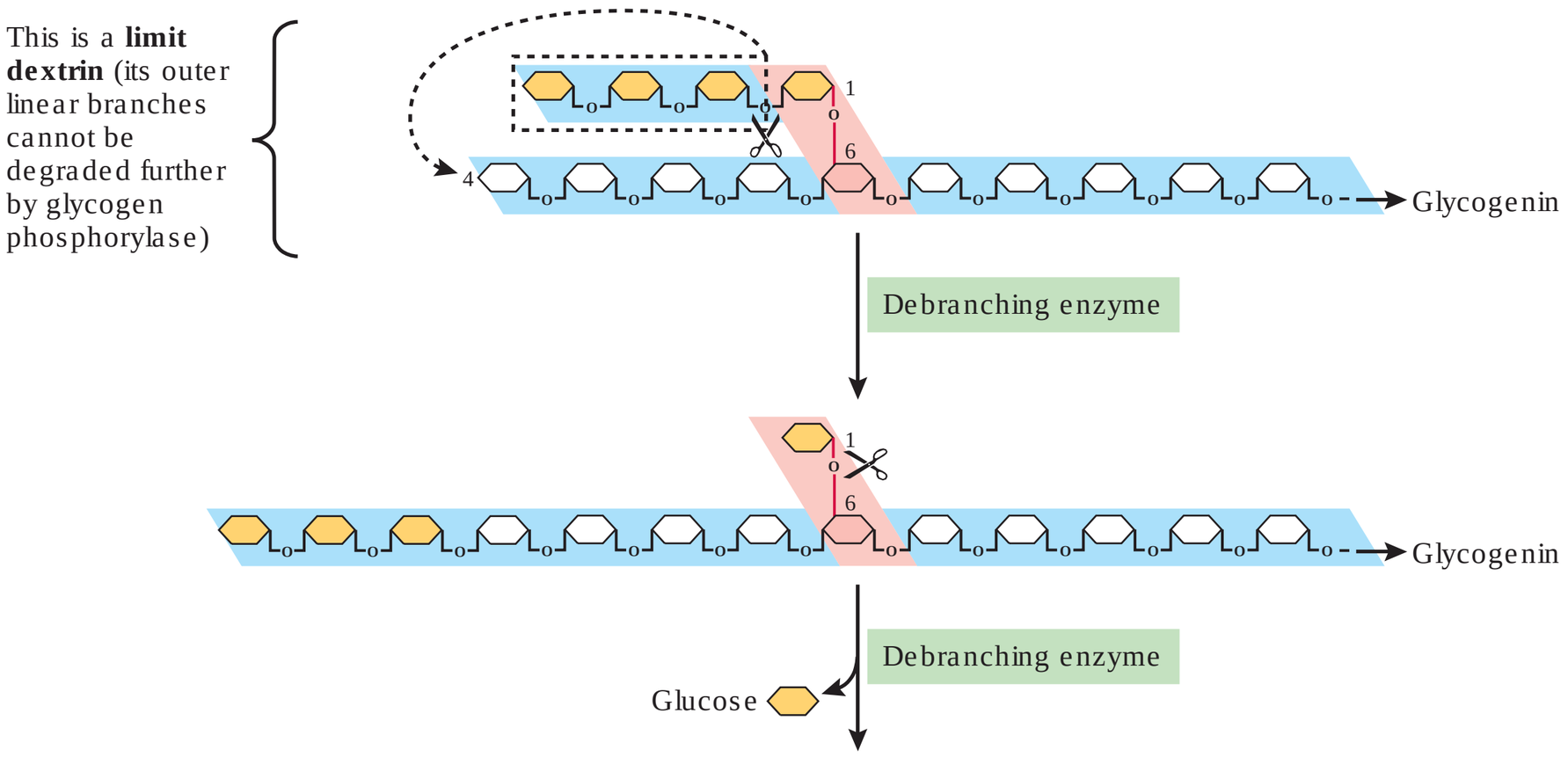


Fig. 24.8 **Mechanism of action of the glycogen debranching enzyme.**

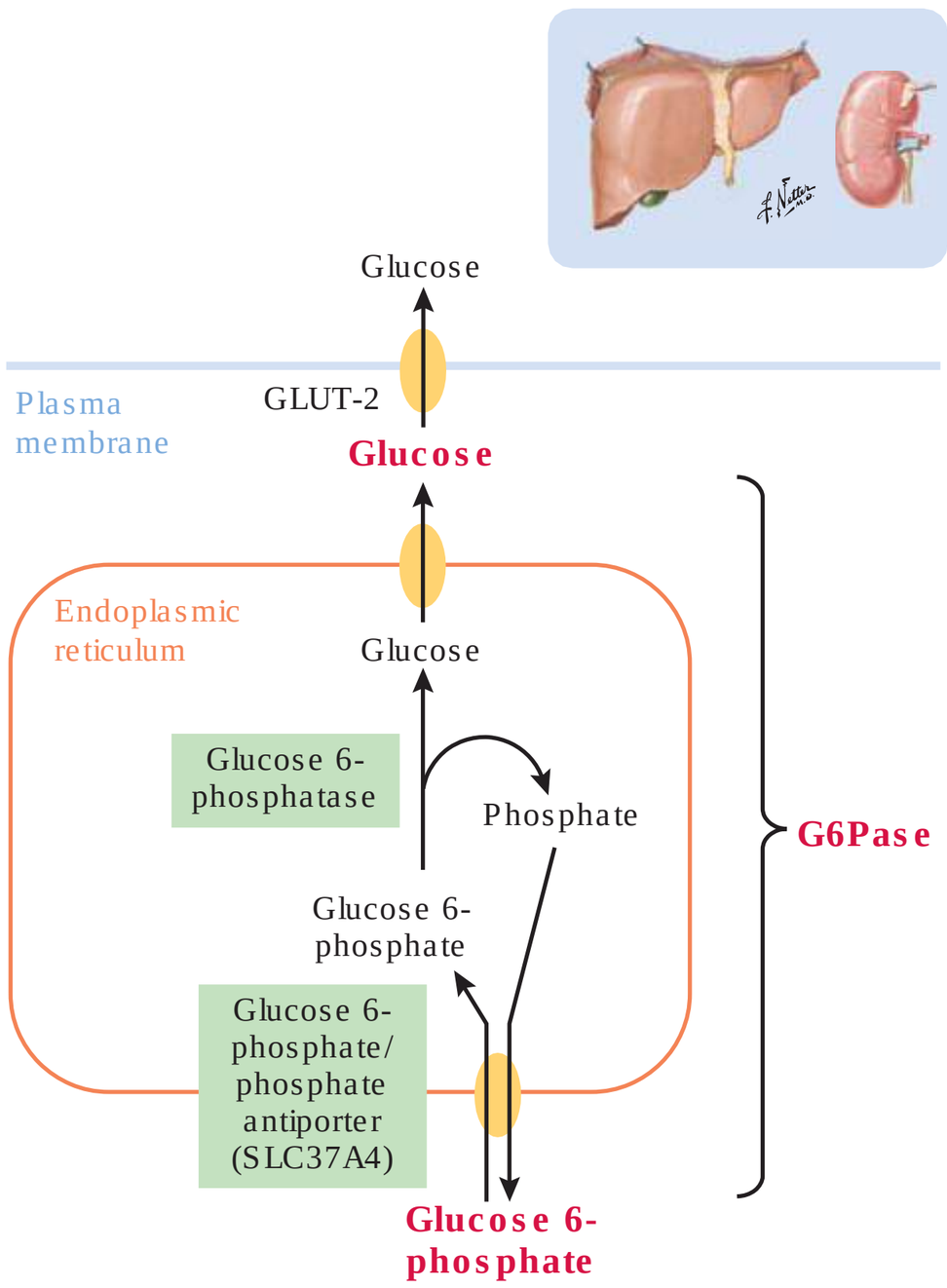


Fig. 24.9 **Production of glucose by glucose 6-phosphatase.** Glucose 6-phosphatase also plays a role in gluconeogenesis (see Chapter 25).

glycolysis. Increased blood flow eventually allows the muscle to use more oxygen and glucose from the blood.

With increasing **duration** of moderate-intensity exercise, muscles shift some of their energy production from carbohydrate to **fatty acid oxidation**. T ese fatty acids derive from increased hydrolysis of circulating very-low-density lipoprotein (VLDL) and intermediate-density lipoprotein (IDL) particles by muscle lipoprotein lipase, and from the increased hydrolysis of triglycerides inside the adipose tissue (see Chapter 28).

Persistent, intense exercise requires that some of the energy be produced from the degradation of muscle glycogen. Without glycogen degradation in muscles, a person's power output is limited to about half the output at the person's maximal oxidative capacity. T is is partly explained by the fact that a given amount of oxygen produces more ATP from glucose than from fatty acids.

With increasing **intensity** of exercise, muscles degrade glycogen at a faster rate. With exercise at less than 25% of a person's maximal aerobic capacity, glycogen use is small and ceases after about 30 minutes. Glycolysis then mainly degrades glucose that is taken up from the blood. In contrast, with exercise at ~80% of a person's maximal aerobic capacity, glycogen degradation after 30 minutes still accounts for about half the calories consumed by muscle. When most of the muscle glycogen is consumed, muscle **fatigue** sets in. T erefore, high-intensity exercise endurance critically depends on the size of muscle glycogen stores.

Skeletal muscles do not have glucose 6-phosphatase and therefore cannot convert glycogen-derived glucose 6-phosphate to glucose. Although glycogen debranching enzyme produces glucose from the (1→6)-branch points, the concentration of glucose in the cytosol of exercising muscle is still lower than in the extracellular space; hence, this glycogen-derived glucose does not leave the muscle (it enters glycolysis).

Within contracting muscle, an increase in the cytosolic concentration of $\mathbf{Ca^{2+}}$ activates glycogenolysis (see Fig. 24.7). T e Ca^{2+} stems from the endoplasmic reticulum in response to neural input.

Table 24.1 Muscle Fiber Types

Fiber Type	Composition and Metabolism	Fiber Speed	Onset of Fatigue	Example
1	Rich in mitochondria; oxidize carbohydrates, fatty acids, ketone bodies to CO_2	Slow twitch	Last	Soleus
2a	Combination of metabolism of type 1 and type 2x fibers	Intermediate twitch	Intermediate	
2x	Few mitochondria; mostly glycolysis	Fast twitch	First	Gastrocnemius

In contracting muscle, an increase in the concentration of adenosine monophosphate (**AMP**) activates both glycogen phosphorylase and glucose transport (see Fig. 24.7). Since contraction uses ATP, the concentrations of ADP and AMP in the cytosol are higher during exercise than at rest. ADP and AMP are in equilibrium via the reaction 2 ADP ↔ AMP + ATP (see Section 1 in Chapter 38). Incorporation of GLUT-4 transporters into the plasma membrane permits increased uptake of glucose from the blood.

During exercise, nerves stimulate the adrenal medulla to release **epinephrine**; epinephrine activates β-adrenergic receptors that in turn lead to increased glycogen phosphorylase activity and decreased glycogen synthase activity (see Figs. 24.5 and 24.7). Within 15 minutes of intense exercise, the concentration of epinephrine in the serum increases by a factor of ~10 (see Fig. 26.8).

Skeletal muscle expresses virtually no **glucagon receptors**; therefore, glucagon has no appreciable ef ect on muscle glycogen metabolism.

In the **heart**, ischemia or an acute increase in workload both stimulate glycogen degradation. At a low workload, the heart mostly uses fatty acids for energy generation, but as the workload increases, the heart uses progressively more glucose and glycogen because ATP generation from glucose requires less oxygen than ATP generation from fatty acids. During ischemia, the heart degrades glucose 6-phosphate from glycogen mostly to lactate.

In the **liver**, as postprandial carbohydrate influx from the intestine fades, liver glycogen increasingly serves as a source of glucose to maintain a physiological concentration of glucose in the blood. After a 15-hour fast, liver glycogenolysis typically accounts for about one-third of the body's glucose production (the other two-thirds stem from gluconeogenesis; see Chapter 25).

Glucagon, **epinephrine**, **norepinephrine**, and extracellular **ATP** stimulate liver glycogenolysis, while **insulin** inhibits it (see Figs. 24.7). A pharmacological dose of glucagon can immediately activate liver glycogenolysis. **Diabetic** patients take advantage of this ef ect to counter insulin-induced hypoglycemia. A glucagon injection can also be used to test whether a patient's liver can degrade glycogen to glucose. Epinephrine and norepinephrine are released from the adrenal glands during exercise or hypoglycemia. Like glucagon, epinephrine is ef ective even in the presence of a significant concentration of insulin (epinephrine is not routinely used to counter hypoglycemia because it also af ects pulse rate and blood pressure). During **exercise**, norepinephrine and ATP are released from splanchnic nerve endings into the extracellular space.

Glucose and **fructose** both inhibit glycogenolysis (see Fig. 24.7). Glucose inhibits glycogen phosphorylase partly through direct allosteric inhibition and partly by creating a glucose-glycogen phosphorylase complex that is more readily inactivated through phosphorylation by protein phosphatase 1. T e fructose ef ect is partly due to direct inhibition of glycogen phosphorylase by fructose 1-phosphate, but a further understanding is currently lacking.

Some glycogen particles are engulfed by **lysosomes** and then degraded by **acid α-glucosidase** (also called **acid maltase**), which hydrolyzes both α(1→4) and α(1→6) glucosidic linkages, thereby exclusively producing glucose. T is process is presumably part of the normal turnover of all components of a cell; it does not contribute significantly to glucose production when glycogenolysis is activated. Lysosomes have a low pH (about 5). T e word "acid" in acid maltase refers to the lysosomal enzyme having optimum activity at a lower pH than does maltase on the brush border of small intestinal enterocytes (see Chapter 18); in fact, the two enzymes are encoded by dif erent genes.

3. DISORDERS OF GLYCOGEN METABOLISM

Diabetes is associated with reduced glycogen synthesis and degradation. Glycogen storage diseases are rare; glucose 6-phosphatase def ciency, debranching enzyme def ciency, and lysosomal α-glucosidase def ciency are the most common o*f* these disorders. Disorders that a*ff*ect the liver result in hepatomegaly and *f*asting hypoglycemia; disorders that a*ff*ect muscle cause weakness and cardiomyopathy.

3.1. Diabetes and Glycogen Metabolism

After a meal, patients with insulin-resistant **type 2 diabetes**, as well as those with **type 1 diabetes** who inject too little insulin, generally form less liver and muscle glycogen than healthy patients. Similarly, in the fasting state, patients with type 1 or type 2 diabetes degrade glycogen at an abnormally low rate.

Patients who are heterozygous for a mutant liver **glucokinase** with physiologically insufficient activity typically have

maturity-onset diabetes of the young subtype 2 (MODY-2) and form less glycogen in the liver (see Chapter 39). Although this is the most common form of MODY, fewer than 1% of patients with diabetes have MODY-2. In accordance with the notion that glucokinase activity in the liver is a key regulator of glycogen synthesis, patients with MODY-2 store glycogen in the liver at a reduced rate. Muscle glycogen synthesis in patients with MODY-2 is not appreciably af ected because muscle normally does not express glucokinase.

3.2. Fructose and Glycogen Metabolism

Patients with **hereditary fructose intolerance** (see Chapter 20) who are given fructose after an overnight fast have a reduced rate of glycogenolysis and become markedly hypoglycemic. T e hypoglycemia is in part due to diminished glycogenolysis, which is in turn due to the inhibition of glycogen phosphorylase by a persistently high concentration of fructose 1-phosphate. Since most phosphate is trapped in fructose 1-phosphate, the intracellular concentration of phosphate is low, which further lowers the activity of glycogen phosphorylase.

Patients with **fructose 1,6-bisphosphatase deficiency** also become hypoglycemic when given fructose after an overnight fast because they have a reduced rate of glycogenolysis and gluconeogenesis. In the fasting state, these patients perform little or no gluconeogenesis (see Chapter 25) and thus depend largely on glycogenolysis for glucose production. Glycogen phosphorylase is inhibited to a dangerous degree by a combination of an abnormally low concentration of free phosphate, a nearly normal concentration of fructose 1-phosphate, and elevated concentrations of fructose 1,6-bisphosphate and glycerol 3-phosphate (both of which are intermediates of gluconeogenesis; see Chapter 25).

3.3. Glycogenoses

In Europe, the combined incidence of all **glycogen storage disorders** (also called **glycogenoses**) is about 1:20,000. Almost all of these disorders are inherited in an autosomal recessive fashion, and the carrier frequency is therefore about 1%. Among patients with glycogen storage diseases, the following enzyme deficiencies make up about 90% of all patients in roughly comparable fractions: glucose 6-phosphatase deficiency, lysosomal acid α-glucosidase deficiency, debranching enzyme deficiency, and liver glycogen phosphorylase or phosphorylase kinase deficiency (Fig. 24.10, shown in red).

Type I glycogen storage disease (synonyms: **von Gierke disease, glucose 6-phosphatase deficiency**) has an incidence of about 1 in 100,000. In the fasting state, patients with glucose 6-phosphatase deficiency still release an appreciable amount of glucose into the blood, in part from yet unknown sources. Nonetheless, starting at a few months of age, af ected patients become severely hypoglycemic in the postabsorptive phase because their liver and kidneys cannot release sufficient glucose (from glycogenolysis or gluconeogenesis) into the blood. Hypoglycemia is particularly dangerous to the brain. During the day, small frequent meals help patients avoid hypoglycemia. At night, patients receive a constant infusion of glucose via a nasogastric tube, or they drink uncooked cornstarch in water every few hours (uncooked cornstarch is slowly hydrolyzed to glucose; see Chapter 18). T e liver has excessive glycogen stores because the elevated concentration of glucose 6-phosphate stimulates glycogen synthesis (Fig. 24.5). Fasting may be accompanied by lactic acidosis because gluconeogenesis is blocked (see Section 4.1.5 in Chapter 25). Similarly, the blockage in gluconeogenesis can generate ATP-consuming futile cycles that lead to hyperuricemia and an increased risk of gout (see Section 4.1 in Chapter 38). Severe

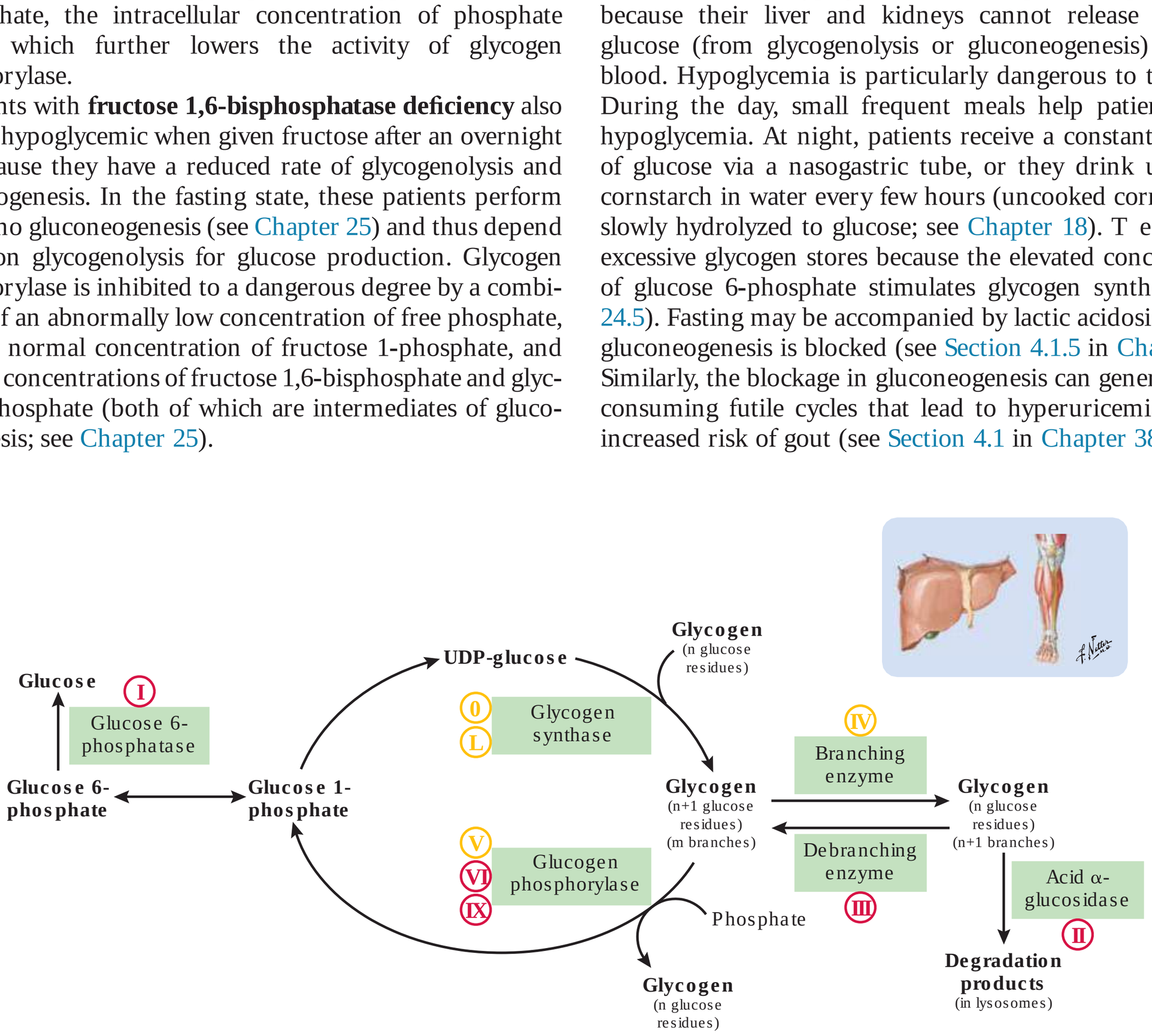


Fig. 24.10 Glycogen storage diseases. Disease types are shown as Roman numerals inside circles, next to the deficient enzyme; L designates Lafora disease. The enzyme deficiencies shown in red together account for about 90% of all cases. Some of the more rare diseases are shown in orange. Types 0, I, VI, and IX affect only the liver; type V affects only muscle. UDP, uridine diphosphate.

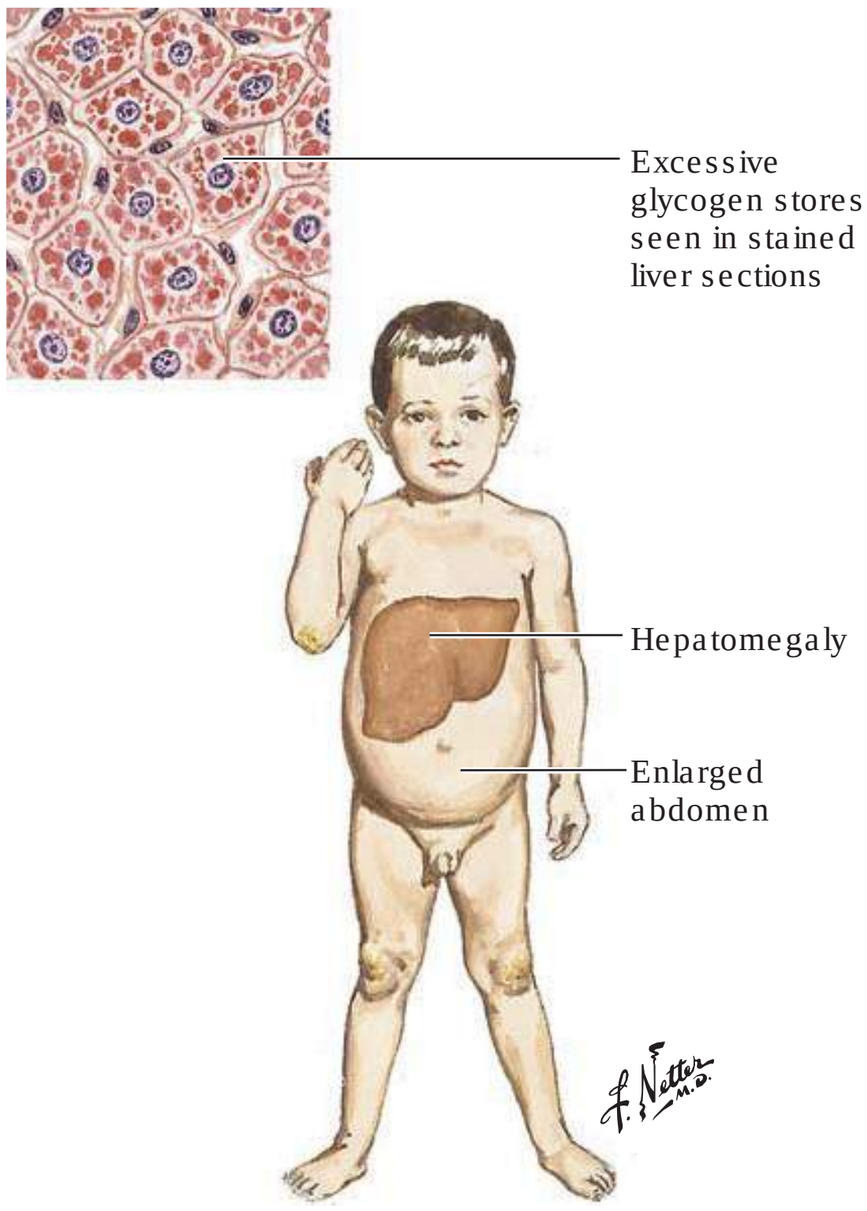


Fig. 24.11 Glucose 6-phosphatase deficiency (type I glycogen storage disease, von Gierke disease). Glycogen is stained with carminic acid, yielding a bright red product.

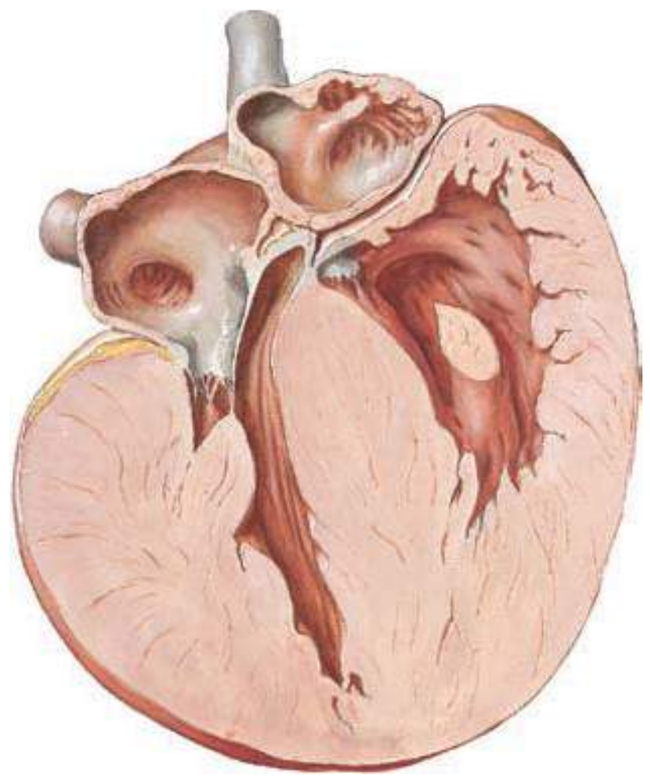


Fig. 24.12 Type II glycogen storage disease (Pompe disease, acid α-glucosidase deficiency, acid maltase deficiency). In classic, infantile Pompe disease, the accumulation of glycogen particles in the lysosomes leads to profound generalized myopathy and cardiomyopathy.

hyperlipidemia and hepatomegaly (Fig. 24.11) are discussed in Section 4.1.5 of Chapter 25.

Type II glycogen storage disease (synonyms: deficiency of lysosomal **α-glucosidase**, deficiency of lysosomal **acid maltase**, **Pompe disease**) has an incidence of about 1 in 40,000. In the infantile-onset form, the heart, liver, and muscles are enlarged (Fig. 24.12) and contain excessive amounts of glycogen in the lysosomes (visible with periodic acid staining; see also Fig. 24.3). Due to generalized muscle weakness, babies are "floppy" and, if not treated, die by age 2 years from cardiorespiratory insufficiency. Glucose metabolism is normal. Creatine kinase activity in the serum is increased due to the loss from damaged muscle. In patients with late onset (≥1 year of age), the heart is less severely affected, but respiratory weakness still leads to premature death. Treatment with alglucosidase alfa, a recombinant glucosidase (administered intravenously), dramatically alters the course of the disease. The enzyme replacement therapy greatly reduces damage to the heart, but the skeletal muscle is less responsive to treatment. A high-protein diet is used to favor maintenance of muscle mass.

Type III glycogen storage disease (synonyms: **debranching enzyme deficiency, Cori disease, Forbes disease, limit dextrinosis**) is the most common glycogen storage disease that affects both the liver and muscle (skeletal and cardiac). Hepatomegaly is common among children but not adults. Starting in childhood, patients have difficulty exercising, but muscle loss and cardiomyopathy often set in only during the 30s or 40s. Fasting hypoglycemia is more moderate than in a glucose 6-phosphatase deficiency because the outer linear branches of glycogen can still be degraded. Compared with a healthy individual, gluconeogenesis (see Chapter 25), lipolysis (see Chapter 28), and ketogenesis (see Chapter 27) are activated abnormally early. Glycogen particles are unusually large because branch points can be created but not degraded. Liver cirrhosis is occasionally seen in adults. Damage to the liver, muscle, and heart is often blamed on the long, poorly water-soluble, linear outer branches of glycogen, because such damage, although more severe, is also seen in the more rare branching enzyme deficiency (i.e., type IV glycogen storage disease, which is not discussed here). Oral glucose tolerance is mildly abnormal because glycogen particles rapidly reach a finite size, to which UDP-glucose can no longer be added. Treatment is largely geared toward avoiding hypoglycemia, which is accomplished with frequent meals containing slowly absorbed carbohydrates, and often also with nocturnal infusions or feedings containing carbohydrates (as in type I glycogen storage disease). In addition, patients are given a diet high in protein

Fig. 24.13 Muscle glycogen phosphorylase deficiency (McArdle disease, type V glycogen storage disease) causes fatigue and cramping several minutes after the start of exercise.

(to minimize muscle protein loss; see also Chapter 35) and low in saturated fatty acids and cholesterol (to lessen the frequently accompanying hypercholesterolemia).

Type V glycogenosis (synonyms: **McArdle** disease, deficiency of **muscle glycogen phosphorylase**) is very rare; it is mentioned here because it illustrates the importance of muscle glycogen in powering muscle contraction. Affected patients (Fig. 24.13) have muscle cramps when they exercise (e.g., sprinting, heavy lifting, walking uphill), often more so during the early phase of exercise, when muscle glycogen is a particularly important contributor of fuel for energy production (see Section 2.2). If vigorous exercise is maintained, rhabdomyolysis sets in with the loss of myoglobin into the blood and from there into the urine (giving urine a burgundy color).

Type VI and **type IX** glycogen storage diseases are due to deficiencies of liver glycogen phosphorylase and its activating enzyme, phosphorylase kinase, respectively. Affected patients usually have hepatomegaly, yet hypoglycemia is mild. Patients avoid episodes of hypoglycemia with small, frequent meals.

Lafora disease (also called **Lafora progressive myoclonus epilepsy**) is often lumped together with the glycogen storage diseases. Lafora disease is due to homozygosity or compound heterozygosity for mutant **laforin** or **malin**. Laforin is a glycogen phosphatase that removes excess phosphate groups from glycogen. Although the origin of phosphate groups on glycogen is not fully understood, recent studies have shown that glycogen synthase can erroneously and very rarely incorporate phosphate groups into glycogen. Malin is an E3-ubiquitin ligase that plays a role in the degradation of laforin. Loss-of-function mutations in laforin or malin lead to accumulation of aberrant glycogen that precipitates in the cytosol of cells, forming Lafora bodies. Such Lafora bodies accumulate in the brain, liver, heart, muscle, and skin. The Lafora bodies contain excessive amounts of unbranched (therefore poorly soluble) and hyperphosphorylated glycogen, and they can be visualized by periodic acid staining (see Section 1.1). The brain is affected foremost, possibly due to a noxious effect of unbranched glycogen. Symptoms typically set in suddenly with apparently healthy teenagers; this is usually followed by myoclonic epilepsy, dementia, and death within about 10 years. Lafora disease is found especially frequently around the Mediterranean, in the Middle East, and in Southeast Asia.

SUMMARY

- Glycogen is a polymer of up to about 60,000 glucose residues that are formed on a side chain of the protein glycogenin. The most appreciable glycogen stores are found in the liver and skeletal muscle.
- The liver synthesizes glycogen after a meal, typically from dietary glucose. Liver glycogen synthesis is largely controlled by the activities of glucokinase and glycogen synthase. Glucokinase is activated by dietary fructose and by insulin. Glycogen synthase is activated by insulin but can be inhibited completely by epinephrine and glucagon.
- The liver degrades glycogen to glucose during the early phases of a fast and also during exercise. The liver releases glucose into the blood; this helps maintain normoglycemia, in the fasting state for the benefit of red blood cells and the brain, and during exercise also for the benefit of the skeletal muscles. Glycogen phosphorylase is the chief controller of glycogen degradation. An increased concentration of glucagon and epinephrine in the blood and increased release of norepinephrine and ATP from the vagus nerve in the liver all lead to an activation of glycogen phosphorylase.
- Skeletal muscles degrade their glycogen during exercise. Glucose 6-phosphate obtained in the degradation of glycogen is particularly important during the first few minutes of exercise when blood flow and glucose uptake are not yet maximal. With increasing duration of mild exercise, skeletal muscles derive more of their energy from glucose and free fatty acids (both taken up from the blood). Once muscle glycogen stores have reached a very small size, fatigue sets in.
- The skeletal muscles synthesize glycogen mainly after a meal from glucose that they take up from the blood. Prior exercise and depletion of glycogen stores render skeletal muscle cells especially sensitive to insulin.

FURTHER READING

- Ørtenblad N, Westerblad H, Nielsen J. Muscle glycogen stores and fatigue. J Physiol. 2013;591:4405-4413.
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Review Questions

1. In skeletal muscle, glycogenolysis is stimulated by an elevated concentration of which one of the following?

   A. AMP
   B. Glucagon
   C. Glucose 6-phosphate
   D. Insulin

2. A 10-year-old boy has signs of a muscle disorder. His lung function and his muscle strength are also decreased. He has difficulty getting up and walking. A muscle biopsy shows that the glycogen is of normal structure, and the size of the glycogen particles is within the normal range. In an oral glucose tolerance test, the patient's 0-, 1-, and 2-hour blood glucose values were all within the range of values seen in 10 healthy volunteers. T is patient could have a deficiency of which one of the following enzymes in his muscles?

   A. Debranching enzyme
   B. Glycogen branching enzyme
   C. Glycogen synthase
   D. Lysosomal acid α-glucosidase

3. A 5-month-old boy is found to have hepatomegaly, fasting hypoglycemia, and high levels of free fatty acids in his blood. His liver glycogen content was found to be high, but the glycogen had a normal structure. After an overnight fast, there was no detectable increase in the serum glucose concentration after an oral administration of galactose (which gives rise to glucose 6-phosphate). T e disease is most likely the result of a deficiency of which one of the following enzymes?

   A. Glucokinase
   B. Glucose 6-phosphatase
   C. Glycogen debranching enzyme
   D. Glycogen synthase

Chapter

25 Gluconeogenesis and Fasting Hypoglycemia

SYNOPSIS

- Gluconeogenesis is a process by which lactate, many amino acids (chiefly alanine and glutamine), and glycerol give rise to glucose. Gluconeogenesis takes place in the liver and the kidneys. Gluconeogenesis benefits glucose-dependent tissues, such as the brain, red blood cells, and exercising muscle.
- Gluconeogenesis proceeds via the reversible reactions of glycolysis and via unique, irreversible reactions that bypass the irreversible reactions of glycolysis.
- Gluconeogenesis depends on the breakdown of body protein (mostly muscle protein) or, in persons who eat a high-protein, low-carbohydrate diet, on the breakdown of dietary protein. Gluconeogenesis also depends on an adequate supply of adenosine triphosphate (ATP), which stems from the β-oxidation of fatty acids.
- Gluconeogenesis is activated by glucagon, epinephrine, and cortisol; it is inhibited by insulin. As a result, gluconeogenesis is most strongly suppressed after a meal, and it is near-maximally active after a 2-day fast, as well as during prolonged, intense exercise.
- Gluconeogenesis is excessive in patients who secrete too little insulin or who secrete too much cortisol, thyroid hormone, epinephrine, norepinephrine, or glucagon.
- Gluconeogenesis can be inadequate in patients who are intoxicated with alcohol, who are hyperinsulinemic, who release too little cortisol, or who have an inherited metabolic defect in the gluconeogenic pathway.

LEARNING OBJECTIVES

For mastery *of* this topic, you should be able to do the *f*ollowing:

- Describe the reactants, products, and tissue distribution of gluconeogenesis.
- Describe the roles of protein degradation and fatty acid oxidation vis-à-vis gluconeogenesis.
- Compare and contrast glycolysis and gluconeogenesis with regard to reactants, products, pathways, and regulation.
- Explain the contribution of gluconeogenesis to blood glucose homeostasis.
- Explain the pathogenesis of lactic acidosis and hyperalaninemia in patients who have a deficiency of one of the enzymes of gluconeogenesis.
- Explain the pathologic alterations of gluconeogenesis in patients who have diabetes, Cushing syndrome, a pheochromocytoma, a glucagonoma, Addison disease, severe liver dysfunction, or a glucose 6-phosphatase deficiency.
- Describe the effect of metformin on gluconeogenesis.
- Discuss abnormalities of gluconeogenesis in newborns.

1. PATHWAY OF GLUCONEOGENESIS

Gluconeogenesis is a process in which lactate, glycerol, or amino acids are turned into glucose. T e energy *f*or this process is derived chie*fl*y *f*rom the oxidation o*f f*atty acids. As part o*f* gluconeogenesis, pyruvate is carboxylated inside mitochondria to oxaloacetate, which in turn is converted to phosphoenolpyruvate in the cytoplasm. From phosphoenolpyruvate, glucose is synthesized via the reversible reactions o*f* glycolysis and the irreversible reactions that are unique to gluconeogenesis. T e liver and the kidneys are the two main organs that are known to carry out gluconeogenesis. T ere is some evidence that the intestine also per*f*orms gluconeogenesis.

In the transition from the fed to the fasting state, the body reduces its **glucose consumption**. After a meal, many organs consume glucose at a high rate. Muscle and liver store some glucose as glycogen, and the liver converts a small amount of glucose into fatty acids. In the presence of a high concentration of insulin, the body can use more than 100 µmol glucose/kg/min (i.e., ~1.3 g/min for a 70-kg person). In contrast, in the fasting state, the body uses only ~10 µmol glucose/kg/min because many organs produce ATP through the oxidation of fatty acids and ketone bodies rather than glucose.

Some cells, such as neurons in the brain, red blood cells, cells in the medulla of the kidney, and cells in the dermis of the skin, need glucose even in the fasting state. T is glucose derives from **glycogenolysis** in the liver and from **gluconeogenesis** in the liver and in the kidney cortex (the kidney cortex does not store a significant amount of glycogen).

During an extended fast, gluconeogenesis accounts for almost all of the endogenous glucose production. Fig. 25.1A shows the time course of glucose production by glycogenolysis and gluconeogenesis during a 2-day fast. In the evening of day 1, volunteers consumed a standardized meal followed by an overnight fast. In the morning of day 2, measurements were started and continued until almost noon on day 3. By that point, glycogenolysis produced virtually no glucose, and gluconeogenesis accounted for almost all the endogenous glucose production.

After a fast, the intake of food leads to a decrease in glucose production from glycogenolysis and gluconeogenesis. Fig. 25.1B shows the time course of an experiment with healthy, adult volunteers who were treated similarly to those described above. After fasting, the volunteers were given 75 g of glucose in water by mouth (similar to a standard oral glucose tolerance test; see Chapter 39). After 3 hours, about half of the glucose had been transported from the intestine into the blood. Over the same period, glucose production from glycogenolysis and

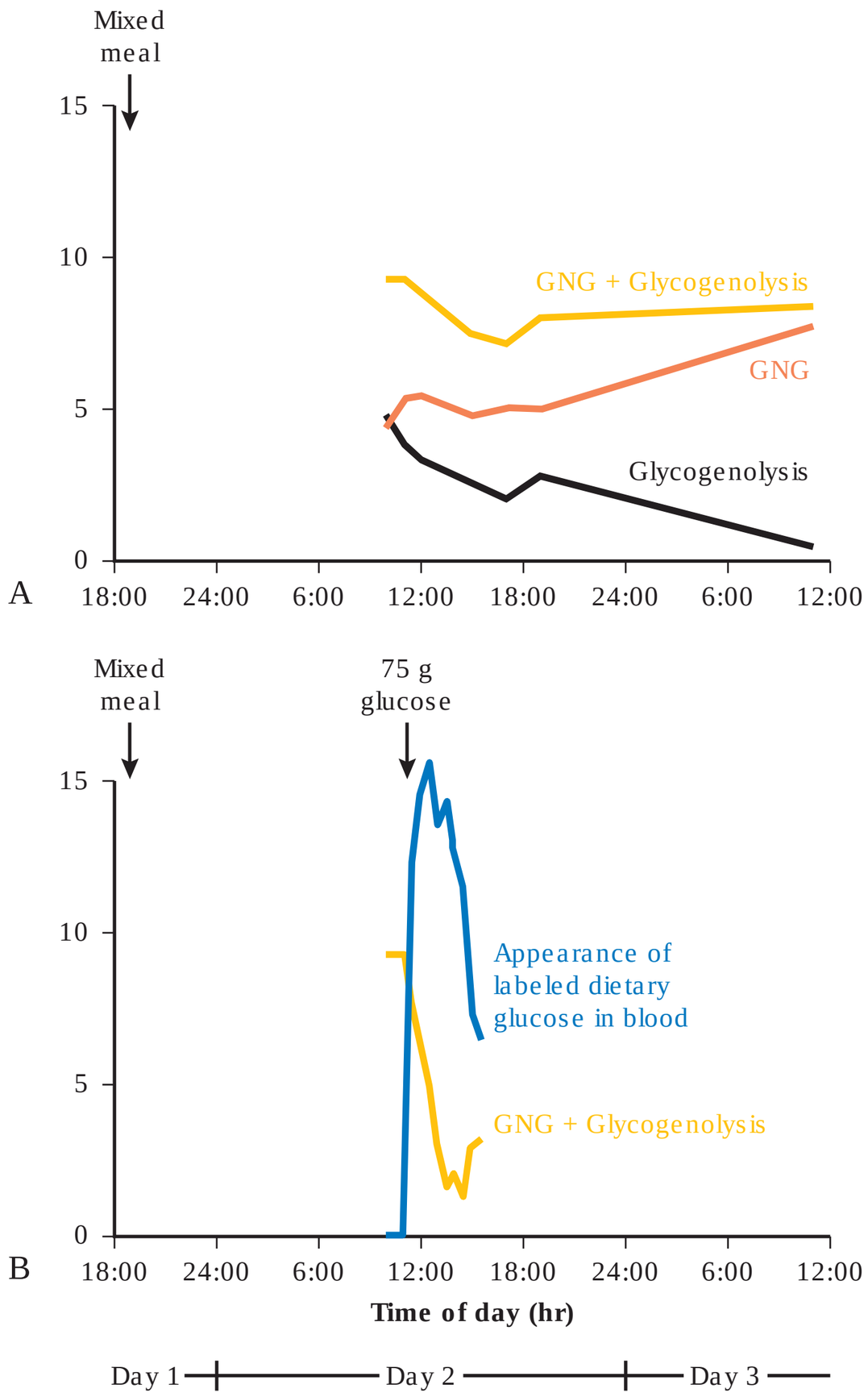
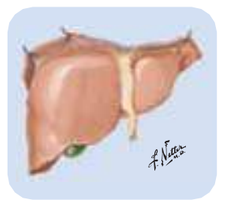
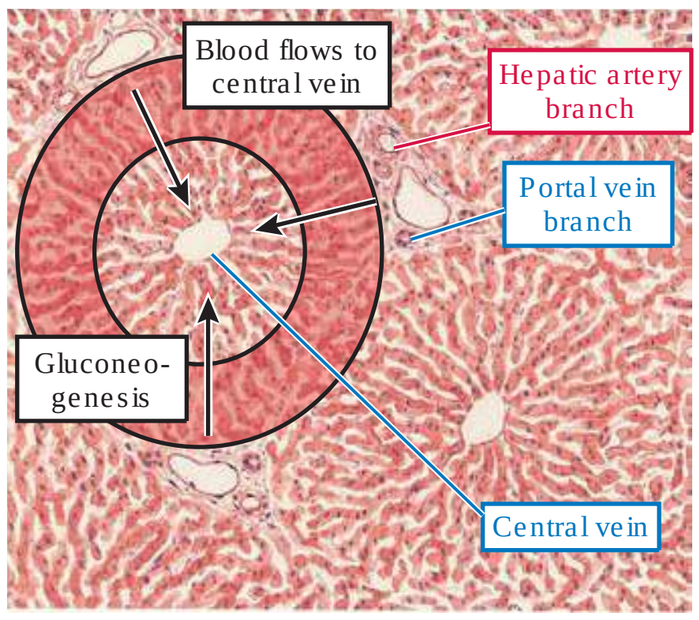


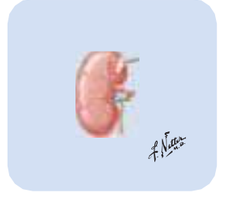
Fig. 25.1 Effect of fasting and feeding on endogenous glucose production. (A) Endogenous glucose production from glycogenolysis and gluconeogenesis (GNG), as measured with various tracer methods. (B) Appearance of dietary glucose and suppression of endogenous glucose production. (Based on data of Bisschop PH, Pereira Arias AM, et al. The effects of carbohydrate variation in isocaloric diets on glycogenolysis and gluconeogenesis in healthy men. *J Clin Endocrin Metabol.* 2000;85:1963-1967; Kunert O, Stingl H, Rosian E, et al. Measurement of fractional whole-body gluconeogenesis in humans from blood samples using 2H nuclear magnetic resonance spectroscopy. *Diabetes*. 2003;52:2475-2482; Boden G, Chen X, Capulong E, Mozzoli M. Effects of free fatty acids on gluconeogenesis and autoregulation of glucose production in type 2 diabetes. *Diabetes*. 2001;50:810-816; Wajngot A, Chandramouli V, Schumann WC, et al. Quantitative contributions of gluconeogenesis to glucose production during fasting in type 2 diabetes mellitus. *Metabolism*. 2001;50:47-52; Katz J, Tayek JA. Gluconeogenesis and the Cori cycle in 12-, 20-, and 40-h-fasted humans. *Am J Physiol.* 1998;275: E537-E542; and Meyer C, Woerle HJ, Dostou JM, et al. Abnormal renal, hepatic, and muscle glucose metabolism following glucose ingestion in type 2 diabetes. *Am J Physiol.* 2004;287:E1049-E1056.

gluconeogenesis declined to about one-fourth of its initial value. Most of this decrease is due to a decreased rate of glycogenolysis.

Gluconeogenesis takes place in the well-oxygenated **periportal cells** of the **liver** and the **cortical cells** of the **kidneys**







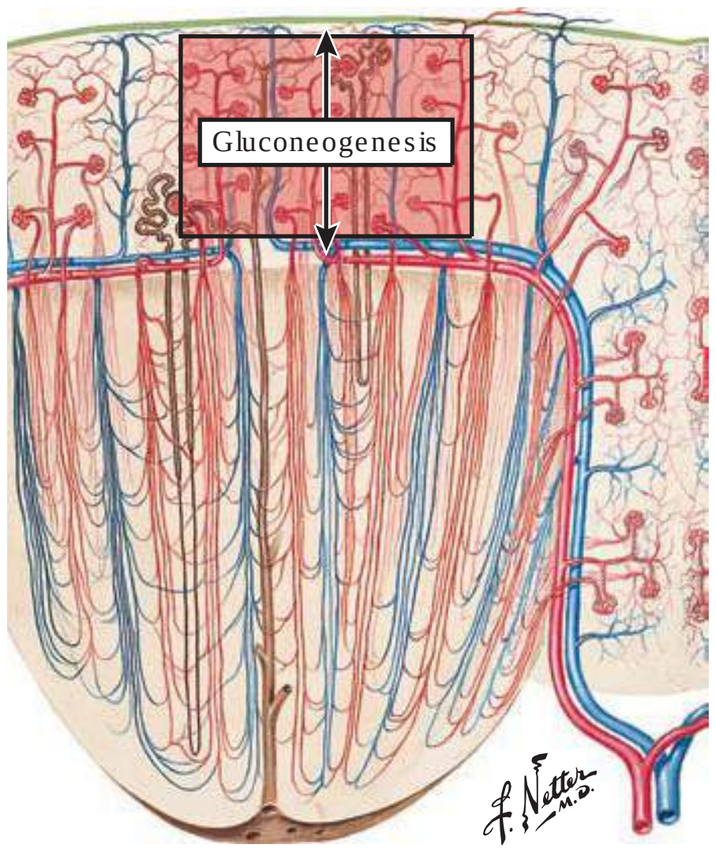


Fig. 25.2 Gluconeogenesis takes place in the liver and the kidneys. *Top,* Hematoxylin and eosin-stained thin section of the liver. Each lobule consists of plates of cells that resemble a stack of pancakes. Blood flows from the periphery to the center of the stack. Gluconeogenesis takes place in the well-oxygenated peripheral portion of the lobules indicated by the red ring, and glycolysis predominates in the central portion of the lobule. *Bottom,* Structure of a pyramid and the associated cortex in the kidney. Gluconeogenesis takes place in the well-oxygenated cortex indicated by the red rectangle.

(Fig. 25.2). T e liver and the kidneys are heterogeneous in that some cells produce glucose, while others consume it. Periportal cells of the liver and cortical cells of the kidneys both have sufficient oxygen to oxidize fatty acids to produce ATP for gluconeogenesis. In contrast, perivenous cells of the liver and cells in the medulla of the kidneys operate at lower concentrations of oxygen, depend at least partially on anaerobic glycolysis for ATP production, and cannot carry out gluconeogenesis.

The **small intestine** expresses all enzymes of gluconeogenesis; however, little is known about the small intestine's contribution to gluconeogenesis under physiological conditions.

The **reactions of gluconeogenesis** start with lactate, alanine, various other amino acids, or glycerol (Fig. 25.3). Several steps in gluconeogenesis require energy in the form of guanosine triphosphate (GTP) or ATP.

The physiologically **irreversible reactions** of gluconeogenesis (see Fig. 25.3) differ from those of glycolysis, whereas the reversible reactions are the same as for glycolysis (see Fig. 19.2) and they are also catalyzed by the same enzymes. The physiologically irreversible reactions of glycolysis are not used for gluconeogenesis. The physiologically irreversible reactions of gluconeogenesis are pyruvate → phosphoenolpyruvate (in several steps, two of which are irreversible), fructose 1,6-bisphosphate → fructose 6-phosphate, and glucose 6-phosphate → glucose.

Pyruvate is converted to **phosphoenolpyruvate** in several enzyme-catalyzed steps that take place in the mitochondria and the cytosol (see Fig. 25.3). Pyruvate enters the mitochondria, where **pyruvate carboxylase** carboxylates it to oxaloacetate. This is the same reaction that also supplies the citric acid

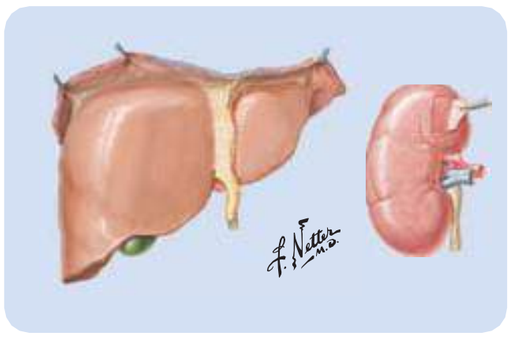


Fig. 25.3 Pathway of gluconeogenesis. The direction of pathway flow is from the bottom to the top. Compounds with carbon skeletons that give rise to glucose are shown in blue. Further details about the amino acids that give rise to pyruvate or feed into the citric acid cycle are shown in Fig. 25.5.

cycle with oxaloacetate (see Section 3 in Chapter 22). A high concentration of acetyl-coenzyme A (CoA) stimulates pyruvate carboxylase. Mitochondria do not have a transporter for oxaloacetate. Hence, oxaloacetate is converted to either aspartate or malate, which can be exported into the cytosol. The choice of export system depends on the need for NADH in the cytosol. In the cytosol, both aspartate and malate give rise to oxaloacetate. Oxaloacetate is then converted to phosphoenolpyruvate by **phosphoenolpyruvate carboxykinase** (**PEPCK**).

2. SUBSTRATE AND ENERGY SOURCES FOR GLUCONEOGENESIS

The substrates of gluconeogenesis are, in decreasing order of quantity used, lactate, alanine, glutamine, glycerol, and other glucogenic amino acids. Lactate stems from red blood cells, the skin, the intestine, and exercising muscle. Alanine, glutamine, and other glucogenic amino acids are derived from skeletal muscle protein or the diet. Glycerol results from the hydrolysis of adipose tissue triglycerides, which also yields fatty acids. Energy for gluconeogenesis stems from the β-oxidation of fatty acids inside the mitochondria.

2.1. Lactate

In the course of a day, a sedentary adult produces about 115 g of lactate. The major producers of lactate are, in decreasing order, red blood cells, skin, brain, skeletal muscle type 2X fibers, kidney medulla, and the intestine. The liver, kidney cortex, and skeletal muscle type 1 fibers oxidize most of the lactate (lactate → pyruvate → acetyl-CoA → CO_2). The liver uses about 20% of the daily lactate production for the synthesis of glucose via gluconeogenesis.

The term **Cori cycle** refers to the cycling of carbon skeletons between glucose and lactate via glycolysis and gluconeogenesis (Fig. 25.4).

The fate of lactate depends on the hormonal state of the body. Shortly after a meal, most of the lactate is oxidized in the citric acid cycle. Conversely, during a long-term fast or strenuous exercise most of the lactate that reaches the liver is converted to glucose via gluconeogenesis.

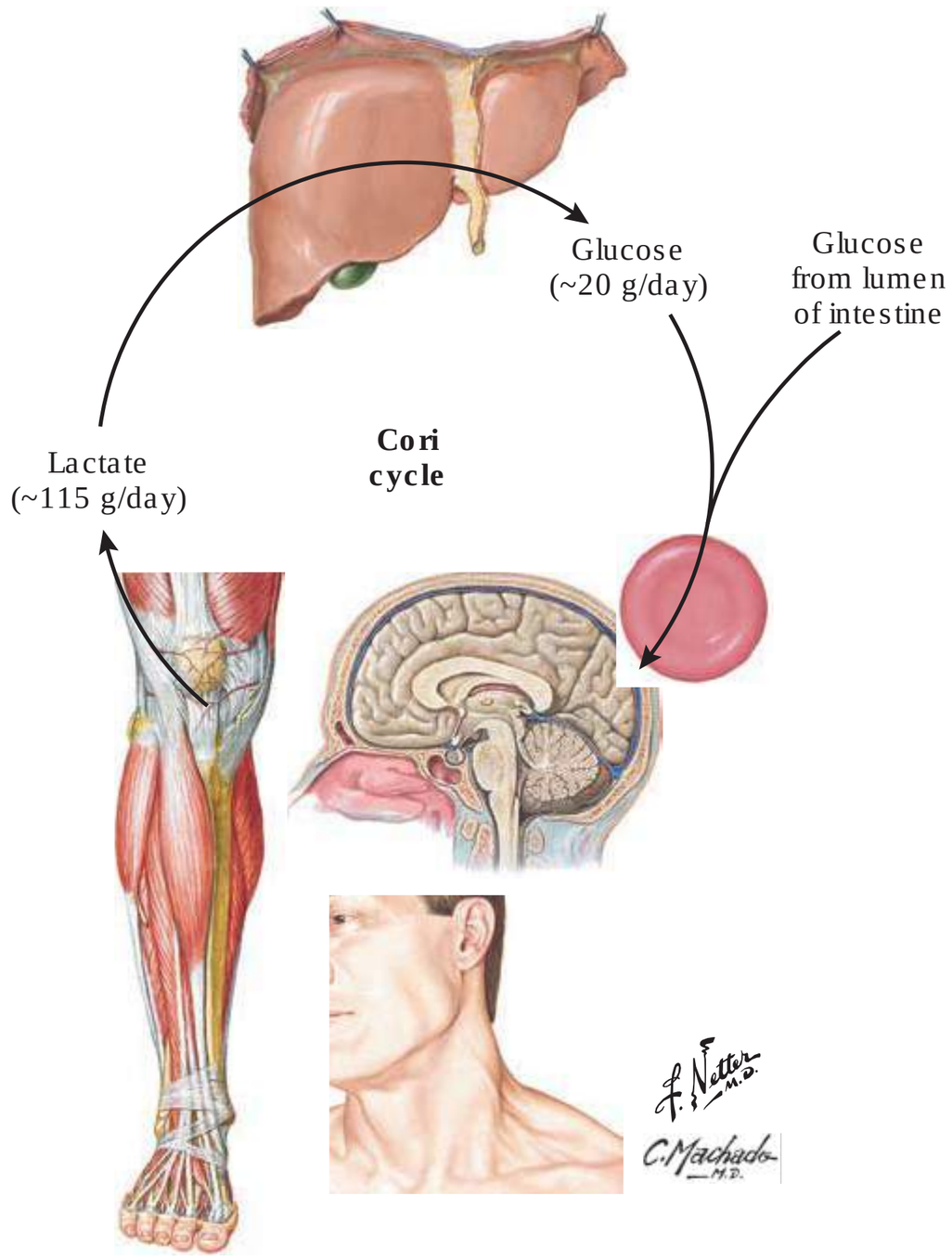


Fig. 25.4 The Cori cycle.

2.2. Amino Acids

Glucogenic amino acids are amino acids from which net glucose synthesis is possible via gluconeogenesis (see also Chapter 35). These amino acids are shown in Fig. 25.5. All of these amino acids can eventually give rise to oxaloacetate, from which phosphoenolpyruvate is made (see Fig. 25.3). It is not possible to net produce oxaloacetate from acetyl-CoA.

Amino acids that are used for gluconeogenesis can stem from the diet but, in the long run, they are derived from the degradation of skeletal muscle protein. Cortisol stimulates proteolysis in muscle, while **insulin** inhibits it (see Chapter 35). Cortisol also stimulates the transcription and translation of transaminases that transfer amino groups from amino acids

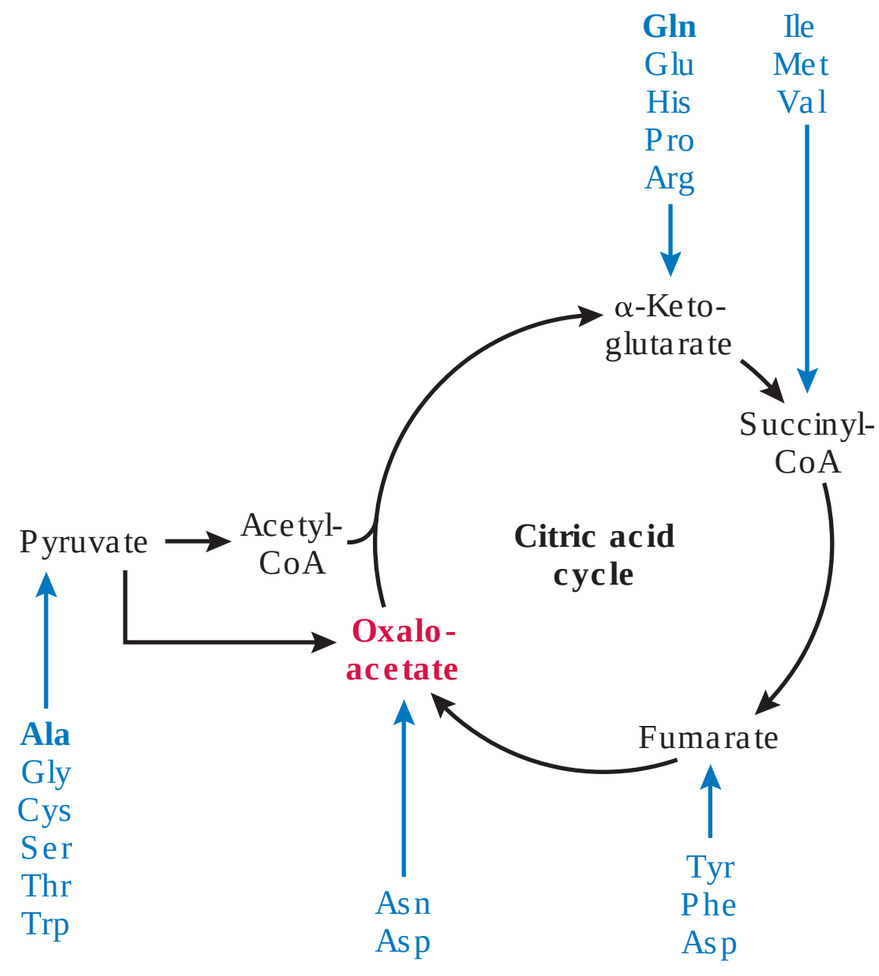


Fig. 25.5 Amino acids that can serve as substrates for gluconeogenesis. Among the 20 genetically encoded amino acids, only leucine and lysine cannot serve as substrates for gluconeogenesis. Quantitatively the most important glucogenic amino acids are alanine and glutamine. (Aspartate is listed twice because it can give rise to either oxaloacetate or fumarate.)

to pyruvate and glutamate. Muscle exports mostly **alanine** and **glutamine** (Fig. 25.6; see also Fig. 35.3 and Section 2 in Chapter 35).

The term **glucose-alanine cycle** refers to the pathway in which muscle exports alanine and the liver takes up alanine and converts it to glucose; the liver then releases glucose into the blood, and muscle takes up a portion of this glucose from the blood (Fig. 25.6).

Glutamine can also give rise to glucose. In the fasting state, glutamine in the blood stems mainly from muscle (see Fig. 25.6). The small intestine converts some of this glutamine to alanine. The liver uses this alanine to synthesize glucose via gluconeogenesis. The kidneys also take up glutamine, but they convert it to α-ketoglutarate and then, via a portion of the citric acid cycle, to oxaloacetate (see Fig. 25.3), which is used for the synthesis of glucose via gluconeogenesis. Both the liver and the kidneys release glucose from gluconeogenesis into the blood.

2.3. Glycerol

Glycerol stems from the hydrolysis of triglycerides (see Section 5 in Chapter 28). The liver converts glycerol to dihydroxyacetone phosphate, which is an intermediate of gluconeogenesis (see Fig. 25.3). Glycerol is a precursor of quantitatively minor importance. Thus, after a 16-hour fast, only ~10% of the glucose produced by gluconeogenesis stems from glycerol.

2.4. Fatty Acids as a Source of ATP

The oxidation of **fatty acids** provides ATP but not carbon skeletons for gluconeogenesis. Glucose can be converted into fatty acids (see Section 2 in Chapter 27), but **fatty acids cannot be converted into glucose**. There are two reasons for this: (1) acetyl-CoA cannot be converted to pyruvate (this reaction is physiologically irreversible and proceeds only from pyruvate to acetyl-CoA), and (2) net production of a citric acid cycle intermediate from acetyl-CoA alone is impossible (oxaloacetate is required to feed acetyl-CoA into the citric acid cycle, and acetyl-CoA is not entirely lost before oxaloacetate is reformed).

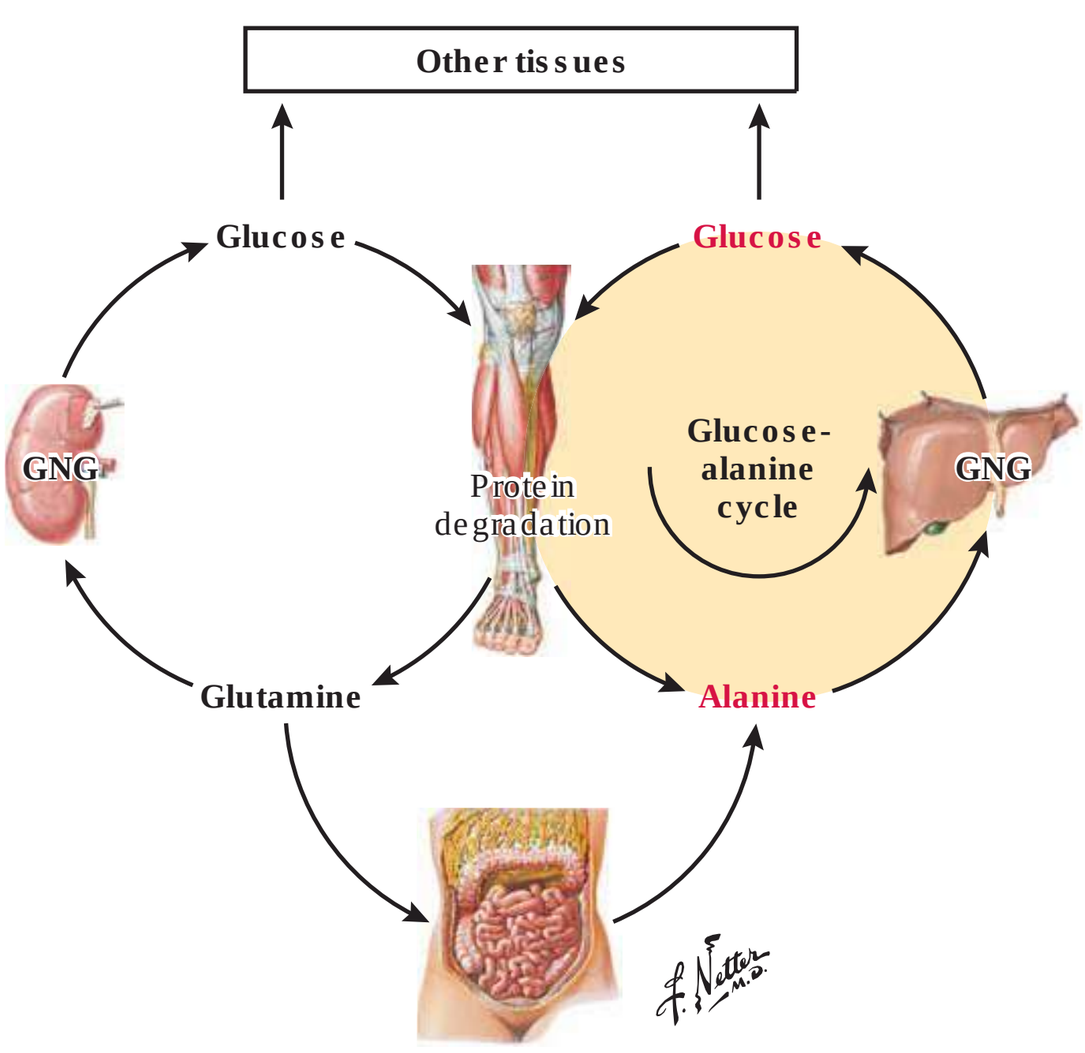


Fig. 25.6 **Major interorgan flux of amino acids when gluconeogenesis (GNG) is active.**

3. REGULATION OF GLUCONEOGENESIS

The rate of gluconeogenesis is lowest after a high-carbohydrate meal and highest during prolonged strenuous exercise and prolonged fasting. Flux through gluconeogenesis changes largely as a result of long-term controls, which include an effect of hormones on the production of transaminases, PEPCK, and glucose 6-phosphatase. Normally, PEPCK activity exerts the strongest control over the rate of gluconeogenesis. Short-term controls have modest effects and include an effect of insulin, glucagon, and epinephrine on the activity of fructose 1,6-bisphosphatase, as well as an allosteric effect of acetyl-CoA on pyruvate carboxylase.

Gluconeogenesis must be regulated to avoid excessive substrate cycling with glycolysis, quickly correct hypoglycemia and support ongoing strenuous exercise, avoid the excessive consumption of amino acids from body protein, and accommodate the input of different substrates. As a consequence, the regulation of gluconeogenesis is complex; Fig. 25.7 shows a simplified version of it. The regulated enzymes are pyruvate carboxylase, PEPCK, fructose 1,6-bisphosphatase (FBPase), and glucose 6-phosphatase (G6Pase), all of which catalyze physiologically irreversible reactions.

The long-term rate of gluconeogenesis is regulated chiefly via changes in the rate of transcription of transaminases, PEPCK, and G6Pase. It takes about 30 minutes from the time transcription starts to the time these enzymes are synthesized de novo and thus become active. The half-lives of these enzymes are on a scale of hours. Changes in the amount of PEPCK exert the main control over the rate of gluconeogenesis. Transaminase activity is important for the export of amino acids (mostly alanine and glutamine) from muscle and the import of amino acids into the liver, the kidney cortex, and the intestine (see Fig. 25.6).

Short-term, gluconeogenesis is regulated via phosphorylation/dephosphorylation and allosteric regulators of enzymes. FBPase is largely controlled by this mechanism (see Fig. 25.7).

During the transitions between feeding and fasting, glycolysis and gluconeogenesis are both appreciably active in the liver. This state permits the fine and rapid control of glucose production, but it wastes ATP due to metabolite cycling between glycolysis and gluconeogenesis.

Glucagon, **epinephrine**, **cortisol**, and **thyroid hormone** activate gluconeogenesis (see Fig. 25.7). In contrast, **insulin** and adenine monophosphate (**AMP**) or **AMP-dependent protein kinase** (**AMPK**) inhibit gluconeogenesis.

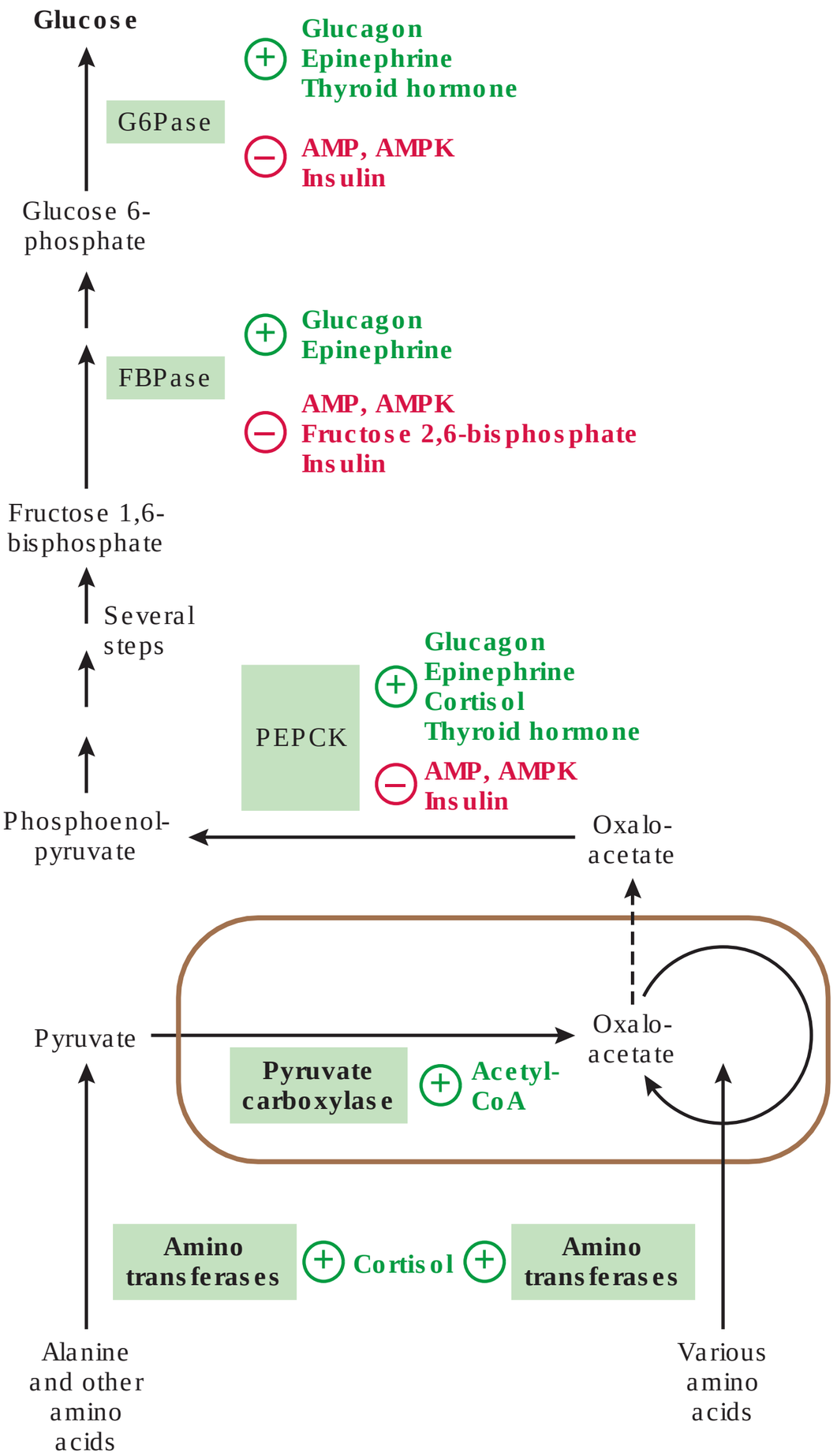


Fig. 25.7 Regulation of gluconeogenesis. Phosphoenolpyruvate carboxykinase (PEPCK) has the greatest control strength over the rate of gluconeogenesis. AMPK, adenine monophosphate (AMP)-activated protein kinase; CoA, coenzyme A; FBPase, fructose 1,6-bisphosphatase; G6Pase, glucose 6-phosphatase.

Details about the regulation of gluconeogenesis by **hormones** are as follows. Pancreatic α-cells secrete **glucagon** in response to hypoglycemia (see Chapter 26). Glucagon stimulates gluconeogenesis by binding to glucagon receptors. T e medulla of the adrenal glands secretes **epinephrine** in response to hypoglycemia or exercise (see Chapter 26). Epinephrine stimulates gluconeogenesis via β-adrenergic receptors. T e cortex of the adrenal glands secretes **cortisol** in a diurnal pattern; the concentration of cortisol in the blood is lowest in the early evening and highest in the early morning (see Chapter 31). Fasting enhances cortisol release most pronouncedly in the afternoon and during the night. T e concentration of cortisol also increases with intense exercise. Cortisol activates glucocorticoid receptors, which in turn increase transcription of genes that are associated with a promoter that contains a glucocorticoid response element. Cortisol notably leads to increased synthesis of transaminases. **Triiodothyronine** (T3, the active metabolite of thyroid hormone) activates thyroid hormone receptors in the nucleus, which in turn increases the transcription of genes that are associated with a thyroid hormone response element–containing promoter. T e concentration of T3 gradually decreases with long-term fasting. Pancreatic β-cells secrete **insulin** in response to hyperglycemia (see Chapter 26), and insulin binds to insulin receptors, which lead to a decreased rate of gluconeogenesis.

Gluconeogenesis is never completely suppressed, but it is least active 1 to 2 hours after a high-carbohydrate **meal** and most active 2 to 3 days into a prolonged **fast**. Gluconeogenesis is also very active during prolonged strenuous **exercise**. For instance, after an overnight fast, 1 hour of strenuous exercise approximately doubles the rate of gluconeogenesis.

Persons who previously exclusively consumed a **very-low-carbohydrate diet** (e.g., an **Atkins**-type diet) show a somewhat increased rate of gluconeogenesis after an overnight fast. T is reflects a partial compensation of a decreased rate of glycogenolysis.

An increased concentration of epinephrine, in conjunction with a decreased concentration of insulin, not only promotes gluconeogenesis but also stimulates **lipolysis** of triglycerides in the adipose tissue (see Chapter 27). As a result, the adipose tissue releases **fatty acids** and **glycerol** into the blood. Mitochondrial oxidation of fatty acids provides **ATP** for gluconeogenesis, and it also raises the concentration of **acetyl-CoA**, which in turn activates pyruvate carboxylase (see Fig. 25.7). When periportal hepatocytes or cells in the kidney cortex cannot produce enough ATP, they do not participate in gluconeogenesis. In the liver, the concentration of acetyl-CoA is highest during a long-term fast, when the rate of fatty acid β-oxidation is high and the need for acetyl-CoA oxidation in the citric acid cycle is limited (see Chapter 27).

T e **liver autoregulates** the balance between hepatic glycogenolysis and gluconeogenesis. T us, in a healthy person, an increase in the concentration of fatty acids or of precursors for gluconeogenesis (lactate, amino acids, or glycerol) in the blood leads to an increase in the rate of gluconeogenesis and a concomitant decrease in the rate of glycogenolysis. T ese changes cannot be explained by altered concentrations of hormones alone. T e mechanism by which the liver autoregulates its glucose production is unknown.

While the rate of gluconeogenesis is high in the long-term fasting state, the rate of **glycolysis** is low (see Sections 3 and 5 in Chapter 19). In the fasting state, **glucokinase** in the liver is inactive because it is inhibited by its regulatory protein GKRP and is sequestered in the nucleus (see Section 5.6 in Chapter 19). **Phosphofructokinase 1** (PFK 1) has low activity due to a lack of its powerful allosteric activator, fructose 2,6-bisphosphate. **Pyruvate kinase** has low activity because of glucagon-induced phosphorylation.

4. DISEASES ASSOCIATED WITH AN ABNORMAL RATE OF GLUCONEOGENESIS

Gluconeogenesis is impaired and can be a cause of hypoglycemia in patients with insufficient cortisol. Gluconeogenesis is also impaired in patients who have impaired ATP production from fatty acids (e.g., alcohol-intoxicated patients, patients who have deficient fatty acid β-oxidation), and in patients who have a hereditary deficiency of an enzyme of gluconeogenesis. Deficiencies in enzymes of gluconeogenesis between pyruvate and glucose lead to life-threatening lactic acidosis and hypoglycemia in the fasting state. On the other hand, gluconeogenesis is inappropriately elevated and a cause of hyperglycemia in patients with insufficient insulin secretion (diabetes), excessive thyroid hormone (hyperthyroidism), excessive cortisol (Cushing syndrome), or excessive glucagon (glucagonoma).

4.1. Diseases Associated With Inadequate Gluconeogenesis

4.1.1. General Comments

Inadequate gluconeogenesis during a fast leads to **hypoglycemia**, which is particularly damaging to the brain.

Knowledge of the regulation of gluconeogenesis (see Fig. 25.7) suggests that an excessive concentration of insulin or abnormally low concentrations of cortisol, thyroid hormone, epinephrine, or glucagon can lead to an abnormally low rate of gluconeogenesis. In addition, impaired gluconeogenesis can also be the result of liver dysfunction or impaired enzyme activity. Diseases that lead to such impairment of gluconeogenesis are described in detail below.

Inadequate gluconeogenesis causes hypoglycemia as soon as glycogenolysis can no longer provide glucose at an adequate rate. Patients with chronically impaired gluconeogenesis need to consume carbohydrates with adequate frequency. **Low-carbohydrate meals**, **dieting**, and extended periods of **fasting** may be life threatening. Intense, prolonged **exercise** likewise requires that these patients frequently take in extra carbohydrates. **Newborns** and **children** are at a greater risk of hypoglycemia than adults due to the large size of their brain relative to the size of their liver and kidneys.

4.1.2. Hyperinsulinemia

Because insulin stimulates glucose use and exerts a dominant inhibitory effect over glycogenolysis and gluconeogenesis, an excessive concentration of insulin can lead to hypoglycemia. The concentration of insulin is excessive in patients with **diabetes** who inject too much insulin (causing an **insulin reaction**; see Chapter 39), in **newborns** of mothers who had **chronic gestational hyperglycemia** (see Chapter 39), in patients who have an **insulinoma** (a tumor of the pancreas that secretes insulin; see Section 6.1.1 in Chapter 26), and in patients who have persistent hyperinsulinemia due to a **β-cell defect** (see Section 6.1.3 in Chapter 26).

4.1.3. Hypocortisolism

Patients who take a large dose of **exogenous glucocorticoids** should **taper** them of gradually to avoid developing hypocortisolism. The exogenous glucocorticoids suppress the secretion of adrenocorticotrophic hormone (ACTH) from the pituitary gland, which normally stimulates cortisol release from the adrenal glands (see Fig. 31.12). ACTH secretion adjusts over a period of days.

Toddlers and young children sometimes have delayed development of **cortisol** production. Cortisol is needed to stimulate muscle protein breakdown and induce the transcription of transaminases in muscle and the liver, which convert pyruvate to alanine and vice versa (see Section 2.3 and Chapter 35). With a prolonged fast (e.g., ≥15 hours), these children experience life-threatening hypoglycemia. At the same time, they also show hypoalaninemia and ketosis (a consequence of increased lipolysis; see Chapters 27 and 28). This is delayed maturation of cortisol production is also known as **ketotic hypoglycemia of childhood**. The disease is usually apparent at 2 to 5 years of age and remits spontaneously by 10 years of age.

Patients who have **Addison disease** chronically produce insufficient quantities of **glucocorticoids**, of which cortisol is the major one (see Fig. 31.20 and Section 4.2 in Chapter 31). In Europe, about 1 in 10,000 persons has a diagnosis of Addison disease. Patients with Addison disease lose the function of their adrenal cortex slowly, so that the disease is often discovered only during a crisis. If there is also a mineralocorticoid deficiency, there is a decrease in blood pressure. If patients with Addison disease fast for a prolonged period (e.g., due to illness or following surgery), they may become severely hypoglycemic and ketotic. Treatment with glucocorticoids and mineralocorticoids is essential for these patients.

4.1.4. Liver Disease

Severe dysfunction of the liver leads to hypoglycemia. This can happen in patients with toxic **hepatitis**, fulminant viral hepatitis, or **sepsis**.

4.1.5. Impaired Production of ATP and Intermediates of Gluconeogenesis (Impaired Oxidation of Fatty Acids, Alcohol Intoxication, and Enzyme Deficiencies)

Patients who have impaired fatty acid β-oxidation (e.g., **medium-chain acyl-CoA dehydrogenase** [**MCAD**] deficiency; see Section 7.1 in Chapter 27) develop hypoglycemia in the fasting state due to excessive use of glucose and insufficient flux in gluconeogenesis. Gluconeogenesis is impaired because β-oxidation does not permit adequate ATP production, and because the concentration of acetyl-CoA is too low to inhibit pyruvate dehydrogenase and activate pyruvate carboxylase.

Consumption of **excess alcohol** leads to a decrease in the rate of gluconeogenesis (see Section 3.1 in Chapter 30). The rate of gluconeogenesis is low because the concentration of

pyruvate is low and that of AMP is high. Early on, due to the autoregulation of glucose production by the liver, endogenous glucose production is maintained through an increase in the rate of glycogenolysis. In a later phase, glycogen stores are depleted and hypoglycemia sets in, particularly in patients who consume large quantities of alcohol without any food.

Newborns, especially **preterm infants**, frequently have hypoglycemia during the first day or so of life. Several factors appear to be the cause. One is the delayed expression of enzymes of gluconeogenesis, including glucose 6-phosphatase (which is also needed for glucose release from glycogenolysis).

During a fast, patients who have an inherited deficiency of an **enzyme** that catalyzes one of the irreversible steps in gluconeogenesis develop **hyperalaninemia** and **lactic acidosis**. The hyperalaninemia is due to decreased use of alanine in gluconeogenesis and increased production of alanine in muscle (secondary to a low concentration of insulin because of hypoglycemia). The lactic acidosis also has two causes: the decreased use of lactate in gluconeogenesis and the increased formation from glycolysis (activated by elevated concentrations of intermediates and by AMP from ATP-consuming cycles between gluconeogenesis and glycolysis). Reduced secretion of insulin and increased secretion of epinephrine also lead to increased lipolysis and hence increased ketone body production (see Chapter 27). The high concentrations in the blood of lactic acid, acetoacetic acid, and β-hydroxybutyric acid in turn impair the renal excretion of uric acid (see Section 2.3 in Chapter 38). Hence, **hyperuricemia** often accompanies hereditary deficiencies of enzymes of gluconeogenesis.

Hereditary **glucose 6-phosphatase deficiency** (**von Gierke disease**, **glycogen storage disease type I**) affects glucose production from both glycogenolysis (see Section 3.3 in Chapter 24) and gluconeogenesis. In the fasting state, the disease is accompanied by severe lactic acidosis (see above). A glucose 6-phosphatase deficiency leads to increased concentrations of all metabolites between phosphoenolpyruvate and FBPase (see Fig. 25.7). In turn, this leads to a high concentration of glycerol 3-phosphate. The severe fasting hypoglycemia leads to increased release of fatty acids into the blood (see Section 5 in Chapter 28) and hence an increased concentration of fatty acids inside hepatocytes. The combination of increased intracellular concentrations of glycerol 3-phosphate and fatty acids favors the excessive formation of triglycerides in the fasting state, causing **hypertriglyceridemia** and hepatomegaly.

Hereditary **FBPase deficiency** presents much like a glucose 6-phosphatase deficiency, except that affected patients develop hypoglycemia more slowly. The disease is rare, and the mortality rate is high early in life. As survivors age, the weight ratio of glucose producing organs/glucose-consuming organs becomes more favorable, and episodes of hypoglycemia are milder and occur less frequently. For glucose homeostasis, children with FBPase deficiency depend on glycogenolysis to an unusually high degree. Because the metabolism of dietary fructose normally leads to an inhibition of glycogenolysis, patients with hereditary FBPase deficiency should refrain from consuming fructose, sucrose, or sorbitol (see Section 3.3 in Chapter 20).

4.2. Diseases Associated With Excessive Gluconeogenesis

4.2.1. General Comments

Excessive gluconeogenesis causes **hyperglycemia**, which can lead to diabetes and its concomitant long-term complications (see Chapter 39).

As can be expected from knowledge of the regulation of gluconeogenesis (see Fig. 25.7), excessive gluconeogenesis is seen in patients who secrete too little insulin or do not respond well to circulating insulin and in patients who have excessive concentrations in the blood of cortisol, thyroid hormone, epinephrine, or glucagon. These diseases are described below.

4.2.2. Insulin Deficiency With Diabetes

Patients who have **diabetes** (see Chapter 39) and who are insulin deficient have an excess rate of gluconeogenesis. This applies to patients with type 1 diabetes who inject too little insulin, particularly when they are ill or stressed (when extra epinephrine, glucagon, and cortisol are released). It also applies to patients with type 2 diabetes who are insulin resistant and show absolute or relative insulin deficiency. Both an inordinately low concentration of insulin and an inadequate response of cells to circulating insulin (i.e., insulin resistance) cause excessive glucose production via gluconeogenesis (see Fig. 25.7).

To normalize the rate of glucose production from gluconeogenesis with drugs, patients with type 1 diabetes can be treated with an adequate amount of exogenous insulin; patients with type 2 diabetes can be treated with exogenous insulin, drugs that boost insulin secretion, drugs that increase insulin sensitivity, or, as is currently most common, with the hypoglycemic agent **metformin**. Metformin leads to an activation of AMPK, thereby inhibiting gluconeogenesis at the level of PEPCK, FBPase, and G6Pase (see Fig. 25.7).

In patients who have type 2 diabetes and who develop the acute **hyperosmolar hyperglycemic state** (see Chapter 39), gluconeogenesis plays a special role in causing severe hyperglycemia and dehydration. Over the course of a few days, a persistently low concentration of insulin leads to an abnormally increased rate of gluconeogenesis, decreased glucose use, and a mildly increased rate of lipolysis (not enough to cause pronounced ketoacidosis). The concentration of glucose in the blood reaches such a high concentration that a massive loss of glucose and water in the urine ensues, which eventually causes life-threatening dehydration and hyperosmolarity.

4.2.3. Cushing Syndrome

Patients who have **Cushing syndrome** secrete an excessive amount of **cortisol** (see Fig. 31.16) and therefore have an increased rate of gluconeogenesis. The abnormally high

concentration of cortisol stimulates the breakdown of muscle protein to amino acids. The increased availability of amino acids leads to an increased rate of gluconeogenesis. Due to autoregulation by the liver, this in turn decreases the rate of glycogenolysis. The abnormally high concentration of cortisol also leads to a poor response to insulin (i.e., insulin resistance); the mechanism of this alteration remains unknown. Hence, patients with untreated Cushing syndrome tend to be glucose intolerant and develop diabetes.

Patients who receive long-term treatment with high doses of a **glucocorticoid**, such as dexamethasone or prednisone, show symptoms similar to patients with Cushing disease. Thus, they show excessive degradation of muscle protein with concomitant muscle weakness, they become insulin resistant, and tend to have hyperglycemia and diabetes.

4.2.4. Hyperthyroidism

Patients who have pronounced **hyperthyroidism** (e.g., in thyrotoxicosis or thyroid storm; Fig. 25.8) are hyperglycemic. These patients have elevated concentrations of cortisol and epinephrine in the blood, which stimulate glycogenolysis and gluconeogenesis. Furthermore, thyroid hormone makes the liver more sensitive to epinephrine. Epinephrine inhibits insulin secretion. As a result, endogenous glucose production is increased, whereas glucose use is decreased.

4.2.5. Pheochromocytoma

Patients who have a **pheochromocytoma** (see Fig. 22.13), an uncommon chromaffin cell tumor that secretes epinephrine and norepinephrine, can have life-threatening episodes of hypertension and heart disease. Elevated concentrations in the blood of epinephrine and norepinephrine may also cause chronic hyperglycemia (in the liver, norepinephrine binds to the same receptor as epinephrine, though with lower affinity). Pheochromocytomas occur at various sites in the body.

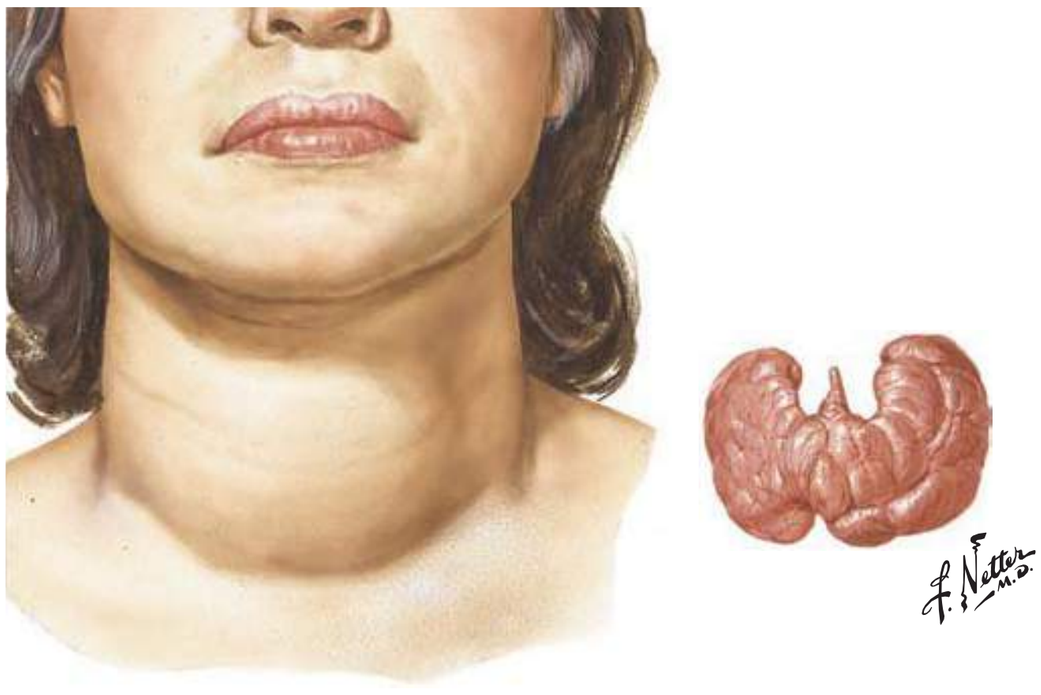


Fig. 25.8 Pronounced hyperthyroidism is typically associated with an increased rate of gluconeogenesis that leads to hyperglycemia.

4.2.6. Glucagonoma

Patients who have a **glucagonoma**, a rare tumor of the pancreatic islets that produces predominantly glucagon, have an excessive rate of gluconeogenesis and readily develop diabetes. These patients come to medical attention due to diabetes or a migratory skin rash. The rash appears to be due to **hypoaminoacidemia**. The concentration of glucagon is usually elevated approximately 20-fold. Glucagon primarily stimulates gluconeogenesis (and glycogenolysis) in the liver and, at a high concentration, lipolysis in adipose tissue. Muscle does not have many glucagon receptors. Patients with a glucagonoma have mild hyperglycemia that is caused by a combination of increased gluconeogenesis, as well as increased lipolysis and decreased tissue glucose use (largely due to the glucose-sparing effect of the increased concentration of circulating fatty acids that accompany the increase in lipolysis). The hyperglycemia necessitates increased insulin secretion and, within months, is accompanied by β-cell failure and diabetes. The hypoaminoacidemia appears to be due to persistent, increased use of amino acids for gluconeogenesis and the concomitant loss of muscle protein (patients lose both lean body mass and fat). The loss of muscle protein is probably a consequence of hypoaminoacidemia.

SUMMARY

- Gluconeogenesis produces glucose from lactate, glycerol, alanine, glutamine, and other glucogenic amino acids.
- Gluconeogenesis takes place in the periportal cells of the liver and the cortex of the kidneys.
- Gluconeogenesis requires ATP, which is normally derived from the β-oxidation of fatty acids.
- Insulin and cortisol control the degradation of muscle protein into amino acids. Cortisol controls the synthesis of transaminases, which transfer amino groups from various amino acids onto pyruvate or glutamate so that muscle chiefly exports alanine and glutamine.
- The hormones insulin, glucagon, epinephrine, cortisol, and thyroid hormone control the synthesis of phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase, which catalyze irreversible steps in gluconeogenesis. In an instant, insulin and glucagon control the activity of FBPase. PEPCK activity is the most important determinant of the rate of gluconeogenesis.
- Gluconeogenesis is impaired in patients who have hyperinsulinemia, hypocortisolism, severe liver dysfunction, a deficiency of an enzyme of gluconeogenesis, deficient fatty acid β-oxidation, or alcohol intoxication along with low carbohydrate intake. In the fasting state, an inadequate rate of gluconeogenesis leads to hypoglycemia. Patients with a deficiency of an enzyme of gluconeogenesis also develop lactic acidosis during fasting.
- Gluconeogenesis is inappropriately high and hence a cause of hyperglycemia in patients who have poorly controlled diabetes, have a high concentration of circulating

cortisol (i.e., patients with Cushing disease or those who are treated with high doses of glucocorticoids), or have pronounced hyperthyroidism, a pheochromocytoma, or a glucagonoma.

FURTHER READING

- Jitrapakdee S. Transcription factors and coactivators controlling nutrient and hormonal regulation of hepatic gluconeogenesis. Int J Biochem Cell Biol. 2012;44:33-45.
- Mitrakou A. Kidney: its impact on glucose homeostasis and hormonal regulation. Diabetes Res Clin Pract. 2011;93(suppl 1):S66-S72.

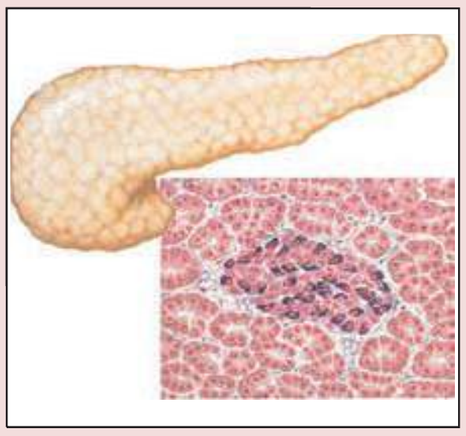
Review Questions

1. An abnormally high rate of gluconeogenesis is observed in patients with which one of the following abnormalities?

   A. Acute alcohol intoxication
   B. Addison disease
   C. Fulminant viral hepatitis
   D. Glucagonoma
   E. Insulin reaction and type 1 diabetes

2. In the fasting state, infants who have a glucose 6-phosphatase deficiency develop which one of the following?

   A. Hyperglycemia
   B. Hyperinsulinemia
   C. Hypoalaninemia
   D. Lactic acidosis



Chapter

26

Insulin and Counterregulatory Hormones

SYNOPSIS

- The body uses insulin, glucagon, epinephrine, and cortisol to control the flow, storage, and use of fuels. Cells in the pancreas secrete insulin and glucagon, depending on the concentration of glucose and other fuels. Cells in the intestine secrete incretins, which modify insulin and glucagon secretion. Cells in the brain secrete hormones that regulate the secretion of epinephrine and cortisol from the adrenal glands.
- Glucagon, epinephrine, norepinephrine, and cortisolare "counter-regulatory hormones" because they increase the concentration of glucose in the blood in contrast to insulin, which lowers it.
- The pancreas contains islets, which are small nests of cells that secrete insulin, glucagon, and other hormones into the blood-stream. Islet β-cells secrete insulin in response to an elevated concentration of glucose, and this secretion is enhanced by amino acids, fatty acids, and ketone bodies. Epinephrine inhibits insulin secretion. Islet α-cells secrete glucagon in response to amino acids or epinephrine, and hypoglycemia enhances this effect.
- Inherited and acquired defects of β-cell fuel sensing can lead to life-threatening hypoglycemia, neonatal diabetes, maturity-onset diabetes of the young (MODY), or other forms of diabetes.
- In response to food, the intestine secretes incretins, which enhance glucose-induced insulin secretion. Patients who receive glucose as part of parenteral nutrition may need to be given exogenous insulin, in part because the bypassed intestine does not secrete incretins.
- Insulin can stimulate glucose uptake, glucose use, glycogen synthesis, fatty acid synthesis, triglyceride deposition, protein synthesis, and cell growth. Insulin can inhibit glycogenolysis, lipolysis, and gluconeogenesis.
- Tissues of patients who are pregnant or obese or who have polycystic ovary syndrome show a diminished response to insulin.
- Glucagon favors the release of glucose from the liver. The liver makes glucose via glycogenolysis or gluconeogenesis.
- Insulin-secreting tumors are uncommon and cause hypo-glycemia. Glucagon-secreting tumors are very rare and lead to hypoaminoacidemia and hyperglycemia.

LEARNING OBJECTIVES

For mastery *of* this topic, you should be able to do the *following*:

- Explain how glucagon, glucagon-like peptide 1 (GLP-1), and insulin are synthesized, processed, and stored.
- Compare and contrast how glucose, amino acids, ketone bodies, epinephrine, and GLP-1 affect glucagon and insulin secretion, relating hormone secretion to food intake, exercise, and fasting.
- Describe the basic mechanism by which sulfonylurea and glinide hypoglycemic drugs work, noting their most common side effect.
- Explain why C-peptide is a useful measure of endogenous insulin secretion.
- Outline the molecular events that are set in motion after insulin, glucagon, epinephrine, and cortisol bind to their respective receptors.
- Explain the mechanism of action and pharmacologic use of GLP-1 receptor agonists.
- Explain the mechanism of action and pharmacologic use of dipeptidylpeptidase-4 inhibitors.
- Describe the effect of polycystic ovary syndrome on insulin signaling.
- Characterize the clinical presentation of patients who have an asymptomatic insulinoma; do the same for glucagonoma.
- Describe abnormalities of β-cell proteins that cause hypo-glycemia; do the same for hyperglycemia.
- Describe the effects of adrenal insufficiency, a pheochromocy-toma, or Cushing syndrome on plasma glucose.

1. STRUCTURE OF THE HUMAN PANCREAS

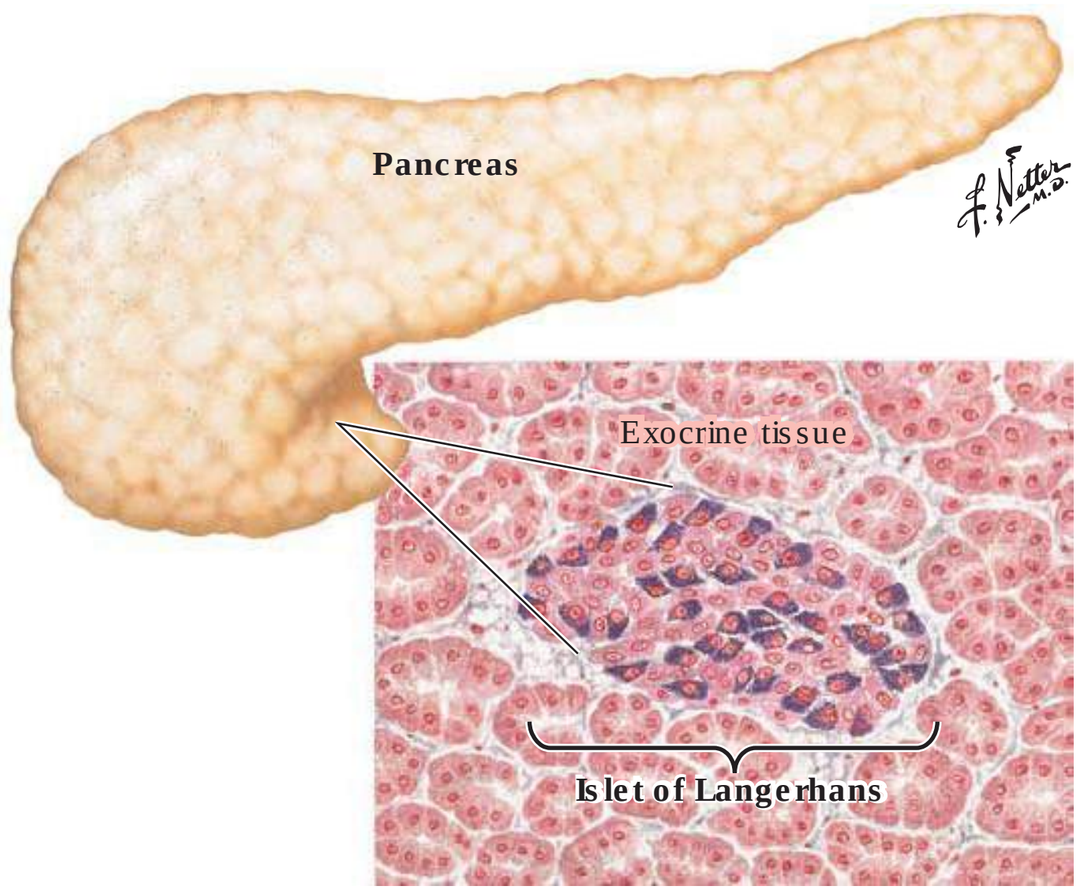
T e pancreas contains islets o*f* Langerhans. T ese islets contain α-cells that store glucagon and β-cells that store insulin inside secretory vesicles.

T e human pancreas consists of an **exocrine** and an **endo-crine** portion. T e exocrine cells make up about 99% of the volume of the pancreas and secrete digestive enzymes via the pancreatic duct into the lumen of the intestine. T ese digestive enzymes are composed of amylase, lipases, nucleases, and pro-teases or precursors of proteases (see Chapters 18, 28, and 34). T e endocrine cells of the pancreas account for about 1% of the volume of the pancreas and secrete hormones into the bloodstream; these hormones control fuel metabolism and growth.

T e endocrine cells occur in nests of cells called **islets of Langerhans** (Fig. 26.1). Each such islet contains **β-cells** (pre-viously called B-cells, but not to be confused with B-lymphocytes) that secrete insulin and some amylin, **δ-cells** (previously called D-cells) that secrete somatostatin, and either **α-cells** (previously called A-cells) that secrete glucagon, or **PP-cells** (F-cells) that secrete pancreatic polypeptide (PP). Some islets contain both α- and PP-cells. T e average human islet contains about 2,000 cells, but individual islets may contain a half a dozen cells to tens of thousands of cells. T e entire pancreas contains roughly 1 million islets.

2. SYNTHESIS OF GLUCAGON, GLUCAGON-LIKE PEPTIDES, INSULIN, EPINEPHRINE, AND CORTISOL

In α-cells, the proteolytic processing o*f* preproglucagon gives rise to glucagon; in intestinal L-cells, it gives rise



Cell type	Fraction of islet volume	Hormone content
α-cells	~20%	Glucagon
β-cells	~65%	Insulin, C-peptide, amylin
δ-cells	~10%	Somatostatin
PP-cells	~5%	Pancreatic polypeptide

Fig. 26.1 Structure of the human pancreas and an islet of Langerhans. In this islet, the β-cells appear deep purple from an aldehyde fuchsin stain.

to glucagon-like peptides 1 and 2. Proteolytic processing of preproinsulin gives rise to insulin, which consists of disulfide-linked A- and B-chains, as well as C-peptide. In the adrenal glands, epinephrine is synthesized from tyrosine, and cortisol is made from cholesterol.

2.1. Synthesis of Glucagon and Glucagon-Like Peptides

The glucagon gene encodes **glucagon**, **glucagon-like peptide 1** (**GLP-1**), and **glucagon-like peptide 2** (**GLP-2**). GLP-1 and GLP-2 have appreciable amino acid sequence homology to glucagon, but they activate different receptors and have different effects. Glucagon and the GLPs evolved via DNA sequence duplication.

Glucagon and the glucagon-like peptides are synthesized from **preproglucagon**. The **"pre" sequence**, or **signal sequence,** of preproglucagon ensures the insertion of the nascent peptide into the endoplasmic reticulum (see Chapter 7). After cleavage of the "pre" sequence, **proglucagon** (containing the sequences for glucagon, GLP-1, and GLP-2) is sorted through the Golgi apparatus and ends up in **secretory vesicles** (also called **secretory** granules). Several different tissues produce proglucagon.

Inside secretory vesicles, proglucagon is proteolyzed to tissue-specific products (Fig. 26.2). α-Cells in the pancreas contain prohormone convertase 2 (PC2) and process proglucagon into **glucagon**. **L-cells** in the small and large intestine

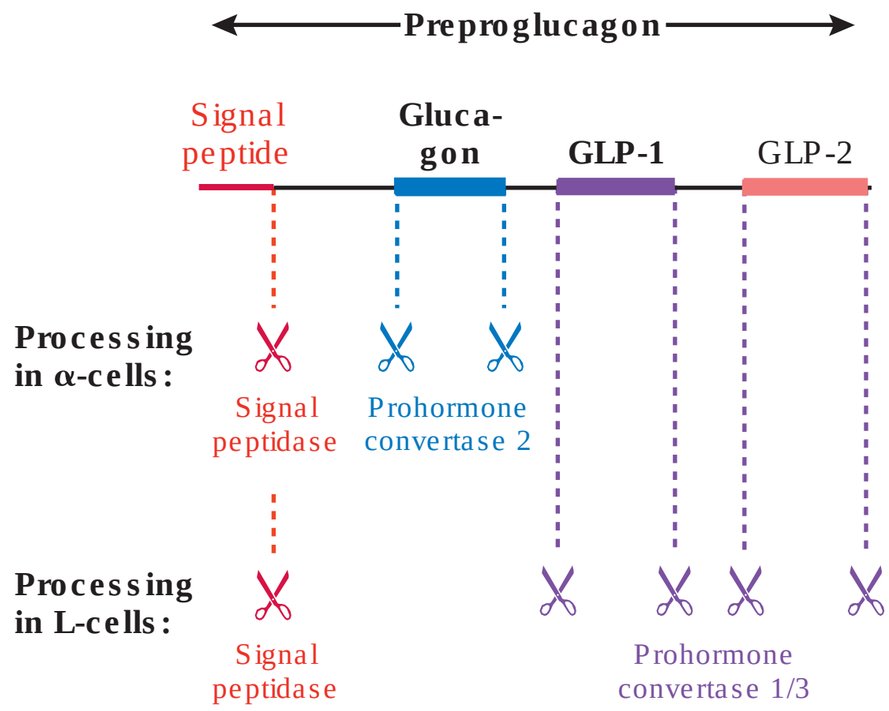


Fig. 26.2 Tissue-specific processing of proglucagon. GLP, glucagon-like peptide.

instead contain prohormone convertase 1/3 (PC1/3, meaning PC1 is identical with PC3) and process proglucagon into GLP-1 and GLP-2.

2.2. Synthesis of Insulin and Amylin

Insulin consists of two peptides, the **A-chain,** and the **B-chain**, that derive from a single precursor, **preproinsulin** (Fig. 26.3), which derives from the insulin gene. The insulin gene is almost exclusively transcribed in pancreatic β-cells. Translation of insulin mRNA gives rise to preproinsulin. The "pre" sequence ensures the insertion of the nascent peptide into the endoplasmic reticulum and is cleaved (see also Chapter 7). The remaining peptide, **proinsulin**, contains the A- and B-chains, as well as a connecting peptide, called **C-peptide**. The A- and B-chains contain cysteine residues that form disulfide bridges. These bridges form properly in high yield only from folded proinsulin but not from isolated A- and B-chains. Proinsulin is transported through the Golgi apparatus and ends up in secretory vesicles.

The secretory granules inside β-cells contain the **prohormone convertases PC1/3** and **PC2**, which hydrolyze proinsulin into an A-chain, a B-chain, and C-peptide. The A- and B-chains remain disulfide-linked and together are called **insulin**. Insulin binds **zinc** (see Fig. 26.3) and crystallizes inside the granules. **C-peptide** remains soluble and is secreted together with insulin.

In patients who have diabetes and inject pharmaceutical-grade insulin, measurement of the concentration of **C-peptide in blood** plasma provides information about the patient's insulin secretion. Although commercially available insulins are generated from proinsulins, they do not contain the C-peptide.

β-Cells are packed with about a week's supply of insulin-containing secretory vesicles. When a patient's pancreas cannot adequately control the concentration of glucose in the blood, it is almost never due to a lack of insulin inside β-cells; rather, it is due to a problem with the number of β-cells (e.g., type 1 diabetes) or the response of β-cells to physiological stimuli (e.g., MODY and type 2 diabetes; see Chapter 39).

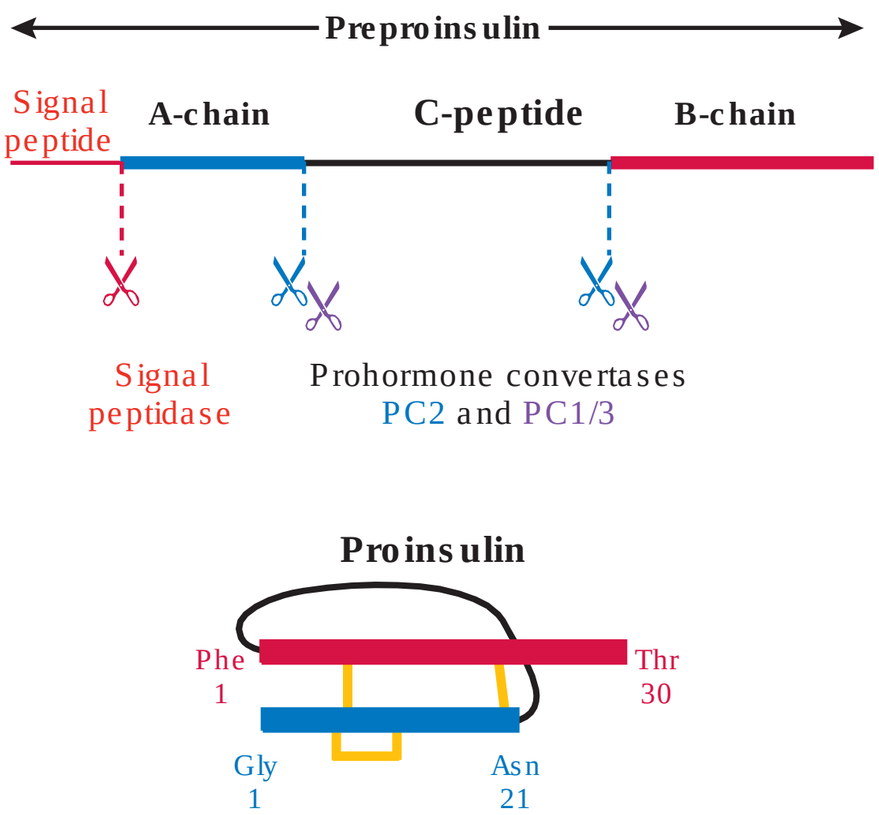


Fig. 26.3 Synthesis of insulin in pancreatic β-cells. Yellow lines indicate disulfide bonds. (Based on Protein Data Bank [www.rcsb.org] file 1MSO from Smith, GD, Pangborn WA, Blessing RH. The structure of T6 human insulin at 1.0 A resolution. *Acta Crystallogr D Biol Crystallogr.* 2003;59:474-482.)

Besides insulin, pancreatic β-cells also synthesize a small amount of **amylin**. Amylin is also called **islet amyloid polypeptide** (**IAPP**). Amylin is a small peptide that derives from preproamylin. On a molar basis, the β-cell secretory granules contain up to 100 times more insulin than amylin. The pancreatic β-cells are the primary but not the exclusive producer of amylin. Because the secretory granules of pancreatic β-cells contain both insulin and amylin, these two hormones are also secreted at the same time.

Amylin is found as part of extracellular **amyloid** deposits (see Chapter 9) in the islets of patients who secrete a large amount of insulin, commonly because of insulin resistance or an insulinoma (see Section 6 below).

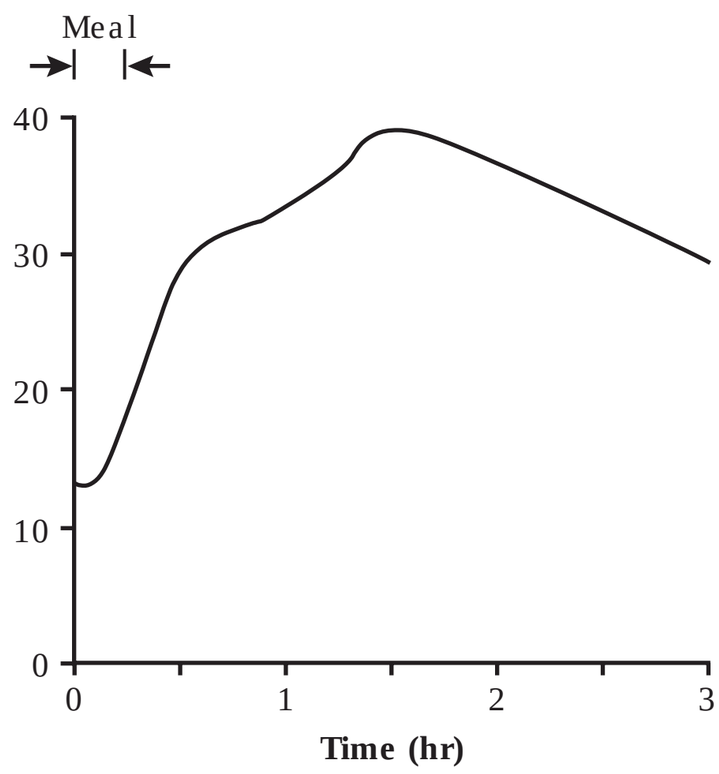


Fig. 26.4 Effect of a mixed meal on plasma glucagon-like peptide 1 (GLP-1). Volunteers consumed a 450-kcal breakfast after an overnight fast. (Data from Orskov C, Rabenhøj L, Wettergren A, Kofod H, Holst JJ. Tissue and plasma concentrations of amidated and glycine-extended glucagon-like peptide I in humans. *Diabetes*. 1994;43:535-9; and Højberg PV, Vilsbøll T, Zander M, et al. Four weeks of near-normalization of blood glucose has no effect on postprandial GLP-1 and GIP secretion, but augments pancreatic B-cell responsiveness to a meal in patients with type 2 diabetes. *Diabet Med*. 2008;25:1268-1275.)

2.3. Synthesis of Epinephrine and Cortisol in the Adrenal Glands

The adrenal glands sit on top of the kidneys (see Fig. 31.12 and 31.15) and contain a medulla (inner region) that synthesizes norepinephrine and epinephrine from tyrosine (see Section 4.2 in Chapter 35), and a cortex (outer region) that synthesizes cortisol from cholesterol (see Section 3 in Chapter 31). Norepinephrine and epinephrine are both **catecholamines** (dopamine is another catecholamine). The adrenal glands store catecholamines inside secretory vesicles. Cortisol is membrane permeable and therefore cannot be stored in secretory granules. Instead, it is synthesized as needed.

3. SECRETION OF GLUCAGON, GLUCAGON-LIKE PEPTIDES, INSULIN, EPINEPHRINE, AND CORTISOL

α- and β-cells in the islets of the pancreas secrete glucagon and insulin, respectively, in response to nutrients, incretins, and epinephrine, depending on the prevailing concentration of glucose. L-cells in the intestine secrete GLP-1 in response to nutrients in the diet. The hypothalamus and anterior pituitary gland control the secretion of epinephrine and cortisol.

3.1. Secretion of Glucagon-Like Peptide 1

L-cells in the distal ileum and colon secrete GLP-1 in response to fats and sugars, whereas amino acids have little effect. Fig. 26.4 shows the effect of a mixed meal on the concentration of GLP-1 in plasma.

3.2. Secretion of Glucagon

Amino acids are the principal fuel stimulus of glucagon secretion from α-cells. α-Cells appear to recognize amino acids much like β-cells do (see Fig. 26.7). **Glucose** decreases amino acid–induced glucagon secretion (the mechanism for this is still debated). Physiologically, the concentration of glucose is a major regulator of glucagon secretion. For instance, at the 1-hour time point of a 75-g **oral glucose tolerance test** given to fasting volunteers, the concentration of glucagon in peripheral blood reached a low of about 60% of the pretest concentration.

Acting through the β_2-adrenergic receptors, **epinephrine** and **norepinephrine** stimulate glucagon secretion from α-cells. T is occurs through the production of cyclic adenosine monophosphate (cAMP), activation of protein kinase A (PKA), and subsequent potentiation of exocytosis.

Glucagon from pancreatic islets enters the hepatic portal vein; the liver therefore experiences the highest concentration of glucagon. Under physiological conditions, changes in the concentration of glucagon in the peripheral circulation are small.

Fig. 26.5 shows the concentration of glucagon in the peripheral blood in response to a mixed meal. T e time course of the glucagon concentration is determined by a combination of decreased glucagon secretion due to meal-induced hyperglycemia and increased glucagon secretion due to the presence of amino acids. T e data make it apparent that glucagon secretion is ongoing, and that metabolism is regulated by small changes in glucagon concentration rather than an absence or presence of this hormone.

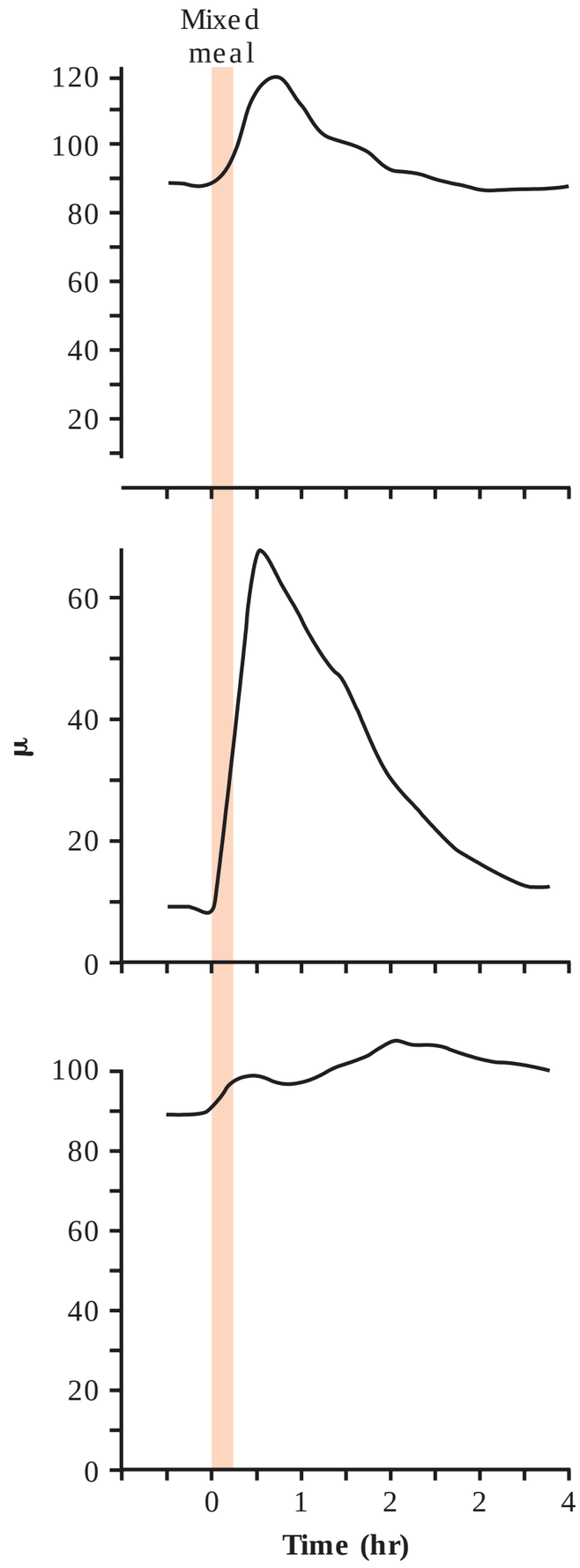


Fig. 26.5 Effect of a mixed meal on plasma glucose, insulin, and glucagon concentrations. Lean volunteers aged ~27 years were given a mixed meal of 400-500 kcal, of which approximately 50% was from carbohydrates and 17% from protein. (Data from Gerich JE, Lorenzi M, Karam JH, Schneider V, Forsham PH. Abnormal pancreatic glucagon secretion and postprandial hyperglycemia in diabetes mellitus. *JAMA*. 1975;234:159-165; and Cooperberg BA, Cryer PE. β-Cell-mediated signaling predominates over direct α-cell signaling in the regulation of glucagon secretion in humans. *Diabetes Care*. 2009;32:2275-2280.)

3.3. Secretion of Insulin

3.3.1. Stimulatory Effect of Glucose

T e principal stimulus for insulin secretion is an elevated concentration of glucose in the blood. Fig. 26.6 shows the relationship between the steady-state concentrations of glucose and insulin in blood plasma in overnight-fasted volunteers.

Inside β-cells, **glucokinase** serves as a glucose sensor (Fig. 26.7). GLUT-2 glucose transporters equilibrate glucose between blood plasma and the cytoplasm of β-cells. Glucokinase shows a small degree of cooperativity toward glucose, and it is half-maximally active at about the same concentration of glucose that half-maximally stimulates insulin secretion (i.e., ~10 mM or ~180 mg glucose/dL). Mutations that increase the affinity of glucokinase for glucose cause hypoglycemia (see Section 6.1), whereas mutations that decrease the affinity for glucose cause diabetes (MODY-2; see Section 6.2 and Chapter 39). Unlike hepatocytes, β-cells do not express the glucokinase regulatory protein (GKRP; see Chapter 19).

Pancreatic β-cells contain adenosine triphosphate (**ATP)-sensitive K^+-channels** (**K_{ATP}-channels**) that regulate insulin secretion (see Fig. 26.7). T e pore of these channels oscillates between open and closed states. T ese channels conduct more K^+ when the concentration of ATP is relatively low and the concentration of adenosine diphosphate (ADP) is relatively high. At a very low concentration of glucose, the concentration of ADP inside β-cells is relatively high and that of ATP slightly low. Under these conditions, K_{ATP} channels pass enough K^+ that they can polarize β-cells to about −70 mV; such polarized cells do not secrete insulin. In contrast, at a high concentration of glucose, the concentration of ADP is low and that of ATP is normal. T e K_{ATP} channels are therefore almost always closed now in place of K^+ flowing through K_{ATP}-channels, yet uncharacterized currents play a greater role in determining the membrane potential, and these currents depolarize the plasma membrane. Once the membrane

potential reaches about −40 mV, voltage-sensitive Ca^{2+} channels open and allow **Ca^{2+}** to flow from the extracellular space into the cytoplasm. T ere, the elevated concentration of Ca^{2+} activates **exocytosis**, which moves insulin-containing granules to the plasma membrane.

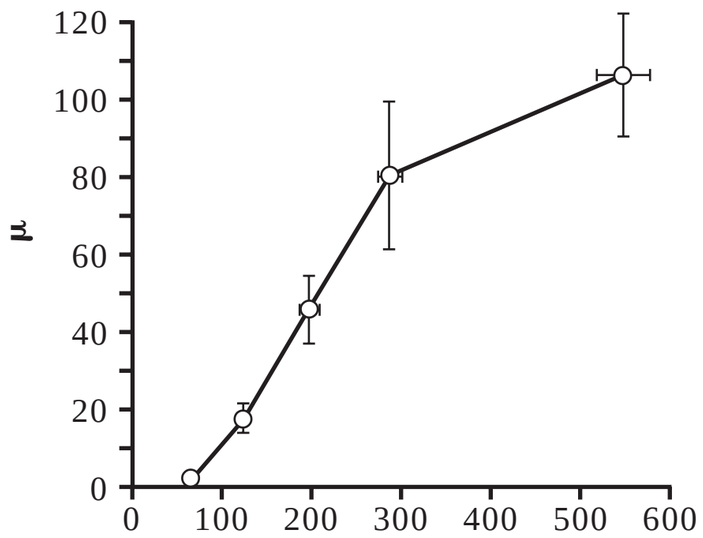


Fig. 26.6 Relationship between the steady-state concentrations of glucose and insulin in blood plasma. Volunteers were infused with glucose at various rates to near steady state. Blood samples were drawn and the concentrations of glucose and insulin determined. (Modified from Cerasi E, Luft R, Efendic S. Decreased sensitivity of the pancreatic beta cells to glucose in prediabetic and diabetic subjects; a glucose dose-response study. *Diabetes*. 1972;21:224-234.)

K_{ATP} channels are inhibited pharmacologically by the **sulfonylurea** and the **glinide anti-diabetes drugs** that are sometimes used to treat patients with type 2 diabetes (see Chapter 39). Each β-cell K_{ATP} channel consists of Kir6.2 peptides that form a K^+-selective pore, and sulfonylurea-receptor 1 (SUR1) peptides that regulate the opening and closing of the K^+ pore. T e sulfonylureas and the glinides bind to the SUR1 peptides of the K_{ATP} channels. T ese drugs boost insulin secretion by reducing the probability that K_{ATP} channels are open (hyperglycemia normally has this same ef ect). Obviously, a dangerous side ef ect of these drugs is excessive insulin secretion that leads to severe **hypoglycemia**.

Diazoxide, a potassium channel–opening drug, also binds to the SUR1 peptides of K_{ATP} channels in β-cells, but it increases the probability that the K_{ATP} channels are open. Consequently, diazoxide inhibits insulin secretion. Diazoxide is used in the rare patient who has persistent hypoglycemia from secretion of excessive amounts of insulin, most commonly as a result of heritable faulty sensing of amino acids (see Section 6.1).

During hormone secretion, secretory vesicles dock to the plasma membrane and empty their contents. Docking is aided by a protein on the vesicle surface and two proteins on the cytosolic plasma membrane surface that contain one or two **SNARE sequences**, which form a coiled coil structure (see Fig. 9.7).

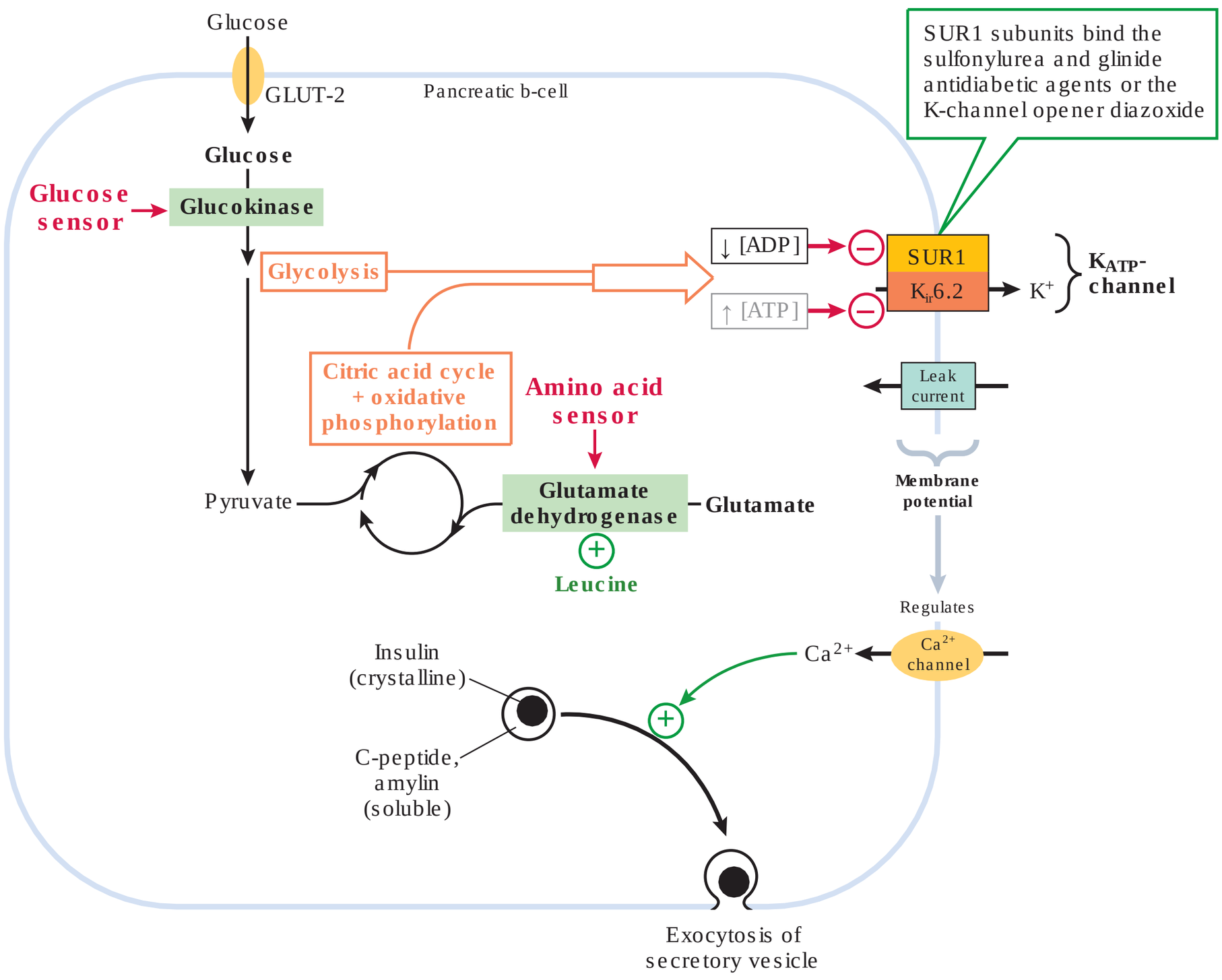


Fig. 26.7 Regulation of the membrane potential of pancreatic β-cells by adenosine triphosphate (ATP)-sensitive K^+-channels (K_{ATP} channels).

3.3.2. Amplification of Glucose-Induced Insulin Secretion

Incretins, amino acids, fatty acids, and ketone bodies can amplify glucose-induced insulin secretion, but by themselves, they cannot induce sustained insulin secretion.

The incretins **GLP-1** and **GIP** (gastric inhibitory peptide, glucose-dependent insulinotropic peptide) boost glucose-induced insulin secretion. Incretins are defined as hormones that are secreted from the intestine and regulate insulin secretion. GLP-1 and GIP are secreted when the gastrointestinal tract contains nutrients. GIP is secreted from **K-cells** in the duodenum and upper jejunum. GLP-1 is secreted mainly from L-cells in the ileum. GLP-1 and GIP increase insulin secretion only when the concentration of glucose is above ~90 mg/dL (~5 mM).

If a patient receives an intravenous infusion of glucose, incretins are not secreted. As a result, the same dose of glucose given intravenously results in lower insulin secretion than the same dose given orally.

In **parenteral nutrition,** insulin is sometimes infused together with glucose to increase glucose utilization and diminish hyperglycemia.

Some patients who have type 2 diabetes are treated with **GLP-1 receptor agonists.** These agonists are peptides and must be injected.

In the bloodstream, GLP-1 and GIP are rapidly degraded by dipeptidylpeptidase-4 (DPP-4). **DPP-4 inhibitors,** known as gliptins, are used to treat type 2 diabetes (see Chapter 39).

Among amino acids, the combination of **leucine** and **glutamine** is particularly effective at potentiating glucose-induced insulin secretion. Leucine is an essential amino acid. Its concentration in the blood rises significantly after a protein meal; this increase may serve as a signal of protein intake. (In the fasting state, muscle cells degrade protein and release amino acids into the blood, but they largely transaminate leucine and release only its corresponding ketoacid, α-ketoisocaproic acid.) Glutamine is the most abundant amino acid in the blood. Inside β-cells, glutamine is deaminated into glutamate. In the mitochondria, glutamate dehydrogenase, allosterically activated by leucine, converts glutamate to α-ketoglutarate, which is part of the citric acid cycle (see Fig. 26.7 and Fig. 22.7). In pancreatic β-cells, glutamate dehydrogenase is a sensor of amino acids; excessive activity of this enzyme leads to excessive insulin secretion and concomitant severe hypoglycemia (see Section 6.1.3). Besides insulin secretion, leucine and glutamine regulate other processes as well as insulin secretion. Thus, leucine also stimulates protein synthesis in skeletal muscle (see Chapter 34). Similarly, glutamine affects gene expression, protein synthesis, metabolism, and cell survival in many tissues of the body.

Besides leucine and glutamine, **arginine** and **lysine** also stimulate insulin secretion. The most commonly invoked explanation for the effects of these positively charged amino acids on insulin secretion is that their uptake depolarizes the β-cell plasma membrane and thus stimulates insulin secretion.

As evident from the above discussion, an elevated concentration of amino acids stimulates both insulin and glucagon secretion. As will become evident below, insulin stimulates not only the use of amino acids for protein synthesis, but also the removal of glucose from the blood; glucagon counteracts this last effect by favoring glucose production. The net result is the removal of amino acids and maintenance of normoglycemia.

Fatty acids and **ketone bodies** each have only a mild stimulatory effect on insulin secretion, but this effect is crucial in attenuating adipose tissue lipolysis in the fasting state to prevent ketoacidosis (see Chapter 27).

3.3.3. Inhibition of Insulin Secretion by Catecholamines

Epinephrine and norepinephrine potently inhibit insulin secretion, regardless of the β-cell stimulus. Epinephrine and norepinephrine both work through α_2-adrenergic receptors. Pancreatic β-cells are exposed to increased concentrations of epinephrine and norepinephrine during exercise, hypoglycemia, trauma, or stress.

Fig. 26.5 shows the concentrations of glucose and insulin in healthy volunteers in response to a mixed meal. Glucose in the meal is the principal stimulus for insulin secretion, and both incretins and amino acids enhance glucose-induced insulin secretion. Fatty acids and ketone bodies significantly stimulate insulin secretion only in the fasting state. Fig. 39.6 shows 1-day profiles of the concentrations of glucose and insulin in volunteers who consumed three mixed meals.

3.4. Secretion of Epinephrine and Norepinephrine

During exercise or hypoglycemia, nerves from the sympathetic division of the autonomic nervous system stimulate chromaffin cells in the medulla of the adrenal glands to secrete epinephrine and norepinephrine.

Fig. 26.8 shows the effects of short duration, high-intensity **exercise** on the plasma concentrations of glucose, insulin, glucagon, and epinephrine. During intense exercise, the concentration of glucose rises somewhat, but an increased concentration of epinephrine ensures that insulin secretion decreases. The concentration of glucose in the blood reflects the balance of glucose production and consumption by muscles. Glucose enters the blood from the intestine after a meal; otherwise, the liver produces glucose from glycogenolysis, and both the liver and the kidneys produce glucose from gluconeogenesis. During the recovery phase, glucose production initially far surpasses glucose consumption, thus increasing the concentration of glucose in the blood. In response to the elevated concentration of glucose and no longer inhibited by a high concentration of epinephrine, insulin is secreted. Insulin then attenuates glucose production.

For type 1 diabetic patients who no longer secrete insulin, it is challenging to manage blood glucose during and after exercise, which they must do by adjusting carbohydrate intake and the size of the subcutaneous insulin depot.

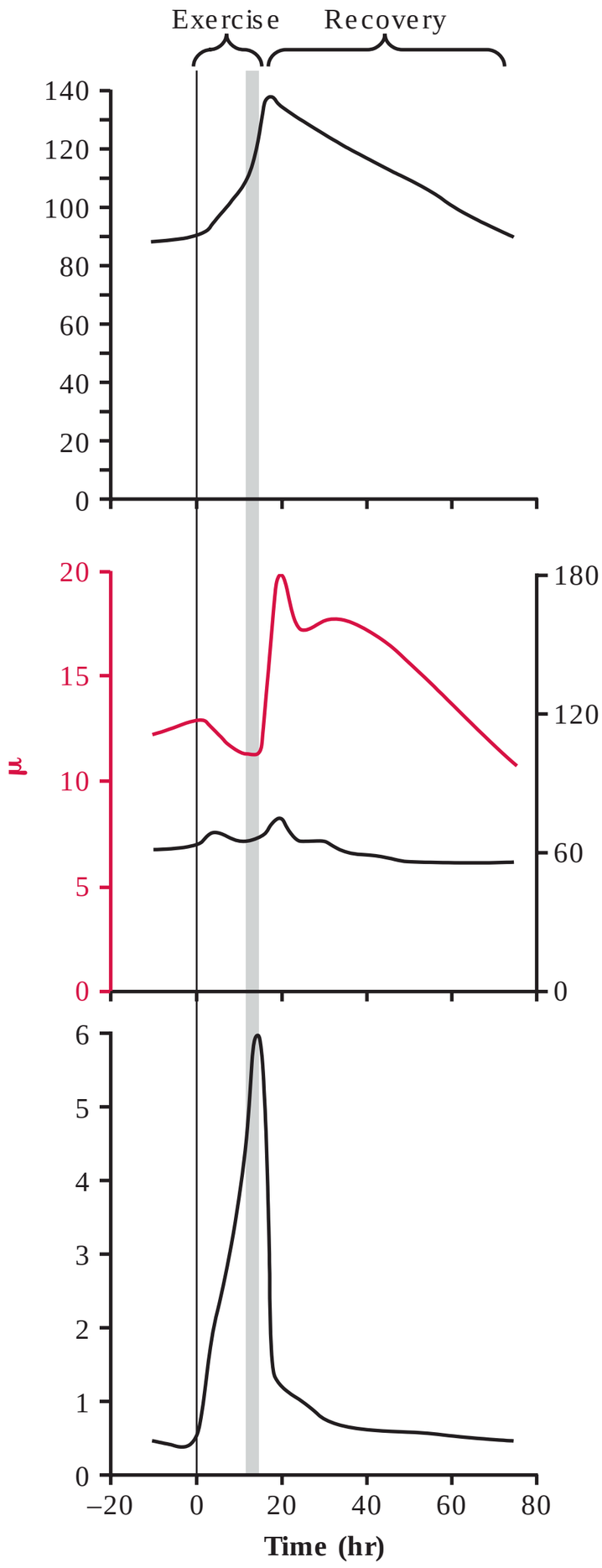


Fig. 26.8 Glucose, insulin, glucagon, and epinephrine in volunteers who exercised to exhaustion. Exhaustion occurred after 12-16 minutes of exercise (range indicated by gray bar). (Modified from Sigal RJ, Fisher SJ, Manzon A, et al. Glucoregulation during and after intense exercise: effects of alpha-adrenergic blockade. *Metabolism*. 2000;49:386-394.)

3.5. Secretion of Cortisol

The hypothalamus and the pituitary gland regulate the secretion of cortisol form the adrenal glands (see Chapter 31). The hypothalamus secretes corticotropin-releasing hormone (CRH), which stimulates the pituitary gland to secrete adrenocorticotropic hormone (ACTH). ACTH stimulates the synthesis and secretion of cortisol.

Cortisol is secreted in a diurnal pattern (see Fig. 31.13). With a normal sleep cycle, the lowest concentration of cortisol is observed around the time of sleep onset, and the highest shortly after awakening in the morning. In addition, long-term stress increases cortisol secretion (short-term stress increases the secretion of epinephrine and norepinephrine).

4. EFFECTS OF INSULIN AND COUNTERREGULATORY HORMONES ON TISSUES

Insulin lowers the concentration of glucose in the blood by affecting multiple metabolic pathways that consume glucose. The binding of insulin to insulin receptors activates intracellular signaling pathways that dephosphorylate certain enzymes of metabolism and alter the rate of transcription of certain genes. Glucagon increases the concentration of glucose in the blood by stimulating glycogenolysis and gluconeogenesis in the liver. Activated glucagon receptors signal through G-proteins that lead to altered rates of transcription of certain genes and phosphorylation of certain enzymes for metabolism. Epinephrine and norepinephrine signal through G protein–coupled receptors, and cortisol exerts its effects through receptors that are transcription factors.

4.1. Biological Effects of Glucagon-Like Peptides

GLP-1 potentiates glucose-induced insulin secretion, stimulates the growth of pancreatic β-cells, slows gastric emptying, decreases food intake, and favors glycogen synthesis in the liver rather than in muscle.

GLP-1 exerts its biological effects via a G protein–coupled **GLP-1 receptor** (see Chapter 33). In pancreatic β-cells, binding of GLP-1 to the GLP-1 receptor leads to an increased concentration of **cAMP** and activation of **protein kinase A (PKA)**, which enhances glucose-induced insulin secretion.

DPP-4 cleaves two amino acids from GLP-1 and thus renders it inactive. DPP-4 is present as a soluble protein in blood and as an integral membrane protein on the surface of many cells. **DPP-4 inhibitors** are used in the treatment of type 2 diabetes (see Chapter 39).

GLP-2 stimulates the growth of intestinal cells and is useful in the treatment of patients who have short bowel syndrome.

4.2. Biological Effects of Glucagon

Glucagon receptors are G protein–coupled receptors that signal via **cAMP** (similar to GLP-1 receptors; Fig. 26.9; see also Chapter 33). cAMP activates **PKA**, which phosphorylates various enzymes of metabolism, thereby either increasing or decreasing their activity. In addition, cAMP binds to **cAMP-response element-binding (CREB) protein**, which in turn binds to the promoter of certain genes and thereby alters the rate at which they are transcribed.

In the liver, glucagon stimulates **glycogenolysis** and **gluconeogenesis** while inhibiting **glycolysis** (see Chapters 24 and 25).

Glucagon and **GLP-1** receptors are each highly selective for glucagon and GLP-1, respectively, but they are not completely specific for either peptide due to peptide homology.

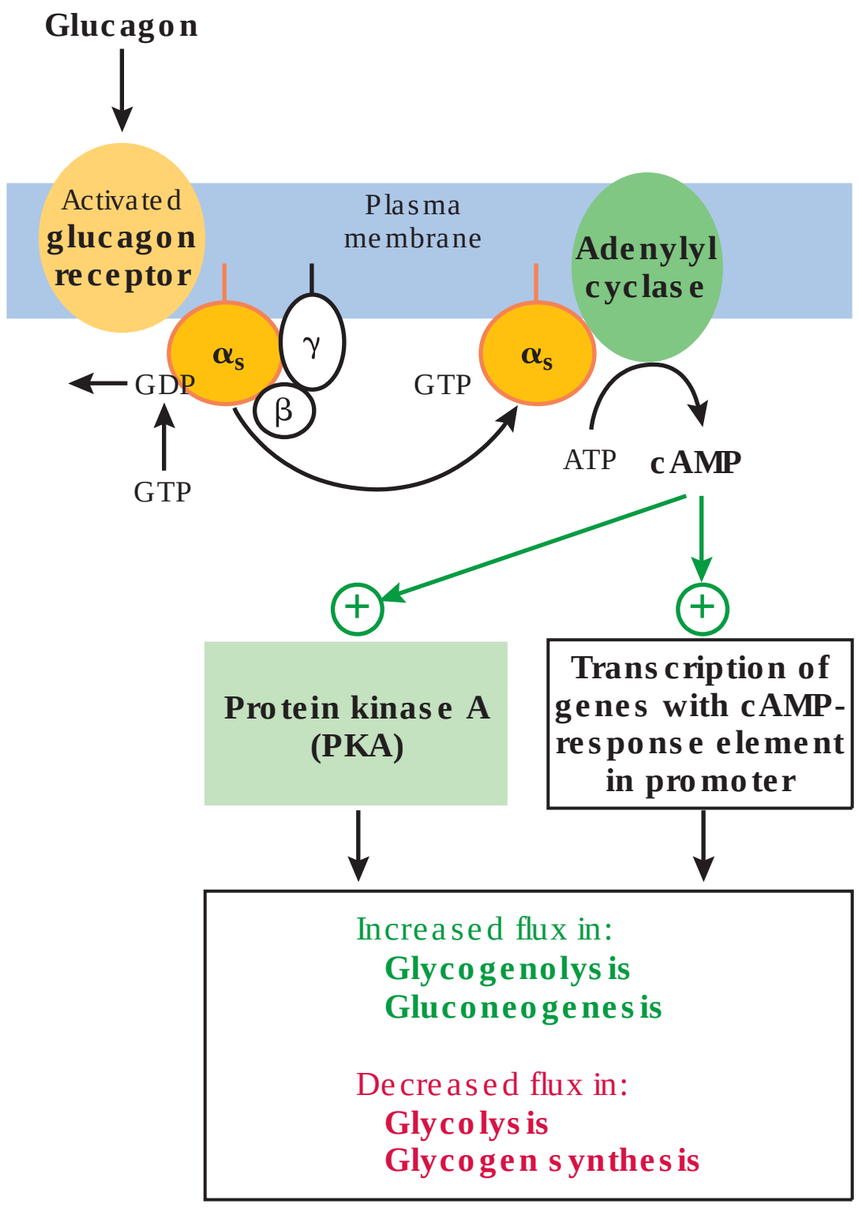


Fig. 26.9 **Overview of glucagon signaling.**

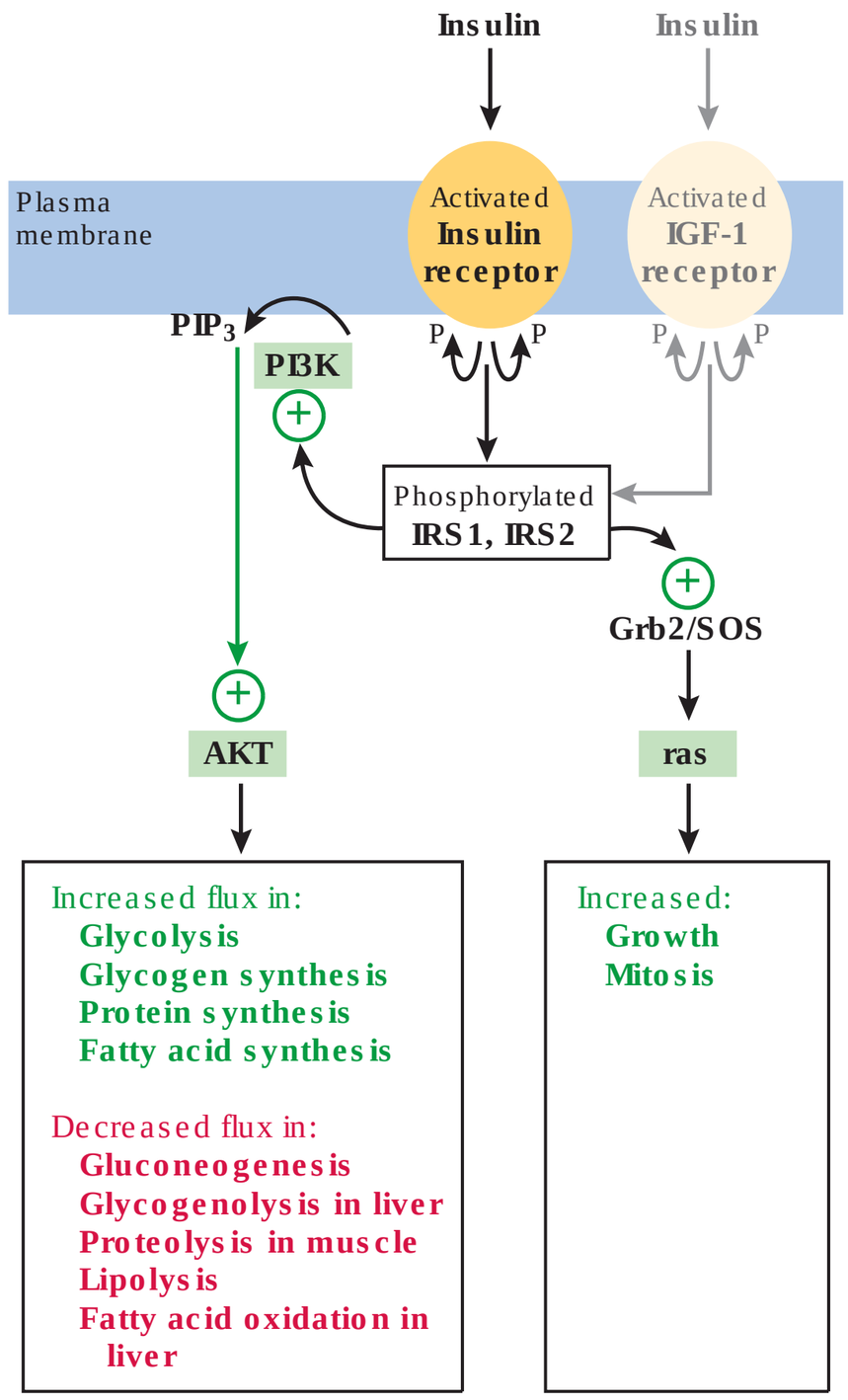


Fig. 26.10 **Biological effects of insulin.**

Glucagon is the most important hormone in the body's defense against hypoglycemia; epinephrine is the second most important such hormone. T e hormones glucagon, epinephrine, norepinephrine, and cortisol are called **counterregulatory hormones**.

Radiologists often use glucagon injected intravenously to relax and dilate the small intestine and reduce bowel motion.

Type 1 diabetic patients sometimes use glucagon to counteract **hypoglycemia**. Regardless of the concentration of insulin in the blood, glucagon stimulates glycogenolysis in the liver, which leads to an increase in blood glucose.

Once released into the blood, glucagon has a half-life of about 6 minutes. Glucagon is degraded by the liver, the kidneys, and enzymes in the blood vessels (mostly by DPP-4, the same enzyme that also degrades GLP-1).

4.3. Biological Effects of Insulin

Almost all cells have insulin receptors because insulin is a regulator of metabolism as well as cell proliferation. However, the number of insulin receptors varies among tissues.

With its diverse ef ects on almost all tissues, insulin takes a prominent position in hormone signaling. Insulin is a **growth factor** (see Chapter 8), promotes protein synthesis (see Chapters 7 and 34), and regulates metabolism. Fig. 26.10 lists the major ef ects of insulin on metabolism. Further details on these metabolic pathways are given in separate chapters. T e balance of metabolic and mitogenic ef ects of analogs of human insulin is of concern in the treatment of diabetes (see Chapter 39).

T e insulin receptor is a tetramer of two α- and two β-subunits. Proteolytic processing of the insulin receptor precursor gives rise to one α- and one β-subunit. T e α- and β-subunits aggregate to form active insulin receptors that span the plasma membrane.

When the insulin receptor binds insulin, it has tyrosine kinase activity, it phosphorylates itself, and it also phosphorylates **insulin receptor substrate** (**IRS**) proteins. T en, phosphorylated IRS acts as a signal and activates enzymes in two pathways: phosphatidylinositol 3-kinase (PI3K) and Grb2-SOS (a complex with guanine nucleotide exchange factor activity). PI3K phosphorylates the phospholipid phosphatidylinositol 4,5-bisphosphate (PIP2) to produce PIP3, which attracts protein kinase B (AKT, PKB) to the membrane and activates it. AKT, in turn, af ects the activity of various enzymes of metabolism. Grb2-SOS activates Ras in the ERK1/2 pathway, which alters the rate of transcription of certain genes.

Although not shown in Fig. 26.10, each signaling branch is also subject to stimulation and inhibition by other signaling pathways. Furthermore, each cell type has a tailored network of signaling pathways, thanks to the cell-specific expression of signaling proteins.

In response to a rising concentration of insulin, enzymes that play a role in fuel metabolism are usually **dephosphorylated**. In contrast, an increase in the concentration of glucagon

or epinephrine often leads to the phosphorylation of enzymes of metabolism (see below).

Insulin and **insulin-like growth factor 1** (**IGF-1**) can have similar biological effects. IGF-1 is normally derived mainly from the liver and circulates in the blood, along with insulin. IGF-1 is predominantly a growth factor. Insulin receptors prefer to bind insulin over IGF-1, and the reverse is true for IGF-1 receptors. Insulin and IGF-1 receptors signal in a similar, though not identical, fashion. Furthermore, cells can form heterodimeric insulin/IGF-1 receptors. For this reason, patients who have a tumor that secretes IGF-1 may have hypoglycemia. Furthermore, synthetic analogs of insulin used in the treatment of diabetes may be more mitogenic (and thus possibly tumorigenic) than normal insulin.

Signaling by insulin receptors is modulated by internalization and by the phosphorylation state of several residues. Occupation of the insulin receptor by insulin leads to the **internalization** of the receptor. Internalized receptors can either be returned to the plasma membrane or be degraded. **Phosphotyrosine phosphatases** dephosphorylate tyrosine-phosphorylated insulin receptors and thus render them inactive. Various **protein kinases** phosphorylate the insulin receptor on certain serine residues and thus render it less active.

In the blood, insulin has a half-life of about 4 minutes. After endocytosis of an insulin receptor-insulin complex, insulin is mostly degraded intracellularly by insulin-degrading enzyme and other enzymes. By the time blood from the hepatic portal vein reaches the hepatic vein, the liver has extracted approximately half of the insulin. The other half is removed principally by the kidneys and further passages through the liver.

4.4. Biological Effects of Epinephrine and Norepinephrine

Epinephrine and norepinephrine bind to α- and β-adrenergic G protein–coupled receptors, and these receptors and their subtypes couple to different α-subunits of heterotrimeric G proteins (see Chapter 33). α_2-Adrenergic receptors inhibit insulin secretion from β-cells by activating G_i and G_0 proteins. β-Adrenergic receptors enhance glycogenolysis and gluconeogenesis in the liver, lipolysis in adipose tissue, and glucagon secretion from α-cells.

Cells, particularly in the liver, take up circulating catecholamines and then inactivate them by methylating norepinephrine to **normetanephrine** and epinephrine to **metanephrine**; some of these metabolites end up in the urine. Measurement of normetanephrine or metanephrine in urine and/or blood plasma is part of the diagnosis of **pheochromocytoma** (a tumor that secretes mostly epinephrine and a lesser amount of norepinephrine; see Fig. 22.13).

4.5. Biological Effects of Cortisol

Cortisol crosses membranes and binds to the glucocorticoid receptor in the cytosol, which then moves into the nucleus, binds to a glucocorticoid response element, and thus stimulates transcription (see Chapters 6 and 31).

Cortisol enhances the transcription of transaminases, which help export alanine and glutamine from muscle and import these amino acids into the intestine and the liver (see Figs. 35.4 and 35.10). In muscle, transaminases facilitate the amination of pyruvate (producing alanine) and α-ketoglutarate (producing glutamate, which gives rise to glutamine). In the intestine and liver, transaminases facilitate the reverse processes. Transfer of amino acids from muscle to the liver is essential for gluconeogenesis (see Chapter 25).

5. PHYSIOLOGICAL AND PATHOLOGICAL CHANGES IN INSULIN SENSING

Insulin resistance is a state of diminished cellular responses to circulating insulin. Because pancreatic β-cells attempt to maintain the concentration of glucose in the blood at a normal concentration, β-cells in an insulin-resistant person must secrete more insulin. Insulin resistance is seen in normal pregnancy, in obese persons, and in those who have polycystic ovary syndrome.

5.1. General Comments About Insulin Resistance

The term **insulin resistance** refers to a state of poor response to insulin. The terms insulin resistance and insulin insensitivity mean the same, whereas the term insulin sensitivity means the opposite of insulin resistance. In clinical practice, insulin resistance refers to the effect of insulin on glucose transport; other effects of insulin on a patient's cells are currently measured only rarely. Compared with a patient with a normal response to insulin, an insulin-resistant patient needs a higher concentration of insulin to move a given amount of glucose out of the blood. The insulin resistance may be due to a problem with insulin receptors or with the insulin receptor–activated signaling pathway.

Insulin resistance can be organ specific or affect multiple organs. In humans, there is evidence that common forms of "whole body" insulin resistance are associated with insulin resistance of at least the liver, muscles, and adipose tissue.

Puberty is associated with mild insulin resistance. Insulin resistance is most pronounced around Tanner stage III. Girls are more insulin resistant than boys.

Pregnancy is associated with marked insulin resistance. Pregnant women in their third trimester secrete about eight times more insulin than nonpregnant women, although the mass of pancreatic β-cells increases by only about 25% with pregnancy. If the pancreas does not provide the required extra insulin, **gestational diabetes** ensues (see Chapter 39).

Pharmacological doses of **corticosteroids** induce insulin resistance. Patients who take corticosteroids for years are at an increased risk of developing diabetes.

In developed countries, **obesity** is the most common cause of insulin resistance (see Chapter 39). T e cause of this association is still debated. It may be that triglyceride-laden adipocytes have altered secretion of hormones and fatty acids. Furthermore, triglyceride accumulation inside muscle and the liver may impair signaling from activated insulin receptors.

Severe insulin resistance is often accompanied by **acanthosis nigricans** (thickening and darkening of the skin, most often in the axillae and the skin folds of the neck and groin Fig. 26.11); **ovarian dysfunction**, **hyperandrogenism**, and **hirsutism** (male-pattern hair growth; Fig. 26.12); and **lipoatrophy.**

Exercise depletes the glycogen stores of skeletal muscle; as a consequence, after a meal, more glucose can be deposited as glycogen in exercised than in unexercised muscle. Persons who exercise regularly are less likely to be insulin resistant than **sedentary** persons. Most insulin-resistant persons can increase their insulin sensitivity with exercise.

In medical practice, insulin sensitivity, if quantified, can be estimated in one of the following ways.

1. In patients who have **type 2 diabetes** and treat their disease with insulin, the **daily dose** of insulin required for blood glucose control gives the treating physician an idea of the patient's insulin sensitivity. A lean adult without β-cells requires about 30 units of insulin per day.
2. Glucose and insulin can be measured in plasma after an overnight fast, and an insulin sensitivity index can then be calculated.
3. Glucose and insulin in plasma can be measured before and during an oral glucose tolerance test, and the data can be used to calculate another insulin sensitivity index.
4. Rarely, an **insulin tolerance test** is applied, which consists of measuring the degree of hypoglycemia after an injection of insulin. At first, a fasting patient is injected with only a small amount of insulin (often ~0.1 U/kg body weight). If hypoglycemia does not occur, the patient is injected with increasingly higher amounts of insulin. Insulin-resistant patients need an abnormally large amount of insulin to cause hypoglycemia. Unfortunately, there are no generally accepted ranges that define normal insulin sensitivity.

Only a minority of patients with **hereditary severe insulin resistance** have mutant insulin receptors. Instead, they are likely to have mutations in other proteins that are involved in insulin signaling.

Fig. 26.11 **Acanthosis nigricans.**

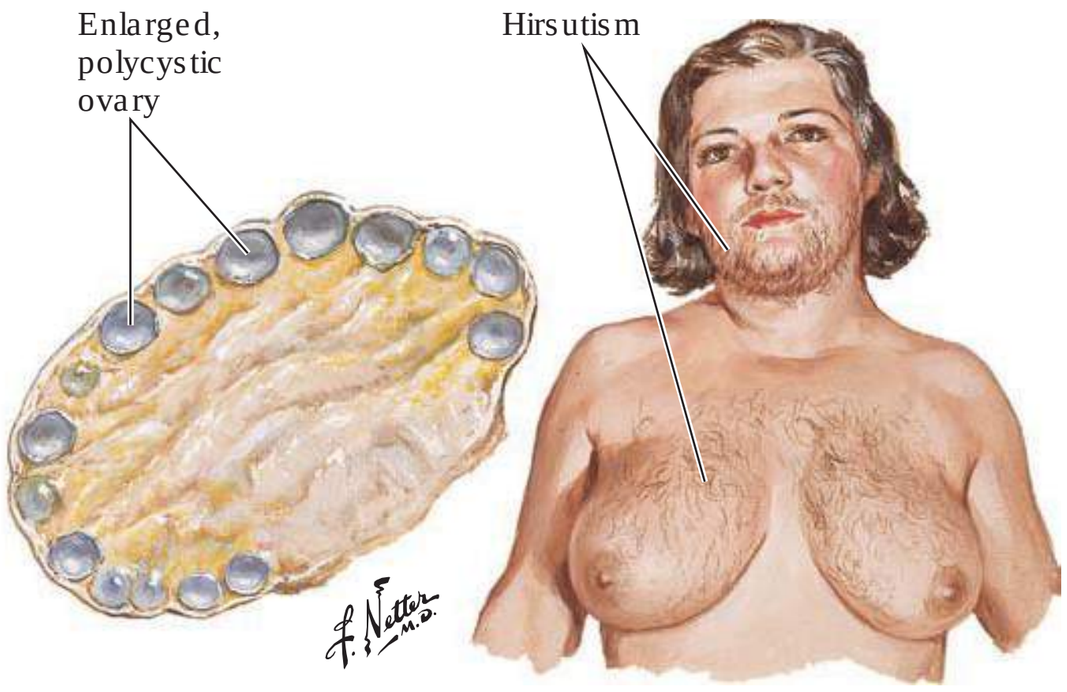


Fig. 26.12 **Patient with polycystic ovary syndrome.**

5.2. Polycystic Ovary Syndrome

Polycystic ovary syndrome (PCOS) af ects about 5% to 10% of women during their reproductive years. In women who do not take birth control pills, a polycystic ovary is defined as an ovary that has a volume greater than 10 mL and/or contains 12 or more follicles 2 to 9 mm in diameter (see Further Reading for a reference to the currently used 2003 Rotterdam criteria). T e ovarian dysfunction is commonly associated with an abnormally high concentration of androgens in the blood (see Chapter 31). PCOS may be accompanied by irregular menses, infertility, obesity, and hirsutism (i.e., male-pattern hair growth; see Fig. 26.12). About 40% of patients with PCOS have impaired glucose tolerance or diabetes.

PCOS most likely represents a family of diseases of yet unknown cause. T e syndrome shows multigenic inheritance with a strong environmental component.

Among patients with PCOS, **insulin resistance** is common, even though this is not part of the diagnosis. If insulin resistance is assessed, the measurements usually refer only to the relationship between insulin and glucose metabolism, whereby metabolism in skeletal muscle contributes the most. How these measurements relate to the insulin sensitivity of the androgen-producing theca cells in the ovaries is uncertain.

In overweight or obese patients with PCOS, an increase in insulin sensitivity can be achieved with weight loss and exercise. T is is accompanied by increased fertility. Oral contraceptives with progestin and estrogen can be used to treat the hyperandrogenism and hirsutism.

6. PATHOLOGY OF THE SECRETION OF INSULIN AND COUNTERREGULATORY HORMONES

Insulin-secreting tumors occasionally develop in middle-aged patients and cause hypoglycemia. Multiple endocrine neoplasia (MEN-1) is a syndrome of tumor formation in two or more endocrine organs. Mutations in one of several proteins that are involved in β-cell development or insulin secretion can give rise to either insufficient or excessive insulin secretion (i.e., to hypoglycemia or hyperglycemia). Adrenal insufficiency can give rise to hypoglycemia, and an excess of epinephrine or cortisol can cause hyperglycemia.

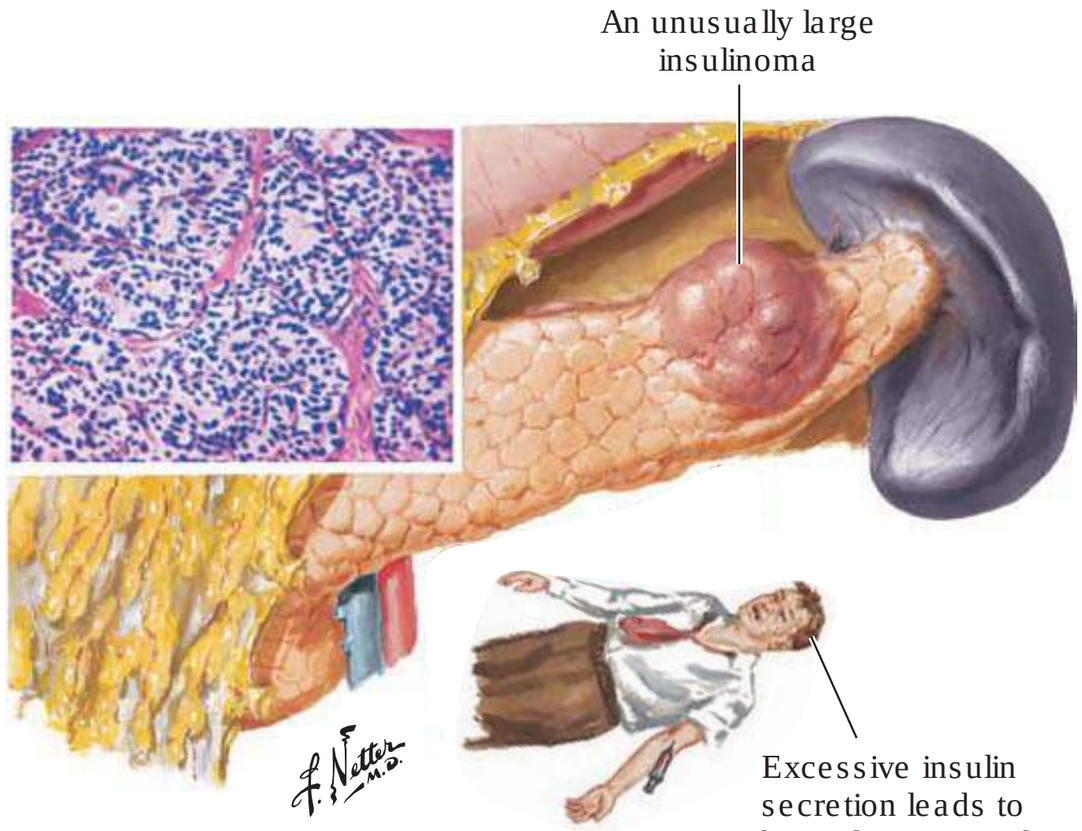


Fig. 26.13 **Patient with an insulinoma.**

6.1. Disorders Associated With Hypoglycemia

When determining the cause of hypoglycemia, physicians sometimes first distinguish between ketotic and nonketotic hypoglycemia.

In **ketotic hypoglycemia**, the concentration of insulin must be low because it is a prerequisite for a high rate of lipolysis that, in turn, gives rise to the conversion of fatty acids to ketone bodies (see Chapter 27). Hence, in ketotic hypoglycemia the problem is usually with glucose production, (i.e., in glycogenolysis and/or gluconeogenesis; see Chapters 24 and 25).

In **nonketotic** or **hypoketotic hypoglycemia**, there is usually a problem with excessive insulin (which inhibits lipolysis and glucose production) or a problem with the oxidation of fatty acids (see Chapter 27).

6.1.1. Insulinoma

Many pancreatic endocrine tumors secrete a variety of hormones, but the secretion of one hormone usually far outpaces that of all others.

Insulinomas cause hypoglycemia. Excessive secretion of insulin from an insulinoma is usually due to an abnormal regulation of insulin secretion by glucose. T e response of the nervous system to hypoglycemia causes patients to be hungry, sweaty, and anxious and have a tremor. Food intake temporarily alleviates the hypoglycemia; many patients who have an insulinoma therefore become overweight or obese. Marked hypoglycemia impairs primarily the central nervous system and can manifest itself in confusion, unusual behavior, visual disturbances, and eventually seizures and loss of consciousness.

Despite the hypoglycemia, patients with an insulinoma do not show ketosis. T e high concentration of insulin inhibits lipolysis (see Chapter 28). When the concentration of free fatty acids in the blood is low, the liver does not produce ketone bodies.

Insulinomas are commonly diagnosed based on symptoms of hypoglycemia in the fasting state that are accompanied by measurable hypoglycemia and excessive concentrations of both insulin and C-peptide in the blood whereby infusion of glucose or glucagon provides rapid relief of symptoms. Diagnosis of an insulinoma also requires ruling out the surreptitious administration of an anti-diabetes drug, such as a sulfonylurea.

Surgical removal of the insulinoma usually cures the hypoglycemia. About 90% of all insulinomas are benign. About 95% of insulinomas are sporadic, and about 5% are associated with MEN-1 (see below). Perhaps 1 in 4,000 persons develops an insulinoma during his or her lifetime. T e median age at diagnosis is about 50 years. T e median diameter of insulinomas is only about 1.3 cm, and they are therefore often difficult to locate (Fig. 26.13). Patients who cannot undergo surgery can be treated with **diazoxide** (a K^+ channel opener and activator of K_{ATP} channels; see Fig. 26.7).

6.1.2. Multiple Endocrine Neoplasia

MEN-1, also called **Wermer syndrome**, af ects at least 1 in 30,000 persons. Af ected patients most often inherit one nonfunctional copy of the tumor suppressor and transcription factor menin. T e physiological role of menin is only poorly understood. In persons with MEN-1, the function of the normal menin allele is lost sporadically in endocrine glands, particularly the parathyroid glands, pancreatic islets, and anterior pituitary. Neoplasms may then develop by additional mutations in other genes (see Chapter 8). By 40 years of age, almost all af ected patients develop symptomatic hyperplasias and adenomas. Patients often have multiple adenomas, which

complicate surgical excision. Pancreatic islet adenomas secrete multiple hormones and cause hypoglycemia only if they secrete sufficient insulin. Islet adenomas are often malignant and deadly. However, of all patients who have an insulinoma, only a minority have MEN-1.

6.1.3. Congenital Hyperinsulinism

Known heritable abnormalities of β-cell proteins are very rare, but they tell us a lot about the mechanisms that control insulin secretion. Four such abnormalities cause excessive insulin secretion and thus pose a risk of deadly hypoglycemia: mutations that affect K_{ATP} channels, glucokinase, glutamate dehydrogenase, or a monocarboxylate transporter.

Patients with insufficiently active K_{ATP} channels due to autosomal recessive mutations in the Kir6.2 or SUR1 subunits cannot adequately suppress insulin secretion during hypoglycemia. This disease is present in utero and causes macrosomia by excessive insulin stimulation of fatty acid synthesis and triglyceride deposition in the adipose tissue. Shortly after birth, affected infants become severely hypoglycemic. The hypoglycemia must be counteracted with infusions of glucose. Insulin secretion can be inhibited by a calcium channel inhibitor, epinephrine, or a somatostatin analog. The excessive concentration of circulating insulin can be partially balanced with an infusion of glucagon or epinephrine. In most patients, the K_{ATP} channel opener diazoxide is ineffective. Many affected patients require partial pancreatectomy to control blood glucose, but this means that they may later develop diabetes. For reasons that are not understood, the hyperinsulinemia abates with age.

Patients who are heterozygous for a mutant **glucokinase** that is overly sensitive to glucose secrete insulin even during hypoglycemia. This is disease is usually evident in the newborn period. Many affected patients can be successfully treated with diazoxide, which opens K_{ATP} channels in pancreatic endocrine cells (see Fig. 26.7).

Patients with overly active, mutant **glutamate dehydrogenase** may secrete an excessive amount of insulin in response to elevated concentrations of leucine and glutamine in the blood. The disease is inherited in autosomal dominant fashion. Hypoglycemia most commonly sets in after a high-protein, low-carbohydrate meal, but it also occurs during an extended period of fasting. Some patients show hypoglycemia already in the newborn period, whereas others receive a diagnosis only as adults. Patients with mutant glutamate dehydrogenase also can be treated with diazoxide (an opener of K_{ATP} channels). These patients should avoid long fasts or high-protein, low-carbohydrate meals.

Some patients have **exercise-induced hypoglycemia** due to inappropriate expression of the **monocarboxylate transporter 1**, which moves lactate and pyruvate across the β-cell plasma membrane. The mutation is in the promoter region of the SLC16A1 gene, which encodes the monocarboxylate transporter. As a result, the β-cells secrete insulin when the concentration of pyruvate or lactate in the blood is increased (e.g., due to exercise). Within 30 minutes of a short bout of anaerobic exercise, affected patients become hypoglycemic. The disorder is inherited in a dominant fashion.

6.1.4. Adrenal Insufficiency

Patients who have adrenal insufficiency, especially children, often develop hypoglycemia in the fasting state.

6.2. Disorders Associated With Hyperglycemia

6.2.1. Diabetes Due to Heritable β-Cell Abnormalities

As described in Section 5, insulin resistance requires a compensatory increase in insulin secretion. It is unclear how insulin resistance eventually leads to β-cell failure. This section describes causes of abnormal insulin secretion that originate in the *β*-cell.

Infants who are diagnosed with diabetes during the first 6 months of life are said to have **neonatal diabetes**, which is usually due to heritable abnormalities in β-cell proteins. Table 44-1 in Chapter 39 provides more details.

In Europe, roughly half of the infants diagnosed with neonatal diabetes are heterozygous for mutations that lead to overactive **K_{ATP} channels**. When K_{ATP} channels are overly active, they keep the β-cells overly polarized, and the β-cells therefore do not secrete enough insulin (see Fig. 26.7). These patients can be treated with insulin, but many achieve better control of blood glucose if they are given a relatively high dose of a sulfonylurea drug.

Heritable mutations in genes that code for yet other proteins that are important for β-cell development, insulin synthesis, or insulin secretion cause **MODY**. This form of diabetes is due to heritable mutations in proteins of the liver and/or β-cells that are essential to glucose homeostasis. MODY resembles type 2 diabetes, which is typically seen in older (i.e., mature) patients, but MODY can present at any age (including newborns). There is some overlap in the genes that are mutated in neonatal diabetes and MODY. Patients with MODY make up a few percent of all patients with diabetes, at most. Section 6 in Chapter 39 provides further details about the most common subtypes of MODY. These phenotypes of these mutations confirm and shape our concepts of the mechanism of pancreatic hormone secretion.

6.2.2. Hyperglycemia Due to Glucagonoma

Glucagonomas are rare, usually malignant, and often recognized only after metastases have formed. The very high concentration of glucagon excessively stimulates gluconeogenesis and to some extent, lipolysis. The elevated rate of lipolysis leads to a high concentration of free fatty acids in the blood. The excessive rate of gluconeogenesis leads to hyperglycemia and hypoaminoacidemia. The hypoaminoacidemia decreases the synthesis of new protein in muscle. Patients with a glucagonoma typically lose weight due to a diminishing mass of both adipose tissue and skeletal muscle. The low concentration of amino acids in the blood often gives rise to a **migratory**

skin rash, which frequently brings patients to medical attention. Other patients first come to medical attention because of type 2 diabetes. Diabetes is thought to be due to β-cell failure after chronic stimulation of β-cells by hyperglycemia.

6.2.3. Hyperglycemia Due to Pheochromocytoma or Cushing Syndrome

Due to the overproduction of epinephrine and norepinephrine, some patients with a **pheochromocytoma** (see Fig. 22.13) also have hyperglycemia (but hypertension is the main presenting symptom).

Hyperglycemia is quite common in patients with **Cushing syndrome** (i.e., hypercortisolism; see Chapter 31). Cortisol favors funneling amino acids into gluconeogenesis, thus increasing glucose production. In addition, cortisol induces insulin resistance, which is initially overcome by increased insulin secretion but eventually leads to impaired glucose-induced insulin secretion.

SUMMARY

- Endocrine cells, organized into islets, make up about 1% of the pancreas. They produce insulin, amylin, and glucagon as well as other peptide hormones and store them in secretory vesicles. These peptides are derived from larger precursors (preprohormones) by proteolysis. Signal peptide peptidase in the endoplasmic reticulum cleaves the signal sequence, whereas prohormone convertases PC1/3 and PC2 in secretory vesicles cleave prohormones.
- Glucose (≥5 mM, or 90 mg/dL) by itself stimulates insulin secretion. Amino acids, fatty acids, and ketone bodies all potentiate glucose-induced insulin secretion. The gastrointestinal tract secretes the incretins glucagon-like peptide 1 (GLP-1) and gastric inhibitory peptide (GIP), which also amplify glucose-induced insulin secretion. Epinephrine inhibits insulin secretion.
- Pancreatic β-cells use glucokinase as a glucose sensor. They also use glutamate dehydrogenase as a leucine- and glutamine-dependent sensor of amino acids. ATP-sensitive K^+ channels (K_{ATP} channels) sense cytosolic ADP and ATP. When active, K_{ATP} channels polarize the plasma membrane and prevent insulin secretion. When inactive, K_{ATP} channels permit membrane depolarization, which is followed by calcium influx and the exocytosis of secretory granules. Certain anti-diabetes drugs (e.g., sulfonylureas, repaglinide, and nateglinide) decrease the opening frequency of K_{ATP} channels and thus boost insulin secretion.
- Amino acids, epinephrine, and hypoglycemia-sensing neuronal pathways stimulate glucagon secretion. In contrast, glucose inhibits glucagon secretion.
- Insulin binds to a receptor in the plasma membrane of a cell. All cells have insulin receptors, although at different densities. The cytoplasmic portion of the insulin receptor has tyrosine kinase activity that phosphorylates insulin receptor substrate (IRS). Grb2-SOS and phosphatidylinositol 3-kinase (PI3K) bind to phosphorylated IRS and thereby become active themselves. Signaling from Grb2-SOS stimulates cell growth and facilitates cell survival. Signaling from PI3K via AKT eventually leads to dephosphorylation of enzymes of metabolism. Some enzymes are activated by dephosphorylation, and others are inactivated. In muscle and adipose tissue, PI3K-derived signals also lead to the insertion of insulin-sensitive glucose transporters (GLUT-4) into the plasma membrane.
- The liver is physiologically the most important target of glucagon. Muscle cells have only an insignificant number of glucagon receptors. Glucagon binds to a G-protein–coupled receptor in the plasma membrane that leads to signaling via cAMP, protein kinase A (PKA), and cAMP-response element-binding (CREB) protein. Activation of glucagon receptors leads to the phosphorylation of target enzymes of metabolism and to increased transcription of genes that are linked to a promoter that has a cAMP-response element.
- Patients who are insulin resistant require a higher concentration of insulin to control the concentration of glucose in the blood than patients who have a normal response to insulin. Puberty is associated with mild insulin resistance. Pregnancy and obesity cause moderate insulin resistance. Polycystic ovary syndrome (PCOS) is usually associated with insulin resistance.
- Patients with insulinomas have episodes of nonketotic hypoglycemia. Patients with inactivating mutations in K_{ATP} channels, or with activating mutations in glucokinase or glutamate dehydrogenase, may have congenital persistent or episodic nonketotic hypoglycemia. Activating mutations in K_{ATP} channels and inactivating mutations in glucokinase, as well as mutations in other factors that impair β-cell development, insulin synthesis, or insulin secretion may cause hyperglycemia and thus diabetes.
- Patients with glucagonoma are hyperglycemic and hypoaminoacidemic. They also frequently have a migratory skin rash and diabetes.

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- T e Rotterdam ESHRE/ASRM-sponsored PCOS consensus workshop group. Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome (PCOS). Hum Reprod. 2004;19: 41-47.
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Review Questions

1. Under physiological circumstances, which of the following increase both insulin and glucagon secretion?

   A. Amino acids
   B. Epinephrine
   C. Fatty acids
   D. GLP-1
   E. Glucose

2. A 28-year-old woman has hyperglycemia (160 mg/dL, or 8.9 mM). Immune assays show that the patient has extreme hyperinsulinemia. When injected with a dose of a recombinant human insulin analog that induces mild hypoglycemia in insulin-sensitive persons, this patient develops a similar mild hypoglycemia. T is patient most likely has an inborn abnormality of which of the following?

   A. Glucose transporter
   B. Grb2/SOS
   C. Insulin
   D. Insulin receptor
   E. Phosphatidylinositol 3-kinase

Chapter

27

Fatty Acids, Ketone Bodies, and Ketoacidosis

SYNOPSIS

- Fatty acid synthesis occurs mostly in the liver after a carbohydrate-rich meal, stimulated by insulin. The lactating mammary glands also synthesize fatty acids.
- Fatty acids are synthesized in two-carbon steps. To this end, glucose gives rise to malonyl-CoA in the cytosol (Fig. 27.1). Condensation of several malonyl-CoA yields long-chain fatty acids of about 16 carbons. These fatty acids, along with dietary fatty acids, are stored as triglycerides in adipose tissue.
- Many cells can elongate long-chain fatty acids and introduce double bonds in the cis configuration in certain places.
- Particularly in the fasting state and during prolonged exercise, but to a small degree also on an ongoing basis, the adipose tissue hydrolyzes triglycerides and releases fatty acids into the blood (see Fig. 27.1). Many different tissues oxidize these fatty acids to acetyl-CoA in their mitochondria. The liver also converts acetyl-CoA to ketone bodies that the brain and muscle oxidize to acetyl-CoA. Fatty acids and ketone bodies are the principal fuel for energy production during prolonged starvation; this helps the body conserve glucose for cells that depend on it, such as erythrocytes and the central nervous system.
- Patients who have a disorder of fatty acid oxidation may develop hypoglycemia when fasting and show dysfunction of the heart and skeletal muscle.
- Fatty acids with double bonds in the trans configuration are found in foods that are derived from ruminants or contain chemically partially hydrogenated oils.

LEARNING OBJECTIVES

For mastery *of* this topic, you should be able to do the *f*ollowing:

- Interpret common chemical notations pertaining to fatty acids, such as 18:2, Δ^9, ω-3, and n-6.
- List common sources of cis and trans unsaturated fatty acids.
- Describe the overall purpose of fatty acid synthesis as well as its reactants and products, cellular location, tissue distribution, and regulation.
- Describe the elongation and desaturation of fatty acids as well as their reactants and products, cellular location, and tissue distribution.
- Define the term "essential fatty acids," list two classes of essential fatty acids, and provide an example of a fatty acid in each class.
- Describe the overall purpose of fatty acid β-oxidation as well as its reactants and products, cellular location, tissue distribution, and regulation.
- Describe the overall purpose of ketone body synthesis and ketone body oxidation as well as their reactants and products, cellular location, tissue distribution, and regulation.
- Explain the terms ketosis, ketoacidosis, ketonemia, and ketonuria. Compare and contrast the metabolic basis of ketosis and ketoacidosis.
- Describe the blood glucose concentration and the rate of endogenous glucose production in the fed and fasting state of a patient who has a deficient rate of fatty acid β-oxidation.
- Describe the β-oxidation of very-long-chain fatty acids in peroxisomes and compare it with β-oxidation of long-chain fatty acids inside mitochondria.
- Name the cause of X-linked adrenoleukodystrophy and the cause of Zellweger syndrome.
- Describe the link between oxidative phosphorylation and fatty acid β-oxidation, and predict the change in the rate of β-oxidation when a tissue becomes hypoxic.

1. USE AND NOMENCLATURE OF FATTY ACIDS

Fatty acids consist o*f* a hydrophilic –COOH group and a hydrophobic tail that may contain one or more double bonds, mostly in the cis conf guration. Partial chemical hydrogenation o*f* oils generates some trans *f*atty acids. Fatty acids are needed *f*or the synthesis o*f* eicosanoids, phospholipids, and triglycerides.

Table 27.1 lists physiologically important fatty acids along with their structure, use, and properties.

T e body uses fatty acids to **anchor** certain proteins in the membrane and to form **phospholipids** for membranes, **eicosanoids** and **docosanoids** for signaling, or **triglycerides** as an energy store. Fig. 27.2 shows the structures of these compounds. Most of the fat we eat is in the form of triglycerides.

Short-chain fatty acids contain 5 or fewer carbons, **medium-chain** contain ~6 to 12 carbons, **long-chain** contain ~14 to 20 carbons, and **very-long-chain** fatty acids contain 22 or more carbons.

Fatty acids are **detergents** and can therefore be detrimental to cell function. Hence, fatty acids are mostly bound to **albumin** in the blood and **fatty acid binding proteins** inside cells (see Figs. 9.6 and 9.8).

Fatty acids without double bonds are called **saturated**, those with one double bond are called **monounsaturated** (**MUFA**), and those with two or more double bonds are called **polyunsaturated** (**PUFA**).

T e location of **double bonds** in fatty acids can be described according to the classical chemical method for acids or a special **omega** terminology. A notation of 18:1 indicates that a fatty acid has 18 carbons and one double bond. T e location of the double bond is often indicated by a notation such as Δ^9, which indicates that the double bond is between carbons 9 and 10, with carbon 1 being the carbon of the carboxyl (–COOH) end of the fatty acid. In contrast, the C at the end of the hydrophobic tail of a fatty acid is called ω-1, omega-1, or n-1. For a discussion of fatty acid metabolism, the omega numbering

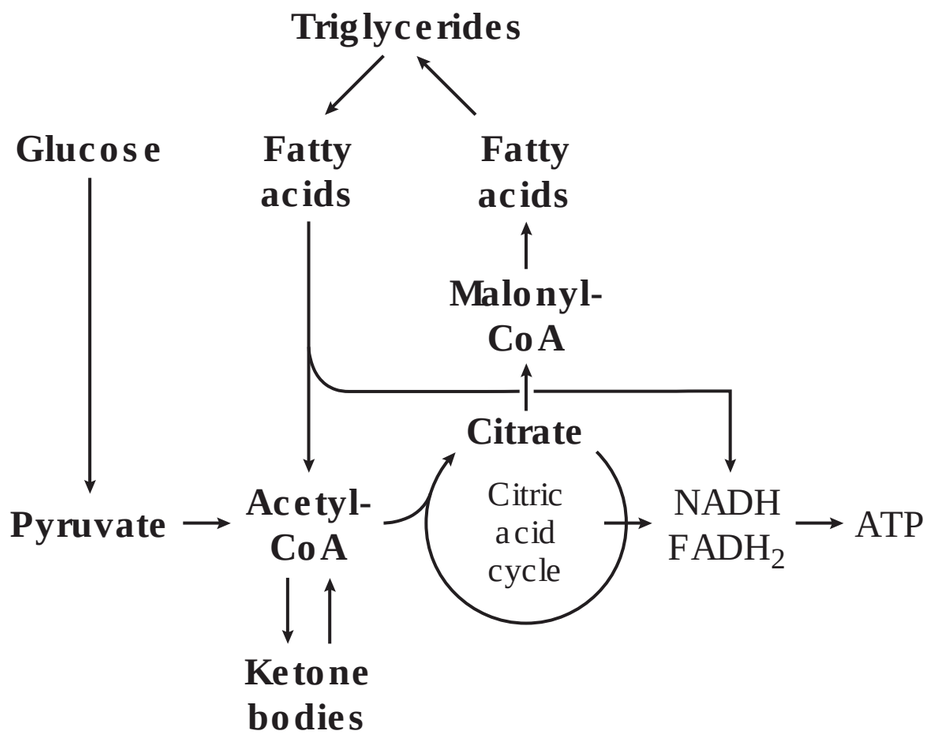


Fig. 27.1 **Overview of the synthesis, storage, mobilization, and oxidation of fatty acids.**

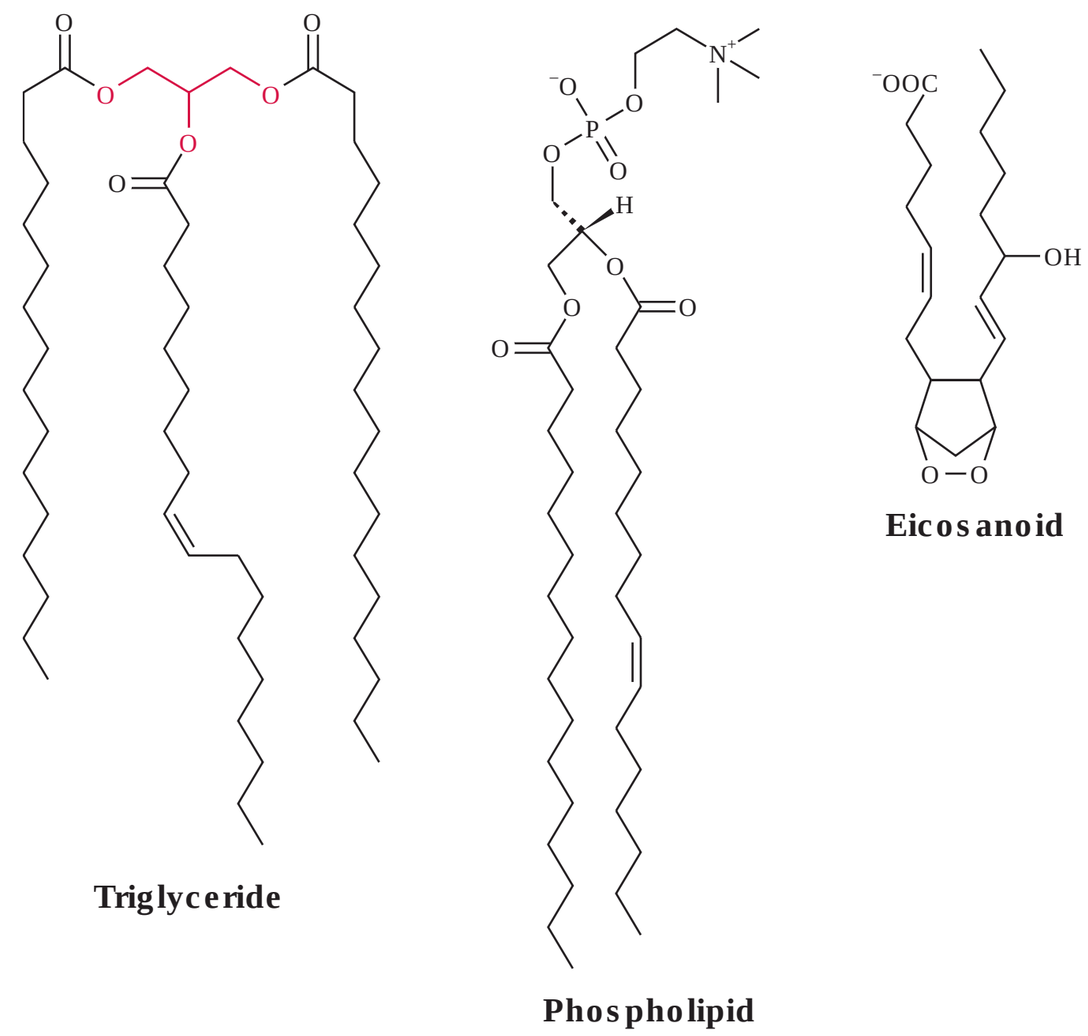


Fig. 27.2 **Structures of physiologically important lipids derived from fatty acids.**

Table 27.1 Physiologically Important Fatty Acids

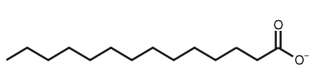
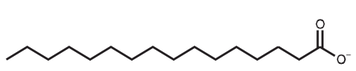
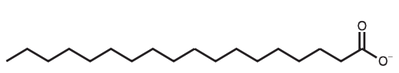
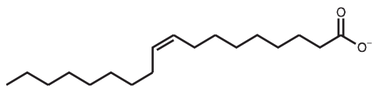
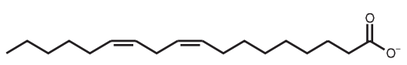
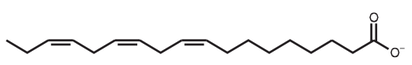
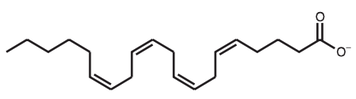
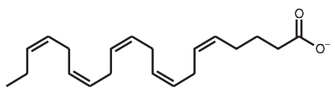
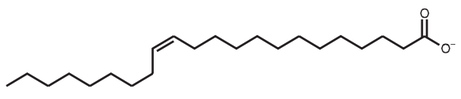
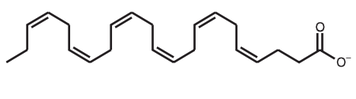
Structure	Fatty Acid	Number of Carbons	Number of Double Bonds	Comments
	Myristic acid	14	0	Membrane anchor for some proteins
	Palmitic acid	16	0	Saturated, abundant in triglycerides, a membrane anchor for some proteins
	Stearic acid	18	0	Saturated, abundant in triglycerides
	Oleic acid	18	1	Monounsaturated, abundant in triglycerides, the main fatty acid in olive oil
	Linoleic acid	18	2	Polyunsaturated, ω-6
	α-Linolenic acid	18	3	Polyunsaturated, ω-3
	Arachidonic acid	20	4	Polyunsaturated, ω-6, a precursor for ω-6 eicosanoids
	Eicosapentaenoic acid	20	5	Often abbreviated as EPA, used as ω-3 fatty acid supplement, precursor for ω-3 eicosanoids
	Erucic acid	22	1	Triglycerides containing oleic and erucic acid make up “Lorenzo’s oil”
	Docosahexaenoic acid	22	6	Often abbreviated as DHA, used as ω-3 fatty acid supplement, precursor for ω-3 eicosanoids

Table 27.2 Fatty Acid Composition of Some Foods

Food	Saturated	Monounsaturated	Polyunsaturated	Trans
Canola oil	7%	63%	28%	0%
Margarine	19%	48%	30%	18%
Butter	63%	26%	4%	4%
Cheddar cheese	57%	25%	4%	3%
Milk, 2% fat	63%	28%	4%	4%
Eggs	33%	38%	20%	0%
Potato chips	10%	56%	24%	0%

Values are derived from the United States Department of Agriculture National Nutrient Database for Standard Reference Release 27.

is more convenient than the standard chemical numbering because of the way cells elongate fatty acids and introduce double bonds (see Section 3 and Figs. 27.6 and 27.7).

Double bonds in naturally occurring fatty acids are typically in the **cis** configuration, which places a kink in the hydrophobic chain (see Table 27.1 and Figs. 27.2, 27.7, and 27.8). As a result, triglycerides or phospholipids that contain cis unsaturated fatty acids have lower **melting points** than those that contain only saturated fatty acids. Trans fatty acids (see below) have physical properties that are similar to those of saturated fatty acids.

Chemically **partially hydrogenated fats** (e.g., margarine) and unsaturated fats that are heated to high temperatures (e.g., during frying) contain a mixture of cis and **trans** double bonds. The body metabolizes fatty acids with either cis or trans double bonds, but cis and trans fatty acids can affect the membrane structure, signaling pathways, and lipoprotein metabolism differently. The degree of fatty acid saturation affects the shelf life and physical characteristics of fat-containing foods. Chemical **hydrogenation** of unsaturated fatty acids reduces the number of double bonds. Fully hydrogenated fatty acids are saturated and have no double bonds. Partially hydrogenated fatty acids contain some double bonds in the trans configuration due to a side reaction that occurs during hydrogenation. In place of using partially hydrogenated oils, manufacturers can obtain desired physical properties of fats by blending fully hydrogenated and natural unsaturated oils; the resulting mixture is free of trans fatty acids. Alternatively, manufacturers can use enzymes to switch fatty acids with cis double bonds among triglycerides from a liquid oil and a solid fat; this process is called **interesterification**.

Table 27.2 lists the fatty acid composition of some foods.

2. FATTY ACID SYNTHESIS

After a carbohydrate-rich meal, the liver synthesizes fatty acids from excess glucose. Glycolysis gives rise to acetyl-coenzyme A (CoA), which is converted to citrate inside mitochondria. Citrate is exported into the cytosol, where it gives rise to malonyl-CoA. Fatty acid synthase uses malonyl-CoA to generate fatty acids. This process requires NADPH, which derives from the pentose phosphate shunt and malic enzyme. Fatty acid synthesis is controlled largely by insulin, which favors glycolysis and synthesis of malonyl-CoA. Besides the liver, the lactating mammary glands can also synthesize fatty acids.

In humans, nearly all fatty acid synthesis takes place in the **liver** and the **lactating mammary glands**. The adipose tissue carries out only a minor amount of fatty acid synthesis, but it imports fatty acids and stores them as triglycerides (see Chapter 28). A part of the **epidermis** also seems to be able to synthesize fatty acids for local use. Little is known about the regulation of fatty acid synthesis in human mammary glands and epidermis. For this reason, the following discussion is focused on the liver.

The liver synthesizes fatty acids in the fed state by using glycolysis, the oxidative branch of the pentose phosphate pathway, and a part of the citric acid cycle. These pathways provide acetyl-CoA, malonyl-CoA, and NADPH for fatty acid synthesis. Here, fatty acid synthesis is presented in two steps: first, the synthesis of malonyl-CoA, and second, the synthesis of fatty acids by fatty acid synthase.

In the liver, stimulated by **insulin**, excess dietary glucose gives rise to **malonyl-CoA** (Fig. 27.3). Insulin stimulates the formation of fructose 2,6-bisphosphate, which in turn activates phosphofructokinase 1 (see Chapter 19). This enables the liver to increase glycolysis beyond the rate that would be required for adenosine triphosphate (ATP) production alone. Pyruvate from glycolysis enters mitochondria. About half of this pyruvate is converted to acetyl-CoA and the other half to oxaloacetate; together, acetyl-CoA and oxaloacetate form **citrate**. Citrate is exported into the cytosol, where it gives rise to **acetyl-CoA**. This is production of acetyl-CoA is also useful for the synthesis of cholesterol (see Chapter 29). Stimulated by insulin, **acetyl-CoA carboxylase** (**ACC**) converts

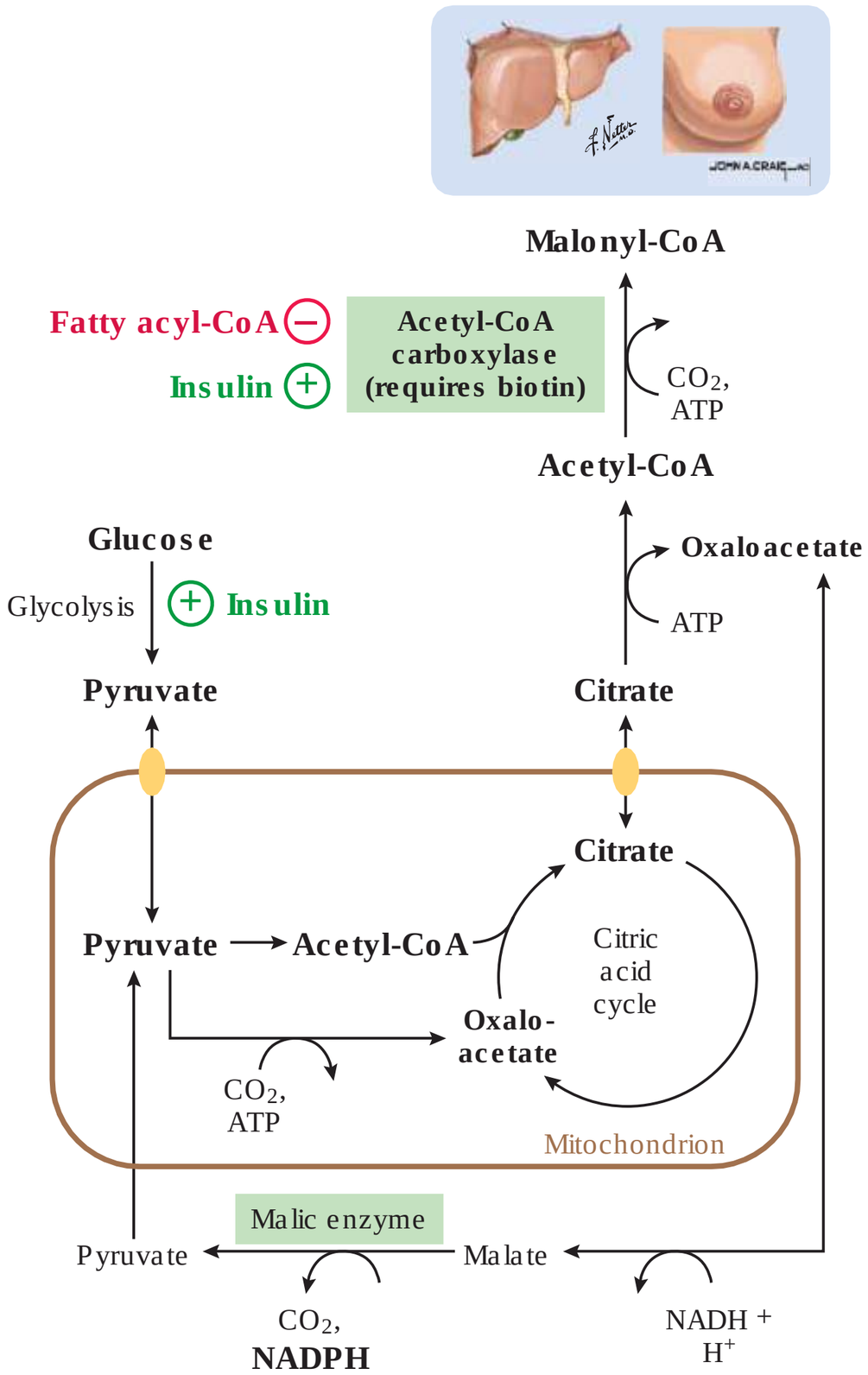


Fig. 27.3 conversion of glucose to malonyl-coenzyme A (CoA) as part of fatty acid synthesis. Oxaloacetate in the cytosol can be moved back into mitochondria after conversion to pyruvate with the production of NADPH. Alternatively, malate can enter mitochondria via the malate-aspartate shuttle (not shown).

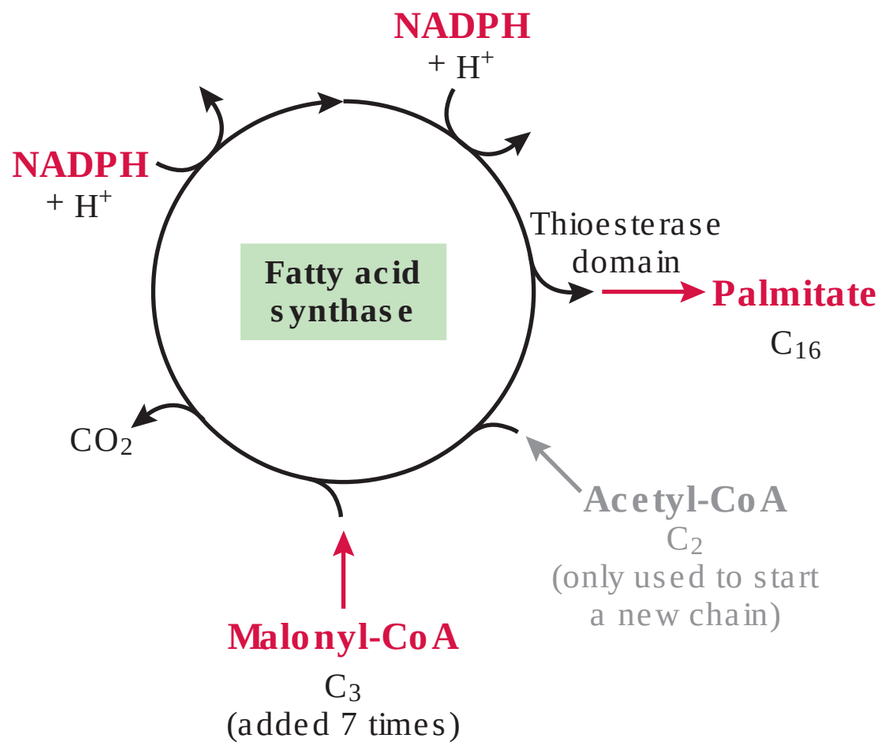


Fig. 27.4 Synthesis of fatty acids from malonyl-coenzyme A (CoA). Synthesis of palmitate requires acetyl-CoA plus seven cycles of malonyl-CoA addition.

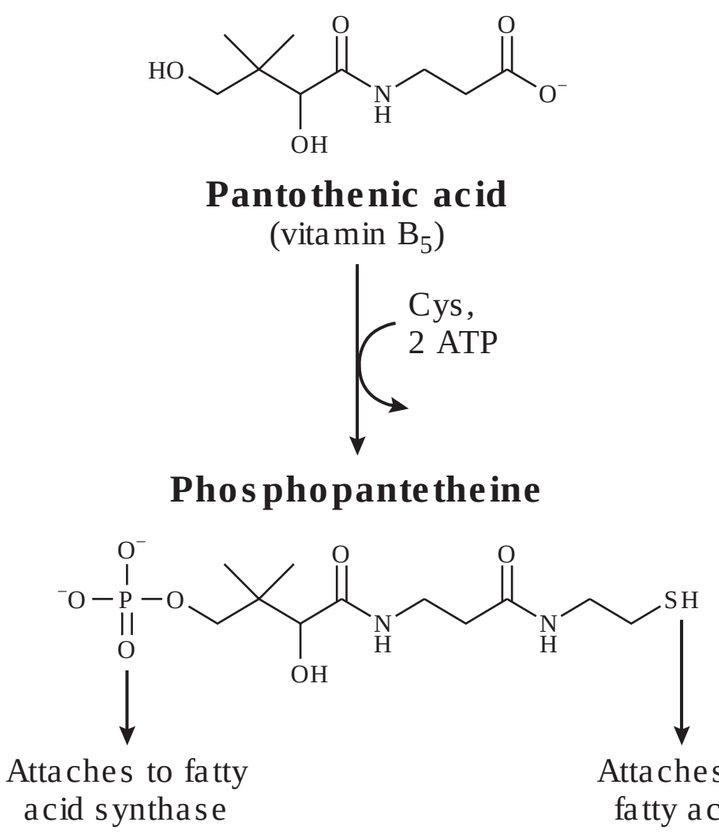


Fig. 27.5 Role of pantothenic acid in fatty acid synthesis.

acetyl-CoA to malonyl-CoA. Like other carboxylases, ACC requires the vitamin **biotin**. ACC activity is the main determinant of the rate of fatty acid synthesis. In the fasting state, lack of stimulation by insulin and inhibition by cytosolic fatty acyl-CoA attenuate ACC activity.

Fatty acid synthase in the cytosol produces fatty acids by the sequential addition of two-carbon units (Fig. 27.4). Fatty acid synthase is a single protein that contains multiple enzymatic activities. Synthesis starts with acetyl-CoA (two carbons), to which malonyl-CoA is added (three carbons). Decarboxylation, hydration, and reduction with NADPH yields a four-carbon fatty acid that remains bound to fatty acid synthase. Malonyl-CoA is added in further such steps. T ereby, the concentration of malonyl-CoA limits the rate of fatty acid synthesis. An arm-like domain of the synthase, called **acyl-carrier protein** (**ACP**), contains a **phosphopantetheine** prosthetic group (Fig. 27.5) that binds the first acetyl group and then presents the growing fatty acid chain to the dif erent enzyme domains of the fatty acid synthase. T is arrangement is thought to greatly enhance the catalytic efficiency of the enzyme. Phosphopantetheine is derived from the vitamin **pantothenic acid**. Pantothenic acid is also part of CoA (see Fig. 22.3). A thioesterase activity of the fatty acid synthase cleaves the bond between the ACP and the fatty acid when the growing fatty acid chain is about 16 carbons long.

NADPH for fatty acid synthesis stems from the oxidative branch of the **pentose phosphate pathway** (see Fig. 21.3) and from **malate dehydrogenase (decarboxylating)**, which is often called **malic enzyme** (see Fig. 27.3). Malic enzyme is located in the cytoplasm.

Tracer-based measurements in nonlactating humans have so far shown only a **small rate** of fatty acid de novo synthesis. T is small rate was highest when volunteers consumed a diet that provided more than 80% of calories from carbohydrates.

Many **tumor** cells express markedly more fatty acid synthase than their normal counterparts. Ef orts are underway to

test whether inhibition of fatty acid synthase should be part of the treatment of neoplasms.

3. FATTY ACID ACTIVATION, ELONGATION, AND DESATURATION

For metabolism, *f*atty acids must be activated with CoA to *f*orm *f*atty acyl-CoA. Fatty acyl-CoA can be elongated, and double bonds can be inserted between carbons that are 5, 6, or 9 carbons *f*rom the COOH end. Essential *f*atty acids cannot be synthesized by humans because o*f* the position o*f* their double bonds. Humans need these essential *f*atty acids *f*or the synthesis o*f* eicosanoids and docosanoids, which participate in short-distance signaling (see Chapter 32).

Metabolic reactions involving fatty acids require that the fatty acids be converted to **fatty acyl-CoAs**. T is is necessary for elongation, desaturation, β-oxidation (see Section 4 below), and the synthesis of triglycerides (see Chapter 28), phospholipids, and glycolipids. T e formation of acyl-CoA is catalyzed by acyl-CoA synthetase and requires ATP (Fig. 27.6).

T ere are multiple acyl-CoA synthase isozymes that dif er in subcellular location and specificity for the fatty acid chain length. An isozyme on the cytosolic surface of the endoplasmic reticulum facilitates fatty acid elongation and desaturation. Other isozymes of acyl-CoA synthetase are involved in fatty acid oxidation (see below) and are located on the mitochondrial outer membrane, as well as on the inside of the inner mitochondrial membrane.

Most cells can modify fatty acids by elongation, desaturation, or a combination of elongation and desaturation in any order. Both newly synthesized fatty acids and fatty acids acquired from the diet can be modified after activation with fatty acyl-CoA.

Elongation of existing fatty acids occurs by the sequential addition of two carbon units that are derived from malonyl-CoA (see Fig. 27.6) and is catalyzed by elongases on the cytosolic face of the endoplasmic reticulum. Humans have seven elongase isozymes. T e structure of these enzymes likely resembles that of fatty acid synthase. Most tissues elongate fatty acids to 18 to 24 carbons, mainly for use in phospholipids and glycolipids. T e skin, brain, retina, and sperm synthesize small amounts of fatty acids with up to 40 carbons; the function of these lipids is largely a mystery.

Desaturation can occur at carbons 5, 6, or 9 from the carboxyl end of fatty acids (see Fig. 27.7). Most double bonds are introduced into C-9 of palmitate (16:0) or stearate (18:0), thus giving rise to palmitoleate (16:1, Δ^9) and oleate (18:1, Δ^9), respectively. In the entire body, about 25% of the fatty acids are palmitate (16:0) and about 50% are oleate (18:1). Like fatty acid elongases, fatty acid desaturases are bound to the cytosolic face of the endoplasmic reticulum.

Since fatty acid synthase produces mostly 16-carbon saturated fatty acids, and because desaturases cannot insert double bonds beyond carbon 9, humans cannot synthesize long-chain unsaturated fatty acids with a double bond near the omega end. T ese fatty acids are therefore essential and must be taken up from the diet. **Linoleic acid** (18:2, $\Delta^{9,12}$) and **α-linolenic acid** (18:3, $\Delta^{9,12,15}$) are both **essential fatty acids**. Linoleic acid is an **ω-6 fatty acid**, and α-linolenic acid is an **ω-3 fatty acid** (see Fig. 27.8). Although α-linolenic acid has a double bond at position ω-6, it is never called a ω-6 fatty acid. In other words, only the double bond closest to the ω-end is considered when classifying essential fatty acids into ω-3 and ω-6.

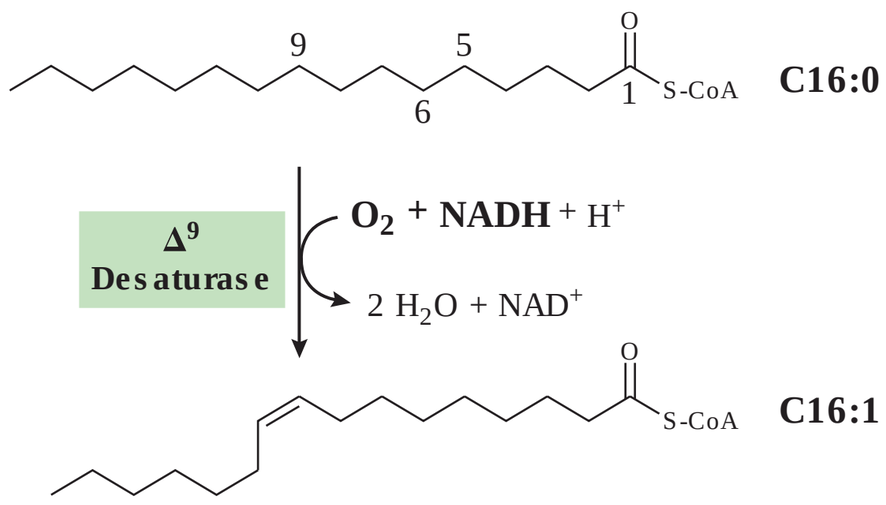


Fig. 27.7 **Desaturation of fatty acids.**

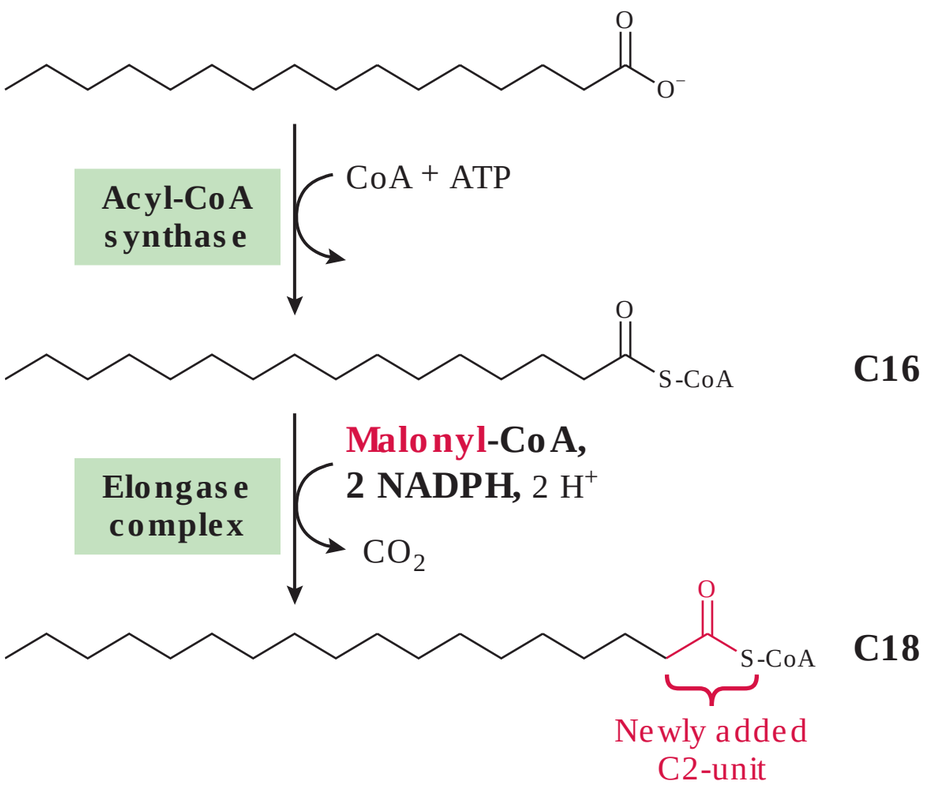


Fig. 27.6 **Activation and elongation of fatty acids.**

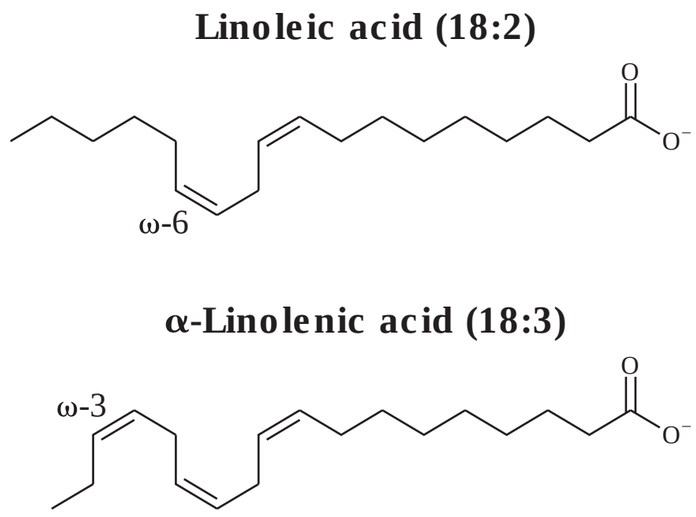


Fig. 27.8 **Essential fatty acids linoleic and α-linolenic acid.** While this figure shows the fatty acids in extended conformations, polyunsaturated fatty acids that are part of phospholipids in membranes also assume looped conformations. The transitions between these conformations occurs rapidly.

Bacteria, **algae**, and **plants** make some ω-3 fatty acids and lots of ω-6 fatty acids, and we acquire these essential fatty acids either directly from food or indirectly via the food chain. **Table 32.1** shows the ω-3 and ω-6 fatty acid content of various foods, and **Section 1** in Chapter 32 discusses the recommended intake of these fatty acids.

Essential fatty acids can be desaturated and elongated like nonessential fatty acids. In this fashion, linoleic acid gives rise to **arachidonic acid** (20:4, still an ω-6 fatty acid; see structure in Table 27.1). Similarly, α-linolenic acid gives rise to **eicosapentaenoic acid** (**EPA**; 20:5) and **docosahexaenoic acid** (**DHA**; 22:6, still an ω-3 fatty acid). Since elongation occurs at the carboxyl-end, an ω-3 fatty acid always remains an ω-3 fatty acid, and an ω-6 fatty acid always remains an ω-6 fatty acid. The same is true for desaturation because human desaturases cannot introduce double bonds beyond carbon 9. Metabolites of ω-3 and ω-6 fatty acids act as lipid messengers or as short-lived local hormones (see Chapter 32).

Some use the term essential fatty acid only for linoleic and linolenic acid; others use it more broadly for all ω-3 and ω-6 fatty acids in the body.

4. FATTY ACID OXIDATION

To enter the mitochondria, long-chain fatty acids must be converted from acyl-CoA to acyl-carnitine, a process that is inhibited by malonyl-CoA. As a result, the liver moves fatty acids into mitochondria only when the concentration of insulin is low, and muscle does it only when the concentration of adenosine monophosphate (AMP) is elevated. Mitochondria oxidize fatty acids to produce acetyl-CoA, reduced flavin adenine dinucleotide ($FADH_2$), and NADH. $FADH_2$ and NADH enter oxidative phosphorylation. Acetyl-CoA enters the citric acid cycle. The liver can also convert acetyl-CoA to ketone bodies (see Section 5 below).

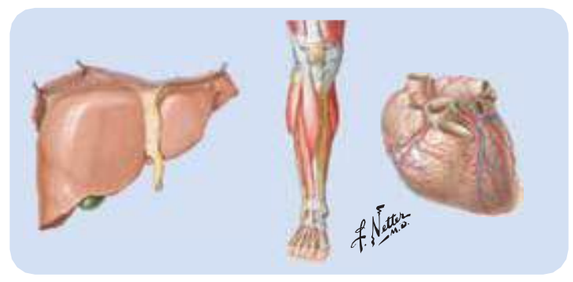
In the fasting state, the adipose tissue hydrolyzes stored triglycerides and releases the fatty acids (also called **free fatty acids** [**FFAs]** or **nonesterified fatty acids** [**NEFAs]**) into the blood, where they bind to **albumin** (see Chapter 28). These fatty acids can be oxidized by a variety of cells.

The blood contains triglyceride-rich lipoprotein particles that can also give rise to fatty acids for use as fuel. Triglycerides are mostly contained in chylomicrons and very-low-density lipoprotein particles (see Chapter 28). These triglycerides can be hydrolyzed by lipoprotein lipase, which is tethered to the wall of capillaries in the adipose tissue and muscle and by hepatic lipase in the capillaries of the liver. Most of the resulting fatty acids enter the tissues in which they are hydrolyzed, but perhaps ~20% enter the general circulation. The rate of hydrolysis of triglycerides in lipoprotein particles changes markedly with feeding and fasting, as does the use of the resulting fatty acids. Details are provided in Chapter 28.

Fatty acid oxidation occurs primarily in muscle and liver cells, which extract the fatty acids from the blood. Fatty acid oxidation in the **liver** is maximal during a prolonged fast; in **muscle**, it is maximal during endurance exercise.

Fatty acid transporters enhance the transport of fatty acids across plasma membranes, and some of the transporters likely play a role in forming fatty acyl-CoA. As with glucose transporters (see Chapter 18), some fatty acid transporters are always inserted into the plasma membrane, and others are inserted only on demand (e.g., in the heart, when AMP-dependent protein kinase [AMPK] is active). Fatty acids that contain eight or fewer carbons can efficiently cross membranes without the need for a transporter.

Inside cells, the concentration of **malonyl-CoA** controls the uptake of fatty acids into mitochondria (Fig. 27.9). Fatty acids are transported through the cytosol bound to fatty acid–binding proteins. At the cytosolic face of the mitochondrial outer membrane, an acyl-CoA synthetase activates fatty acids to fatty acyl-CoAs. Fatty acyl-CoAs pass freely through pores in the mitochondrial outer membrane, but they do not cross the inner mitochondrial membrane. For fatty acids to cross the inner membrane, **carnitine palmitoyltransferase I** (**CPT-I**) must convert fatty acyl-CoAs to fatty acyl carnitines. After transport into the mitochondrial matrix, fatty acyl carnitines are converted back into fatty acyl-CoAs. Malonyl-CoA inhibits CPT-I and thus prevents the oxidation of fatty acids. As shown in Section 2, malonyl-CoA is also a substrate of fatty



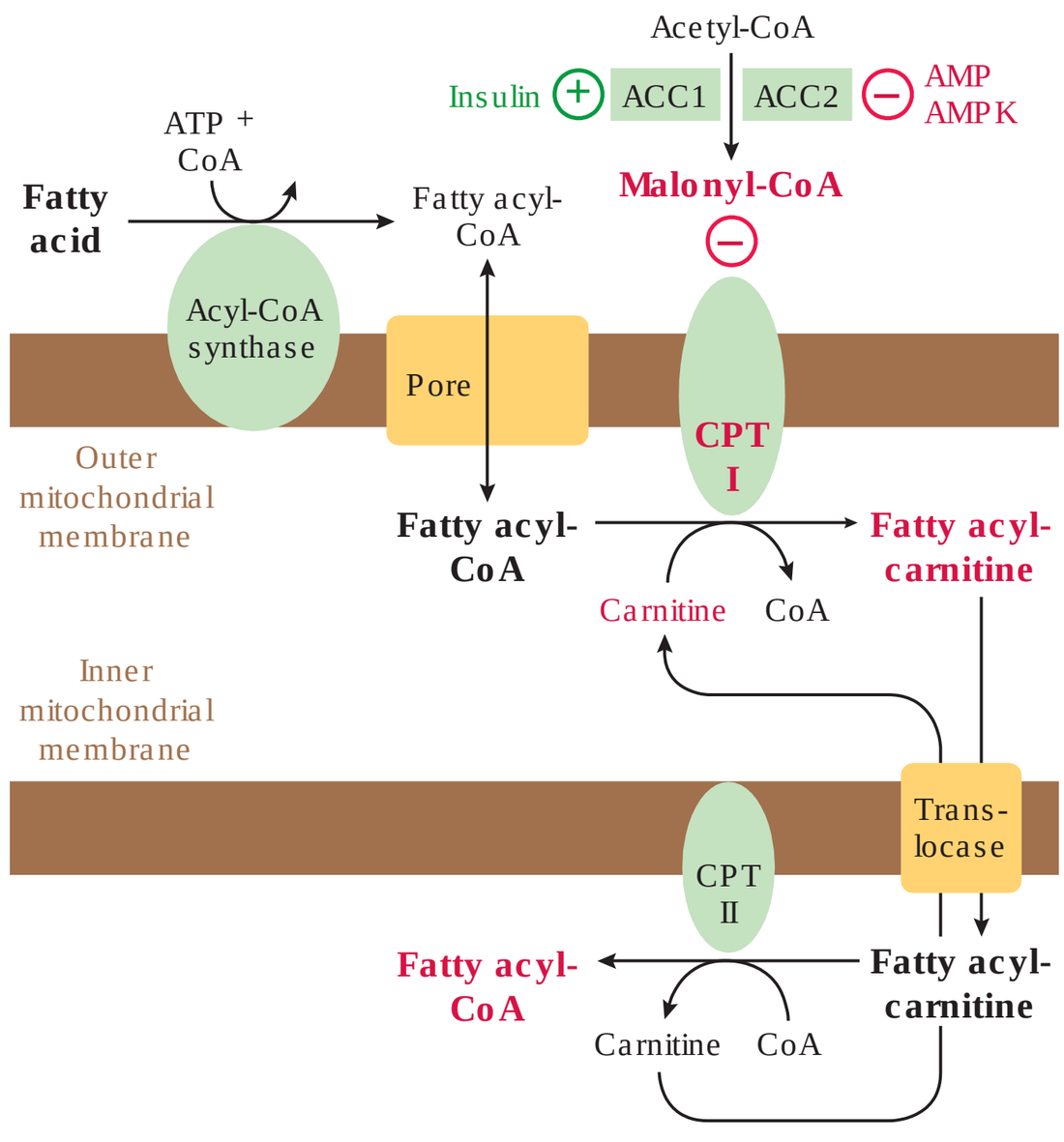


Fig. 27.9 Malonyl-coenzyme A (CoA) regulates the transport of fatty acids across the inner mitochondrial membrane.

acid synthase and via its concentration determines the activity of fatty acid synthase.

In the liver, the concentration of malonyl-CoA depends on **insulin**, while in other cells, it depends on the cell's **energy state** (see Fig. 27.9). In the liver, insulin stimulates the formation of malonyl-CoA via **acetyl-CoA carboxylase 1** (**ACC1**). Hence, insulin inhibits the uptake of fatty acids into mitochondria and thus prevents the oxidation of fatty acids. In the fed state, newly synthesized fatty acids can therefore not enter mitochondria. In all other cells that synthesize malonyl-CoA, there is no significant de novo fatty acid synthesis, and malonyl-CoA is simply a regulator of fatty acid transport into mitochondria. In these cells, **acetyl-CoA carboxylase 2** (**ACC2**) produces malonyl-CoA depending on the energy state of the cell, as reflected in the concentration of **AMP** and the activity of **AMPK**. Muscle also has an AMPK-activated malonyl-CoA decarboxylase that destroys malonyl-CoA.

Carnitine (Figs. 27.9 and 27.10) stems from the degradation of proteins that contain trimethylated lysine residues. T ese proteins are found both in the human body and in dietary meats. Vegans consume virtually no carnitine in their diet. Under normal circumstances, humans can synthesize enough carnitine and also recover enough of it from the glomerular filtrate in the kidneys; carnitine is therefore not a vitamin. Carnitine is available commercially as a supplement.

In medicine, carnitine supplementation is occasionally used in the treatment of diseases in which excess acyl-CoA depletes free CoA in a harmful way (see Section 7.1). Carnitine then leads to the formation of acyl-carnitines with a concomitant increase in available free CoA.

T e **β-oxidation** of fatty acyl-CoA involves the sequential removal of two-carbon units, yielding acetyl-CoA, NADH, and $FADH_2$ (Fig. 27.11). NADH and $FADH_2$ both enter oxidative phosphorylation (see Fig. 23.3 and Section 1 in Chapter 23) and thus provide about one-third of the ATP that can be derived from the complete oxidation of fatty acids to CO_2. Acetyl-CoA can enter the citric acid cycle and thereby give rise to yet more NADH and $FADH_2$, as well as some guanosine triphosphate (GTP). β-Oxidation of fatty acids can occur in all cells that have mitochondria.

T ere are four isozymes of acyl-CoA dehydrogenase (see Fig. 27.11) that dif er with respect to the range of acyl chain lengths they recognize: **very-long-chain acyl-CoA dehydrogenase** (**VLCAD**), **long-chain acyl-CoA dehydrogenase** (**LCAD**), **medium-chain acyl-CoA dehydrogenase** (**MCAD**), and **short-chain acyl-CoA dehydrogenase** (**SCAD**). Deficiencies of these enzymes are described in Section 7.

Fibrates are a class of drugs that increase the rate of fatty acid β-oxidation and are used to lower the concentration of triglycerides in the blood. Fibrates act on the transcription factor **PPARα** (**peroxisome proliferator-activated receptor α**), which stimulates the proliferation of peroxisomes and transcription of genes that encode proteins that play a role in fatty acid oxidation, such as CPT-I (see Fig. 27.9).

When a cell is **hypoxic**, it has a decreased capacity to oxidize fatty acids and glucose and must resort to anaerobic glycolysis (see Chapter 19). During hypoxia, the concentrations of NADH and $FADH_2$ increase, and those of NAD^+ and FAD decrease, which reduces the rate of fatty acid β-oxidation.

T e β-oxidation of **unsaturated fatty acids** proceeds similar to that of saturated fatty acids, except that NADH is needed to reduce the double bond in additional, enzyme-catalyzed steps.

T e β-oxidation of fatty acids with an **odd number of carbons** also proceeds similar to that of saturated fatty acids, but it yields a final propionyl-CoA (three carbons), which is then converted to succinyl-CoA (see Fig. 36.12). Odd-chain fatty acid metabolism produces only a minor amount of propionyl-CoA; a much greater amount is produced from the metabolism of isoleucine, valine, and methionine.

Very-long-chain fatty acids of 22 or more carbons are oxidized to medium-chain fatty acids in **peroxisomes** and then transferred to mitochondria (Fig. 27.12). Very-long-chain fatty acids are present primarily in neural tissue. T ese fatty acids enter peroxisomes, are activated, and get shortened to medium-chain acyl-CoA, thereby giving rise to acetyl-CoA and NADH. In a poorly understood manner, the

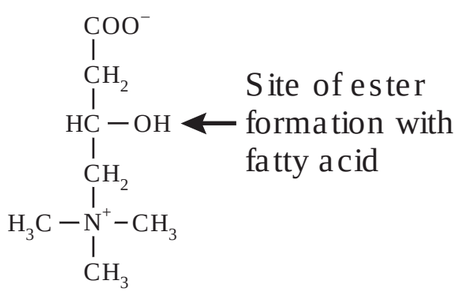


Fig. 27.10 **Carnitine.**

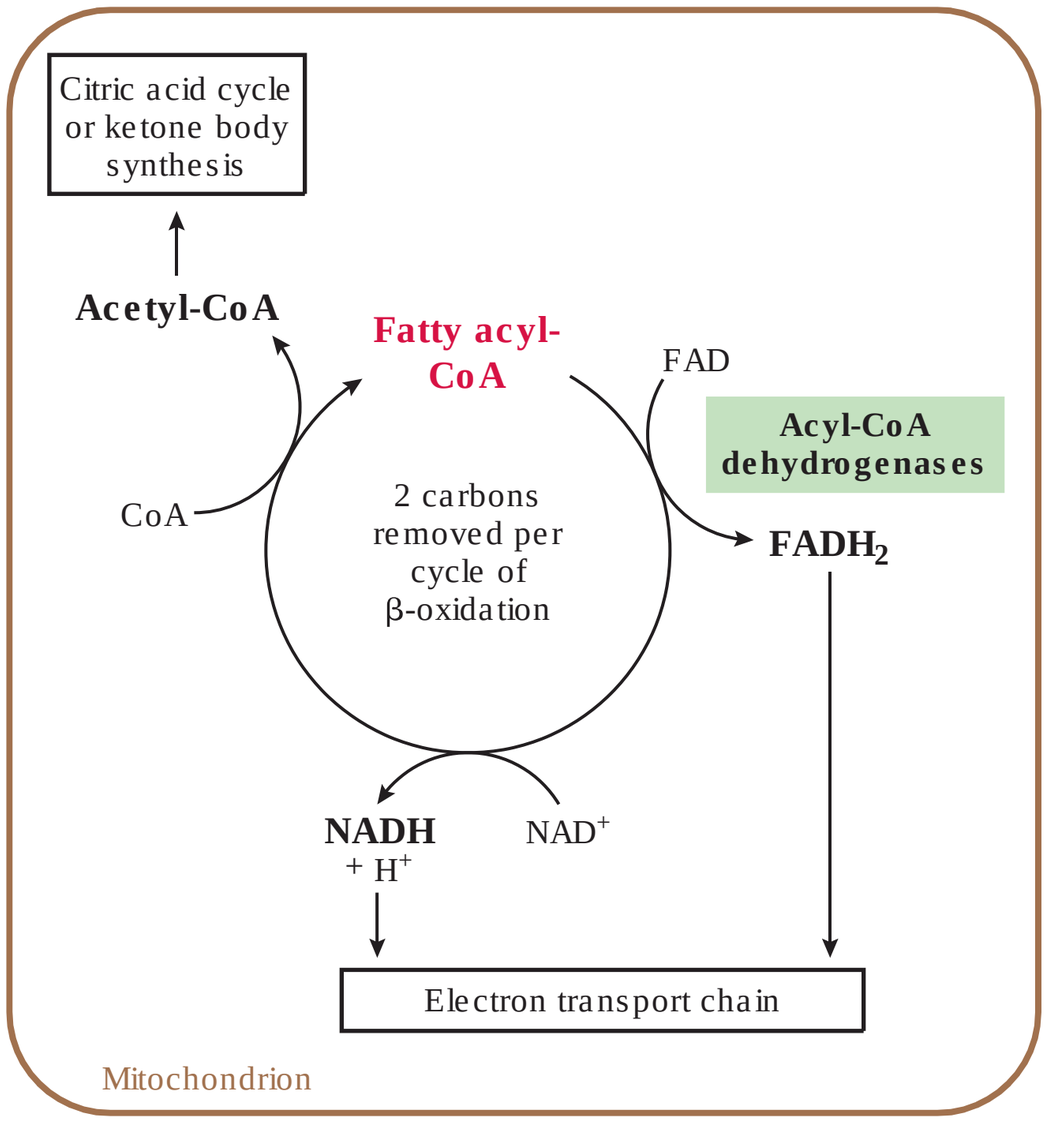


Fig. 27.11 **β-Oxidation of fatty acids.**

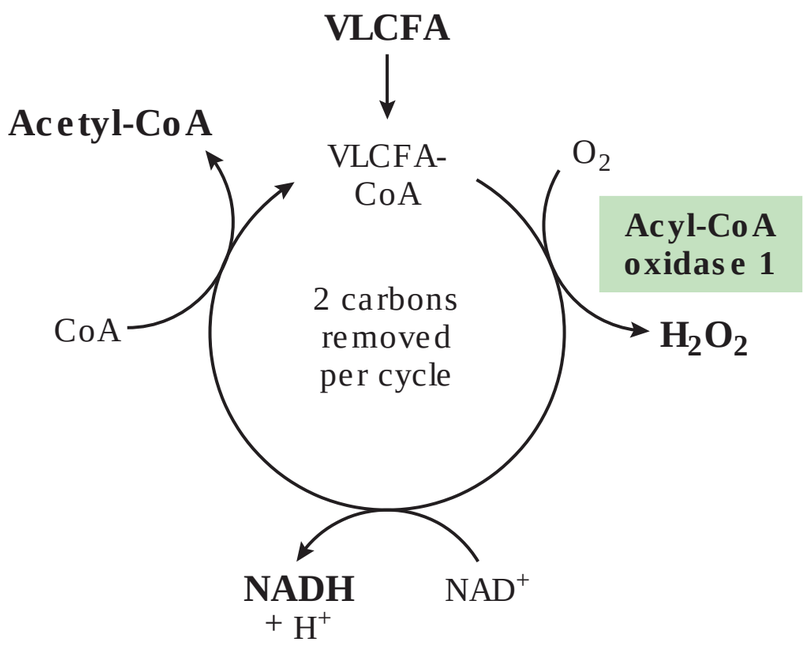


Fig. 27.12 β-Oxidation of very-long-chain fatty acids (VLCFA) in peroxisomes. β-Oxidation of VLCFA only proceeds to a medium-chain length.

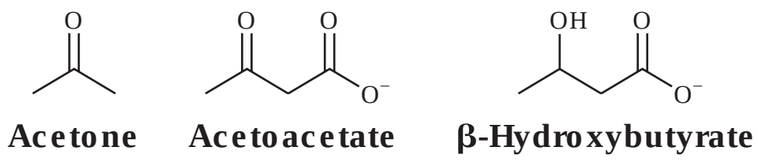


Fig. 27.13 Ketone bodies.

medium-chain fatty acids get transferred to mitochondria for β-oxidation as described above. The reducing power of NADH is also transferred from the peroxisomes to the mitochondria (the exact mechanism is not well understood).

5. SYNTHESIS AND DEGRADATION OF KETONE BODIES

Mitochondria in the liver synthesize the ketone bodies acetoacetate and β-hydroxybutyrate when the concentration of fatty acids in the blood is elevated. During starvation, ketone bodies are an important source of energy for several extrahepatic tissues, especially the brain, thereby reducing the body's need for glucose. Dipstick tests can provide an indication of the concentration of acetoacetate. More refined laboratory tests report the concentration of β-hydroxybutyrate.

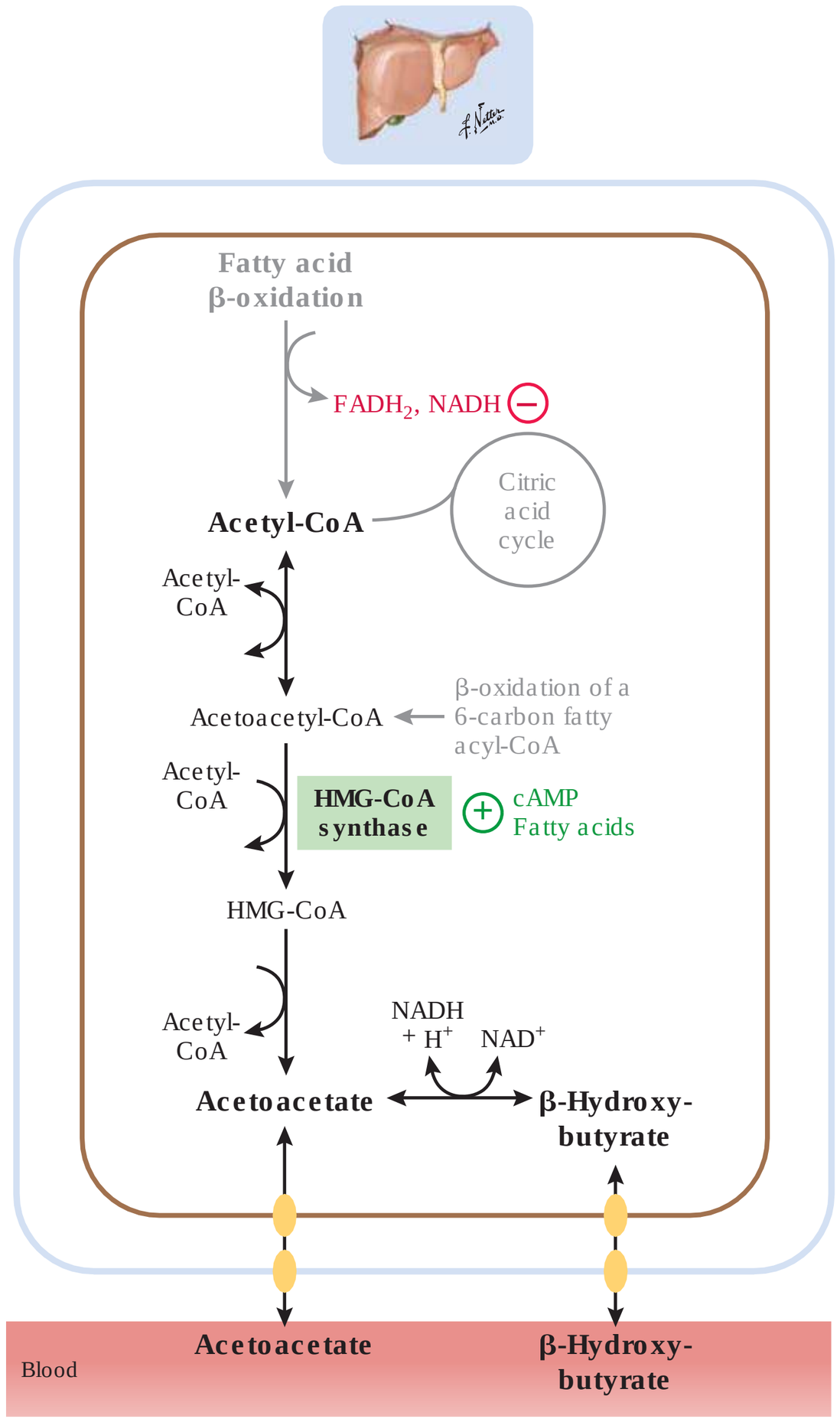


Fig. 27.14 Synthesis of ketone bodies. HMG-CoA, hydroxymethylglutaryl-CoA.

5.1. Ketone Body Synthesis (Ketogenesis)

The term ketone bodies encompasses the compounds **β-hydroxybutyrate** (also known as **3-hydroxybutyrate**), **acetoacetate**, and **acetone** (Fig. 27.13). The acetone is produced nonenzymatically from acetoacetate through the loss of CO_2. Acetone is not used by the body. The liver is the only organ that produces an appreciable amount of ketone bodies.

During starvation, liver mitochondria convert some of the acetoacetyl-CoA and acetyl-CoA from the β-oxidation of fatty acids to ketone bodies (Fig. 27.14). In the liver, fatty acid oxidation occurs at a relatively rapid pace. After a 1- to 2-day fast, β-oxidation alone can supply most of the $FADH_2$ and NADH that oxidative phosphorylation needs. As a result, NADH is present at a relatively high concentration and inhibits the citric acid cycle (see Chapter 22). Then, acetyl-CoA from β-oxidation does not enter the citric acid cycle and instead gives rise to ketone bodies.

In liver mitochondria, two acetyl-CoA give rise to acetoacetyl-CoA, and the addition of a third acetyl-CoA yields HMG-CoA (3-hydroxy-3-methylglutaryl-CoA; see Fig. 27.14). Hydrolysis of HMG-CoA then returns an acetyl-CoA and gives rise to acetoacetate. NADH is used to reduce acetoacetate to β-hydroxybutyrate, such that the ratio β-hydroxybutyrate/acetoacetate reflects the NADH/NAD^+ ratio inside mitochondria.

There are two distinct pools of **HMG-CoA** in liver cells: a pool in mitochondria that leads to ketone body synthesis and a pool in the **cytosol** that leads to **cholesterol** synthesis (see Chapter 29).

The concentration of **acetyl-CoA** in liver mitochondria is the main controller of ketone body synthesis. The

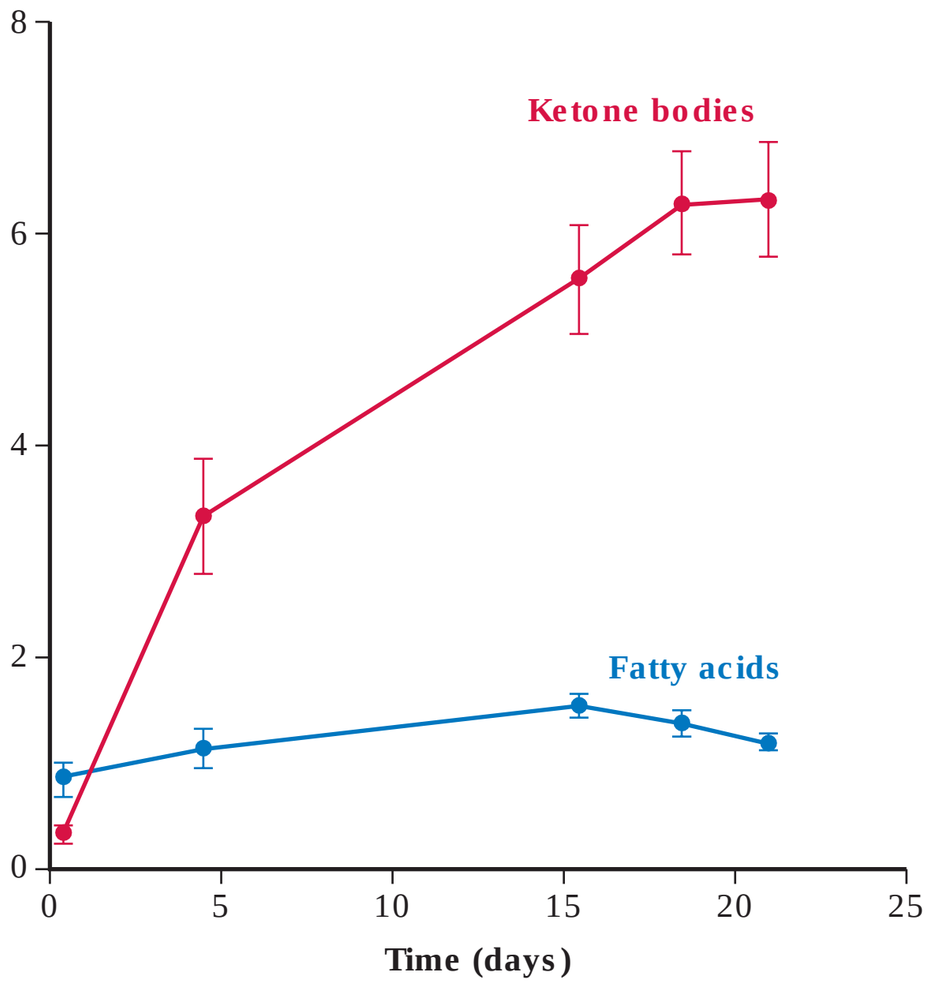


Fig. 27.15 Plasma fatty acids and ketone bodies during prolonged fasting. The graph shows means ± standard error of means for five obese volunteers who were studied after longer than a 12-hour fast. (Data from Owen OE, Smalley KJ, D'Alessio DA, Mozzoli MA, Dawson EK. Protein, fat, and carbohydrate requirements during starvation: anaplerosis and cataplerosis. *Am J Clin Nutr.* 1998;68:12-34.

concentration of acetyl-CoA in the liver is considerably higher in the fasting state than in the fed state. In addition, an elevated concentration of **fatty acids** or **cyclic adenosine monophosphate** (**cAMP**) increases the transcription of HMG-CoA synthase, the rate-limiting enzyme of ketone body synthesis.

In persons who consume a typical Western diet (roughly 30% of calories from fat, 15% from protein, and 55% from carbohydrates), the concentration of ketone bodies in the plasma substantially increases only with a fast of more than 1 day (Fig. 27.15).

5.2. Oxidation of Ketone Bodies by Extra-Hepatic Tissues

The brain, heart, muscle, and kidneys are particularly active in using ketone body oxidation (also called ketolysis) for ATP production. Degradation of ketone bodies generates acetyl-CoA, which enters the citric acid cycle, and NADH, which delivers reducing power to oxidative phosphorylation (Fig. 27.16). The rate of ketone body oxidation is roughly proportional to the ketone body concentration in the blood (see Fig. 27.15). The liver does not use the ketone bodies that it synthesizes because it does not possess the enzyme that transfers CoA from succinyl-CoA to acetoacetate.

Glucose cannot be generated from ketone bodies. Ketone body metabolism leads solely to the production of acetyl-CoA, and the carbon skeleton of acetyl-CoA cannot give rise to the carbon skeleton of glucose (see Chapter 25).

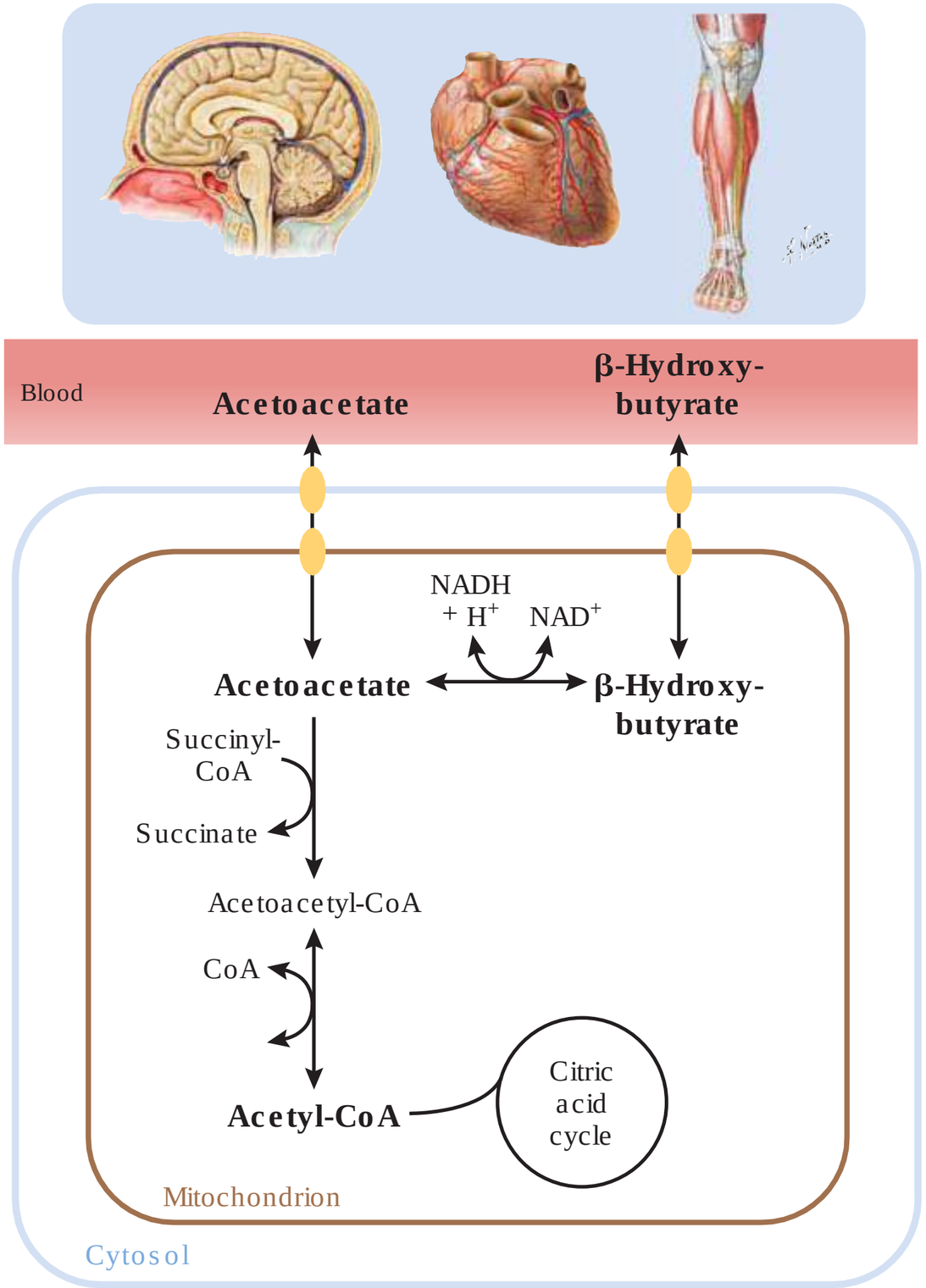


Fig. 27.16 Oxidation of ketone bodies.

5.3. Laboratory Tests for Ketone Bodies

Tests for ketone bodies measure the concentration of either acetoacetate or β-hydroxybutyrate. Most tests based on nitroprusside (sodium nitroferricyanide) are predominantly sensitive to acetoacetate. **Dipsticks** and **tablets** based on this test are widely available for detecting acetoacetate in the urine, based on the production of a dark-red color. **Clinical laboratories** measure β-hydroxybutyrate and acetoacetate separately. There are also **test strips** for β-hydroxybutyrate that use a small amount of capillary blood and are analyzed in a hand-held meter.

The concentration ratio **β-hydroxybutyrate/acetoacetate** in the blood is proportional to the concentration ratio NADH/NAD^+ in mitochondria. During a normal fast in a healthy person, there is approximately three times more β-hydroxybutyrate than acetoacetate. Conditions that lead to ketoacidosis, particularly alcoholic ketoacidosis, typically lead to an increase in the NADH/NAD^+ ratio, and β-hydroxybutyrate then makes up an even greater fraction of ketone bodies. Hence, a test that measures only the acetoacetate has to be interpreted with caution.

5.4. Ketosis, Ketonemia, and Ketonuria

Ketosis is a state of increased production of ketone bodies; this can be normal or abnormal.

In persons who consume a typical, weight-maintaining Western diet (more than about 50% of calories from carbohydrates), ketosis occurs physiologically only with prolonged **fasting** (see Fig. 27.15). Persons who consume a diet that contains little carbohydrate (e.g., an **Atkins**-type diet), particularly if it is associated with weight loss, have ongoing ketosis.

If ketosis is stable for a prolonged period, the control of lipolysis by **insulin** is usually intact.

Ketosis occurs abnormally rapidly in patients who have deficient **glycogenolysis** or **gluconeogenesis** while fasting.

Ketonemia is a readily detectable concentration of ketone bodies in the blood; this can be normal or abnormal.

Ketonuria is a readily detectable concentration of ketone bodies in the urine; this too can be normal or abnormal. Ketonuria occurs during ketosis when the rate of filtration of ketone bodies in the kidneys exceeds the rate of recovery of ketone bodies from the filtrate. Ketone bodies in the urine increase the loss of Na^+, K^+, and water in the urine.

Ketoacidosis is discussed in Section 7.3.

6. OVERVIEW OF FUEL USE BY TISSUES

Fuel use by most tissues is highly complex, depending on cell type, energy output, time, circulating hormones (especially insulin), and concentrations of circulating glucose, fatty acids, and ketone bodies. Hence, the following descriptions are simplified.

The **heart** has a relatively high need for ATP production. Between rest and exercise, this need varies about fivefold. In the heart of a resting person, typically about 60% of the ATP is produced from the β-oxidation of fatty acids, 20% from the oxidation of lactate, and 20% from the oxidation of glucose. After a meal, when the concentration of insulin is high, the heart increases its oxidation of glucose. Oxidation of ketone bodies depends on availability. Compared with other tissues, the heart has a higher rate of ketone body oxidation per gram of tissue. Oxidation of ketone bodies reduces the oxidation of fatty acids and glucose by the heart. As the heart enters high contractile activity, it makes more ATP from the oxidation of glucose than from fatty acids, because oxygen becomes limiting and ATP production from glucose requires less oxygen than ATP production from fatty acids.

Skeletal muscles during rest and sustained aerobic exercise use mostly fatty acids for ATP production. In contrast, the early phase of exercise is fueled mostly by the metabolism of blood glucose and muscle glycogen. Later, some of the blood glucose derives from liver glycogen and from gluconeogenesis in both the liver and kidneys.

The **brain** uses a substantial amount of energy, accounting for approximately 20% of total body oxygen use of a person who is at rest. ATP use by neurons is roughly comparable to ATP use by intensely exercising muscle. The brain contains neurons and glial cells, which support the neurons. There is some evidence that glial cells convert glucose to lactate and transfer the lactate to neurons, which oxidize lactate via acetyl-CoA in the citric acid cycle. Contrary to earlier beliefs, fatty acids readily cross the blood brain barrier. Most of the brain β-oxidation of fatty acids takes place in astrocytes, a type of glial cell that is abundant. Still, compared to the heart, the rate of fatty acid β-oxidation is low, and it is not a major source of energy for the brain. This seems to be due to low CPT-I activity (therefore a low rate of fatty acid uptake into mitochondria) and a very low activity of one of the enzymes of fatty acid oxidation. Both glial cells and neurons can oxidize ketone bodies, which can meet up to about 75% of the caloric needs of the brain; glucose must provide most of the balance of calories.

The **liver** mainly oxidizes glucose, lactate, and fatty acids; it cannot oxidize ketone bodies. Glucose oxidation is highest in the postprandial period, and fatty acid β-oxidation is highest during a prolonged fast. In the fasting state, ATP from fatty acid β-oxidation is needed to power gluconeogenesis.

Brown fat oxidizes both glucose and fatty acids. These adipocytes are brown because they contain a significant amount of mitochondria. Brown fat stores triglycerides. When the body temperature needs to increase, mitochondria in brown fat are partially uncoupled to generate heat in place of ATP (see Chapter 23).

7. METABOLIC DISTURBANCES OF FATTY ACID AND KETONE BODY METABOLISM

A severe deficiency of any one of the enzymes of fatty acid or ketone body oxidation can produce a metabolic disorder. Of these disorders, the most common are carnitine deficiency and MCAD deficiency. X-linked adrenoleukodystrophy results from an inability to import very-long-chain fatty acids into peroxisomes for oxidation. The buildup of very-long-chain fatty acids then affects myelination, leading to neurological symptoms. In patients who have ketoacidosis, ketone body production significantly exceeds ketone body use. This life-threatening condition most commonly occurs in patients who have type 1 diabetes.

7.1. Hypoketotic Hypoglycemia and Disorders of Fatty Acid Oxidation

When trying to determine the cause of hypoglycemia, physicians often first distinguish **ketotic hypoglycemia** from **hypoketotic** (or **nonketotic**) **hypoglycemia**. Patients who have ketotic hypoglycemia can evidently perform lipolysis and produce ketone bodies, which rules out excess insulin as a cause of the hypoglycemia. Patients with ketotic hypoglycemia may have a problem with glucose production. In contrast, patients who have hypoketotic hypoglycemia may have an excessive concentration of circulating insulin or a defect in lipolysis, fatty acid β-oxidation, ketone body synthesis, or

ketone body oxidation. An excess of insulin is most common (see Sections 4.3, 5.3, and 7 in Chapter 39), and a defect in β-oxidation is the second most common.

Most patients who have impaired fatty acid β-oxidation develop **rhabdomyolysis** after sustained exercise. T ose with more severe disease may also have hypoketotic hypoglycemia in childhood, along with liver dysfunction; those with the most severe disease may have **cardiomyopathy** at birth and die at a young age.

Patients who cannot gain much energy from fatty acid oxidation have a compensatory increase in **glucose oxidation** and impaired **gluconeogenesis**. T e impairment of gluconeogenesis is due to a combination of inadequate ATP synthesis and an unusually low concentration of acetyl-CoA, which thus fails to activate pyruvate carboxylase (see Fig. 25.3). Af ected patients should therefore avoid long fasts because they lead to severe nonketotic or hypoketotic hypoglycemia.

An **acquired carnitine deficiency** may develop in patients who receive an inadequate amount of carnitine in parenteral nutrition (particularly in newborns) and in patients who lose acyl-carnitines in their urine due to a disorder of fatty β-oxidation (see below).

T e following disorders of fatty acid β-oxidation are inherited in an autosomal recessive fashion (i.e., patients are homozygous or compound heterozygous for pathogenic mutant alleles).

Primary carnitine deficiency is caused by mutant alleles of the SLC22A5 gene, which encodes the organic cation transporter 2 (OCTN2). OCTN2 transports carnitine from the extracellular space into the cytosol. T e prevalence is about 1 in 50,000 worldwide but about 1 in 300 on the Faroe Islands. Af ected patients are treated with supplementary carnitine, which is then transported into cells by other organic cation transporters, although less efficiently.

Mitochondrial trifunctional protein deficiency is due to homozygosity or compound heterozygosity for mutant alleles of the HADHA or HADHB gene and impairs the oxidation of long-chain fatty acids. T e HADHA and HADHB genes encode the α- and β-subunit, respectively, of the mitochondrial trifunctional protein.

Multiple acyl-CoA dehydrogenase deficiencies (**MADD**) is caused by a deficiency of electron transport flavoprotein (ETF) dehydrogenase, the subunits of which are encoded by the genes E*T*FA, E*T*FB, and E*T*FDH.

Very-long-chain acyl-CoA dehydrogenase deficiency (**VLCADD**) is due to mutant ACADVL alleles. About 1 in 50,000 people have this deficiency. In newborn screening (of blood) with tandem mass spectroscopy, it is detectable based on the amount of C14:1 acyl-carnitines.

Medium-chain acyl-CoA dehydrogenase deficiency (**MCADD**) is due to mutant ACADM alleles and has a prevalence of about 1 in 15,000, but some of these patients are asymptomatic. Fever often leads to metabolic decompensation, in part because the mutant enzyme shows an excessive loss of activity with increased temperature. Patients with MCADD should avoid prolonged fasting. When they are sick, they should be hospitalized and infused with glucose. In newborn screening, the acyl-carnitine ratios C8/C10 and C8/C2 are the most reliable markers. Patients are supplemented with carnitine to normalize the concentration of free carnitine.

Short-chain acyl-CoA dehydrogenase deficiency (**SCADD**) is due to mutant ACADS alleles. T e enzyme dehydrogenates butyryl-CoA (C4), and SCADD leads to an accumulation of butyric acid and butyryl-carnitine. Among newborns, the prevalence is about 1 in 45,000.

7.2. Diseases of Very-Long-Chain Fatty Acid Oxidation in Peroxisomes

Zellweger syndrome is due to a **deficiency in peroxisome biogenesis**. T is deficiency causes several problems, including defective oxidation of very-long-chain fatty acids. Most af ected infants die by age 6 months. T e prevalence is about 1 in 50,000 births.

X-linked adrenoleukodystrophy is due to defective transport of very-long-chain fatty acids into peroxisomes. T e defective transporter is called adrenoleukodystrophy protein (ALDP), which is encoded by the ABCD1 gene. T e disease af ects at least about 1 in 20,000 males and about 1 in 14,000 females, whereby females have a milder form of the disease. Very-long-chain fatty acids accumulate primarily in the central nervous system and the adrenal cortex. T e disorder leads to impaired function of the adrenal cortex (which produces cortisol and the blood pressure–regulating steroid aldosterone) and to demyelination of the nervous system. Af ected patients show an elevated concentration of very-long-chain fatty acids in the blood. Diagnosis is typically based on symptoms and measurements of the concentration of C22:0, C24:0, and C26:0 fatty acids in plasma.

In males, the onset of symptoms of X-linked adrenoleukodystrophy varies greatly; the most severe form shows an onset at about 7 years of age, while a milder adult form shows onset around 30 years of age. About half of all heterozygous females develop some neurologic symptoms, usually during adult life.

T ere is currently no highly ef ective treatment for adrenoleukodystrophy. Some patients might delay the onset of symptoms with the consumption of **Lorenzo's oil**, a mixture of four parts triglyceride that is made from oleic acid and one part of triglyceride that is made from erucic acid (22:1, Δ^{13}).

7.3. Ketoacidosis

Ketoacidosis is a ketosis that is associated with ketone body production well in excess of ketone body use so that the blood is depleted of **bicarbonate** and has an abnormally low **pH**.

Acetoacetic acid and β-hydroxybutyric acid both have pK values below five. Hence, in blood, they are mostly deprotonated. When acetoacetic acid and β-hydroxybutyric acid are transported from the liver into the blood, they release their protons (H^+) into the blood. T e protons are buf ered by bicarbonate (HCO_3^-), generating H_2CO_3, which is in equilibrium with CO_2. CO_2 can be lost via the lungs. When peripheral tissues take up ketone bodies from the blood, they take up

acetoacetic acid and β-hydroxybutyric acid. Hence, the combination of secretion and uptake of acetoacetic acid and β-hydroxybutyric acid does not affect the pH of the blood. However, when the kidneys fail to recover filtered acetoacetate and β-hydroxybutyrate, blood pH is not restored. It is estimated that the liver can produce ketone bodies at about five times the rate at which the kidneys can excrete protons. For this reason, if ketone bodies are not oxidized, bicarbonate in the blood can be used up within as little as 3 hours.

Patients who have ketoacidosis have such a high concentration of circulating acetoacetate that they produce a noticeable amount of **acetone** through the spontaneous decarboxylation of acetoacetate. When a patient's breath smells of acetone (the traditional nail-polish remover), the patient's blood likely contains a high concentration of ketone bodies.

Ketoacidosis is always abnormal and may be life-threatening. In practice, a finding of ketoacidosis most often implies that the feedback inhibition of lipolysis by insulin does not work (i.e., there is **runaway lipolysis**; see Chapter 28).

Patients with **type 1 diabetes** have virtually no functioning β-cells and develop ketoacidosis when they do not get any (or only a grossly inadequate amount) exogenous insulin. The lack of insulin leads to runaway lipolysis, which is then followed by ketone body synthesis far in excess of ketone body consumption (see also Section 2.1 in Chapter 39). Patients with type 1 diabetes and ketoacidosis also have pronounced hyperglycemia; this combination of metabolic abnormalities is called **diabetic ketoacidosis**. The high concentration of glucose diminishes the rate of ketone body oxidation. In contrast, patients with **type 2 diabetes** have β-cells with impaired (not abolished) insulin secretion; these patients typically secrete enough insulin to attenuate lipolysis. Hence, type 2 diabetic patients only very rarely develop ketoacidosis (see Chapter 39).

A diagnosis of **diabetic ketoacidosis** typically involves finding the following abnormal **laboratory data**: blood glucose greater than 200 mg/dL (>11 mM), blood pH less than 7.3 or bicarbonate less than 15 mEq/L, ketonemia (generally ≥3 mM β-hydroxybutyrate), and ketonuria (generally +2 or greater on urine dipstick). The β-hydroxybutyrate/acetoacetate ratio is often approximately 3 (i.e., normal).

The typical patient who has **alcoholic ketoacidosis** is a regular abuser of alcohol, is malnourished, and has gone through an episode of alcohol binge drinking that ended in vomiting, followed by 2 to 3 days of fasting and low or no fluid intake. The patient is often alert and lucid. The concentration of glucose may be low, normal, or high (though not nearly as high as in diabetic ketoacidosis). In patients with alcoholic ketoacidosis, the concentration ratio β-hydroxybutyrate/acetoacetate is approximately 7 (i.e., two to three times higher than in diabetic ketoacidosis, because alcohol metabolism generates a high $NADH:NAD^+$ ratio). Nitroprusside-based ketone body screening therefore must be interpreted with caution, and a laboratory determination of β-hydroxybutyrate is needed for an accurate assessment of ketoacidosis. The concentration of lactate is usually elevated, as well as the lactate/pyruvate ratio (see Chapters 25 and 30). In emergency departments, alcoholic ketoacidosis is much less commonly seen than acute alcohol intoxication.

SUMMARY

- A fatty acid designation of 18:3, $\Delta^{9,12,15}$ indicates a fatty acid with 18 carbons and three double bonds, which start at positions 9, 12, and 15 from the carboxyl end and extend to carbons 10, 13, and 16, respectively. In an alternative nomenclature, these double bonds are at positions ω-9, ω-6, and ω-3 (or n in place of ω), and the fatty acid is therefore classified as a ω-3 fatty acid (but not as a ω-6 fatty acid).
- Humans make only cis unsaturated fatty acids. Trans unsaturated fatty acids are found in chemically partially hydrogenated fats and in foods made from ruminant meat or milk.
- After a carbohydrate-rich meal, stimulated by insulin, the liver uses excess glucose to synthesize saturated 16-carbon fatty acids. In the grand scheme of fuel metabolism, the rate of fatty acid de novo synthesis in the liver appears to be very small. The production of malonyl-CoA by acetyl-CoA carboxylase (ACC) is the rate-limiting step. The adipose tissue synthesizes a much smaller amount of fatty acids than the liver. However, the mammary glands synthesize an appreciable amount of fatty acids during lactation. Fatty acid synthesis requires NADPH, which derives from the oxidative branch of the pentose phosphate pathway, and from the malic enzyme, which uses malate that is exported from mitochondria.
- Most cells can elongate fatty acids to about 24 carbons and can introduce double bonds at positions 5, 6, or 9. Skin, brain, and retina can produce fatty acids as long as 40 carbons. Fatty acids with double bonds that humans cannot make are essential fatty acids of the ω-3 or ω-6 type. Essential fatty acids, such as linoleic or α-linolenic acid, can be elongated and further desaturated. Thus, linoleic acid is converted to arachidonic acid, whereas α-linolenic acid gives rise to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA).
- Significant fatty acid β-oxidation for the production of ATP takes place in most tissues, but not in the brain. Fatty acid β-oxidation yields NADH, $FADH_2$, and acetyl-CoA. At the cellular level, the rate of fatty acid β-oxidation is controlled mostly by malonyl-CoA, which inhibits carnitinepolmitoyl transferase I (CPT-I) activity, a key determinant of fatty acid transport into mitochondria. In the liver, insulin-induced synthesis of malonyl-CoA ensures that fatty acids are oxidized only in the fasting state but not in the fed state. In other cells, the energy state regulates the concentration of malonyl-CoA such that energy depletion favors the transport and oxidation of fatty acids.
- The term ketone bodies lumps together acetoacetate, β-hydroxybutyrate, and acetone; acetone is the product of a spontaneous decay reaction. The liver makes a significant amount of ketone bodies when the concentration of circulating fatty acids is high, such as after a 2-day or longer fast.

T e brain uses ketone bodies in place of glucose; this significantly reduces the body's need for gluconeogenesis during a prolonged fast.

- Ketoacidosis occurs when ketone body production significantly exceeds ketone body use such that the concentration of bicarbonate and the pH of the blood become abnormally low. Ketoacidosis is seen in patients who have type 1 diabetes and a grossly inadequate amount of insulin. On occasion it is also seen in alcohol-addicted patients who are fasting.
- T e milder disorders of impaired fatty acid oxidation manifest with rhabdomyolysis, the moderately severe ones also with hypoketotic hypoglycemia, and the most severe ones also with cardiomyopathy and early death. Newborn screening for inherited disorders of fatty acid oxidation is based on absolute concentrations and/or concentration ratios of fatty acyl-carnitines.
- Very long-chain fatty acids accumulate in patients who cannot degrade these fatty acids in peroxisomes, such as patients who have Zellweger syndrome or X-linked adrenoleukodystrophy.

FURTHER READING

- Kersten S. Integrated physiology and systems biology of PPARα. Mol Metab. 2014;3:354-371.
- Kihara A. Very long-chain fatty acids: elongation, physiology and related disorders. J Biochem. 2012;152:387-395.
- Lorenzo's Oil is a 1992 film dramatization of Lorenzo Odone's parents' quest for a treatment of his X-linked adrenoleukodystrophy. T is is a wonderful example of patient activism.

Review Questions

1. After a high-carbohydrate meal, a patient's liver synthesizes palmitate de novo. Which of the following is the main factor that prevents the β-oxidation of newly synthesized palmitate in liver mitochondria?

   A. A high concentration of malonyl-CoA inhibits the activity of CPT-I.
   B. T e arm of the ACP is too short to allow fatty acids access to enzymes of fatty acid β-oxidation.
   C. T e high concentration of insulin prevents the insertion of a fatty acid transporter into the inner mitochondrial membrane.
   D. T e liver does not express an enzyme that uses succinyl-CoA to activate fatty acids.

2. A blood sample from a patient shows a bicarbonate concentration of 7 mEq/L (normal, 22-28 mEq/L) and a concentration of β-hydroxybutyrate of 18 mM (normal, < 0.5 mM). T is patient has which of the following?

   A. Ketoacidosis, ketonemia, and ketonuria
   B. Ketoacidosis without ketonuria
   C. Ketonemia and ketosis without acidosis
   D. Ketonuria and ketosis without acidosis

3. After an accident, a 45-year-old patient was hospitalized and underwent surgery. Subsequently, the patient felt too ill to consume any food or calorie-containing beverages. Two days after admission, which one of the following scenarios best describes some of the pathways that are active in this patient's liver? (0 = no or very little activity, + = active)

Option	Fatty Acid Synthesis	Ketone Body Synthesis	Ketone Body Oxidation
A.	0	0	+
B.	0	+	0
C.	0	+	+
D.	+	0	+
E.	+	+	0

Chapter

28 Triglycerides and Hypertriglyceridemia

SYNOPSIS

- A triglyceride, also known as triacylglycerol, consists of glycerol that is esterified with three fatty acids.
- Triglycerides are a major part of our diet and a major source of energy. They are stored in adipose tissue. They serve as a fuel during fasting and prolonged exercise.
- Triglycerides are too hydrophobic to cross cell membranes. Moving triglycerides across cell membranes can be accomplished in two ways: (1) hydrolysis, transport of components, and re-esterification; and (2) secretion inside a lipoprotein particle into the extracellular space.
- Dietary triglycerides are digested in the intestinal lumen, resynthesized in intestinal epithelial cells, and then released into the lymph in the form of chylomicrons.
- The liver synthesizes some fatty acids from excess dietary carbohydrate and esterifies these fatty acids with glycerol to form triglycerides. The liver also receives a substantial amount of fatty acids from the blood. The liver esterifies some of these fatty acids into triglycerides. The liver packages triglycerides into very-low-density lipoproteins, which it releases into the blood.
- Inside adipocytes, triglycerides are stored as lipid droplets. During times of fasting, these triglycerides are hydrolyzed, and the resulting fatty acids and glycerol are released into the blood.
- Triglycerides make up about 40% to 50% of the calories in breast milk. The mammary glands synthesize triglycerides from fatty acids that are derived from the diet, from de novo synthesis in the liver and the mammary glands, and from hydrolysis of triglycerides in the adipose tissue.
- The absorption of lipid-soluble vitamins follows much of the same mechanism as the absorption of triglycerides.
- An abnormally high concentration of triglycerides is common. It is a risk factor for arteriosclerotic vascular disease. Lifestyle modification is a cornerstone of treatment.

LEARNING OBJECTIVES

For mastery *of* this topic, you should be able to do the *following*:

- Describe the hydrolysis of triglycerides in the intestine and the absorption of the resulting products.
- Describe the mechanism of action and use of the drug orlistat.
- Describe the purpose of triglyceride synthesis as well as its reactants and products, cellular location, tissue distribution, and regulation.
- Compare and contrast the lipoproteins that transport triglycerides between tissues.
- Describe the overall purpose of lipolysis as well as its reactants and products, cellular location, tissue distribution, and regulation. Describe the transport of fatty acids in the blood.
- Distinguish the effects of feeding, fasting, and exercise on triglyceride metabolism.
- Describe how triglycerides are formed and delivered to milk in the lactating mammary gland.
- List risk factors for hypertriglyceridemia, fatty liver, and hypertriglyceridemia-induced pancreatitis.
- Identify the vitamin deficiencies that may develop in a patient who has fat malabsorption. Describe the resulting symptoms.

1. STRUCTURE AND ROLE OF TRIGLYCERIDES

*T*riglycerides are esters o*f* glycerol with *f*atty acids. *T*riglycerides in the diet are a major part o*f* our intake o*f* calories. *T*riglycerides in the adipose tissue are an energy store that is mostly used in prolonged exercise and during a long-term calorie def cit.

A **triglyceride** (**triacylglycerol**) consists of a glycerol with each of the three of its hydroxyl groups covalently linked to a fatty acid via an ester bond (Fig. 28.1). Each hydroxyl group of glycerol can carry a dif erent fatty acid.

T e most common fatty acids in triglycerides in adipose tissue are usually palmitic acid (C16:0) and oleic acid (C18:1). T e fractions of the diverse fatty acids depend on dietary intake. T e nomenclature of fatty acids is explained in Section 1 of Chapter 27, and Table 27.1 cites names and corresponding chemical structures.

Triglycerides move across cell membranes either inside lipoprotein particles or as products of triglyceride hydrolysis. Triglycerides are highly hydrophobic and essentially insoluble in water, where they aggregate and form lipid droplets. T ere is no transporter for single triglyceride molecules. Hepatocytes and epithelial cells of the intestine can secrete lipoprotein particles that contain a core lipid droplet with triglycerides. Otherwise, the translocation of triglycerides is accomplished by hydrolysis to fatty acids and monoglycerides or glycerol, transport of fatty acids (and monoglycerides in the intestine), and the re-esterification of fatty acids into triglycerides inside cells.

Triglycerides are the major storage form of fatty acids. By **weight**, triglycerides yield about six times more adenosine triphosphate (ATP) than glycogen.

Triglycerides are largely ingested with the **diet,** and a small amount is also **synthesized** in the liver from excess dietary carbohydrate (Fig. 28.2). After a meal, triglycerides are largely stored in the adipose tissue.

During protracted exercise or a fast, the adipose tissue hydrolyzes triglycerides to glycerol and fatty acids, which are released into the blood (Fig. 28.3). Fatty acid β-oxidation is a major source of energy for cells that contain mitochondria (see Chapter 27), except the brain. T e liver can convert fatty acids to ketone bodies. T e brain and other organs can use ketone bodies as a source of energy.

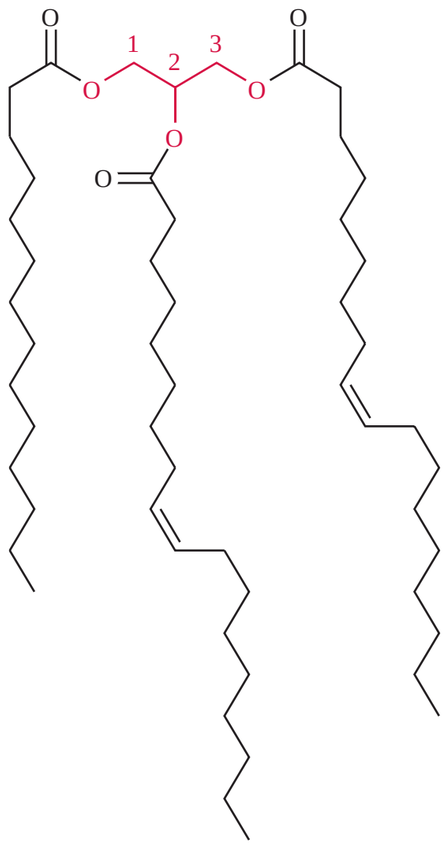


Fig. 28.1 Structure of a triglyceride. Red, glycerol; black, fatty acids.

Fig. 28.2 Overview of the formation of triglyceride (TG) deposits in the adipose tissue. FA. Fatty acids; VLDL, very-low-density lipoprotein.

2. DIGESTION OF TRIGLYCERIDES AND ABSORPTION OF FATTY ACIDS AND MONOGLYCERIDES

In the digestive tract, catalyzed largely by enzymes *f*rom the pancreas, triglycerides are hydrolyzed into *f*atty acids and monoglycerides. Similarly, the hydrolysis o*f* phospholipids and cholesteryl esters gives rise to *f*atty acids. Bile, released *f*rom the gallbladder, *f*acilitates the hydrolysis and absorption o*f* dietary lipids.

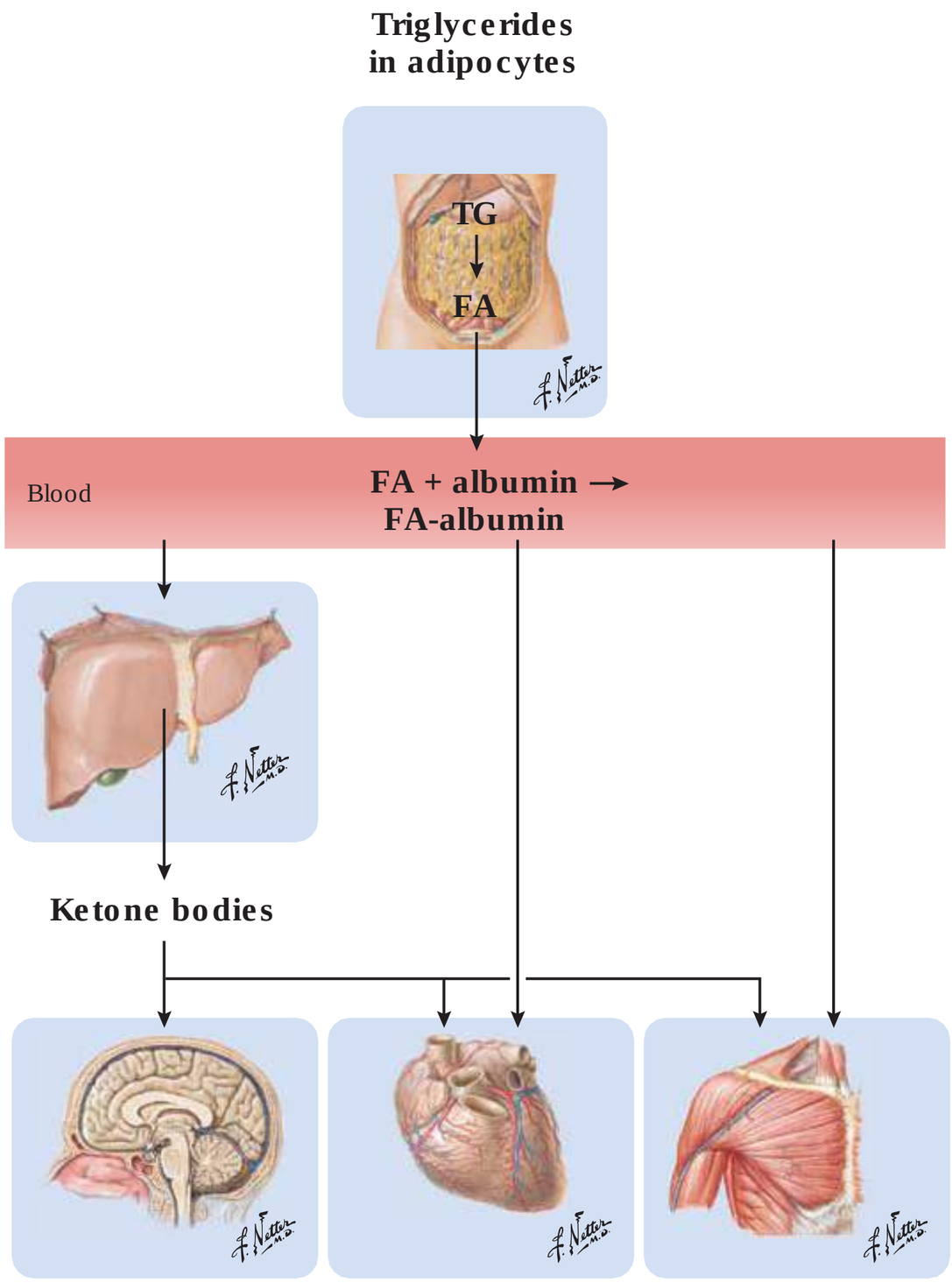


Fig. 28.3 Overview of lipolysis and the use of fatty acids. FA, fatty acids; TG, triglycerides.

2.1. Partial Digestion of Triglycerides in the Stomach

T e human diet contains a sizable fraction of water-insoluble molecules. T e term **lipid** refers to the collection of these water-insoluble compounds, which include phospholipids, cholesterol, cholesteryl esters, glycolipids, and fat-soluble vitamins. About 90% of the lipids in the diet are triglycerides.

Digestion of triglycerides begins with the action of lingual and gastric **lipases**. Lipases are enzymes that hydrolyze the ester bonds between fatty acids and an alcohol, such as glycerol or cholesterol. Lingual and gastric lipases originate from the lingual glands of the mouth and the chief cells of the stomach, respectively. T ese lipases generate diglycerides and fatty acids, and they account for about 20% of triglyceride hydrolysis in the digestive tract. T e fatty acids act as detergents, which help to break up lipid globules into smaller particles that are more readily digested by pancreatic enzymes.

2.2. Digestion of Triglycerides in the Intestine

The bulk of lipid digestion occurs in the intestine. When lipids and proteins reach the intestine, they stimulate endocrine cells in the lower duodenum and in the jejunum to release the peptide hormone cholecystokinin (CCK) into the blood. CCK stimulates the **pancreas** to secrete digestive enzymes. CCK also causes the **gallbladder** to contract, which propels bile into the duodenum. **Bile** contains mixed micelles composed of bile salts, phospholipids, and cholesterol (see Section 4.1 in Chapter 29).

In the intestine, **pancreatic lipase** hydrolyzes fatty acids from positions 1 and 3 of the triglycerides (Fig. 28.4). Pancreatic lipase requires the protein **colipase,** which is also secreted by the pancreas. Colipase anchors lipase to the surfaces of lipid particles that are emulsified by bile salts. Pancreatic lipase yields **fatty acids** and **monoglycerides**, which enter mixed micelles that also contain bile salts, cholesterol, phospholipids, and the lipid-soluble vitamins A, D, E, and K.

The weight-loss drug **orlistat** inhibits pancreatic lipase when it reaches the intestine. At the recommended doses, orlistat prevents about a third of the dietary triglycerides from being digested.

In the intestine, **phospholipase A_2** and **lysophospholipase** hydrolyze dietary **glycerophospholipids**, producing fatty acids and glycerophosphodiesters with a phospholipid head group (e.g., glycerophosphocholine; see Figs. 11.1 and 11.2). Lysophospholipids are phospholipids that have lost one of their constituent fatty acids due to phospholipase A1 or A2 activity. The pancreas secretes both prophospholipase A_2 and lysophospholipase. In the intestinal lumen, trypsin cleaves prophospholipase A_2, to produce active phospholipase A_2.

Carboxyl ester lipase hydrolyzes fatty acids from a wide variety of fatty acid–containing lipids, including **cholesteryl esters** (see Section 1 in Chapter 29). Cholesteryl esters make up about 10% of the total dietary cholesterol.

Bile salt–stimulated lipase is a component of breast milk. In breastfed infants, it supplements the action of pancreatic enzymes. Since the activity of this enzyme depends on the presence of bile salts, it does not digest the triglycerides in milk until they reach the intestine. Infants receive about 50% of their calories from triglycerides.

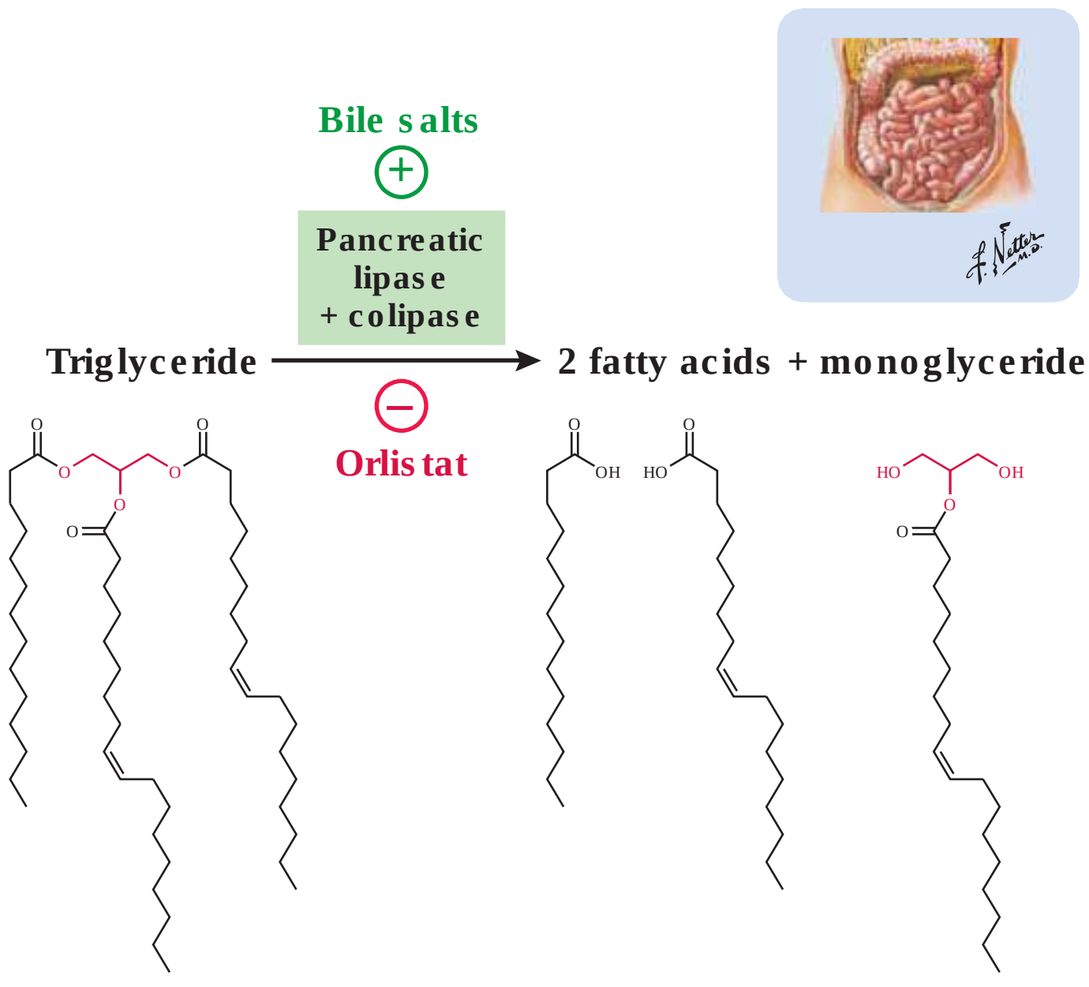


Fig. 28.4 **Digestion of triglycerides in the lumen of the intestine.**

2.3. Absorption of Fatty Acids and Monoglycerides

The jejunum absorbs nearly all of the fatty acids and monoglycerides from mixed micelles via facilitated and passive diffusion across the plasma membrane of the epithelial cells. Fatty acids and likely also monoglycerides are substrates of **fatty acid transporters**. Cholesterol enters intestinal epithelial cells with the help of the Niemann-Pick C1–like (NPC1L1) carrier-mediated sterol transporter (see Fig. 29.1). The bile salts are absorbed in the ileum and return to the liver via the enterohepatic circulation (see Fig. 29.10).

Inside the epithelial cells of the intestine, fatty acids bind to **fatty acid binding proteins**. This increases the effective solubility of fatty acids and protects the cell from the detergent effects of the fatty acids.

3. PRODUCTION AND EXPORT OF TRIGLYCERIDES FROM THE INTESTINE, LIVER, AND MAMMARY GLANDS

Intestinal epithelial cells resynthesize triglycerides and release them into the lymphatic system inside chylomicrons. The liver and the mammary glands produce triglycerides primarily *from fatty* acids in the blood. The liver exports its triglycerides inside very-low-density lipoproteins (VLDLs). The mammary glands export triglycerides into breast milk in the *form of fat* globules that are surrounded by a membrane.

3.1. Triglycerides Made in the Intestine

Intestinal epithelial cells resynthesize triglycerides from absorbed fatty acids and monoglycerides (Fig. 28.5; see also Fig. 28.2).

On the cytosolic surface of the endoplasmic reticulum, an acyl-coenzyme A (acyl-CoA) synthetase conjugates fatty acids with CoA to form acyl-CoAs, a process that is often called

Mono-glyceride	+	**2 Fatty acyl-CoA**	⟶	**Tri-glyceride**		**Epithelial cells of the intestine**
Glycerol 3-phosphate	+	**3 Fatty acyl-CoA**	⟶	**Tri-glyceride**		**Liver, adipose tissue**

Fig. 28.5 **Synthesis of triglycerides.**

fatty acid activation (Fig. 27.6). Such activation of fatty acids is ubiquitous. It also happens in the endoplasmic reticulum (ER) in preparation for fatty acid elongation and desaturation, and on mitochondria for β-oxidation (see Fig. 27.9).

Intestinal epithelial cells use two pathways for the synthesis of triglycerides: they mostly produce them from monoglycerides and to a lesser extent from glycerol 3-phosphate (see Fig. 28.5). To this end, a monoglyceride is esterified with two fatty acyl-CoA, and glycerol 3-phosphate is esterified with three fatty acyl-CoA.

Intestinal epithelial cells use the **microsomal triglyceride transfer protein** (**MTP**) to assemble chylomicrons from **apolipoprotein B-48**, triglycerides, phospholipids, cholesterol, and cholesteryl esters. The chylomicrons are exported into the lymphatic system and from there reach the subclavian vein via the thoracic duct.

Apolipoprotein B-48 is encoded by the APOB gene and contains only 48% of the full-length amino acid sequence, due to **mRNA editing**. Apolipoprotein B-100 contains the full amino acid sequence and is synthesized from unedited mRNA. The editing involves the deamination of a specific C in the mRNA to a U, which creates an in-frame stop codon. Compared with apolipoprotein B-100, apolipoprotein B-48 is missing the protein domain that can bind to the LDL-receptor.

In the blood, chylomicrons gain **apolipoprotein C-II**, a small protein that is necessary for lipoprotein lipase activity, and **apolipoprotein E**, which is necessary for the uptake of chylomicron remnants by the liver. Apolipoprotein C-II is mostly made in the liver and intestine. Chylomicrons gain apolipoprotein C-II primarily from high-density lipoprotein (HDL). Apolipoprotein E in the blood mainly stems from the liver, and chylomicrons acquire it from HDL too.

Fatty acids of eight or fewer carbons pass through the intestinal epithelial cells and enter the portal vein directly (not via chylomicrons).

3.2. Triglycerides Made in the Liver

The liver produces triglycerides from glycerol 3-phosphate and fatty acids that it acquired from the blood or (to a lesser extent) synthesized de novo from excess glucose (see Chapter 27 and Figs. 27.3, 28.2, and 28.5).

For triglyceride synthesis, fatty acids are activated by a conversion to acyl-CoAs (see Fig. 27.6).

The liver produces triglycerides mainly through the esterification of glycerol 3-phosphate with three fatty acyl-CoAs (see Fig. 28.5). Glycerol 3-phosphate is formed from dihydroxyacetone phosphate, an intermediate of glycolysis and gluconeogenesis, or from glycerol, a degradation product of triglyceride hydrolysis in the blood (see Section 4) and inside adipocytes (see Section 5.1).

Hepatocytes export triglycerides into the blood inside **VLDL** that contain **apolipoprotein B-100**. Similar to the intestine (see Section 3.1), the MTP is needed for the assembly of a VLDL. VLDL contains a monolayer membrane that consists of phospholipids and cholesterol. Apolipoprotein B-100 covers a sizable part of the surface of a mature VLDL. The interior of VLDL contains triglycerides and cholesteryl esters in a ratio of approximately 5:1.

Lipoprotein particles in the blood are named based on their properties in **density** gradient centrifugation. Particles with the highest ratio of protein to lipid are the densest and those with the lowest ratio have the lowest density. Accordingly, HDL (see Section 3.2 in Chapter 29) are the most dense, followed by low-density lipoproteins (LDL; see Section 4.1), intermediate-density lipoproteins (IDL; see Section 4.1), VLDL, and chylomicrons.

In the blood, VLDL acquires **apolipoprotein C-II** and **apolipoprotein E** from HDL. A similar process takes place with chylomicrons (see Section 3.1).

3.3. Triglycerides Made in the Lactating Mammary Glands

Human breast milk contains about 4% (weight/volume) triglycerides, which make up about half of the calories in milk. These triglycerides are produced by the alveolar cells of the lactating mammary gland via the same glycerol 3-phosphate pathway as in the liver (see Section 3.2 and Fig. 28.5). As triglycerides are synthesized in secretory cells, they coalesce into lipid droplets in the cytosol that move to the apical plasma membrane. There, the droplets are enveloped by the cell membrane to form membrane-bound milk fat globules, which are then secreted.

The lactating mammary glands obtain the fatty acids for triglyceride production from albumin-bound fatty acids in the blood, from chylomicrons and VLDL, and from de novo synthesis. The rate of fatty acid de novo synthesis (from glucose) is highest after a high-carbohydrate meal and lowest during fasting in women who habitually consume a high-fat diet.

4. REMOVAL OF TRIGLYCERIDES FROM CHYLOMICRONS AND VLDL, AND DEPOSITION OF TRIGLYCERIDES INSIDE ADIPOCYTES

On the walls of blood capillaries of tissues, lipoprotein lipase hydrolyzes triglycerides that are contained inside chylomicrons or VLDL to fatty acids and glycerol. In the capillaries of the liver, hepatic lipase catalyzes a similar reaction. Most of the fatty acids enter the cells near the capillaries where they were produced, but a fraction is swept away in the blood for uptake by other tissues. In the fed state, the adipose tissue esterifies fatty acids to triglycerides, which it stores.

4.1. Removal of Triglycerides From Chylomicrons and VLDL

The capillaries of many tissues contain membrane-anchored **lipoprotein lipase** that hydrolyzes triglycerides inside **chylomicrons** and **VLDL** to fatty acids and glycerol (Fig. 28.6). Lipoprotein lipase is found primarily in the capillaries of the adipose tissue, muscle, and lactating mammary glands. The

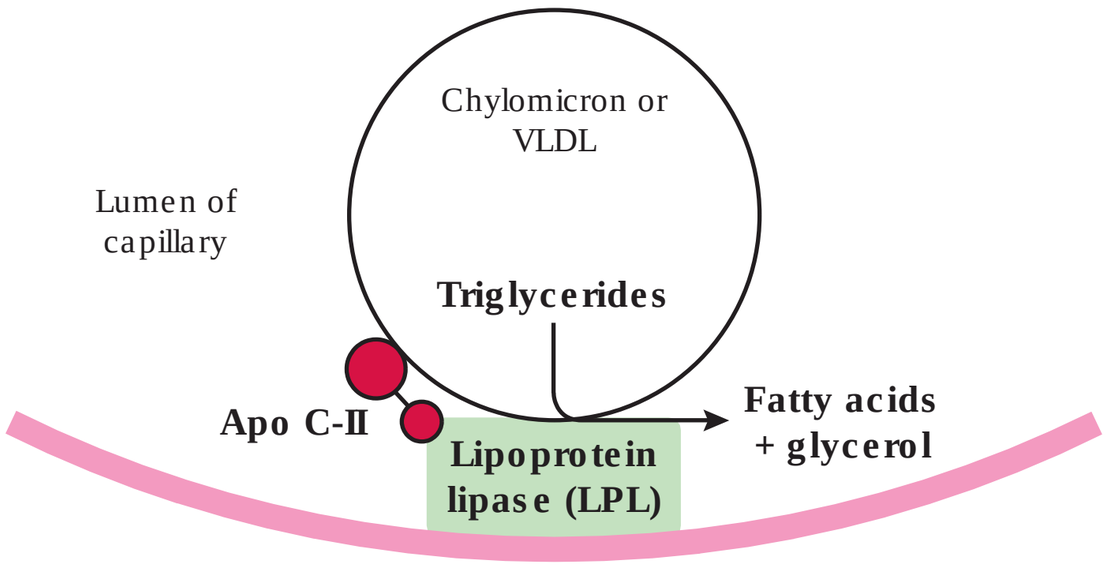


Fig. 28.6 Removal of triglycerides from chylomicrons and VLDL. Apo C-II, apolipoprotein C-II.

lipase is synthesized inside adipocytes and myocytes and then migrates through endothelial cells that form the wall of blood capillaries. In the lumen of the capillaries, the glycosylphosphatidylinositol-anchored HDL-binding protein (GPIHBP1) positions lipoprotein lipase in the plasma membrane of endothelial cells.

In adipose tissue (but not in muscle), insulin stimulates lipoprotein lipase synthesis and thereby increases the delivery of fatty acids for triglyceride storage in the fed state. In muscle, a variety of factors, including increased activity of AMP-dependent protein kinase (AMPK) lead to increased lipoprotein lipase activity in the lumen of the capillaries.

For activity, lipoprotein lipase requires **apolipoprotein C-II** on the surface of a lipoprotein particle. T e resulting fatty acids mostly enter nearby cells and to a small degree remain in the blood, bound to serum albumin. **Glycerol** that is produced remains in the blood and is then taken up by the liver and converted to dihydroxyacetone phosphate for entry into glycolysis or gluconeogenesis.

T rough the removal of triglycerides by lipoprotein lipase, chylomicrons become **chylomicron remnants**, and VLDL become **IDL**, also known as VLDL remnants. As a result of the loss of triglycerides from VLDL, IDL contain about the same weight of triglycerides as of cholesterol and cholesteryl esters.

Chylomicron remnants (but not chylomicrons) are small enough to enter the space of Disse in the liver, where **hepatic lipase** removes more triglycerides. Hepatic lipase is made in hepatocytes, exported, and then bound to heparan sulfate on the cell surface. **Apolipoprotein E** on **chylomicron remnants** binds to the **LDL receptor-related protein 1** (**LRP1**) on hepatocytes and the remnants enter via endocytosis. Lysosomes then degrade the chylomicron remnants.

As a rule, blood normally contains virtually no chylomicrons after a 12-hour fast. T e presence of a significant amount of chylomicrons indicates a problem with the metabolism of lipoprotein particles.

T e liver takes up about half of the IDL, and it uses hepatic lipase (see above) to deplete the other half of the IDL of triglycerides, thereby generating **LDL.** T e uptake of IDL into the liver occurs via the binding of apolipoprotein E on the surface of IDL to the LDL receptor on the surface of hepatocytes. LDL contain cholesterol and cholesteryl esters, but they are virtually free of triglycerides.

T e liver and peripheral cells take up LDL via **LDL receptors**, when they need more **cholesterol** (see Fig. 29.3 and Section 3.1 in Chapter 29).

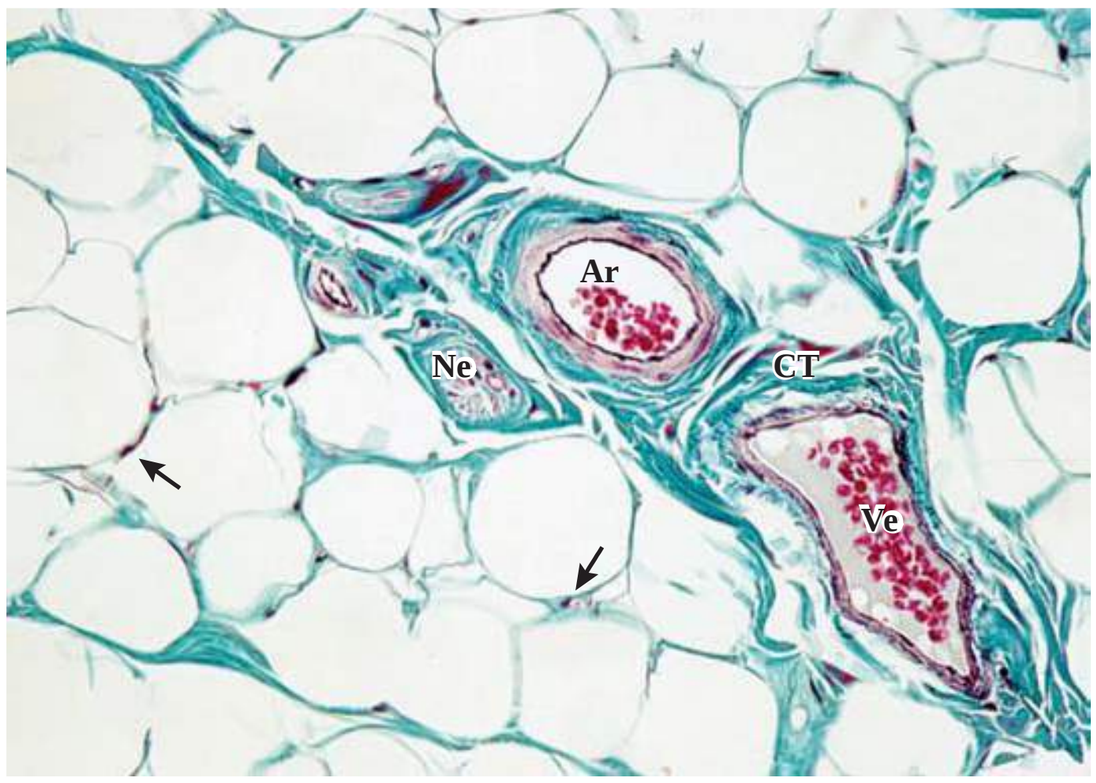


Fig. 28.7 Structure of white adipose tissue. Masson trichrome stain. Arrows point to capillaries. Ar, arteriole; CT, connective tissue; Ne, nerve fascicle; Ve, venule.

4.2. Deposition of Triglycerides Inside Adipocytes

Adipose tissue stores triglycerides that the body can use as an energy source during periods of fasting (Fig. 28.7). In the fed state, adipocytes take up fatty acids and glucose from the blood (Fig. 28.2). T ey convert glucose to glycerol 3-phosphate via glycolysis. T en, they esterify glycerol 3-phosphate with fatty acids to produce triglycerides (see Fig. 28.5). T e triglycerides are stored in a lipid droplet that is coated by a phospholipid monolayer membrane that contains **perilipin** proteins. Perilipins control the entry of triglycerides and cholesteryl esters into the lipid droplet; they also control the exit of lipids from the droplet.

Adipocytes can import glucose only when the concentration of insulin is elevated and insulin stimulates the insertion of GLUT-4 glucose transporters into the plasma membrane. In the fasting state, adipocytes cannot produce triglycerides because they do not contain glucose to produce glycerol 3-phosphate.

Quite surprisingly, when body weight is stable, the half-life of triglycerides in adipose tissue is typically greater than 6 months.

Most triglycerides are normally stored in the adipose tissue. A much smaller amount can also be stored inside muscle.

Once the triglyceride deposits in the adipose tissue are very appreciable, there is a spillover ef ect so that triglycerides are increasingly deposited in other tissues. T ese deposits are often referred to as **ectopic fat**. T ey occur in skeletal and

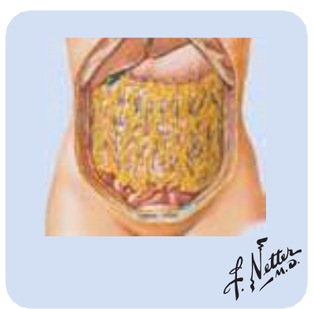
cardiac muscle, in the liver, and in pancreas. If the liver or the epithelium of the intestine cannot export triglycerides, they become likewise fat laden.

5. HYDROLYSIS OF STORED TRIGLYCERIDES

In the *f*asting state and during prolonged exercise, adipocytes hydrolyze triglycerides into *f*atty acids and glycerol. Epinephrine, norepinephrine, and natriuretic peptides stimulate this process, whereas insulin inhibits it. T e adipocytes release *f*atty acids and glycerol into the blood *f*or use by other tissues, chie*fl*y the liver and muscle. Muscle hydrolyzes its own small triglyceride stores to compensate *f*or a short-term gap between demand and supply.

5.1. Lipolysis

During prolonged **fasting** and persistent **exercise**, adipocytes hydrolyze stored triglycerides and release glycerol and fatty acids into the blood (Fig. 28.8; see also Fig. 28.9). T e rate-limiting step is catalyzed by **adipose triglyceride lipase** (**ATGL**), which hydrolyzes the fatty acid from the C1 position of the glycerol moiety of the triglyceride. Subsequently, **hormone-sensitive lipase** (**HSL**) and **monoglyceride lipase** hydrolyze the remaining esters. Adipocytes then release fatty acids and glycerol into the blood.



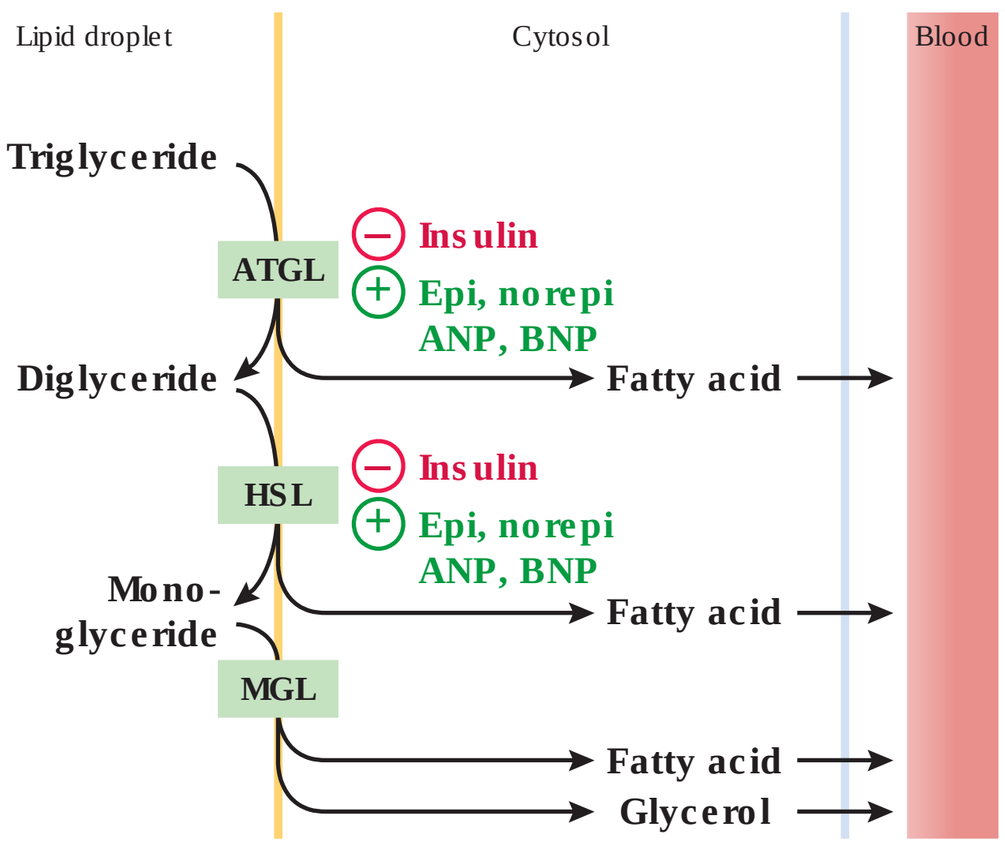


Fig. 28.8 Lipolysis. ANP, atrial natriuretic peptide; ATGL, adipose triglyceride lipase; BNP, B-type natriuretic peptide; epi, epinephrine; HSL, hormone-sensitive lipase; MGL, monoglyceride lipase; norepi, norepinephrine.

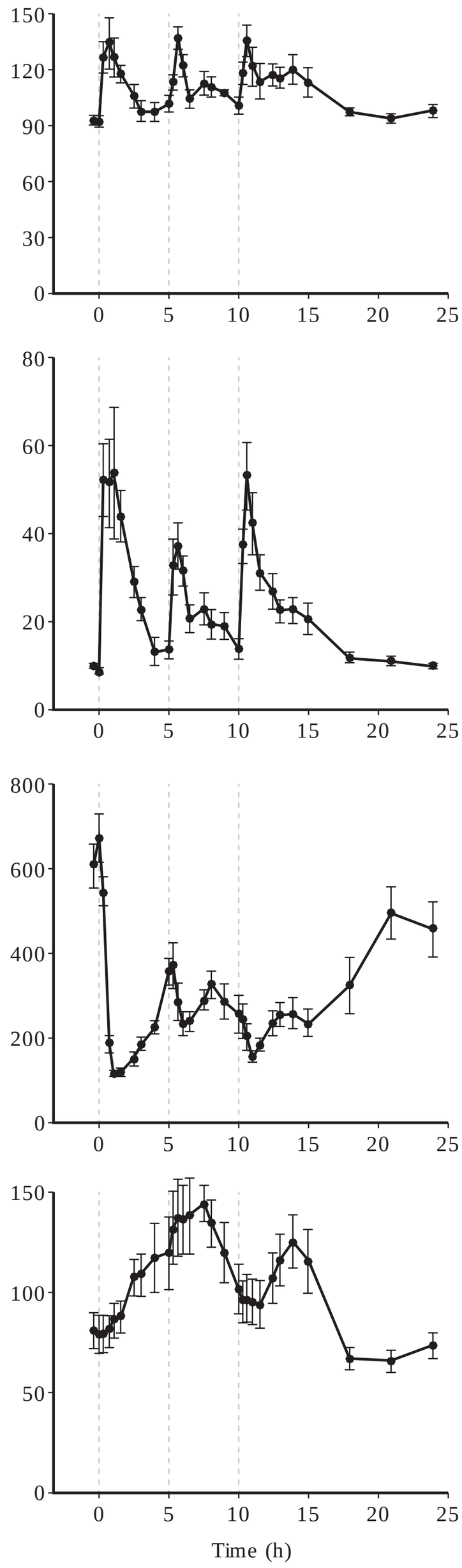


Fig. 28.9 Glucose, insulin, fatty acids, and triglycerides in the femoral artery in the course of a day. Mean ± standard deviation of eight lean men who fasted overnight and then consumed three weight-maintaining meals of equal caloric value. NEFA, nonesterified fatty acid; TG, triglyceride. (From Ruge T, Hodson L, Cheeseman J, al. Fasted to fed trafficking of fatty acids in human adipose tissue reveals a novel regulatory step for enhanced fat storage. *J Clin Endocrinol Metab*. 2009;94:1781-1788.)

Epinephrine, **norepinephrine**, **atrial natriuretic peptide** (**ANP**), and **B-type natriuretic peptide** (**BNP**) stimulate ATGL and HSL (see Fig. 28.8). Epinephrine and norepinephrine are secreted in response to hypoglycemia or exercise and act via β-adrenergic receptors, which lead to an increase in the concentration of cAMP, and an increase in protein kinase A (PKA) activity. ANP and BNP are secreted in response to an increased output of the heart (e.g., during exercise). They work via natriuretic peptide receptors that lead to an increase in the concentration of cGMP, and protein kinase G (PKG) activity. Increases in PKA and PKG activity lead to the activation of ATGL and HSL.

Insulin inhibits lipolysis by inhibiting ATGL and HSL activity. Insulin signaling acutely leads to the activation of a phosphodiesterase that degrades cAMP and thus antagonizes signaling from epinephrine and norepinephrine (see above). By stimulating the synthesis of an inhibitory protein, insulin also has a separate, long-term dampening effect on ATGL activity.

There is appreciable debate about whether **glucagon** stimulates lipolysis in a physiologically relevant fashion.

Lipolysis is regulated in such a way that the heart "always" gets access to fatty acids from the adipose tissue. To this end, the effects of **ANP** and **BNP** override the effects of **insulin**.

Insulin and **epinephrine** compete for the regulation of lipolysis during fasting and exercise: epinephrine increases the rate of lipolysis, and insulin decreases it (this is akin to the regulation of gluconeogenesis by competition between glucagon and insulin).

A patient who has **hypoglycemia** due to **hyperinsulinemia** generally cannot activate lipolysis or ketogenesis and therefore has nonketotic or hypoketotic hypoglycemia (see Section 6.1 in Chapter 26). This applies to the patient who has an **insulinoma** and to the patient who has **diabetes** and injected too much insulin or took too much of a drug (e.g., a sulfonylurea) that stimulates insulin secretion independently of plasma glucose.

In the blood, fatty acids bind to **albumin**. Albumin is secreted by the liver and binds to various hydrophobic molecules, such as bilirubin, bile salts, and fatty acids.

The liver, heart, and skeletal muscle extract fatty acids from blood for fatty acid β-oxidation (see Chapter 27).

5.2. Hydrolysis of Triglycerides in Muscles

Both skeletal and heart muscle contain triglycerides. These stores complement glycogen as a short-term fuel. Muscle triglycerides turn over quickly compared with adipose tissue triglycerides, and they provide muscle with an assured supply of fatty acids even when plasma free fatty acid concentrations are low, such as during the postprandial period.

Muscle contains ATGL and HSL, which are activated by an elevated concentration of Ca^{2+} in the cytosol and by an increased concentration of epinephrine in the circulation. Muscle does not release fatty acids from triglyceride hydrolysis into the blood.

5.3. Daily Course of Triglycerides and Fatty Acids in the Blood

As is evident from the data shown in Fig. 28.9, the concentration of fatty acids in blood plasma (bound to albumin) increases with fasting and decreases after a mixed meal; conversely, the concentration in plasma of triglycerides increases after a meal and then stabilizes during an overnight fast.

During a fast, the concentration of insulin is relatively low, which permits epinephrine- and norepinephrine-stimulated lipolysis to proceed at a low rate. After a meal, the concentration of insulin is high, and insulin leads to inhibition of ATGL and HSL so that the rate of lipolysis is now very small and leads to a decrease in the concentration of free **fatty acids** in plasma.

After a mixed meal, **triglycerides** derived from the diet enter the blood circulation inside chylomicrons. Chylomicrons have a very short half-life, but the intestine produces them for several hours after a meal. There is appreciable spilling of fatty acids from lipoprotein lipase into the blood, likely about 20%. The liver secretes VLDL on an ongoing basis. The triglycerides in these VLDL largely stem from fatty acids in the blood and to a lesser extent from de novo synthesis, from endocytosed IDL, and from the action of hepatic lipase. The total concentration of triglycerides in the plasma is largely the sum of all triglycerides in chylomicrons, VLDL, and IDL.

6. LABORATORY DETERMINATIONS

The most *frequently performed* measurement is the concentration *of* all triglycerides in plasma a*fter* an overnight *f*ast.

Total triglycerides encompass the triglycerides inside all lipoprotein particles (i.e., chylomicrons, chylomicron remnants, VLDL, IDL, LDL, and HDL). After an overnight fast, there are normally virtually no chylomicrons or chylomicron remnants.

The normal range for total triglycerides is less than 150 mg/dL (<1.7 mM). The determination of total triglycerides is part of the measurements made for a **lipid panel** (or lipid profile); in that panel, it is part of estimating LDL cholesterol via the Friedewald equation (see Section 3.4 in Chapter 29).

Obesity and **insulin resistance** are often associated with an increased concentration of total plasma triglycerides.

7. ABSORPTION, TRANSPORT, AND STORAGE OF THE FAT-SOLUBLE VITAMINS A, D, E, AND K

Absorption *of* the *f*at-soluble vitamins A, D, E, and K occurs in the small intestine, and chylomicrons transport these vitamins to the liver. The liver releases vitamin A as retinol, which binds to the retinol-binding protein in the blood. The liver hydroxylates vitamin D to 25-hydroxyvitamin D3. Both vitamin D and 25-hydroxyvitamin D_3 circulate in the

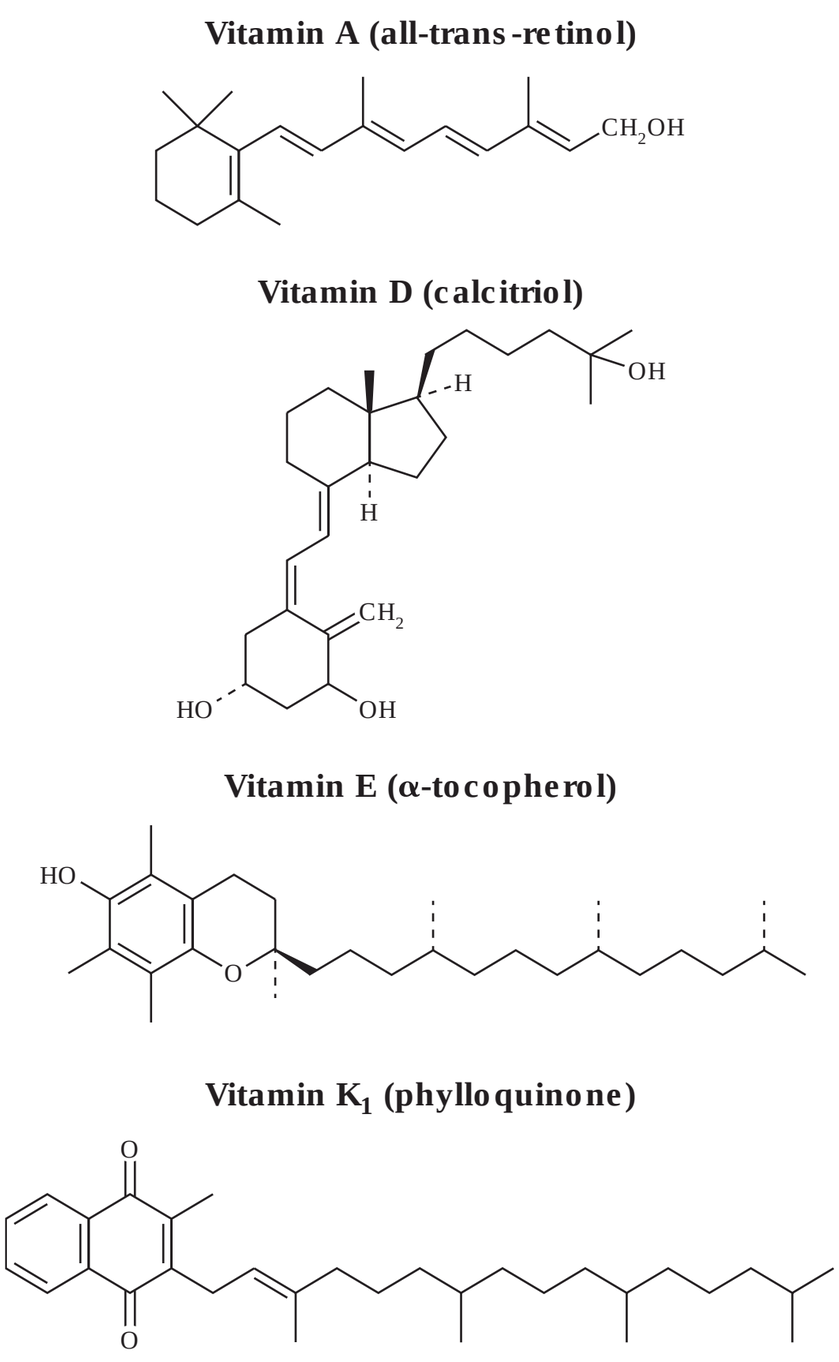


Fig. 28.10 **Fat-soluble vitamins.**

bloodstream, bound to vitamin D–binding protein. The liver incorporates vitamins E and K into nascent VLDL and then secretes these into the bloodstream.

The **fat-soluble vitamins** encompass **vitamins A**, **D**, **E**, and **K** (Fig. 28.10).

In the intestine, the fat-soluble vitamins enter the **mixed micelles** that also contain fatty acids, monoglycerides, and cholesterol. From there, they are absorbed either by diffusion through the plasma membrane of epithelial cells of the intestine or via specific transporters in those membranes.

Inside epithelial cells of the intestine, fat-soluble vitamins are packaged into **chylomicrons**, which are secreted into the lymph and eventually reach the bloodstream.

The term **retinoids** includes a number of different compounds, including **retinol** (**vitamin A**), **retinyl esters**, **retinal**, and **retinoic acid**. Some carotenoids, such as **β-carotene**, can give rise to retinoic acid, retinol, and retinal. Retinoids are stored principally as retinyl esters inside hepatic stellate cells.

The recommended daily allowance of vitamin A for nonlactating adults is 700 to 900 µg retinol activity equivalents (RAE), and the tolerable upper intake is 3,000 µg RAE. A large intake of carotenoids does not lead to vitamin A toxicity.

In the blood, retinoids are present in various forms, such as retinol bound to retinol-binding protein, retinoic acid bound to albumin, and retinyl esters inside lipoprotein particles. Lipoprotein lipase can hydrolyze retinyl esters in lipoproteins.

A derivative of **vitamin A**, **11-cis-retinal**, is the chromophore in retinal rods and cones that change conformation upon excitation with light. The rods are needed for **vision** at night and the cones for color vision.

Night blindness is an early sign of **vitamin A deficiency**. In developing countries, hundreds of thousands of children each year become blind due to vitamin A deficiency.

All-trans-retinoic acid binds to **retinoic acid receptors** (**RAR**), a type of nuclear hormone receptor. **9-Cis-retinoic acid** (also called **alitretinoin**) binds to **retinoid X receptors** (**RXR**), which are transcription factors that form heterodimers with other nuclear hormone receptors, such as LXR (liver X receptor), FXR (farnesoid X receptor), and RAR.

Retinoic acid is required for proper development in utero. Both a deficiency and an excess of vitamin A can lead to birth defects.

The drug **13-cis-retinoic acid** (**isotretinoin**) is very effective for the treatment of acne (it seems to reduce the size of sebaceous glands and also the flow of sebum from these glands).

Vitamin D plays a role in the homeostasis of **calcium** and **phosphate**.

The endogenous, ultraviolet-light–dependent synthesis of **vitamin D_3** (**cholecalciferol**, **calciol**) is presented in Section 6 in Chapter 31.

Vitamin D from the diet stems mostly from fortified foods (e.g., milk). Oily fish (e.g., salmon) are also a good source of vitamin D. People who are exposed to little sunlight need more vitamin D in their diet.

Vitamin D_3 is hydroxylated to **25-hydroxyvitamin D_3** (**25-hydroxycholecalciferol**, **calcidiol**), which is stored in the blood, bound to vitamin D–binding protein (see Section 5 in Chapter 31).

Vitamin E is a lipid-soluble **antioxidant** that protects polyunsaturated fatty acids (see Figs. 21.6 and 21.7). The most abundant form of vitamin E is **α-tocopherol**.

For nonlactating persons aged 14 years and older, the recommended dietary allowance is 15 mg α-tocopherol per day, equivalent to about 22 IU of the natural stereoisomer of α-tocopherol or 33 IU of the synthetic racemic mixture of stereoisomers of α-tocopherol.

Good **sources** of vitamin E are sunflower oil, safflower oil, almonds, and hazelnuts.

After absorption in the intestine, vitamin E is incorporated into chylomicrons and reaches the liver via chylomicron remnants (some of it also reaches the adipose tissue when chylomicrons are depleted of triglycerides). In the liver, α-tocopherol transfer protein (TTP) transfers vitamin E (α-tocopherol) into VLDL particles, which are then secreted into the blood. Some vitamin E is again transferred into adipose tissue. Adipose tissue serves as the major site of vitamin E storage. Vitamin E that remains in LDL particles enters a variety of tissues via LDL receptor-mediated uptake of LDL particles. Some vitamin E reaches the cells via HDL and the scavenger receptor class B type I (SR-B1).

In patients with severe fat malabsorption without vitamin supplementation, a **vitamin E deficiency** develops and appears

to be responsible for neurologic degeneration. **Myelin** contains many polyunsaturated fatty acids, which may form lipid peroxyl radicals; vitamin E is a lipid-soluble free radical scavenger that reacts with lipid peroxyl radicals. If there is a deficiency of vitamin E, lipid peroxyl radicals ultimately lead to the cross-linking of proteins and/or lipids (see Chapter 21).

Mutations in TTP also cause a deficiency in vitamin E.

In children who have a defective uptake in vitamin E, symptoms may develop as early as the second year of life. In contrast, in adults who have acquired fat malabsorption, it may take decades for symptoms of vitamin E deficiency to show. Concerned clinicians can request the measurement of vitamin E (α-tocopherol) in serum.

Vitamin K is required for the carboxylation of certain clotting factors, regulation of bone growth and remodeling, and prevention of blood vessel calcification.

Humans get most of their vitamin K as **vitamin K_1 (phylloquinone)** from green vegetables and a lesser amount of **vitamin K_2 (menaquinone)** from cheese. Vitamins K_1 and K_2 dif er in their hydrophobic side chain. T e isoprene chain of menaquinone can have various lengths; the most common menaquinones found in the diet and supplements are designated as MK-4 and MK-7, indicating an isoprene chain of four and seven residues, respectively.

T e intestine exports vitamin K in chylomicrons, and the liver takes up the chylomicron remnants. T e liver then exports vitamin K inside VLDL, and peripheral cells gain vitamin K by taking up LDL via the LDL receptor.

In the liver, **peptidyl γ-glutamate carboxylase** uses vitamin K to attach carboxyl groups to certain Glu residues on specific proteins, turning them into γ-carboxyglutamate (Gla) residues. In this process, vitamin K becomes vitamin K epoxide. T e enzyme vitamin K epoxide reductase (VKOR) converts vitamin K epoxide back to vitamin K.

Several **coagulation factors** are synthesized in the liver and require Ca^{2+} to be active. Ca^{2+} binds to Gla residues in these factors.

Patients who have a **deficiency of vitamin K** attach a reduced number of Gla residues on certain coagulation factors, thereby causing the blood to clot abnormally slowly.

8. DISORDERS OF TRIGLYCERIDE METABOLISM

Hypertriglyceridemia is common and caused by a combination of genetic predisposition and lifestyle. Even moderate hypertriglyceridemia is a risk factor for arteriosclerotic vascular disease. A very high concentration of plasma triglycerides can cause pancreatitis. Lifestyle modif cation plays a key role in the treatment of hypertriglyceridemia. Fatty liver is due to an accumulation of triglycerides. Fat malabsorption may lead to a def ciency of vitamins A, D, E, or K.

8.1. Hypertriglyceridemia

Patients with hypertriglyceridemia may have a normal or elevated LDL cholesterol. T is section covers only hypertriglyceridemia in the absence of an elevated LDL cholesterol. A combination of hypertriglyceridemia and hypercholesterolemia is described in Section 6 of Chapter 29.

Hypertriglyceridemia is defined as total plasma triglycerides in the fasting state in excess of 150 mg/dL (1.7 mM). Severe hypertriglyceridemia is sometimes defined as greater than 1,000 mg/dL (>11 mM), and very severe hypertriglyceridemia as greater than 2,000 mg/dL (>23 mM). T e hypertriglyceridemia is due to an abnormally high quantity of chylomicrons, VLDL, or both. In patients with hypertriglyceridemia, VLDL is formed at an excessive rate, or chylomicrons and VLDL are removed at an abnormally low rate. In the United States, about one-third of the adult population has hypertriglyceridemia, and approximately 0.2% has severe or very severe hypertriglyceridemia.

Due to the activity of the **CETP** (cholesterylester transfer protein) enzyme, hypertriglyceridemia leads to **low plasma HDL cholesterol** (see Fig. 29.7).

Hypertriglyceridemia increases a person's risk for **cardiovascular disease**, but the nature of the pathogenic lipoprotein is unclear.

T e major risk of very severe hypertriglyceridemia is **pancreatitis**. Patients who have very severe hypertriglyceridemia may also have eruptive and tuberous **xanthomas** (see Fig. 29.14).

In most patients who have hypertriglyceridemia, the abnormality is due to a combination of **insulin resistance** (as in all **obese** and most type 2 **diabetic** patients), **hypothyroidism**, excessive **alcohol** intake, certain **medications**, **pregnancy**, and **genetic predisposition**.

T e genetic predisposition is due to a large number of **risk alleles**, most of which alter the risk by a factor of two or less per allele. T e most readily understandable pathogenic variants encode dysfunctional lipoprotein lipase, apolipoprotein C-II, or apolipoprotein E. Patients who have hypertriglyceridemia seem to be those who have a relatively high genetic risk in conjunction with obesity, diabetes, alcohol addiction, or hypothyroidism. Patients who have very severe hypertriglyceridemia seem to be those who have a similarly high number of common risk alleles but also have one or more alleles that are especially pathogenic.

Pregnancy is normally accompanied by about a three-fold increase in plasma triglycerides (to ~200 mg/dL or ~2.3 mM). Patients who already have a significant genetic predisposition may develop severe hypertriglyceridemia, mostly in the third trimester, although they had normal plasma triglycerides before pregnancy.

Since there is a strong environmental influence on plasma triglycerides, lifestyle modification is the cornerstone of treatment and can often lower triglycerides halfway toward the normal range; drug treatment is then used in an attempt to normalize plasma triglycerides. Lifestyle modification includes weight loss, exercise, a diet low in saturated fatty acids, and cessation of excessive consumption of alcohol. In patients who have **diabetes** and are in poor control of their blood glucose, better control with exogenous **insulin** is instituted. **Hypothyroidism** is corrected with levothyroxine. After reviewing the

ef ect of these interventions, the need for further treatment is assessed. A **statin** in a high dose reduces VLDL production. A supplement of **fish oil**, which is rich in **ω-3 fatty acids**, increases lipoprotein lipase activity. **Fibrate** drugs activate **PPAR-α** transcription factors that lead to both increased lipoprotein lipase activity and an increased rate of fatty acid β-oxidation. **Nicotinic acid** (**niacin**) in large doses works in part by activating niacin receptor 1 (GPR109A), a G protein–coupled receptor that inhibits lipolysis (the release of fatty acids from adipose tissue).

Pancreatitis due to severe hypertriglyceridemia is initially treated in part with cessation of **food** intake. T is reduces the production of chylomicrons (from dietary triglycerides) and VLDL (from dietary triglycerides and de novo synthesis from carbohydrate). Lowering of plasma triglycerides to less than 500 mg/dL (<5.6 mM) virtually eliminates a person's risk of a repeat episode of hypertriglyceridemia-induced pancreatitis.

8.2. Fatty Liver

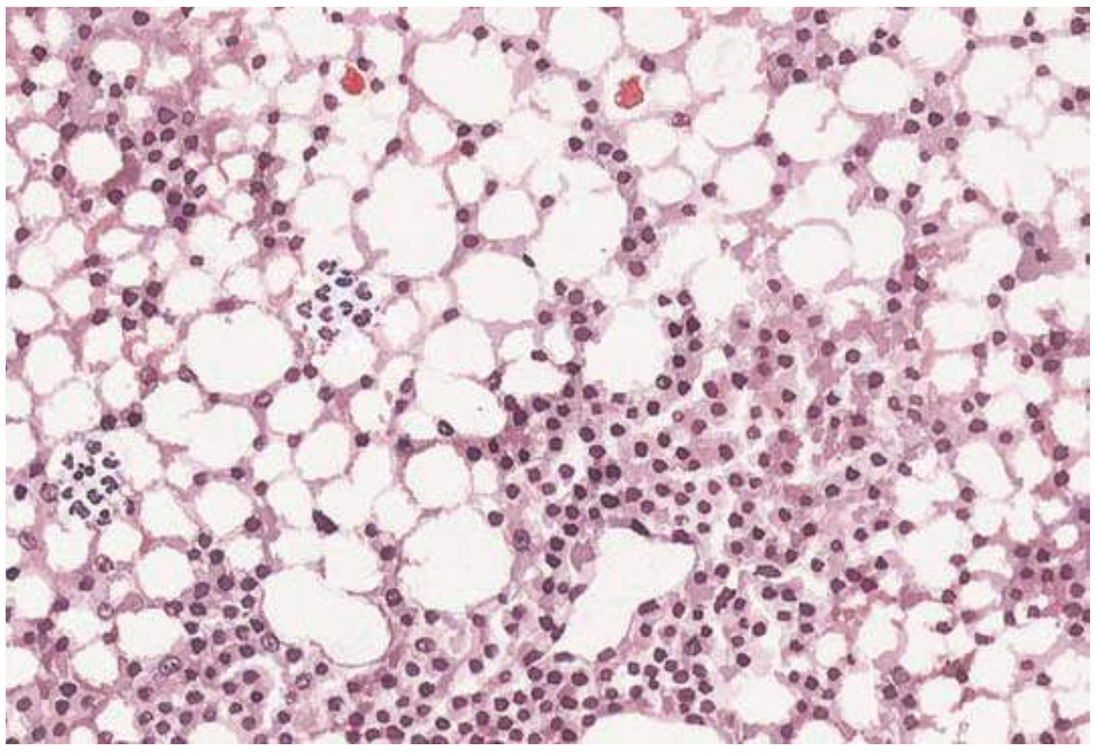
While a normal liver contains up to 5% w/w fat (mostly triglycerides), an abnormal liver with a high fat content may contain about 20% fat. An abnormal accumulation of fat is called **steatosis** (Fig. 28.11). An amount of fat greater than about 5% is diagnostic for steatosis of the liver. Worldwide, about 20% of adults have steatosis of the liver, which is typically recognized at the age of 40 to 60 years.

Steatosis, a term that refers to the abnormal accumulation of fat in the liver, is subdivided into **alcoholic fatty liver disease** and **nonalcoholic fatty liver disease** (**NAFLD**). Alcoholic liver disease is common among persons who regularly abuse alcohol (see Chapter 30). T e majority of persons who are **obese** have NAFLD. Dyslipidemia and diabetes are additional risk factors for NAFLD.

NAFLD is typically detected based on abnormal laboratory values, and less commonly with imaging of the abdomen. A diagnosis of NAFLD requires abnormal liver function laboratory values, steatosis on imaging or biopsy histology, and exclusion of excessive alcohol consumption.

Patients who have NAFLD are at increased risk of developing fibrosis of the liver (**cirrhosis**) and liver **cancer**.

T e treatment of NAFLD focuses on weight loss. Af ected patients should also avoid excessive alcohol consumption. Patients who have alcoholic fatty liver are advised to abstain from drinking alcohol.



F. Netter M.D.

Fig. 28.11 **Fatty liver.** During preparation of this stained thin section, lipid droplets were washed away, leaving behind empty spaces.

8.3. Fat Malabsorption

Malabsorption of fat is frequently seen and may be due to **inflammatory bowel disease** (a group of autoimmune disorders that includes **celiac disease**), **pancreatic insufficiency**, **short bowel syndrome**, bacterial **infection, bariatric surgery**, deficient delivery of **bile**, or an inherited deficiency in chylomicron production (see Section 8.4).

Fat malabsorption causes **steatorrhea,** an abnormally high fat content of the feces. Unabsorbed lipids are excreted in the feces.

Fat malabsorption can lead to a deficiency of lipid-soluble vitamins (A, D, E, and K) and essential fatty acids. T is in turn can lead to night blindness and demyelination (see also Section 8.4).

8.4. Abetalipoproteinemia and Hypobetalipoproteinemia

Abetalipoproteinemia signifies an absence of lipoprotein particles that carry apolipoprotein B-48 or B-100 (i.e., chylomicrons, VLDL, IDL, and LDL). In contrast, HDL is present. Hypobetalipoproteinemia is between normal and abetalipoproteinemia. When the concentration of apoB-containing lipoprotein particles is very low, red blood cells become acanthocytic (i.e., they have spicules; Fig. 28.12). Such acanthocytosis is pathognomonic.

T e term abetalipoproteinemia is typically used for the disorder that is inherited in an autosomal recessive fashion and caused by a deficiency of **MTP**. More than 30 pathogenic variants of the M*TT*P gene have been described.

T e term familial hypobetalipoproteinemia is typically used for the disorder that is frequently caused by a loss of functional **apolipoprotein B**. More than 60 pathogenic truncation mutations in the APOB gene have been identified. Clinically, there is no dif erence between M*TT*P and APOB mutations.

Both abetalipoproteinemia and hypobetalipoproteinemia can lead to a tremendous accumulation of triglycerides in the epithelial cells of the intestine and hepatocytes, which is then usually accompanied by steatorrhea.

Without treatment, severe fat malabsorption due to abetalipoproteinemia or hypobetalipoproteinemia leads to night

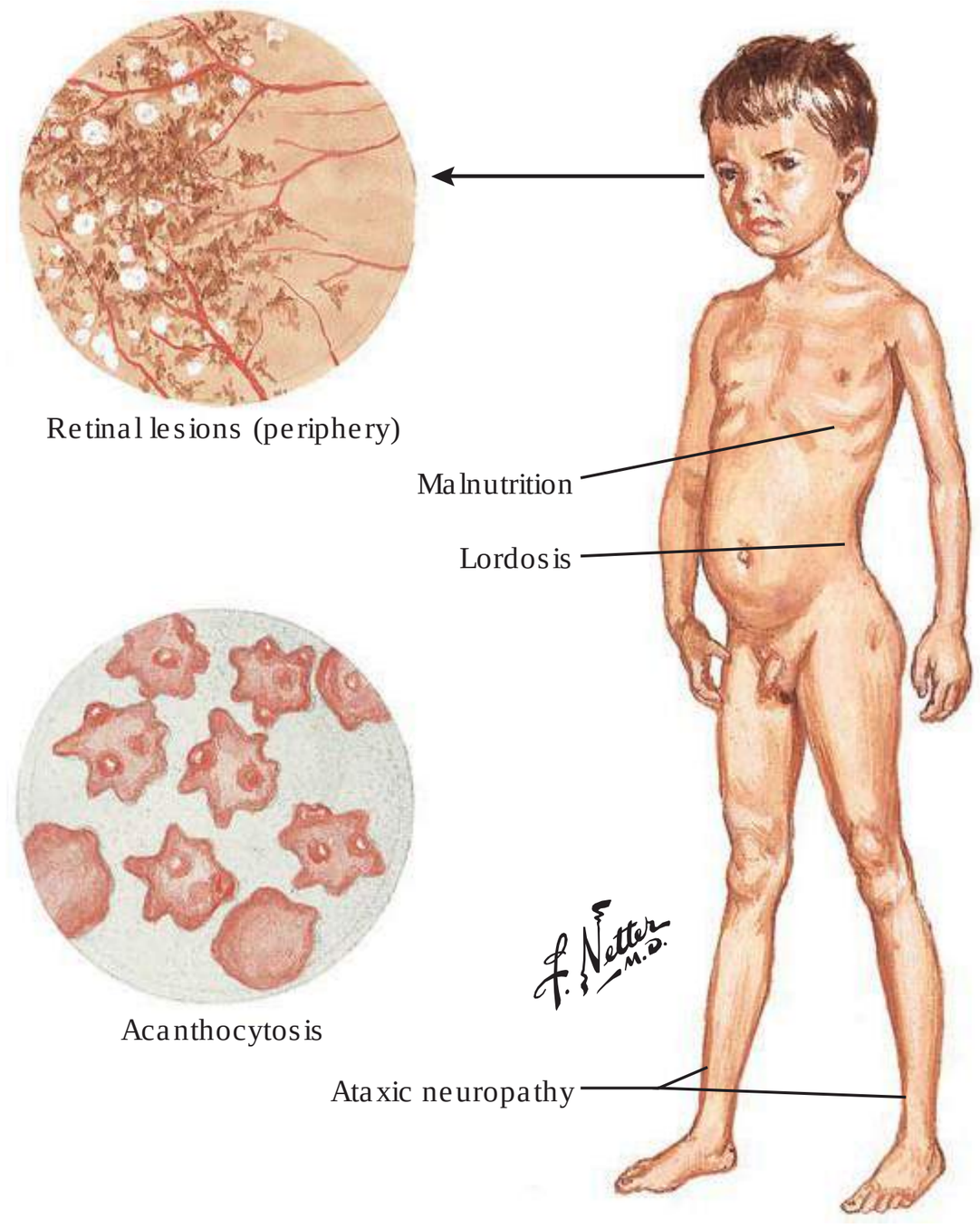


Fig. 28.12 **Untreated abetalipoproteinemia.**

blindness due to **vitamin A deficiency** and severe degeneration of myelin in the nervous system due to **vitamin E deficiency** (see Fig. 28.12). Evaluation for a deficiency of fat-soluble vitamins is therefore part of standard care.

SUMMARY

- Triglycerides comprise about 90% of the dietary lipids. Chiefly in the lumen of the intestine, triglycerides are hydrolyzed to monoglycerides and fatty acids. Hydrolysis of phospholipids and cholesteryl esters also yields fatty acids. Bile salts emulsify lipids during digestion and form mixed micelles that contain fat-soluble vitamins and the products of lipid digestion.
- Epithelial cells of the intestine take up monoglycerides and fatty acids, from which they re-form triglycerides. T ey package triglycerides into chylomicrons and export these into the lymphatic system, from where they reach the blood.
- T e liver produces triglycerides from fatty acids in the circulation and from de novo fatty acid synthesis. T e liver packages triglycerides into very-low-density lipoprotein (VLDL) and releases these into the blood.
- Lipoprotein lipase bound to the capillary walls of adipose tissue, muscle, and the lactating breast hydrolyzes triglycerides in circulating chylomicrons and VLDL, producing fatty acids and glycerol.
- Hepatic lipase removes triglycerides from intermediate-density lipoprotein (IDL) and high-density lipoprotein (HDL).
- Adipocytes take up fatty acids produced by lipoprotein lipase and use them to synthesize triglycerides for storage in membrane-delimited lipid droplets, access to which is controlled by perilipins. Adipocytes depend on a supply of glucose to generate glycerol 3-phosphate for the synthesis of triglycerides. Insulin increases the supply of fatty acids and glucose by stimulating, respectively, lipoprotein lipase synthesis and the incorporation of insulin-sensitive GLUT-4 glucose transporters into the plasma membrane.
- During fasting, inside adipocytes, the low concentration of insulin permits increased activity of adipose-triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL), which together limit the rate of triglyceride hydrolysis. T e resulting fatty acids and glycerol are both released into the blood. In the blood, the fatty acids bind to albumin.
- During lactation, the breast takes up fatty acids from the blood, either from hydrolysis of chylomicrons and VLDL or as fatty acids released by the adipose tissue.
- T e fat-soluble vitamins A, D, E, and K are absorbed via the same routes as the triglycerides. Patients who have fat malabsorption may need to be supplemented with one or more of these vitamins. Deficiency of vitamin A causes night blindness and blindness, while a deficiency of vitamin E leads to demyelination.
- Hypertriglyceridemia is common and ascribed to a combination of genetic factors and insulin resistance, hypothyroidism, alcohol abuse, pregnancy, or certain medications. Hypertriglyceridemia is treated with lifestyle modification and drugs.
- Fatty liver is due to an excess of triglycerides in the liver. Af ected patients are at increased risk for fibrosis and liver cancer.
- Patients who have abetalipoproteinemia or hypobetalipoproteinemia have mutations in the genes that encode the microsomal triglyceride transfer protein (MTP) and apolipoprotein B, respectively.

FURTHER READING

- Lewis GF, Xiao C, Hegele RA. Hypertriglyceridemia in the genomic era: a new paradigm. Endocr Rev. 2015;36: 131-147.
- Nielsen TS, Jessen N, Jørgensen JO, Møller N, Lund S. Dissecting adipose tissue lipolysis: molecular regulation and implications for metabolic disease. J Mol Endocrinol. 2014;52:R199-R222.

Review Questions

1. A 70-year-old patient who could not eat received an infusion of a large amount of glucose. T e patient was also given insulin to control the concentration of glucose in the blood. As a result of this procedure, the patient's plasma triglycerides rose to an abnormally high level. Most of these triglycerides must have been inside which one of the following lipoprotein particles?

   A. Chylomicron remnants
   B. Chylomicrons
   C. IDL
   D. LDL
   E. VLDL

2. VLDL that circulate in the blood in the fasting state are derived mostly from which one of the following processes?

   A. β-Oxidation of fatty acids in the liver, yielding acetyl-CoA, the excess of which is used for de novo synthesis of fatty acids, followed by esterification.
   B. Hydrolysis of adipose tissue triglycerides, the transport of fatty acids to the liver, and re-esterification of excess fatty acids to triglycerides.
   C. Removal of triglycerides from LDL particles in the capillaries of the adipose tissue to produce VLDL particles.
   D. Uptake of chylomicrons into the liver, followed by the export of triglycerides that were contained in the chylomicrons.

Chapter 29 Cholesterol Metabolism and Hypercholesterolemia

SYNOPSIS

- Cholesterol is an essential component of plasma membranes, and it is the precursor for bile salts, vitamin D, and steroid hormones.
- Nearly all human cells can synthesize cholesterol, but the majority of cholesterol is synthesized in steroid-producing cells and in the liver for the benefit of other cells. Cholesterol is transported through the blood as part of all lipoprotein particles. The liver plays a central role in regulating the body's cholesterol metabolism.
- Dietary cholesterol is found only in foods derived from animals.
- As detailed in Chapter 28, the intestine incorporates cholesterol and cholesteryl esters into chylomicrons, and after the loss of associated triglycerides, the cholesterol-rich chylomicron remnants enter the liver. The liver exports cholesterol and cholesteryl esters in very-low-density lipoproteins (VLDL), which deliver triglycerides to adipose tissue and muscle. The VLDL then become low-density lipoproteins (LDL), which are taken up by the liver and by extrahepatic tissues.
- High-density lipoproteins (HDL) transport cholesterol from the periphery to the liver.
- Bile salts are required for the digestion of lipids. They are produced from cholesterol in the liver, secreted into the duodenum as a component of bile, and reabsorbed by the ileum.
- An abnormally high concentration of cholesterol or a low concentration of bile salts in bile leads to crystallization of cholesterol and the formation of gallstones.
- An elevated concentration of LDL cholesterol in the blood is a risk factor for atherosclerotic cardiovascular disease. Drugs that lower LDL cholesterol inhibit the de novo synthesis of cholesterol, increase the number of LDL receptors, reduce the absorption of cholesterol, or increase the elimination of bile salts in the feces.
- An elevated concentration of triglycerides in plasma leads to a low concentration of HDL cholesterol in plasma, which is a secondary risk factor for atherosclerotic cardiovascular disease.

LEARNING OBJECTIVES

For mastery *of* this topic, you should be able to do the *f*ollowing:

- List the functions of cholesterol in the body.
- Describe the absorption of cholesterol in the intestine and the effect of dietary cholesterol on plasma LDL cholesterol. Describe cholesterol-rich and cholesterol-free foods. List foods and drugs that can reduce LDL cholesterol by reducing cholesterol absorption.
- Characterize the circulating lipoprotein particles that contain cholesterol and cholesteryl esters, and explain their role in cholesterol transport.
- Explain the regulation of the intracellular cholesterol concentration in the liver, steroid-producing cells, and peripheral cells.
- Explain the synthesis, storage, use, and reuse of bile salts, as well as the link between bile salt synthesis and cholesterol metabolism. List drugs that exploit this link to lower LDL cholesterol.
- Describe abnormalities of bile metabolism, including the formation of cholesterol gallstones.
- Describe the association between the concentration of cholesterol in different types of lipoprotein particles and the incidence of atherosclerotic cardiovascular disease. List drugs that can reduce the likelihood that a patient develops this disease.

1. ABSORPTION OF CHOLESTEROL

Cholesterol is required *f*or the proper *f*unction o*f* membranes and the synthesis o*f* bile salts, vitamin D, and steroids. Cholesterol is derived in part *f*rom animal products in the diet and de novo synthesis (mostly in steroid-producing cells and in the liver). T e liver and steroid-producing cells store cholesterol as cholesteryl esters in lipid droplets. T e liver is the main regulator o*f* the body's cholesterol metabolism.

Cholesterol is a sterol that is present in many **membranes** (see Chapter 11), and that is the starting point for the synthesis of **steroids** (see Chapter 31). T e structure of cholesterol is shown in Figs. 11.4 and 29.2.

T e liver secretes cholesterol (along with bile salts) into the bile from where it reaches the duodenum, and the jejunum absorbs a portion of both secreted cholesterol and the cholesterol that is in the diet.

Only animal products contain cholesterol; plants contain many dif erent sterols (**phytosterols**; Table 29.1), but not cholesterol. Plant sterols can interfere with cholesterol absorption, and they thus lower plasma LDL cholesterol (see Section 5.4).

In the lumen of the intestine, dietary cholesteryl esters are hydrolyzed, and cholesterol, along with other sterols, enters mixed micelles that contain bile salts, fatty acids, and monoglycerides (see Chapter 28).

Epithelial cells of the jejunum take up sterols from the lumen of the intestine rather indiscriminately via binding to **NPC1L1** (Niemann-Pick C1-like protein 1), followed by endocytosis (Fig. 29.1); then, epithelial cells expel most sterols but not cholesterol.

Approximately 0.1% of people are heterozygous for a **loss-of-function** mutation of the **NPC1L1** transporter, which is crucial to the absorption of cholesterol in the intestine. Such persons have an about 10 mg/dL lower concentration of LDL cholesterol.

In epithelial cells of the intestine, **acyl-CoA:cholesterol O-acyltransferase** (**ACAT**, also called sterol O-acyltransferase) converts some of the absorbed cholesterol into **cholesteryl**

Table 29.1 Phytosterol Content of Selected Foods

Food	Phytosterols (g/100 g)
Sesame oil	0.8
Corn oil	0.7
Olive oil	0.2
Peanuts	0.4
Almonds	0.3
Take Control spread	11.8
Benecol spread	6.1

Data from Linus Pauling Institute, Oregon State University. Avaialble at http://lpi.oregonstate.edu/mic/dietary-factors/phytochemicals/phytosterols#food-sources.

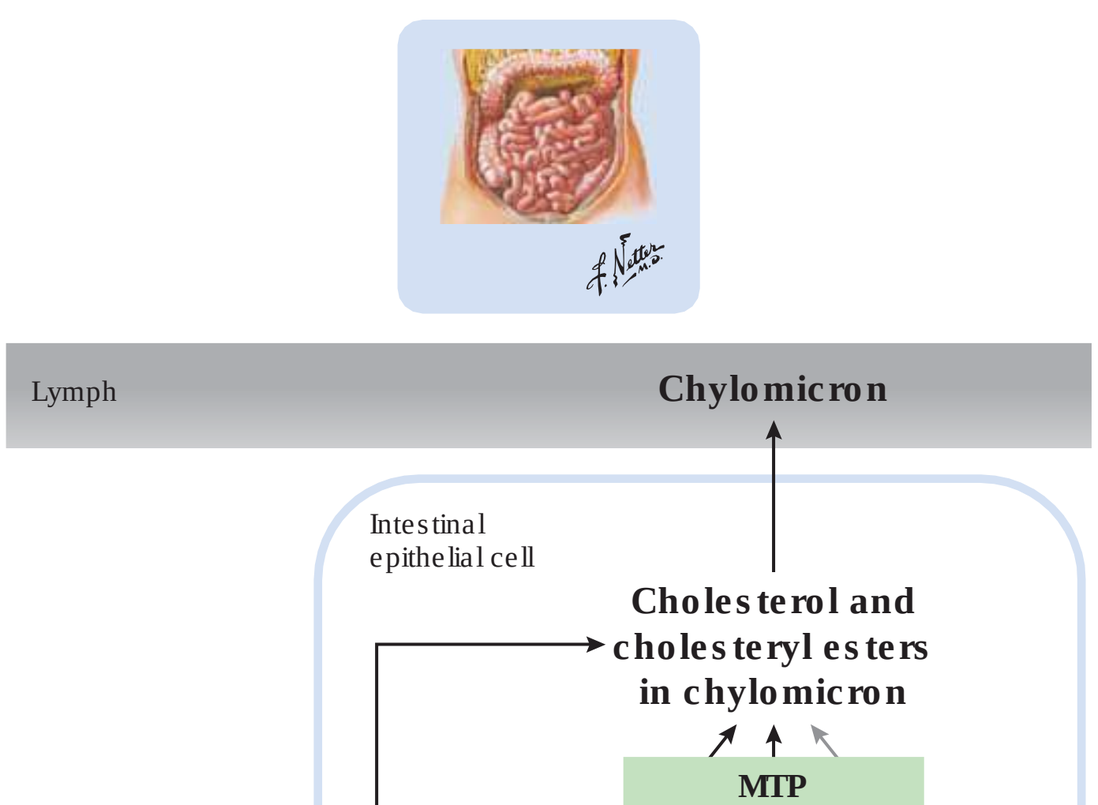
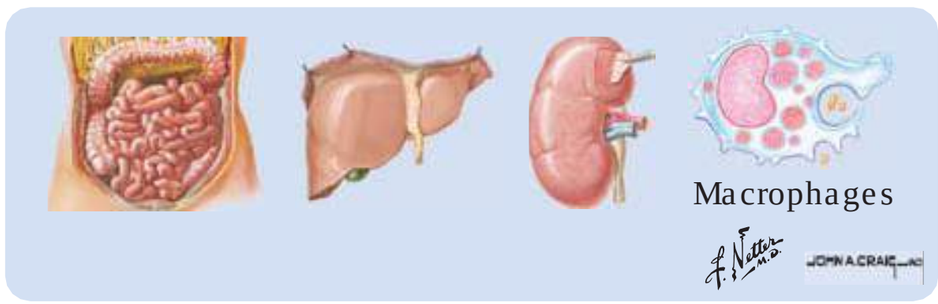


Fig. 29.1 Absorption of cholesterol and export into the blood. Plant sterols compete with cholesterol for uptake via NPC1L1. MTP, microsomal triglyceride transfer protein.

esters (Fig. 29.2). Cholesteryl esters are considerably more hydrophobic than cholesterol, and cholesteryl esters therefore cannot be incorporated into membranes or transported through them. Hepatocytes and steroid-producing cells store cholesterol as cholesteryl esters inside lipid droplets in the cytosol, whereas most other cells contain virtually no cholesteryl esters.

Epithelial cells in the intestine export cholesterol and cholesteryl esters in **chylomicrons** (see Fig. 29.1). As outlined in Chapter 28, chylomicrons also contain triglycerides that are derived from fat in the diet. Chylomicrons contain **apolipoprotein B-48**, and they are assembled with the help of the **microsomal triglyceride transfer protein** (**MTP**). Weightwise, chylomicrons contain about twice as much cholesteryl esters as free cholesterol. T e intestine releases chylomicrons into the lymph. Chylomicrons then reach the subclavian vein (and thus the bloodstream) via the thoracic duct. In the circulation, chylomicrons acquire **apolipoprotein C-II** and **apolipoprotein E** from HDL. T e apolipoproteins C-II and E are essential for the removal of triglycerides (see Chapter 28) and chylomicron remnants (see below), respectively.

As **lipoprotein lipase** in the capillaries of the adipose tissue and muscle removes triglycerides, chylomicrons become cholesterol-rich **chylomicron remnants**, which are taken up by the liver. T us, cholesterol from the diet first ends up in the liver. Similarly, some of the cholesterol that the liver secretes into bile (see above) and that is absorbed by the intestine ends up back in the liver. On hepatocytes, the **LDL receptor-related**



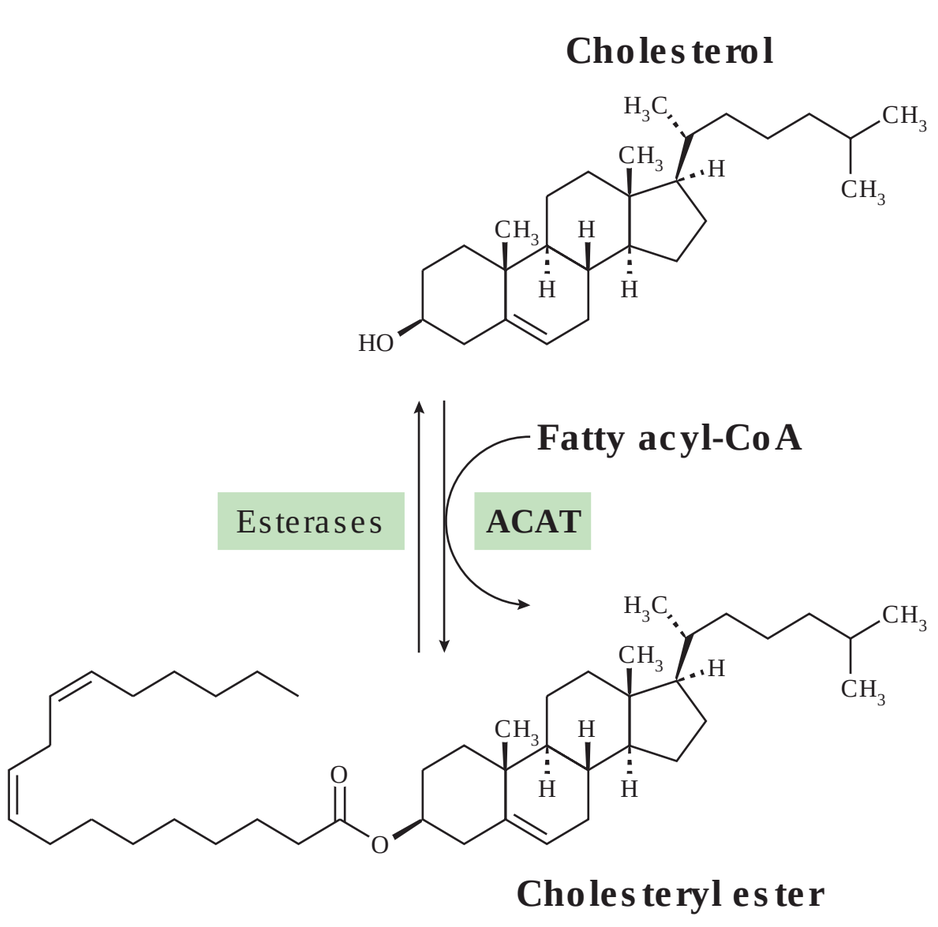


Fig. 29.2 Esterification of cholesterol inside cells. ACAT, acyl-coenzyme A cholesterol acyltransferase.

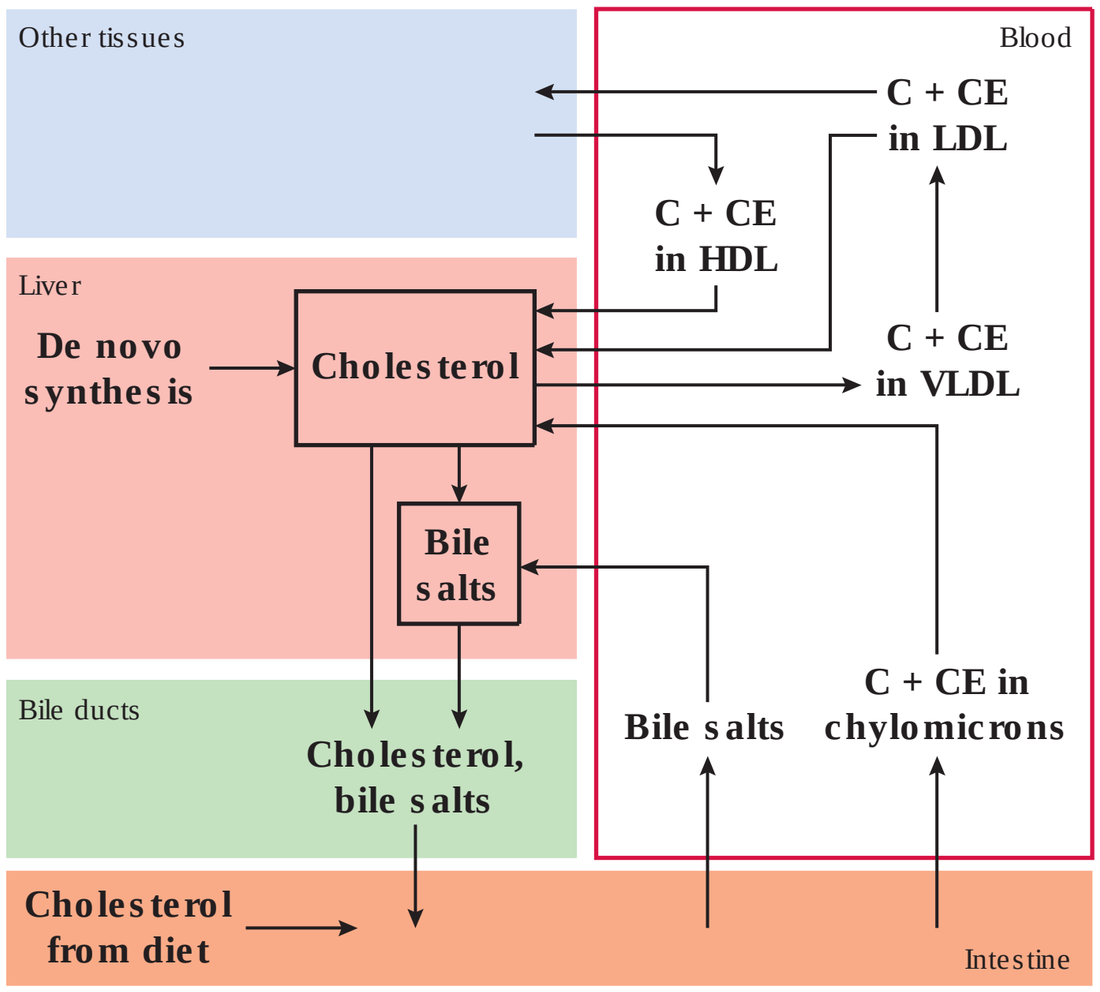


Fig. 29.3 **Central role of the liver in cholesterol metabolism.** C, cholesterol; CE, cholesteryl esters.

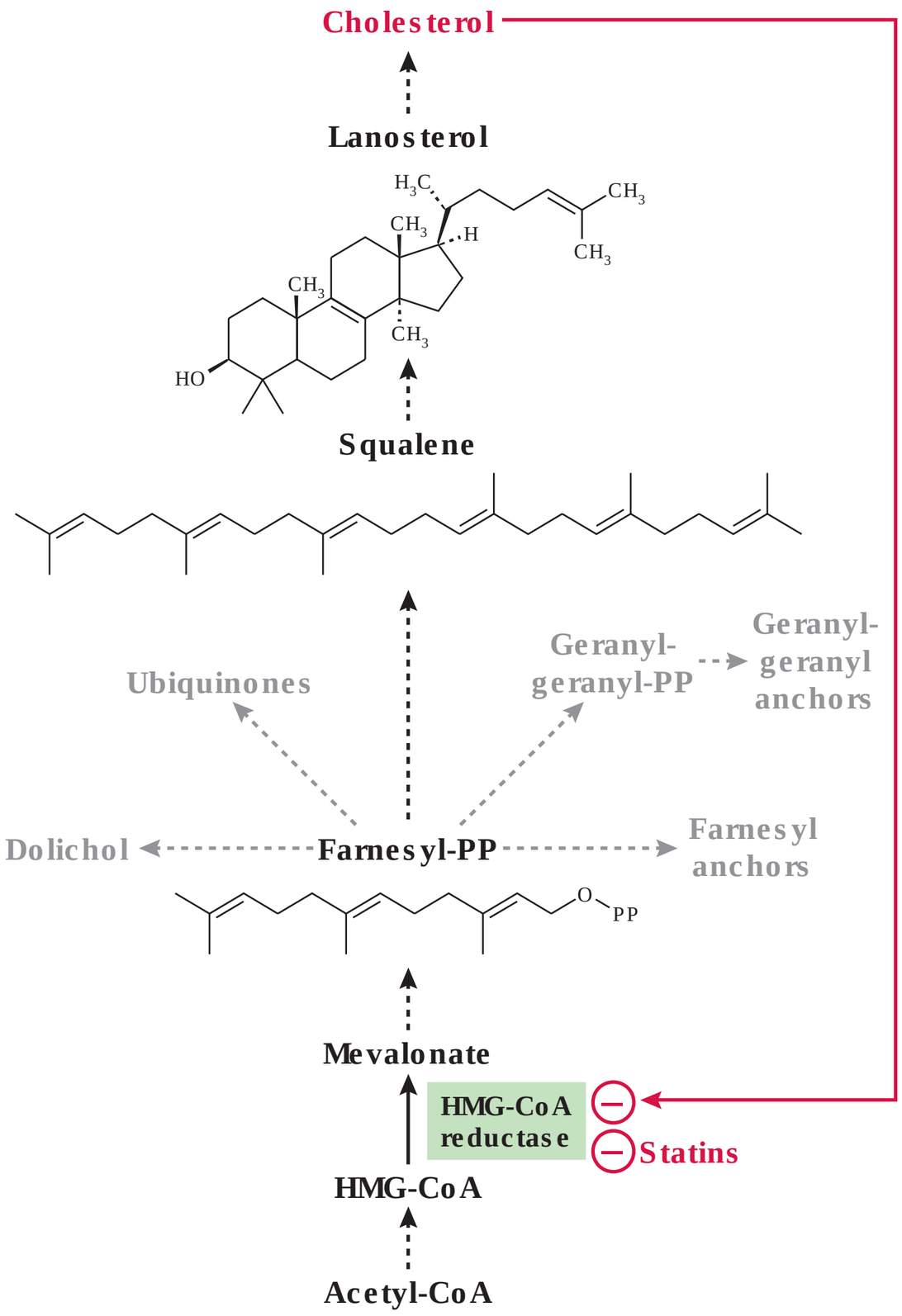


Fig. 29.4 **Synthesis of isoprenes and cholesterol from acetyl-coenzyme A (acyl-CoA).**

protein 1 (**LRP1**) binds to **apolipoprotein E** on chylomicron remnants and then initiates endocytosis of the receptor-chylomicron remnant complex. Subsequently, lysosomes degrade chylomicron remnants.

The liver plays a central role in cholesterol metabolism in that it receives dietary cholesterol, synthesizes additional cholesterol for itself and other tissues as needed, exports cholesterol and cholesteryl esters in VLDL, and secretes both cholesterol and cholesterol-derived bile salts into the bile (Fig. 29.3). A person consuming a typical Western diet takes up about one-third of the daily needed cholesterol and de novo synthesizes the remaining two-thirds.

The autosomal recessively inherited disorder **abetalipoproteinemia** is characterized by an absence of lipoprotein particles that carry apoprotein B-48 or B-100 (i.e., chylomicrons, VLDL, IDL, and LDL). The disease is due to a deficiency of MTP (see Fig. 29.1). Since essentially only HDL are present, these patients have a very low plasma total cholesterol. The disease is described in greater detail in Chapter 28.

Familial hypobetalipoproteinemia is caused by heterozygosity of truncated apolipoprotein B. Patients develop a fatty liver due to reduced export of triglycerides.

2. DE NOVO SYNTHESIS OF CHOLESTEROL

Cholesterol is synthesized in the cytosol *from* HMG-CoA that in turn is derived *from* acetyl-CoA. HMG-CoA reductase catalyzes the rate-limiting step *of* the pathway and is the primary point *of* regulation. The pathway is regulated such that cholesterol synthesis occurs in response to a lower-than-normal concentration *of* cholesterol in the endoplasmic reticulum (ER). An intermediate *of* the pathway, *farnesyl* pyrophosphate, gives rise to several important compounds that are required *for* protein glycosylation, adenosine triphosphate (A*T*P) production, and anchoring *of* proteins in membranes.

2.1. Pathway for the Biosynthesis of Cholesterol

Although every cell that contains mitochondria can make cholesterol, physiologically, cholesterol is predominantly synthesized in **steroid-producing cells** and the **liver** for the benefit of other cells. Every day, we synthesize 1.0 to 1.5 g of cholesterol, adjusting to dietary intake. Humans do not need cholesterol in the **diet**.

The de novo synthesis of cholesterol starts with acetyl-CoA, occurs in the cytosol, and is controlled by the activity of **HMG-CoA reductase** (Fig. 29.4), which yields **mevalonic acid**. The acetyl-CoA for cholesterol synthesis is derived from citrate exported from the mitochondria, as explained in Chapter 27 (the liver also uses this acetyl-CoA for de novo fatty acid synthesis). Chemically, the synthesis of HMG-CoA in the cytosol is similar to the synthesis of HMG-CoA in

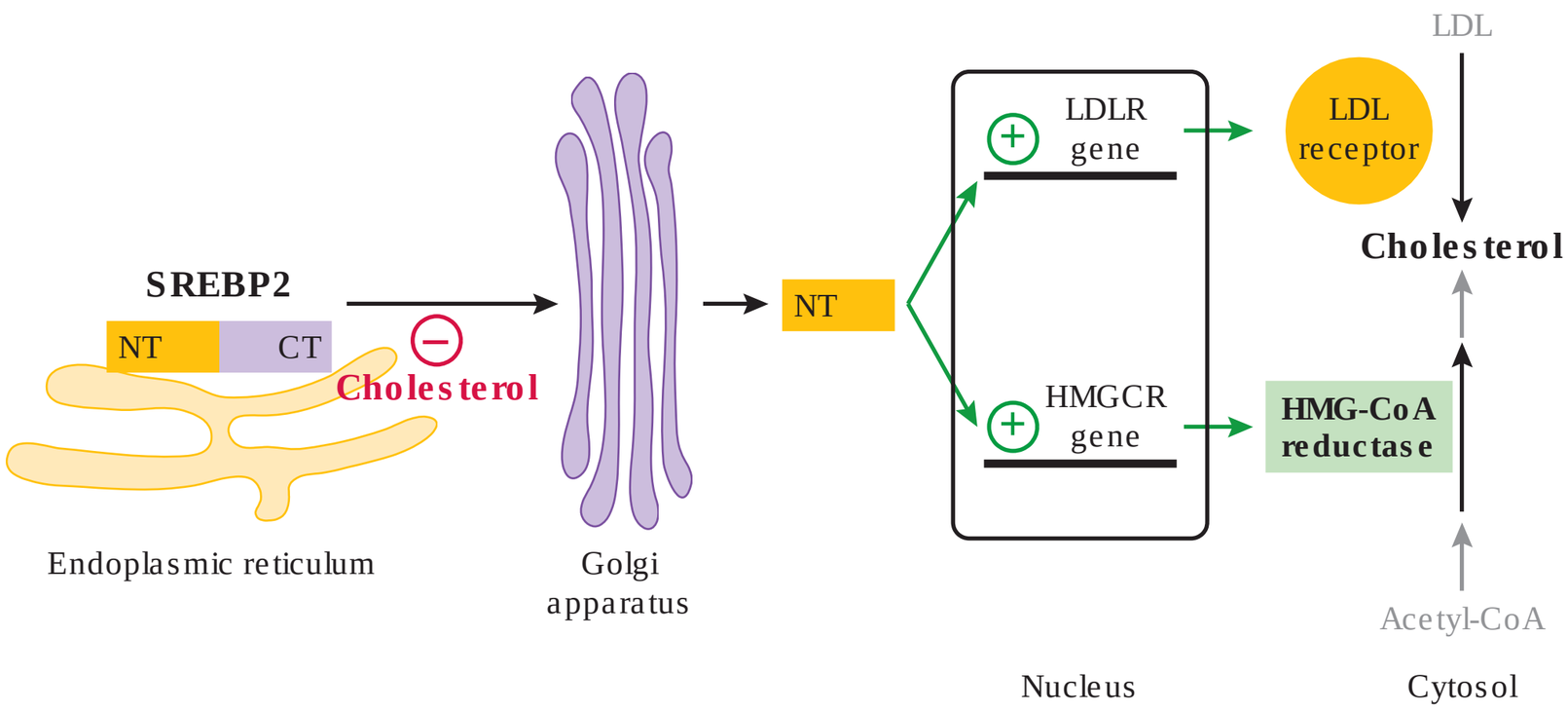


Fig. 29.5 Regulation of cholesterol synthesis by the cholesterol concentration of the endoplasmic reticulum membrane. CT, C-terminal segment; NT, N-terminal segment.

mitochondria as part of the synthesis of ketone bodies, but these processes are catalyzed by isoenzymes.

Mevalonic acid gives rise to the isoprene **squalene**, which "folds up" and through a series of intramolecular reactions gives rise to **lanosterol**; lanosterol, in turn, is converted to cholesterol in many reactions (see Fig. 29.4).

Mevalonic acid gives rise not only to cholesterol, but also to **terpenes** (**isoprenoids**, sometimes also called **isoprenes**) such as farnesyl pyrophosphate, dolichol, ubiquinones, and geranylgeranyl pyrophosphate (see Fig. 29.4). **Farnesyl pyrophosphate** and **geranylgeranyl pyrophosphate** can be conjugated with proteins and then serve as lipid anchors (see Chapter 11). **Dolichyl pyrophosphate** is required for the dolichol pathway of N-linked posttranslational protein glycosylation (see Chapter 7). **Ubiquinones** can be reduced to ubiquinols, which can donate their electrons to the electron transport chain as part of oxidative phosphorylation (see Chapter 23).

Most of the cholesterol in a typical cell is in the plasma membrane; this cholesterol is in equilibrium with the cholesterol in the ER and the Golgi apparatus. Intracellular transport is facilitated by the ATP-driven **ATP-binding cassette transporter A1** (**ABCA1**), which is a floppase (see Chapter 11).

The **statin** drugs inhibit HMG-CoA reductase and thus inhibit the synthesis of terpenes and cholesterol (see Fig. 29.4). Statins are used to lower LDL cholesterol (see Section 5.4). Some of the beneficial effects as well as side effects of statins may be related to altered terpene metabolism.

Among antifungal agents, **fluconazole** is used in the treatment of **candidiasis** and inhibits the conversion of lanosterol to ergosterol in Candida without significantly affecting human cholesterol synthesis. In cell membranes of fungi and protozoa, ergosterol has similar essential functions as cholesterol does in mammals.

2.2. Regulation of Cholesterol Synthesis

Cholesterol synthesis is mainly controlled by the activity of **HMG-CoA reductase**, and **sterol regulatory element–binding protein 2 (SREBP2)**. SREBP2 is a cholesterol sensor that is the main regulator of HMG-CoA reductase activity. There are three different SREBPs. While SREBP2 regulates cholesterol synthesis, SREBP1c regulates fatty acid synthesis, and SREBP1a regulates both cholesterol and fatty acid synthesis.

SREBP2 is an integral membrane protein of the ER that senses the concentration of cholesterol in the ER membrane (Fig. 29.5). The concentration of cholesterol in the ER membrane is rather low, but it is nonetheless reflective of the concentration of cholesterol in the plasma membrane. When the concentration of cholesterol in the ER is abnormally low, SREBP2-containing vesicles form and move to the Golgi apparatus. There, proteases cleave SREBP2. The N-terminal segment of SREBP2 then moves into the nucleus and enhances the transcription of several genes, including the genes for HMG-CoA reductase and LDL receptors. When the concentration of cholesterol is high, there is no such stimulation of transcription by an SREBP2 fragment.

SREBP1c is also in the ER, but it is set loose when the concentration of **insulin** is elevated. A fragment of SREBP1c activates transcription of genes that give rise to proteins that favor **fatty acid synthesis**.

SREBP1a is expressed in growing normal and tumor cells, and it can activate both cholesterol and fatty acid de novo synthesis.

3. TRANSPORT OF CHOLESTEROL VIA THE BLOOD

The liver exports cholesterol and cholesteryl esters in VLDL. Lipases remove triglycerides, thereby converting VLDL to intermediate-density lipoprotein (IDL) and LDL. LDL contain much more cholesterol than triglycerides. When the liver and peripheral cells need more cholesterol, they endocytose the cholesterol-rich LDL. Peripheral cells export cholesterol by adding it to circulating HDL. This cholesterol is delivered mainly to the liver. As part of a "lipid panel," the concentration of total cholesterol and HDL cholesterol is often determined in plasma from fasting patients.

3.1. Transport of Cholesterol From the Liver to Peripheral Cells

The chylomicron-based transport of cholesterol from the intestine to the liver is discussed in Section 1.

The liver exports cholesterol and cholesteryl esters as part of **VLDL**, which contains **apolipoprotein B-100**. VLDL also exports triglycerides from the liver (see Chapter 28). Cholesterol is mainly in the monolayer membrane that delimits VLDL, and cholesteryl esters are in the liquid core of the lipoprotein particle. On a molar basis, a VLDL particle contains about three times more cholesteryl esters than cholesterol.

Lipoprotein lipase in the lumen of the capillaries of the adipose tissue and muscle depletes VLDL of about 80% of its triglycerides, leaving behind all the cholesterol and cholesteryl esters and thus giving rise to **IDL**. The liver expresses **hepatic lipase**, which on one hand depletes IDL of triglycerides and on the other facilitates the receptor-mediated uptake of IDL and LDL into the liver. By weight, VLDL have about a 5:1 ratio of triglyceride/cholesterol, whereas IDL have a ratio of about 1:1 and LDL about 1:10.

Depending on their need for cholesterol, hepatocytes and peripheral cells display **LDL receptors** on their surface, which they use to bind and endocytose LDL via **apolipoprotein B-100**. Cells express LDL receptors based on the concentration of cholesterol that the **SREBP2** senses in the ER membrane. After endocytosis, lysosomal acid lipase hydrolyzes triglycerides and cholesteryl esters in lysosomes. LDL receptors can be recycled to the plasma membrane surface. The liver takes up approximately 70% of all LDL.

The liver secretes the enzyme **PCSK9** into the blood, from where the enzyme reaches LDL receptors, prevents their recycling to the plasma membrane surface, and favors their degradation in lysosomes. This pathway is an integral part of the cholesterol-dependent regulation of the number of LDL receptors.

About 3% of Caucasians are heterozygous for a **loss-of-function mutation** in the PCSK9 gene and therefore have better survival of LDL receptors and about a 15% reduction in LDL cholesterol. About 2% of African-Americans are heterozygous for two other such mutations that result in an ~30% reduction in LDL cholesterol.

Gain-of-function mutations in PCSK9 are uncommon and lead to hypercholesterolemia (see Section 5.2).

3.2. Export of Cholesterol From Peripheral Cells (Reverse Cholesterol Transport)

Transport of cholesterol from the liver to peripheral tissues is often called cholesterol transport; the transport of cholesterol from the peripheral tissues to the liver and steroid-producing tissues is called **reverse cholesterol transport**. Reverse transport is performed by **HDL**.

HDL form chiefly in the liver and intestine by loading phosphatidylcholine, cholesterol, and cholesteryl esters onto **apolipoprotein A-I** (Fig. 29.6). The liver and intestine synthesize apolipoprotein A-I, the chief protein of HDL. These organs use the ABCA1 transporter to enrich discoidal HDL with phosphatidylcholine and cholesterol out of the plasma membrane, thereby generating **preβ-HDL**.

In the blood, **lecithin-cholesterol acyltransferase** (**LCAT**) binds to preβ-HDL and then uses a fatty acid from the phospholipid lecithin (i.e., phosphatidylcholine; made available once again by an ABCA1 transporter) of a peripheral cell to esterify cholesterol to cholesteryl esters, which are stored inside HDL. This lipidation gives rise to mature HDL particles,

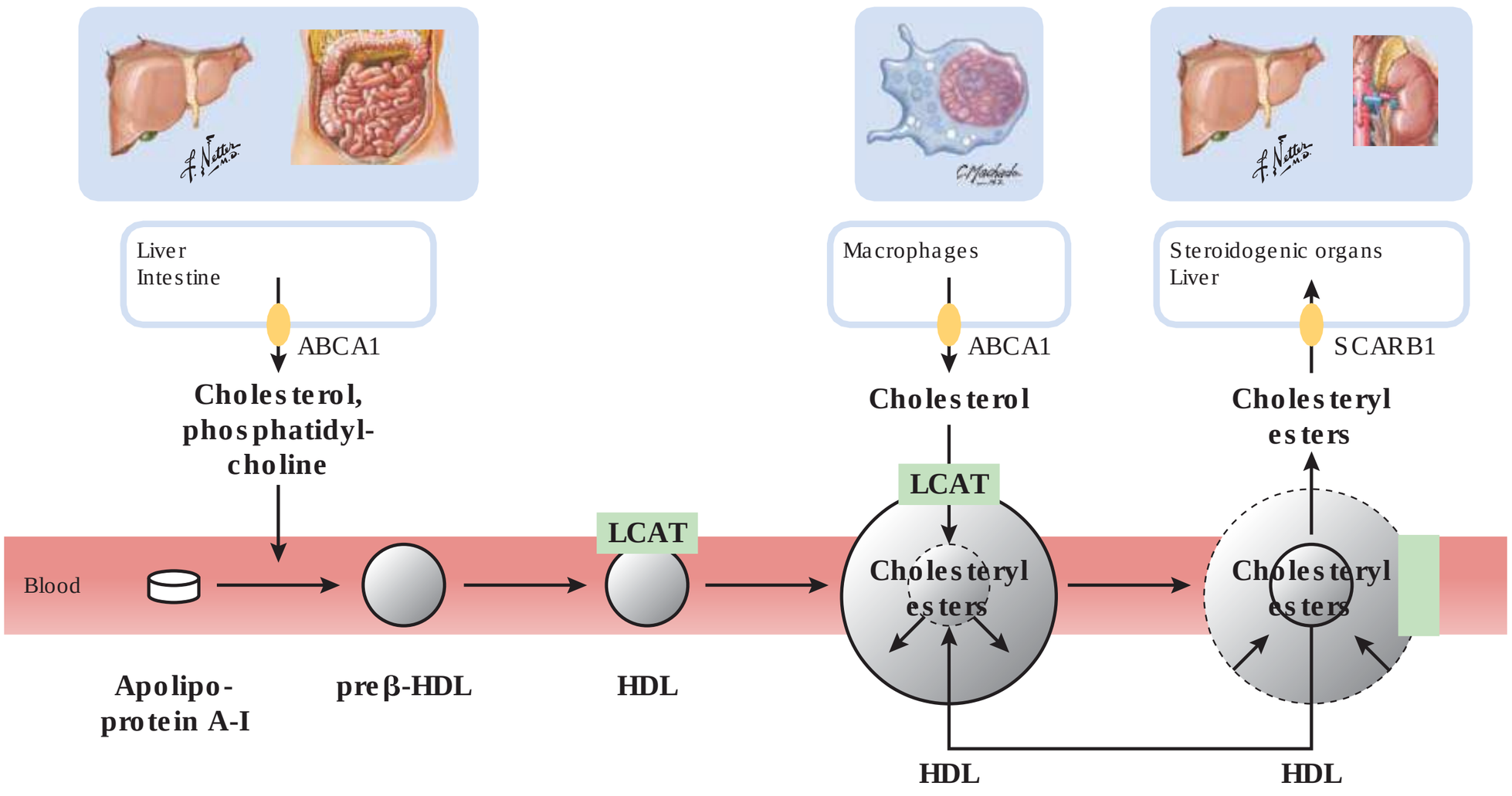


Fig. 29.6 Biology of high-density lipoprotein (HDL). ABCA1, ATP-binding cassette transporter A1; LCAT, lecithin-cholesterol acyltransferase; SCARB1, scavenger receptor class B member type 1.

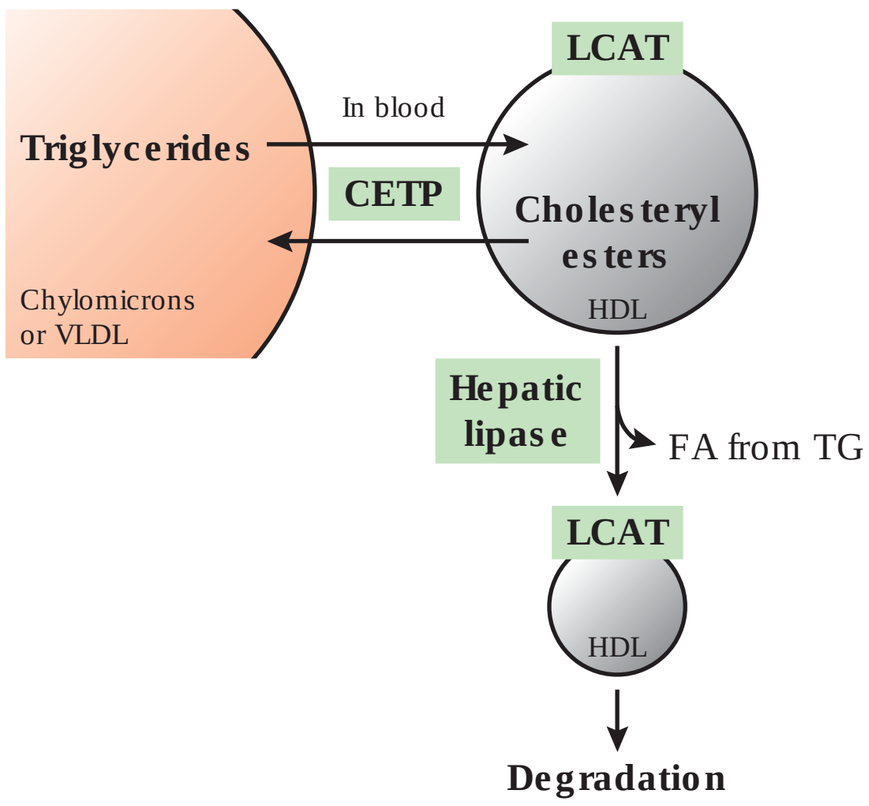


Fig. 29.7 Cholesteryl ester transfer protein (CETP)-mediated exchange of lipids between high-density lipoproteins (HDL) and chylomicrons or very-low-density lipoproteins (VLDL) leads to low HDL cholesterol. FA, fatty acids; LCAT, lecithin-cholesterol acyltransferase; TG, triglycerides.

known as **α-HDL**, which have a much longer half-life than the smaller preβ-HDL. By weight, circulating HDL particles contain about twice as much protein as cholesterol.

HDL offload some of their cholesteryl esters in the liver and in steroidogenic organs, where **scavenger receptor class B member type 1** (**SCARB1, SRB1, SRBI**) equilibrate cholesteryl esters in HDL with cellular cholesteryl esters (see Fig. 29.6). The resulting HDL recirculate and can transport more cholesteryl esters. However, the smaller the HDL, the shorter their lifetime in the circulation.

Catalyzed by the **cholesteryl ester transfer protein** (**CETP**), VLDL exchange lipids with HDL (Fig. 29.7). HDL thus gain triglycerides and lose cholesteryl esters. Subsequently, hepatic lipase removes triglycerides from the HDL. This leaves poorly lipidated HDL, which are then degraded. This series of reactions is clinically important and accounts for the observed low value for HDL cholesterol in patients who have **hypertriglyceridemia**.

3.3. Treatment of a Low Concentration of HDL Cholesterol

Most patients who have HDL cholesterol levels below 20 mg/dL (0.5 mM) have severe **hypertriglyceridemia**. The low HDL is a consequence of **CETP**-catalyzed triglyceride versus cholesteryl ester exchange between VLDL and HDL (see Fig. 29.7). When the triglyceride-enriched, cholesteryl ester–depleted HDL lose their triglyceride, they are poorly lipidated and therefore degraded prematurely. The treatment of hypertriglyceridemia often involves a **statin**, a **fibric acid** drug, and supplementary **ω-3 fatty acids** (see Chapter 32).

Nicotinic acid (**niacin**, **vitamin B_3**) reduces VLDL production and increases HDL cholesterol in part through reduced CETP-mediated lipid exchange between HDL and VLDL, which allows HDL to remain in the circulation longer. Nicotinic acid often has side effects, such as flushing, that prevent patients from taking this drug. Nonsteroidal antiinflammatory drugs (NSAIDs) may reduce the flushing.

In previously sedentary adults, the addition of regular **exercise** increases the plasma HDL cholesterol by a modest ~2 mg/dL (~0.05 mM).

Patients who have HDL cholesterol levels lower than 20 mg/dL (0.5 mM) without marked hypertriglyceridemia may have a **deficiency** of functional **apolipoprotein A-I**, **ABCA1**, or **LCAT**.

About 1 in 400 people are **heterozygous** for an **ABCA1 deficiency**. These individuals have only about two-thirds to one-half of the normal HDL cholesterol, yet they do not have a markedly increased risk of ischemic heart disease.

3.4. Laboratory Measurements of Cholesterol-Containing Lipoproteins

Clinical laboratories routinely measure **total cholesterol, HDL cholesterol,** and **triglycerides** in plasma samples.

LDL cholesterol is usually calculated from measurements of total cholesterol, HDL cholesterol, and total triglycerides, using the plasma from **fasting** patients. The calculation is based on the **Friedewald equation** (all concentrations in **mg/dL**):

$$\text{LDL cholesterol} = \text{total cholesterol} - \text{HDL cholesterol} - (\text{total triglycerides}/5)$$

The equation thus uses an approximation of the triglyceride versus cholesterol content of VLDL, IDL, and LDL. If all concentrations are calculated in units of **mM**, the triglyceride-based correction uses a factor of 0.45 (instead of 0.2 for mg/dL). The Friedewald equation is inaccurate when the concentration of triglycerides is high (>~400 mg/dL) or when there is an appreciable number of chylomicrons. In a fasting plasma sample, LDL cholesterol makes up most of the total cholesterol.

The **non-HDL cholesterol** is calculated as the total cholesterol – HDL cholesterol. Non-HDL cholesterol represents cholesterol in the lipoproteins that contain beta-apolipoproteins (i.e., B-48 or B-100).

Occasionally, the **Castelli risk indices** are used for predicting the risk of vascular disease:

$$\text{Total cholesterol/HDL cholesterol} = \text{Castelli risk index 1}$$

$$\text{LDL cholesterol/HDL cholesterol} = \text{Castelli risk index 2}$$

The **lipid panel** is a group of tests that are often ordered to help assess a patient's risk of heart disease or stroke. The panel includes measurements of **total cholesterol, HDL cholesterol,** and **triglycerides** in a plasma sample. The patient's blood sample is drawn after an overnight fast when chylomicrons have nearly all been cleared.

The U.S. Preventive Services Taskforce recommends that the following persons be screened for lipid disorders: (1) men aged 35 years and older and (2) men aged 20 to 35 years and

women aged 20 years or older who are at an increased risk for coronary heart disease.

The National Cholesterol Education Program's Adult Treatment Panel III (ATP III) recommends that all persons aged 20 years and older have a fasting lipid panel performed every 5 years. More frequent screenings are recommended if the total cholesterol is 200 mg/dL or higher, if the HDL is less than 40 mg/dL, or if the person has risk factors such as cigarette smoking, age older than 45 years for men, age older than 55 years for women, blood pressure higher than 140/90 mm Hg, blood pressure–lowering medication use, or a family history of premature heart disease.

4. BILE METABOLISM

Bile salts are synthesized by the liver and secreted, together with cholesterol and phospholipids, into the bile ducts to form bile. Bile is stored in the gallbladder and then secreted into the duodenum in response to dietary protein and fat. Bile salts solubilize lipids in the intestinal lumen and thus enhance their digestion. Bile salts also form mixed micelles with lipids and thus facilitate their absorption. Insufficient production of bile acids causes lipid malabsorption. Most of the secreted bile salts are reabsorbed by the ileum and returned to the liver. If the liver produces an insufficient amount of bile salts or secretes too much cholesterol, cholesterol gallstones may form in the gallbladder or bile ducts.

4.1. Production of Bile and Recirculation of Bile Salts and Cholesterol

Bile is made in the liver, secreted into bile canaliculi, stored in the gallbladder, and dispensed into the duodenum via the common bile duct (Fig. 29.8). Bile contains micelles of **bile salts**, **cholesterol**, and the phospholipid **phosphatidylcholine** (also called lecithin). Bile salts are detergents that emulsify triglycerides and cholesteryl esters. Bile salts also form mixed micelles with cholesterol, fatty acids, and monoglycerides (see Chapter 28).

The only means of removing cholesterol from the body is via the secretion of cholesterol into bile and conversion of cholesterol into bile acids, followed by the secretion into bile. There is no pathway for the degradation of cholesterol into small molecules.

The liver hydroxylates cholesterol to produce the **bile acids chenodeoxycholic acid** and **cholic acid** (Fig. 29.9); these bile acids are then conjugated with the amino acids **glycine** or **taurine** to yield **primary bile salts**. The rate-limiting enzyme for bile acid production is cholesterol 7α-hydroxylase (CYP7A1, a cytochrome P450 enzyme). Cholic acid has one more hydroxyl group than chenodeoxycholic acid and is therefore more soluble in water. Conjugation of bile acids with glycine or taurine further increases the solubility in water. Bile salts are amphipathic; they have both a hydrophobic and a hydrophilic surface. The amount of bile salts in the bile is an important determinant of the amount of water in bile and thus

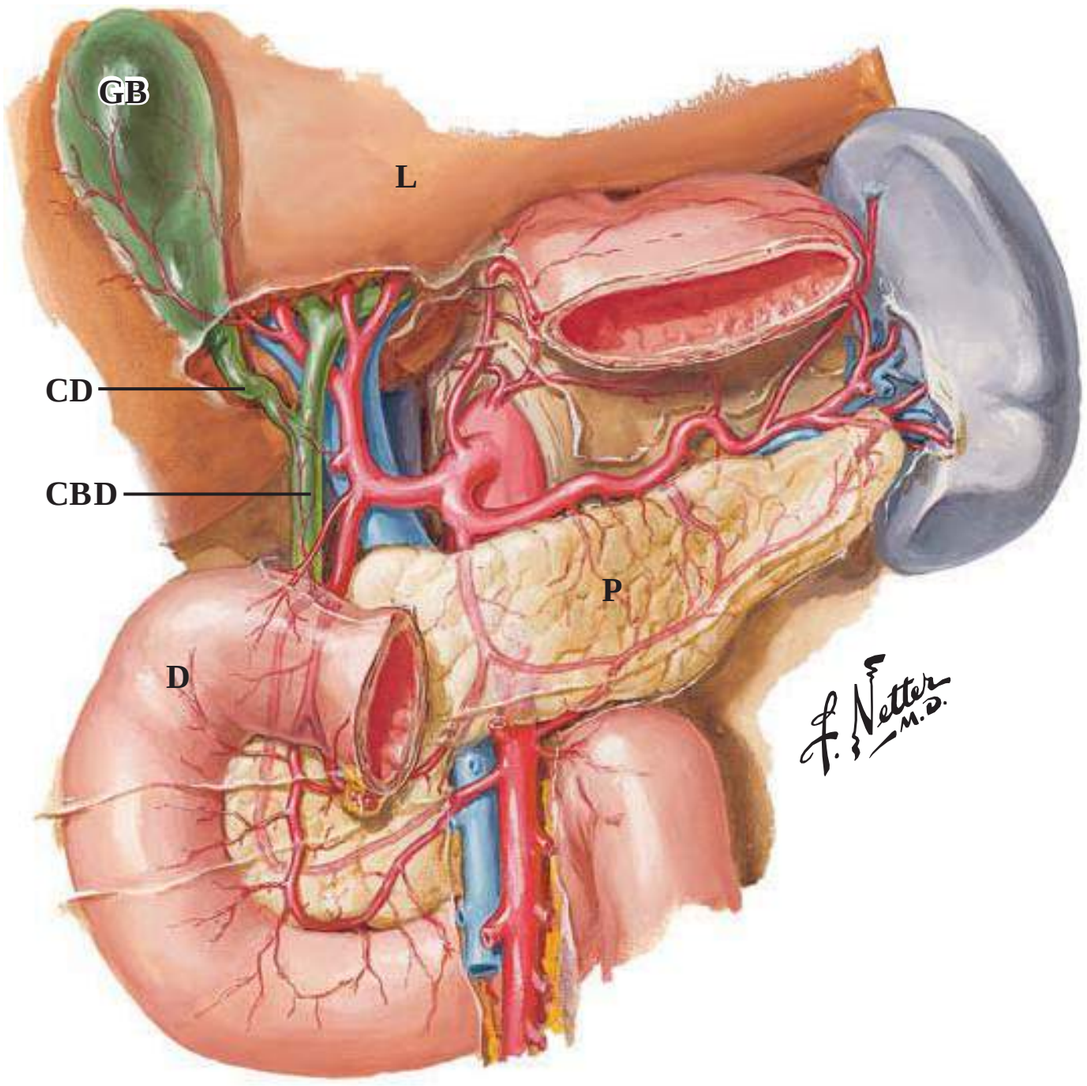


Fig. 29.8 **Biliary system.** Bile is formed in the liver (L), stored in the gallbladder (GB), and secreted into the duodenum (D). CD, cystic duct; CBD, common bile duct; P, pancreas.

the flow of bile out of the liver. The gallbladder then concentrates bile by removing water from it. In the fasting state, most bile salts are stored in the **gallbladder**.

The composition of the micelles in bile is determined by the relative rates of export of bile salts, cholesterol, and phospholipids, each of which has its own export system from the liver (Fig. 29.10). The rate of bile salt export is determined by the activity of the bile salt export pump (**BSEP**, **ABCB11**), which increases in response to elevated concentrations of bile salts inside the cell. Cholesterol is exported from the liver via an ATP-driven transporter (a heterodimer of the ATP binding cassette proteins G5 and G8; **ABCG5/G8**). The phospholipid **phosphatidylcholine** in bile is derived from the cell membrane. The floppase **MDR3** (also called **ABCB4**) moves phosphatidylcholine from the inside of the membrane to the outside.

In response to the influx of chyme that contains protein and fat, I-cells in the duodenum secrete the peptide hormone **cholecystokinin** (**CCK**), which causes the gallbladder to contract and dispense bile into the common bile duct from where it reaches the intestine. CCK also stimulates the secretion of enzymes from the pancreas.

In the lumen of the ileum and colon, **bacteria** can remove the 7α-hydroxyl group from primary bile salts to yield **secondary bile salts** (see Fig. 29.9); bacteria can also deconjugate bile salts to yield bile acids. Both of these modifications decrease the solubility of bile acids and bile salts in the gut.

The distal ileum reabsorbs over 95% of primary and secondary bile salts via a Na^+-driven transporter (ASBT, SLC10A2) and releases the bile salts via the Ostα-Ostβ organic solute transporter into the blood so that they can move back to the

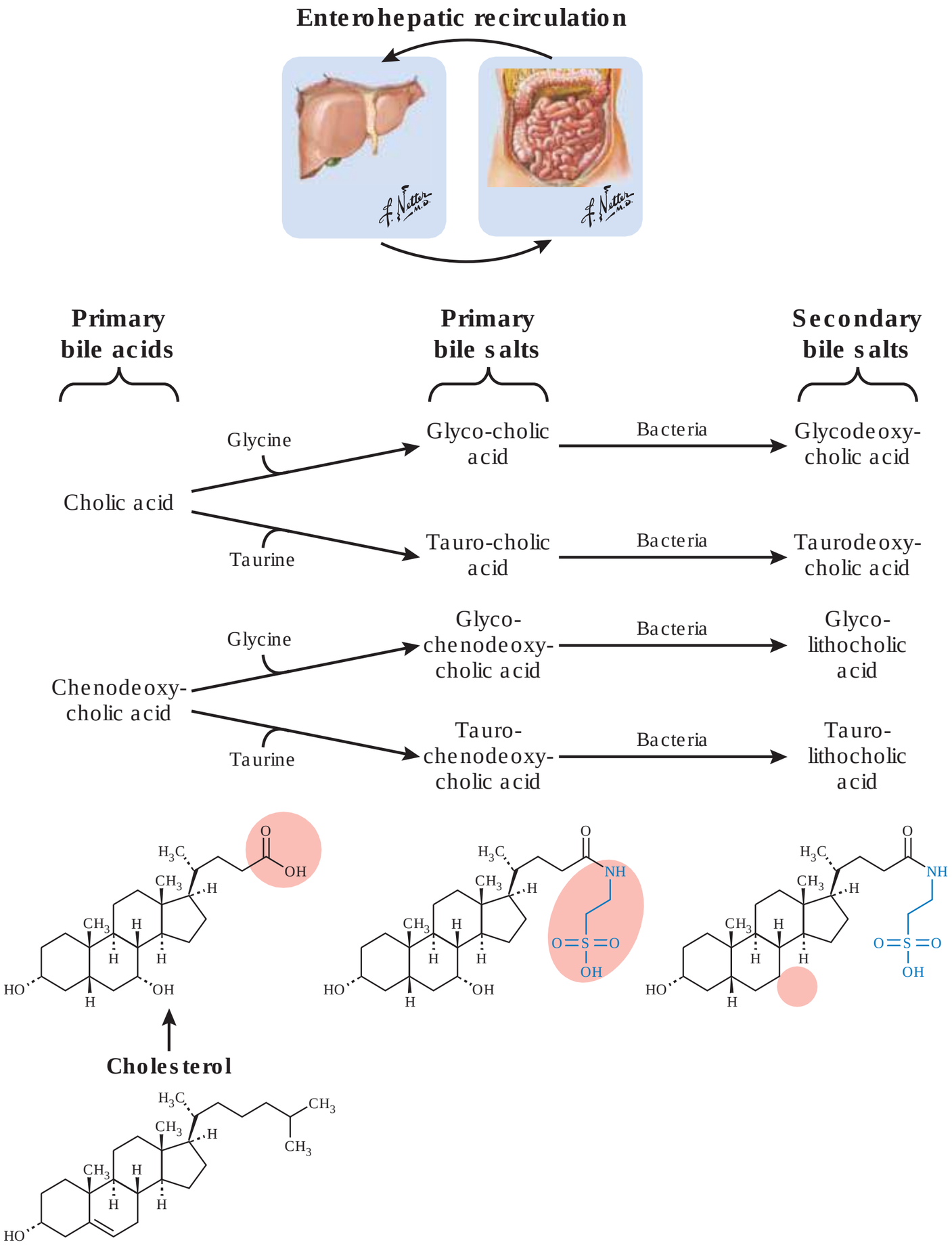


Fig. 29.9 **Production of bile acids and bile salts.**

liver and be resecreted into bile (see Fig. 29.10). In the blood, bile salts bind to albumin. On average, a bile salt makes approximately eight passes per day through this **enterohepatic circulation**. T e total bile salt pool is about 3 g, and the daily loss into the feces is about 0.5 g.

T e conversion of cholesterol to bile salts and the transport of bile salts are in part controlled at the level of transcription by liver X receptors and farnesoid X receptors, each of which forms heterodimers with retinoic acid receptors and function as follows.

Heterodimeric **liver X receptors/retinoic acid receptors** (**LXR/RXRs**) are activated by **oxysterols** (e.g., 27-hydroxycholesterol) and retinoic acid, which cause them to increase the transcription of certain genes so that more cholesterol is exported in the form of bile salts. Fig. 6.5 illustrates the binding of LXR/RXR to DNA. In the absence of an activator, LXR/RXR heterodimers bind to LXR-response elements on DNA and inhibit transcription. Oxysterols that activate LXR/RXRs are intermediates in the conversion of cholesterol to steroids or bile acids. Compared with binding of a single ligand, binding of both an oxysterol and retinoic acid markedly enhances LXR/RXR activity. Some of the genes that have LXR-response elements encode proteins that play a role in cholesterol metabolism, such as CE*T*P (exchanges cholesterol between VLDL and HDL; see Section 3.2), CYP7A1 (the rate-limiting enzyme in bile acid synthesis), ABCA1 (exports cholesterol to HDL; see Section 3.2), ABCG1 (exports cholesterol from macrophages and the liver to circulating lipoproteins), and ABCG8 (exports cholesterol from the liver into bile; see Fig. 29.10). Activated LXR/RXRs also af ect the transcription of some genes that are not associated with an LXR response element.

Heterodimeric **farnesoid X receptors/retinoic acid receptors** (**FXR/RXRs**) are activated by **bile acids** and then increase

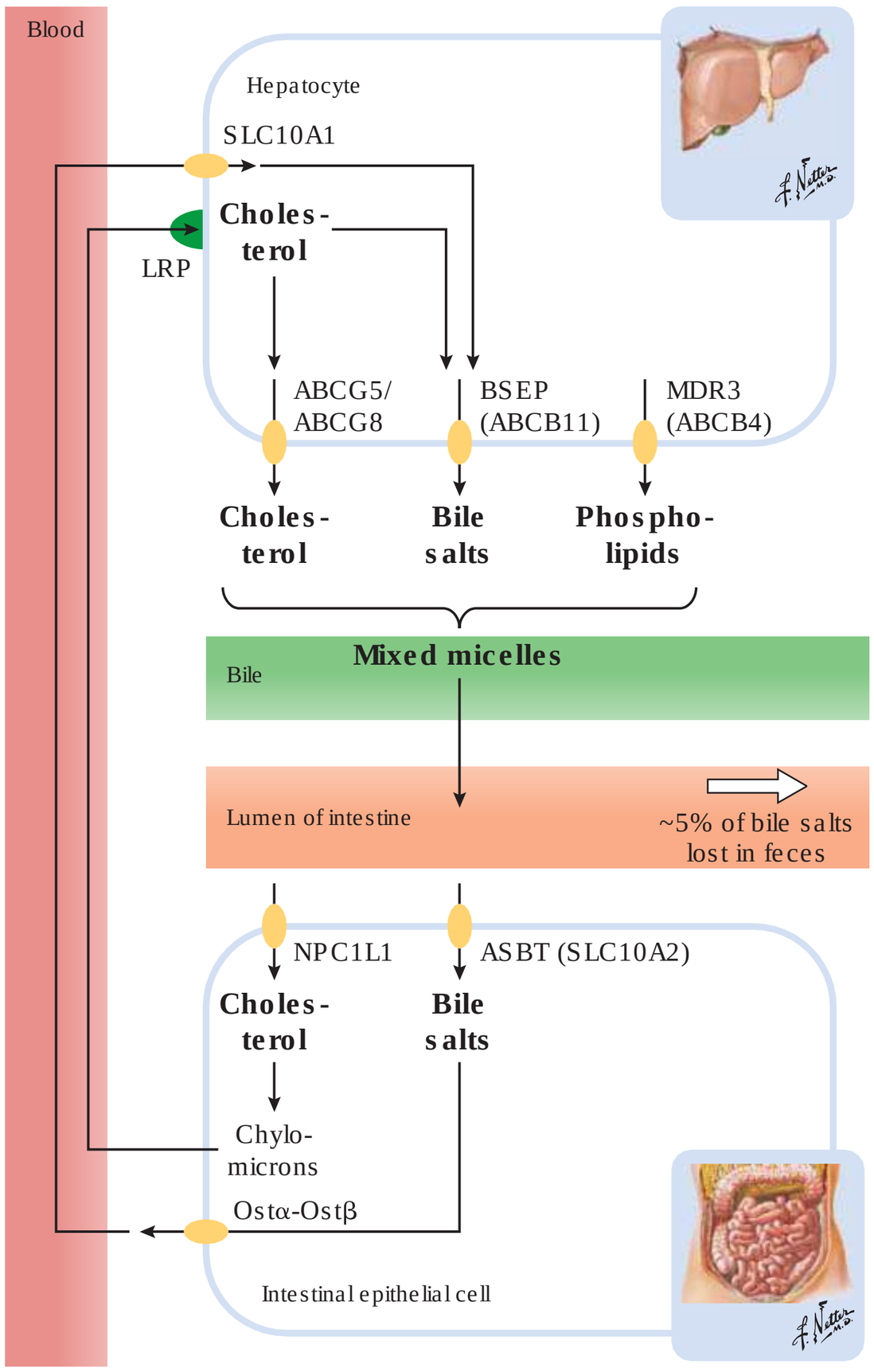


Fig. 29.10 Enterohepatic circulation of bile salts and cholesterol.

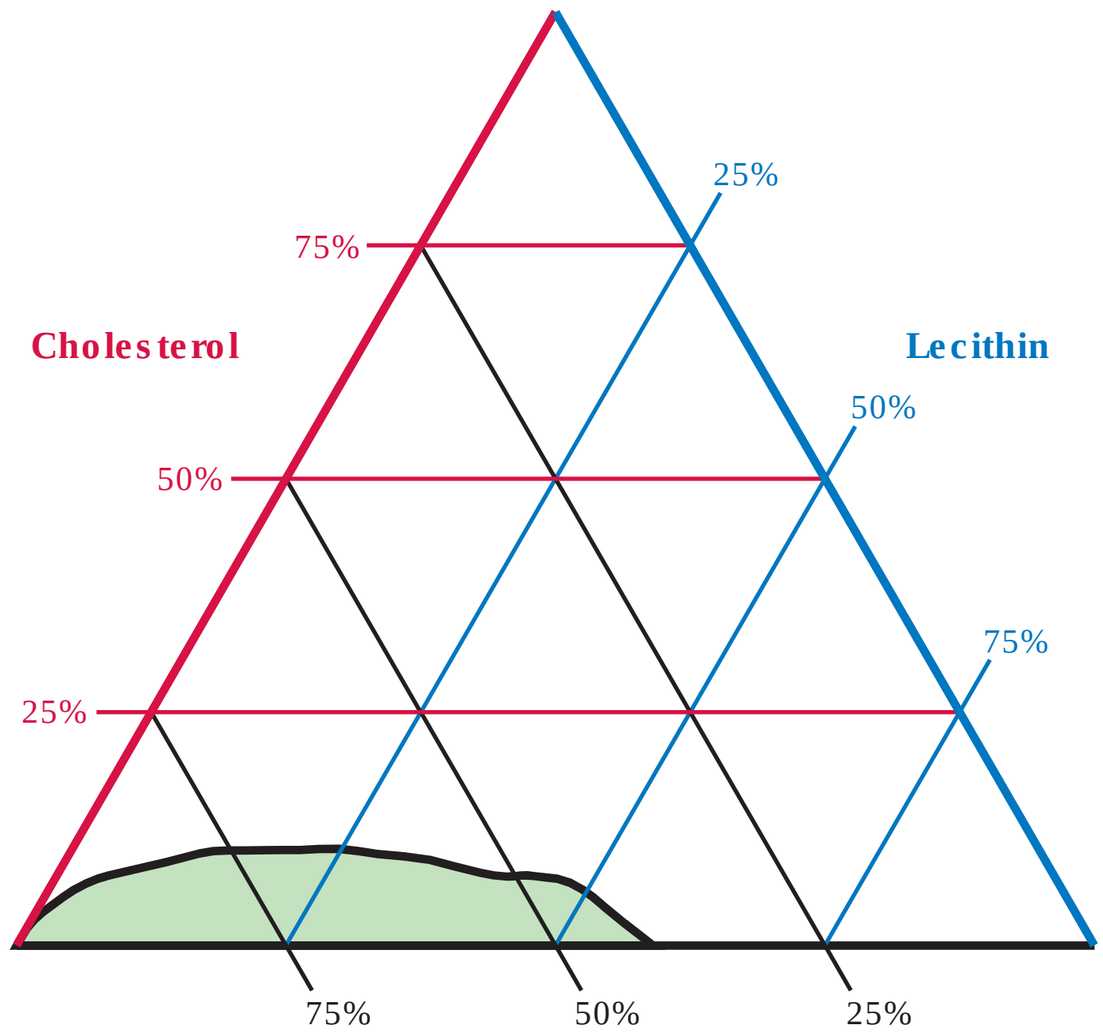


Fig. 29.11 Phase diagram for a model system of cholesterol in micelles in bile. The green area represents the combinations of solutes that give rise to a single phase of micelles. The composition of bile of healthy persons is typically well within the green area, while that of persons with cholesterol gallstones is close to the border of this area or clearly outside of it. The fractions refer to the total moles of solute in a solution that is 90% water and 10% solute (cholesterol + lecithin + bile salts). (Modified from Admirand WH, Small DM. The physicochemical basis of cholesterol gallstone formation in man. *J Clin Invest*. 1968;47:1043-1052.)

the transcription of genes that have a promoter with an FXR-response element. T e products of these genes protect hepatocytes and intestinal epithelial cells from the ef ects of a high concentration of bile acids. In the liver, bile acid–activated FXR/RXRs inhibit bile salt uptake and synthesis and favor bile salt secretion. In intestinal epithelial cells, bile acid–activated FXR/RXRs likewise inhibit bile salt uptake and stimulate bile salt efflux.

4.2. Diseases of Bile Metabolism

For bile to be free of crystals and stones, cholesterol must constitute less than 10% of the total moles of cholesterol, bile salts, and lecithin; furthermore, bile salts must be greater than 40% and lecithin less than 60% of this total (Fig. 29.11). On a molar basis, normal bile contains approximately 6% cholesterol, 74% bile salts, and 20% lecithin.

Gallstones can form when there is an excessive rate of cholesterol secretion or a decreased rate of bile salt secretion. Gallstones are more likely to form when bile gets highly concentrated in the gallbladder, when the gallbladder empties at a low rate, or when the bile system has low motility.

T e presence of gallstones (Fig. 29.12) in the gallbladder (**cholelithiasis**) or bile duct (**choledocholithiasis**) is quite common and usually does not cause symptoms. However, gallstones that block the cystic duct (which connects the gallbladder to the common bile duct) or the common bile duct (see Fig. 29.8) can cause pain. Pain is especially common after a fatty meal because the fat in the duodenum elicits the secretion of the hormone **CCK**, which stimulates the contraction of the gallbladder. Gallstones can lead to cholecystitis (inflammation of the gallbladder), cholangitis (inflammation of the bile duct), jaundice (due to impaired bilirubin excretion), and pancreatitis (due to blockage of the flow of enzymes from the pancreatic duct into the common bile duct).

About 80% of gallstones are due to the precipitation of cholesterol crystals, which appear **yellow**. Precipitates of calcium **bilirubinate** (see Chapter 14), which form **black** stones and are common in persons with a hemolytic disorder, account for the other 20%.

A decrease in bile salt secretion may occur if there is decreased reabsorption of bile salts from the intestine, as is observed in patients with diseases that af ect the ileum, or

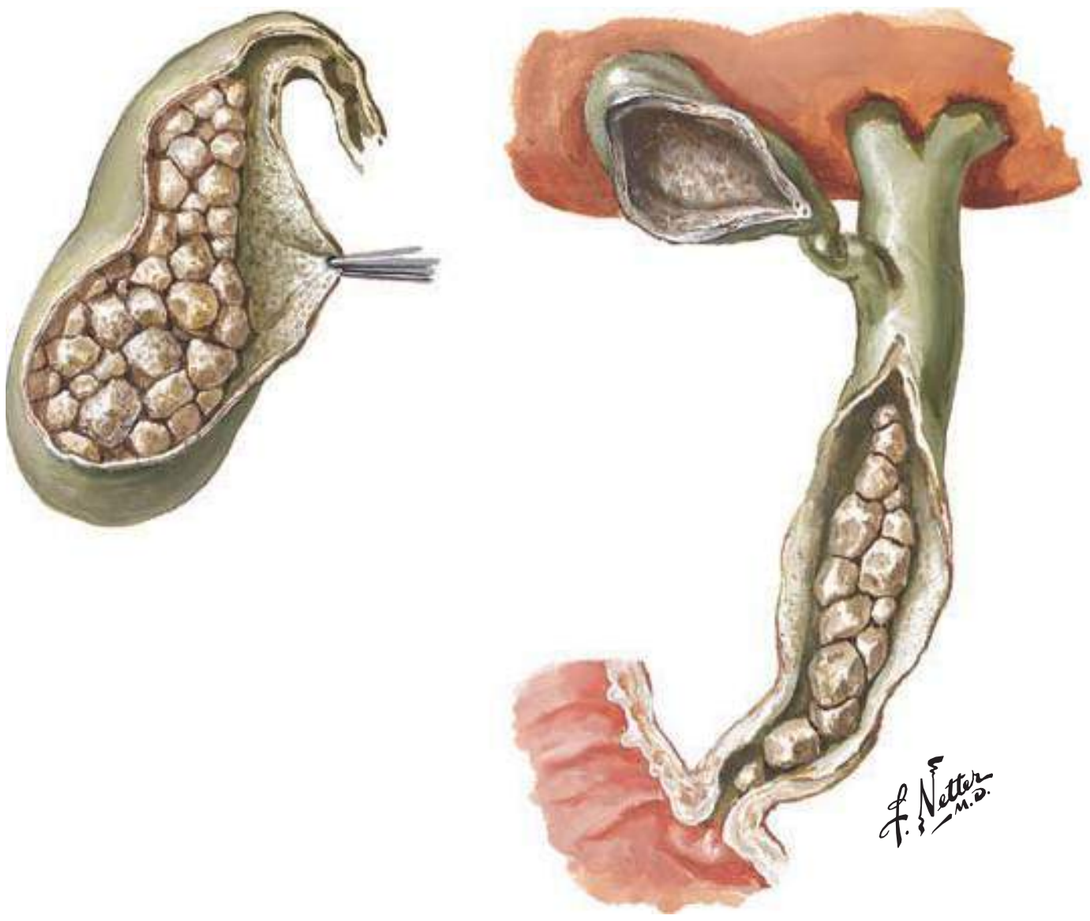


Fig. 29.12 **Multiple cholesterol gallstones in the gallbladder and the common bile duct.**

if there is decreased synthesis of bile salts due to liver dysfunction.

An increase in cholesterol secretion may occur in patients who take one of the **fibrate** drugs (e.g., gemfibrozil or fenofibrate) to lower triglycerides in the blood. Fibrates stimulate the transcription factor PPARα (see Chapter 28), which increases the expression of the ABCG5/G8 proteins, which export cholesterol into the bile (see Fig. 29.10). This increases the risk of gallstone formation.

Noninvasive images of gallstones are usually obtained with **ultrasound**. In plain radiographs, cholesterol stones do not show sufficient contrast because they do not contain heavy elements.

Surgery is currently the preferred treatment of gallstones because drug treatment is mostly unsatisfactory. Over time, the drugs ursodeoxycholic acid and ezetimibe have favorable effects in some patients. **Ursodeoxycholic acid** (**ursodiol**) differs from chenodeoxycholic acid in the orientation of the –OH group at C7. The effect of ursodeoxycholic acid is difficult to understand because it has many diverse effects on cholesterol and bile salt metabolism. **Ezetimibe** inhibits the uptake of cholesterol in the intestine and, as a result, tends to reduce cholesterol secretion from the liver into bile.

Cholestasis (an abnormally low flow of bile from the gallbladder to the duodenum) is often accompanied by **pruritus** (itching), although the pathogenesis of this is not clear. **Bile acid sequestrants** such as cholestyramine and colestipol, which in part reduce the enterohepatic recirculation of bile acids, are a standard treatment for pruritus.

Cholestasis can be induced by an **intrahepatic** problem with bile formation or by **extrahepatic** obstruction. An intrahepatic problem may be due to autoimmune disease, induced by drugs (sometimes dependent on a patient's genetic makeup), or caused by a congenital deficiency in bile formation. An extrahepatic obstruction may be due to gallstones or a tumor.

Progressive familial intrahepatic cholestasis (**PFIC2**) is rare and caused by deficient bile salt export. PFIC is typically inherited in autosomal recessive fashion. The most severe forms show onset in the first year of life, and they lead to malabsorption of fat and fat-soluble vitamins, followed by growth restriction and severe liver disease. Affected patients have defects in the **bile salt export pump** (**BSEP**) **ABCB11**, in the flippase **ATP8B1,** which seems to impair ABCB11 activity, or in the floppase **ABCB4** that exports phosphatidylcholine.

Some women who have **intrahepatic cholestasis of pregnancy** are heterozygotes for a mutation in the ABCB4 gene.

Treatment of cholestasis may include oral ursodeoxycholic acid, diversion of bile, and liver transplantation.

Cerebrotendinous xanthomatosis is caused by deficient activity in cholestanetriol 26-monooxygenase (also called sterol 27-hydroxylase), which catalyzes a step in bile acid synthesis and is encoded by the CYP27A1 gene. About 1 in 25,000 persons has the disorder, which leads to the accumulation of cholesterol and its derivatives in many tissues, often forming xanthomas (see Fig. 29.14). The disorder is particularly devastating to the central nervous system. Ataxia and intellectual decline typically become apparent in adolescence or early adulthood.

5. HYPERCHOLESTEROLEMIA

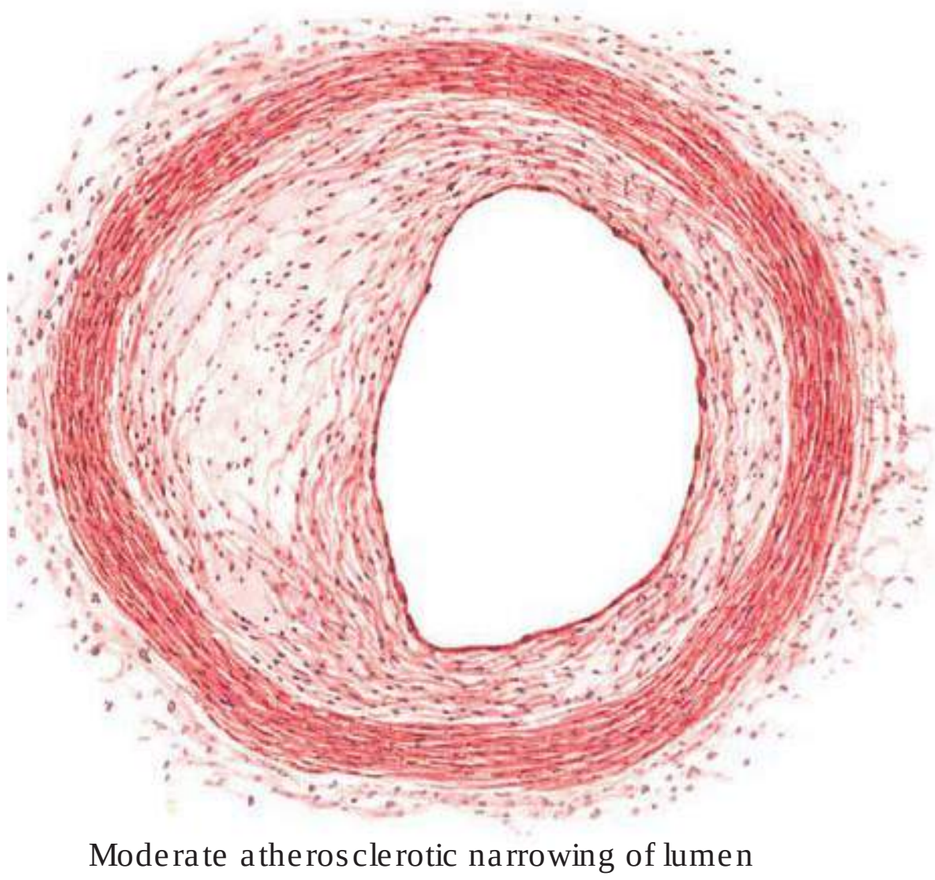
An elevated concentration *of* LDL cholesterol and a low concentration *of* HDL cholesterol in the plasma are risk *f*actors *f*or coronary artery disease (CAD). LDL cholesterol can be high due to genetic *f*actors, diet, obesity, and other *f*actors. LDL cholesterol can be lowered through diet, exercise, and drugs such as statins, ezetimibe, and PCSK9 inhibitors. The concentration *of* HDL cholesterol is most o*ft*en low due to hypertriglyceridemia.

5.1. Blood Cholesterol Concentration and the Risk of Coronary Artery Disease

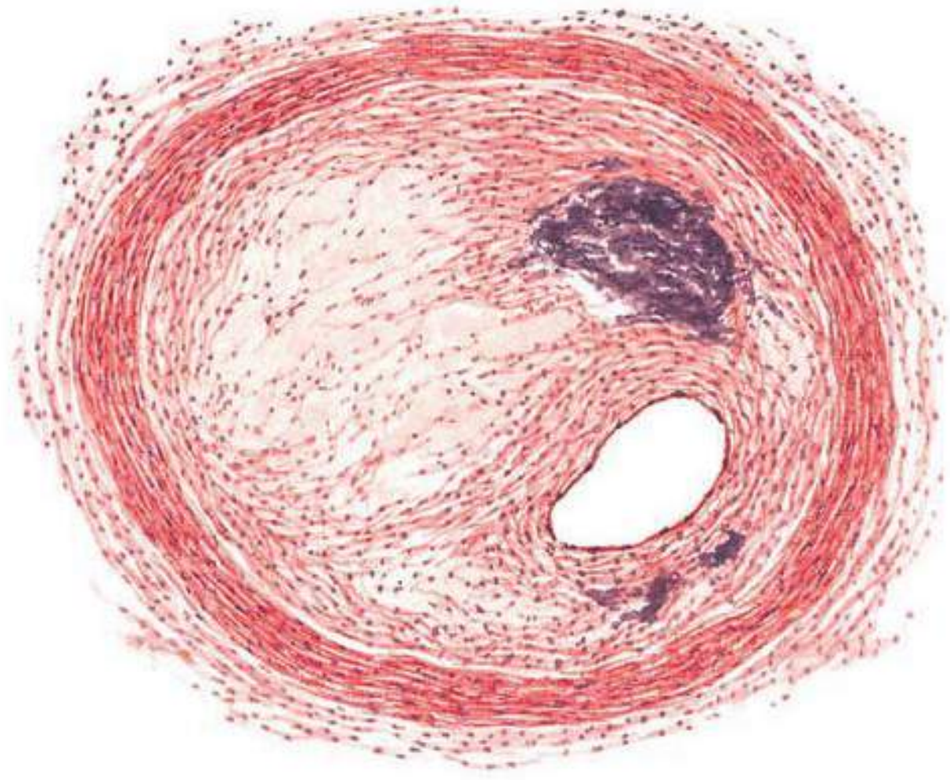
Newborns have plasma **LDL cholesterol** of about 60 mg/dL. The mean serum LDL cholesterol of **adults** is strongly influenced by diet, being on the order of 95 mg/dL in Japanese fishermen who consume a low-fat diet, and about 180 mg/dL in foresters in Finland who consume a fat-rich diet.

In population studies, there is a very strong correlation between plasma **LDL cholesterol** and the incidence of CAD. The lower the LDL cholesterol, the lower the risk for CAD.

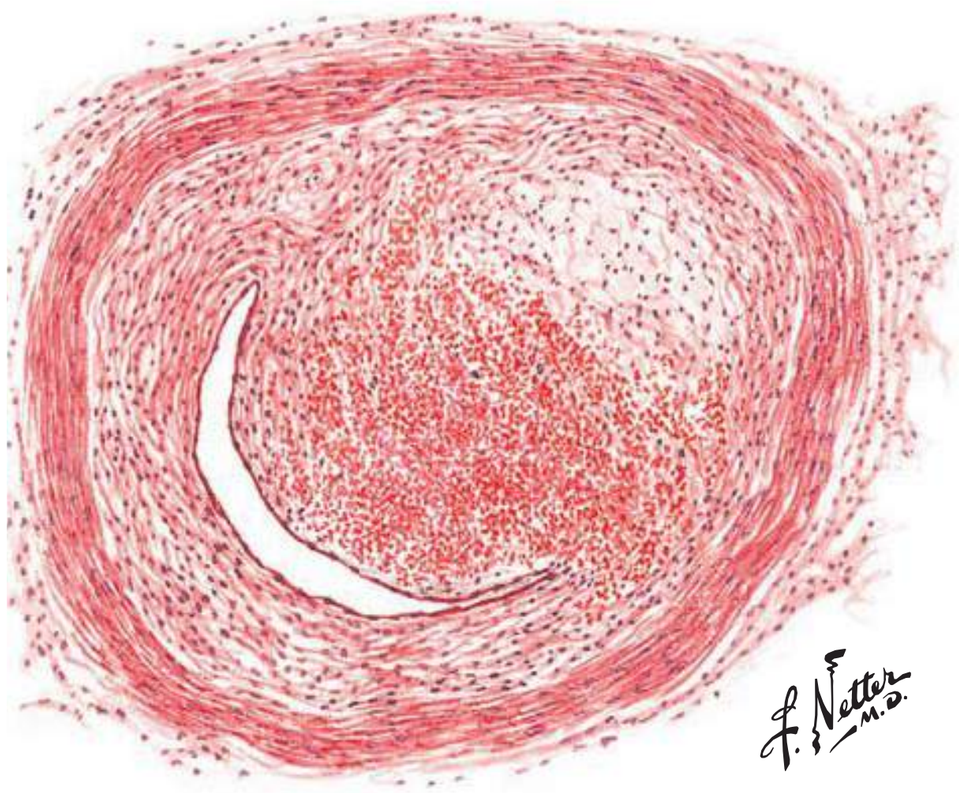
A high concentration of LDL cholesterol is associated with **atherosclerosis** (Fig. 29.13). Plaques are present already in young adults, although they do not yet give rise to clinical symptoms. It is thought that **oxidized LDL** (i.e., LDL with damage to protein and lipids from reactive oxygen species) can enter the walls of arteries and eventually be endocytosed by **macrophages** via the scavenger receptor A (SR-A). These macrophages thus become **foam cells** that secrete cytokines



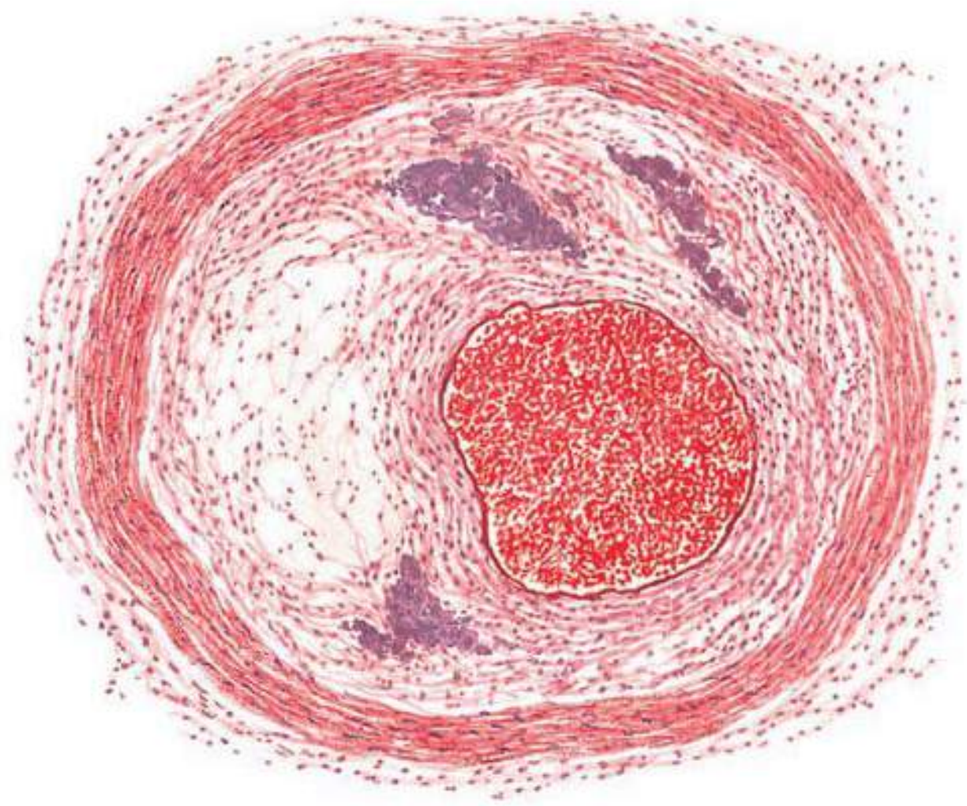
Moderate atherosclerotic narrowing of lumen



Almost complete occlusion by intimal atherosclerosis with calcium deposition



Hemorrhage into atheroma, leaving only a slitlike lumen



Complete occlusion by thrombus in lumen greatly narrowed by atheroma

Fig. 29.13 Progressive narrowing and occlusion of the coronary artery by atherosclerotic plaque and thrombus.

and start an inflammatory reaction. In response, smooth muscle cells proliferate, and plaques grow over time. At some point they rupture and, together with a newly forming blood **clot**, obstruct blood flow.

In industrialized countries, about 25% of all deaths are attributable to atherosclerotic plaques, which mainly cause **heart attacks**.

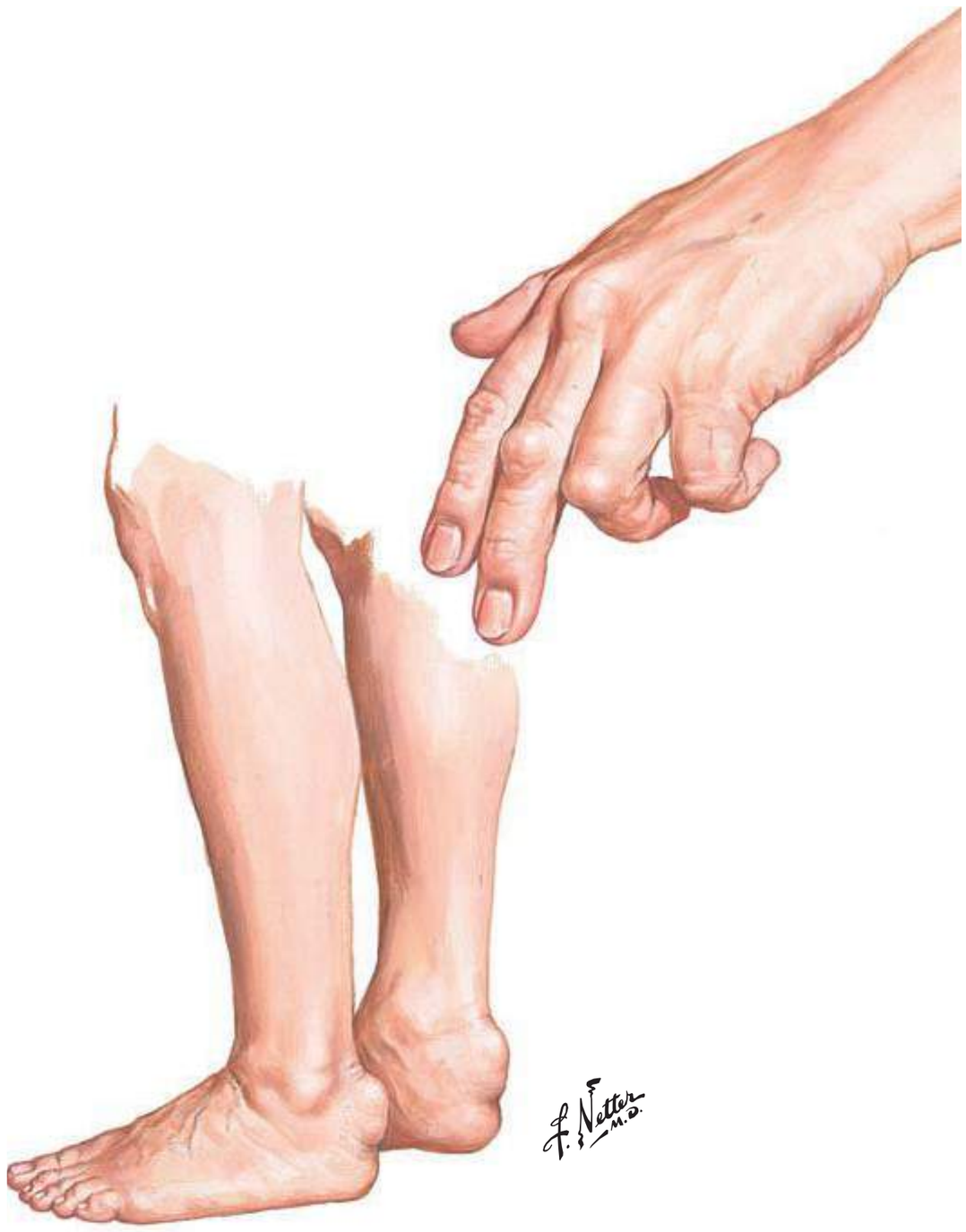
Besides high LDL cholesterol, low **HDL cholesterol** is also positively associated with the incidence of cardiovascular disease. The cause of this association remains to be elucidated. It is puzzling that patients who have monogenic disorders that lead to extremely low HDL cholesterol have no clear-cut increase in CAD risk. This is true for apolipoprotein A-I mutations, Tangier disease due to ABCA1 mutations (ABCA1 exports cholesterol from cells; see Fig. 29.6), and a deficiency of LCAT.

Other risk factors for heart attacks are **smoking**, **hypertension**, and **diabetes**.

5.2. Familial Hypercholesterolemia

Mutations in the genes for the LDL receptor, apolipoprotein B-100, and PCSK9 are currently known to cause congenital hypercholesterolemia. The prevalence is on the order of ~1 in 400. In adulthood, affected persons have elevated LDL cholesterol (>220 mg/dL [>5.7 mM]) and total cholesterol (>300 mg/dL [>7.8 mM]) but a normal plasma concentration of total triglycerides. Without treatment, these persons develop premature **atherosclerosis** (see Fig. 29.13) and often also xanthomas.

Xanthomas (Fig. 29.14) contain cholesterol-laden foam cells, and their location depends on the cause of dyslipidemia.



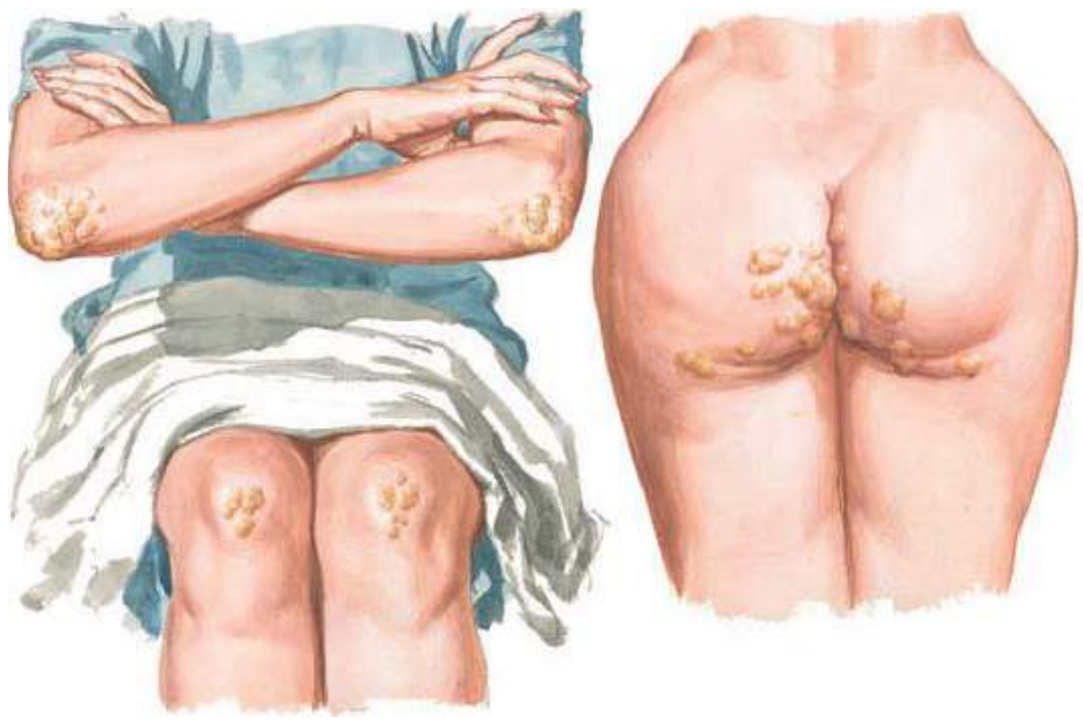


Fig. 29.14 Xanthomas caused by dyslipidemia. Tendon xanthomas (hands and feet), plain and tuberous xanthomas, xanthelasmas of eyelids.

Patients with hypercholesterolemia (no hypertriglyceridemia) have tendon xanthomas, most often on the hands and feet. Patients with combined hypercholesterolemia and hypertriglyceridemia have tuberous xanthomas, which form over joints. Xanthelasmas form under the skin, typically of the eyelid, and mostly occur in patients with high LDL cholesterol.

Most patients who have **heterozygous familial hypercholesterolemia** and a known disease-causing mutation have a mutant allele for the **LDL receptor**. About 5% of these patients have a mutant **apolipoprotein B**, and approximately 2% have an overly active **PCSK9** enzyme. Sometimes, the term heterozygous familial hypercholesterolemia is applied to only those who have a mutant LDL receptor, and carriers of a pathogenic apolipoprotein B mutation are said to have **familial ligand-defective apolipoprotein B-100** (**apoB-100**). All of these patients typically have about twice the usual LDL cholesterol (i.e., 200-400 mg/dL [5-10 mM]), and without treatment they develop CAD before their late 50s.

Homozygous familial hypercholesterolemia has a prevalence of only ~1 in 1 million. Most often, patients with this disorder have two alleles for a defective LDL receptor. T eir LDL cholesterol is approximately tenfold the usual concentration. Without treatment, these patients have heart attacks in their teens and twenties. Less often, patients are homozygous for loss-of-function mutations in apo B-100.

5.3. Other Causes of Hypercholesterolemia

Besides a deficient LDL receptor, uptake of LDL particles into the liver can be impaired by **hypothyroidism** or a diet high in **saturated fats** and **trans fats**. Patients with hypercholesterolemia are often screened for hypothyroidism by measuring thyroid-stimulating hormone (TSH) in serum. T e ef ect of saturated and trans fats on LDL cholesterol depends on **genetic predisposition**.

5.4. Lowering the Concentration of LDL Cholesterol

Atherosclerosis is a process that takes years to develop, and it is not readily reversible. Although plaques occasionally do recede with a dramatic lowering of LDL cholesterol, epidemiologic studies show that repair is typically not sufficient to lower a patient's CAD risk to that of a person with a similarly low LDL cholesterol for decades.

Modification of food intake often lowers plasma LDL cholesterol remarkably. Patients can try the following:

- A **low-calorie diet** to achieve **weight loss** (if overweight or obese)
- A **moderate-fat diet** with some unsaturated fatty acids, especially monounsaturated fats, but low in saturated and trans fats
- A **cholesterol-free diet**, such as a **vegan diet**

- **Plant sterols** (**phytosterols**), which compete with cholesterol for uptake into intestinal epithelial cells via the NPC1L1 (see Fig. 29.1)
- **Fiber**, which binds bile acids and thus prevents their recirculation to the liver

Exercise helps patients lose weight and it also independently leads to a lowering of non-HDL cholesterol by approximately 4%.

Drug treatment guidelines for hypercholesterolemia and the prevention of **atherosclerotic cardiovascular disease** (**ASCVD**) vary by country and ethnic population. Agreement exists mostly on treating patients who have clinically evident ASCVD, diabetes, or familial hypercholesterolemia. Various calculators are used to estimate the 10-year **risk** of ASCVD. These estimators often use gender, age, total cholesterol, HDL cholesterol, systolic blood pressure, diabetes, and smoking history as risk factors. Patients with about a 10% 10-year risk of ASCVD are often advised to take a statin.

Statins (e.g., **rosuvastatin**, **atorvastatin**, **simvastatin**, **lovastatin**, **pravastatin**, and **fluvastatin)** inhibit **HMG-CoA reductase** (see Fig. 29.4), which leads to a decreased concentration of cholesterol in the ER membranes. This stimulates SREBP2-mediated transcription of various genes, including the **LDL-receptor** gene. Increased expression of LDL receptors leads to a decreased number of LDL in the blood, thus lowering LDL cholesterol. While SREBP2 also enhances synthesis of HMG-CoA reductase in the cholesterol biosynthesis pathway, this effect is very modest due to inhibition of HMG-CoA reductase by the statin. Over a 5-year period, lowering of LDL cholesterol by 40 mg/dL with statin therapy reduces the number of major vascular events by about 20%.

Ezetimibe inhibits the **NPC1L1** transporter in intestinal epithelial cells and therefore inhibits the absorption of cholesterol (see Fig. 29.1). Some of this cholesterol is dietary, and some stems from the enterohepatic cholesterol recirculation. For this reason, ezetimibe lowers LDL cholesterol even in persons who adhere to a cholesterol-free vegan diet.

Bile acid sequestrants such as **cholestyramine**, **colestipol**, and **colesevelam** bind bile salts in the intestine and prevent their reabsorption. Because of the lowered amount of reabsorption, bile salt synthesis from cholesterol increases. As more cholesterol is used for bile acid synthesis, the concentration of intracellular cholesterol drops, SREBP2 becomes active, the number of LDL receptors in the plasma membrane increases, and the liver removes more LDL-containing cholesterol from the blood. However, the lowered intracellular concentration of cholesterol also increases cholesterol biosynthesis by increasing the amount of HMG-CoA reductase. **Statins** may be administered in conjunction with bile acid sequestrants to counter this increase in cholesterol synthesis with greater use of cholesterol for bile acid synthesis.

Monoclonal antibodies against PCSK9 (**PCSK9 inhibitors**), such as alirocumab and evolocumab, are approved for patients whose LDL cholesterol cannot be adequately controlled by diet and a statin drug. Alirocumab is injected once every 2 weeks and evolocumab once a month. When the amount of PCSK9 is reduced, there is increased survival of LDL receptors, which leads to greater removal of LDL from the blood (see Section 3.1).

6. COMBINED HYPERLIPIDEMIA

Combined hyperlipidemia refers to elevated levels of triglycerides and cholesterol. Patients are at risk of coronary heart disease, and they may show tuberoeruptive xanthomas.

6.1. Familial Combined Hyperlipidemia

Familial combined hyperlipidemia is a common heritable condition that manifests in elevated plasma triglycerides, elevated total cholesterol, or both of these abnormalities. In patients, these abnormalities may change over time. Laboratory values are often abnormal already in childhood. The cause of the disorder is not known; its prevalence in the general population is approximately 1 in 100, and 1 in 10 among persons who have coronary heart disease or a myocardial infarction before the age of 60 years.

Familial combined hyperlipidemia is primarily treated with diet, exercise, and a statin drug. Overweight or obese patients should lose weight.

6.2. Familial Dysbetalipoproteinemia

The disease is named based on the observation that there is an increased amount of apoB-containing lipoproteins. Total cholesterol and triglycerides are both elevated. The LDL cholesterol is low because LDL is produced at a reduced rate.

This disorder is due to homozygosity or compound heterozygosity for a variant apolipoprotein E that has a decreased affinity for its receptors. Homozygosity for **apolipoprotein E2**, seen in about 0.5% of the population, is the most common cause of familial dysbetalipoproteinemia, although penetrance often requires the presence of obesity, diabetes, high fat intake, high alcohol consumption, or another risk factor. The dyslipidemia is a result of the accumulation of chylomicron remnants and IDL because these cannot be removed efficiently by apolipoprotein E binding to LRP1 or LDL receptors. In the general population, there are three common isoforms of apolipoprotein E: E2, E3, and E4. Most people are homozygous for E3. E4 is a major risk factor for Alzheimer disease.

Patients who have familial dysbetalipoproteinemia often have premature **ASCVD** and tend to have **tuberoeruptive xanthomas** (see Fig. 29.14).

SUMMARY

- Only foods derived from animals contain cholesterol.
- In the intestine, Niemann-Pick C1-like protein 1 (NPC1L1) transports sterols (including cholesterol) from the lumen into intestinal epithelial cells.
- Epithelial cells of the intestine, hepatocytes, steroid-producing cells, and macrophages can esterify cholesterol

with a fatty acid to produce cholesteryl esters, which they can store.

- The intestine exports cholesterol and cholesteryl esters in chylomicrons. Lipoprotein lipase removes triglycerides from chylomicrons, and the liver takes up the resulting cholesterol-rich chylomicron remnants (apoprotein E on the remnants binds to the low-density lipoprotein (LDL) receptor-related protein (LRP) receptor on hepatocytes).
- The liver plays a central role in cholesterol homeostasis in that it acquires dietary cholesterol, synthesizes cholesterol and cholesteryl esters and releases these into the blood in very-low-density lipoprotein (VLDL), takes up a large fraction of (cholesterol-rich) LDL, acquires cholesterol from high-density lipoprotein (HDL), secretes cholesterol into bile, and converts cholesterol into bile salts.
- PCSK9 facilitates the destruction of LDL receptors.
- HDL are formed from apolipoprotein A-I. HDL accept cholesterol from peripheral cells. LCAT on HDL receives cholesterol from a variety of cells and esterifies it for storage inside HDL. HDL transfers cholesterol to the liver.
- Cholesteryl ester transfer protein (CETP) exchanges triglycerides and cholesteryl esters between VLDL and HDL. Hepatic lipase removes triglycerides from such HDL and generates lipid-poor particles that are degraded. These processes account for the low plasma HDL cholesterol that is typical of patients who have hypertriglyceridemia.
- The liver synthesizes bile salts and secretes them into the bile canaliculi. The amount of bile salt secretion determines bile flow. Bile is stored in the gallbladder, which empties in response to dietary fat and protein. In the intestine, bile salts emulsify lipids and form mixed micelles with cholesterol and other lipids. An absence of bile salts causes lipid malabsorption. The ileum takes up bile salts for recirculation to the liver.
- Cholesterol gallstones may be caused by excessive secretion of cholesterol from the liver or by deficient secretion of bile salts.
- Cholestasis often causes pruritus, perhaps due to the accumulation of bile salts. It is treated with certain bile acid sequestrants.
- A lipid panel includes measuring total cholesterol, HDL cholesterol, and triglycerides. From these values, non-HDL cholesterol and LDL cholesterol can be calculated.
- A high concentration of plasma LDL cholesterol is associated with atherosclerosis and cardiovascular disease. Patients with very high LDL cholesterol also have xanthomas.
- Familial hypercholesterolemia can be caused by mutations in the genes for the LDL receptor, apoB-100, and PCSK9.
- Approaches to lowering an elevated LDL cholesterol include weight loss; a diet low in fat and cholesterol but high in fiber and plant sterols; a diet that contains a reasonable amount of unsaturated fatty acids; and drugs such as statins, ezetimibe, PCSK9 inhibitors, and bile acid sequestrants. All currently used drugs have in common that they lead to increased expression of LDL receptors on hepatocytes.
- Statin drugs inhibit HMG-CoA reductase, the enzyme that controls the rate of cholesterol de novo synthesis. This leads to increased expression of LDL receptors and, consequently, a lowering of plasma LDL cholesterol.
- Plant sterols compete with cholesterol for uptake via the NPC1L1, and ezetimibe inhibits transport via the NPC1L1.
- Inhibitors of PCSK9 increase the number of LDL receptors and thus lower the concentration of LDL cholesterol in the plasma.
- Bile acid sequestrants prevent the enterohepatic recirculation of bile acids, thereby increasing the use of cholesterol for bile acid synthesis.
- Low HDL cholesterol (most often due to hypertriglyceridemia) is associated with an increased risk of coronary artery disease (CAD).

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Review Questions

1. Provide a rationale as to why statins lower LDL cholesterol in patients with heterozygous familial hypercholesterolemia but have no or only a minimal effect in patients with the homozygous form of this disease.

2. Ezetimibe can lower LDL cholesterol in patients who consume a vegan diet because ezetimibe diminishes which one of the following?

   A. Absorption of cholesterol that was secreted into bile
   B. CETP-mediated exchange of cholesterol from HDL to VLDL
   C. HMG-CoA reductase activity
   D. PCSK9 activity
   E. Recirculation of bile salts

3. Which one of the following is a likely cause of low serum HDL cholesterol?

   A. CETP deficiency
   B. Cholelithiasis
   C. Deficiency of hepatic lipase
   D. Hypertriglyceridemia
   E. PCSK9 deficiency

4. A 25-year-old patient has a very high serum LDL cholesterol but normal values of plasma triglycerides and HDL cholesterol. Physical examination reveals tendon xanthomata. T e patient's father had premature ASCVD. Heterozygosity for a loss-of-function mutation in the gene for which one of the following proteins is most compatible with these findings?

   A. CETP
   B. LDL receptor
   C. MTP
   D. NPC1L1
   E. PCSK9

Chapter

30 Metabolism of Ethanol and the Consequences of Alcohol Dependence Syndrome

SYNOPSIS

- Excessive alcohol consumption is a huge public health problem. Ethanol acutely impairs the function of the central nervous system; this gives rise to a high rate of accidental deaths and acute alcohol poisoning. Chronic high ethanol intake can lead to cancer, liver disease, heart disease, and the many problems associated with addiction.
- The liver metabolizes most of the dietary ethanol by first converting it to acetaldehyde and then to acetate. Various tissues then oxidize acetate to CO_2. Chronic alcohol consumption leads to increased expression of cytochrome P450 enzymes in the endoplasmic reticulum, which normally oxidize only a small portion of ethanol to acetaldehyde.
- Ethanol acutely suppresses gluconeogenesis and increases the production of triglycerides. In addition, it also increases the production of urate and thus increases the likelihood of an acute episode of gout.
- Alcoholism is a mental disorder with a hereditary component. Chronic high ethanol intake leads to damage of DNA and proteins, and it may induce fatty liver disease, cirrhosis, or cancer (especially of the upper aerodigestive tract). In pregnant women, chronic high ethanol intake may result in offspring with fetal alcohol syndrome; characteristics of this syndrome are abnormalities of the head and problems with behavior.

LEARNING OBJECTIVES

For mastery *of* this topic, you should be able to do the *following*:

- Describe the metabolism of ethanol, including products, cellular location, and tissue distribution.
- List the major harmful effects of ethanol on the human body, and describe the known underlying biochemistry.
- Identify a range of alcohol consumption that is considered acceptable and a range that is indicative of abuse.
- Explain the increased toxicity of acetaminophen and procarcinogens in patients who habitually drink large quantities of alcohol.

1. EFFECTS OF ALCOHOL USE ON THE HEALTH OF THE PUBLIC

Worldwide, excessive alcohol consumption is responsible *for* a large number o*f* injuries and deaths through accidents, an increased prevalence o*f* cancer and cancer-related deaths, disruptions o*f* social interactions, and violence. Alcohol use is currently a *far* larger problem than the use o*f* illicit psychoactive drugs.

Alcohol is used here as a summary term for beverages that contain **ethanol**. Ethanol has the structural formula CH_3CH_2OH.

Beer often contains about 5% ethanol by volume (~860 mM ethanol), **wine** about 12% (~ 2,060 mM), and **vodka** about 40% (~ 6,900 mM; in the United States, 40% by volume is called 80 proof). An alcoholic beverage that contains 10% ethanol by volume contains about 8 g of ethanol per 100 mL of the drink.

Global per capita alcohol consumption (considering people aged 15 years or older) is approximately 6 L of pure alcohol per year, about half of which is consumed as spirits and about one-third as beer. Daily per capita consumption is thus about 275 mL of beer, or 35 mL of spirits, or 13 g of ethanol per day. Consumption is the highest in Europe and lowest in predominantly Muslim populations. In the United States, where per capita ethanol consumption is approximately 35% above the world average, 17% of the population aged 15 years or older binge drink (i.e., females consume ≥4 drinks and males consume ≥5 drinks within a 2-hour period) on at least one occasion per month. Among U.S. adults aged 26 years or older, men binge drink twice as frequently as women.

Ethanol depresses the activity of the cerebral cortex. After consumption of a small amount of alcohol on an empty stomach, the concentration of ethanol in the blood peaks within about 20 minutes; after a large dose, breath alcohol peaks within about 40 minutes. From the blood, ethanol readily dif uses through cell membranes without the need for a transporter; thus, it also readily passes through the blood-brain barrier. People who do not habitually drink alcohol eliminate about 10 g of alcohol per hour.

At low to moderate doses, ethanol exerts its ef ects on the central nervous system in part by enhancing the activity of certain γ-aminobutyric acid (GABA) receptors, which are chloride channels. At low concentrations of ethanol, increased activity of GABA receptors reduces anxiety; at high concentrations of ethanol, GABA receptor activity also induces sedation and anesthesia.

Alcohol is present in blood at relatively high concentrations. At an ethanol concentration above ~25 mg/dL blood (i.e., ~5 mM), a person's reaction time may be decreased and judgment impaired; in addition, the subject may feel that alcohol has taken ef ect. At an ethanol concentration of about 50 mg/dL (~11 mM), a person's reaction time, tracking ability, and vigilance are compromised. At a concentration of about 100 mg/dL (i.e., ~22 mM), balance is impaired. In an alcohol-naive person, at a concentration greater than ~300 mg/dL (i.e., ~65 mM), coma may set in. Moreover, at a concentration of approximately 400 mg or more of ethanol per dL blood (~80 mM), respiratory arrest and death may occur. In many countries, drivers who have a blood alcohol concentration more than 0.05% to 0.1% w/v (i.e., 50–100 mg/dL [11–22 mM])

are considered intoxicated and are forbidden from operating a vehicle.

In many countries, alcohol use leads to many more deaths than the use of illicit drugs. In the United States, an estimated 90,000 alcohol-attributable deaths occur per year; slightly more than 40% of these deaths occur due to acute intoxication. Each alcohol-attributable death reflects about 30 years of life lost. The most common reasons for death due to acute intoxication are motor vehicle accidents, suicides, falls, and being a victim of homicide or poisoning. The most common causes of premature death due to chronic alcohol abuse are liver disease, stroke, and cancer.

2. METABOLISM OF ETHANOL

Ethanol is highly soluble in both water and the lipid phase of membranes. Hence, ethanol readily passes through membranes. Hepatocytes oxidize ethanol in two steps: first to acetaldehyde and then to acetate. The first step can occur in the cytosol or in the endoplasmic reticulum. The second step occurs mostly in mitochondria. The liver exports most of the acetate. Cardiac and skeletal muscles pick up acetate and oxidize it to acetyl-coenzyme A (CoA).

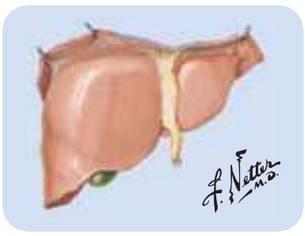
2.1. Metabolism of Ethanol to Acetate

Ethanol is chiefly degraded by a combination of the stomach, intestine, and liver. The liver degrades virtually all of the ethanol that reaches the bloodstream. A low percentage of dietary ethanol is eliminated as ethanol via the urine or expired air. The concentration of ethanol in expired air is a reflection of the concentration of ethanol in blood.

Here, ethanol metabolism is described in three stages: (1) ethanol to acetaldehyde, (2) acetaldehyde to acetate, and (3) acetate to CO_2. The first two reactions occur primarily in the liver; they may also occur in the stomach. Blood vessel walls and cells of the upper digestive tract that are in contact with alcoholic beverages also carry out these reactions. Although this is of minor quantitative importance, it may be important in protecting these cells from the adverse effects of ethanol. The third reaction occurs mostly in muscle and brain.

The reaction ethanol → acetaldehyde is catalyzed independently by both alcohol dehydrogenase in the cytoplasm and by cytochrome P450 enzymes in the endoplasmic reticulum (Fig. 30.1), as follows.

Alcohol dehydrogenase (**ADH**) oxidizes the major portion of dietary ethanol to acetaldehyde in persons who drink alcohol occasionally. Humans synthesize several ADH isoenzymes and express them in a tissue-specific manner. These enzymes play a role in the oxidation of several different alcohols and other compounds (e.g., retinol). A blood ethanol concentration of 5 to 20 mM (~0.025-0.1% weight/volume) impairs a person's judgment and reaction time. ADH isoenzymes that play a physiologic role in removing ethanol have K_m values (K_m values are explained in Chapter 10) in the range 0.05 to 40 mM.



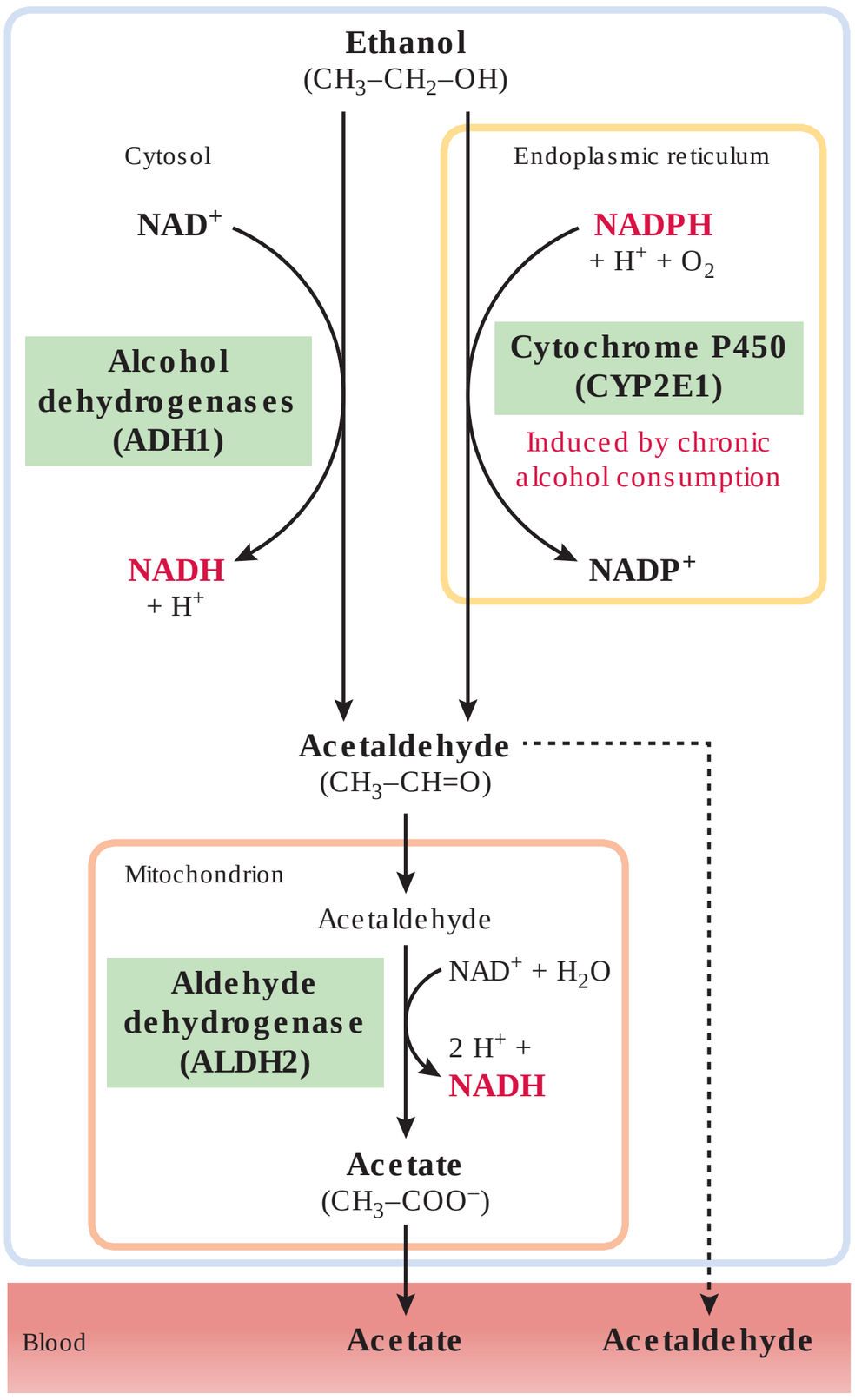


Fig. 30.1 **Metabolism of ethanol to acetate in hepatocytes.**

Individuals may differ considerably in their ability to metabolize ethanol. Homodimers of the common **isoforms** of ADH in the liver exhibit K_m values for ethanol that differ by a factor of greater than 100 and V_{max} values that differ by a factor of greater than 30. **Women** and patients with **gastritis** (e.g., chronic alcohol abusers) express less ADH in the stomach than alcohol-abstaining **men**. Furthermore, aspirin and certain **histamine H2 blockers** (e.g., **cimetidine**) inhibit gastric ADH.

Cytochrome P450 enzymes also oxidize ethanol to acetaldehyde (see Fig. 30.1). The main contributor is **CYP2E1**; minor contributors are CYP1A2 and CYP3A4. Over a period of days to weeks, a persistent ethanol intake is accompanied by increased CYP2E1 activity, which contributes to ethanol tolerance. In habitual drinkers, CYP2E1 becomes the main enzyme that oxidizes ethanol.

Aldehyde dehydrogenase (**ALDH**) inside mitochondria oxidizes acetaldehyde to acetate. Akin to ADH, there are several isoenzymes for ALDH, which in turn oxidize various aldehydes. In the liver, ALDH1 is present in the cytosol and ALDH2 in mitochondria; the enzyme in mitochondria has a much higher affinity for acetaldehyde and oxidizes the lion's share of acetaldehyde.

Acetaldehyde, like ethanol, crosses membranes by dif usion (i.e., without the help of a transporter). Some acetaldehyde leaks from the liver into the blood.

Many persons of **East Asian** heritage (e.g., Japanese, Korean, or Han Chinese) do not efficiently metabolize acetaldehyde. T ese individuals are heterozygous or homozygous for a variant mitochondrial acetaldehyde dehydrogenase that has little enzymatic activity. As a consequence, the concentration of acetaldehyde in the blood is increased, causing nausea, headache, and flushing of the face and upper chest (flushing is due to temporary vasodilation).

Disulfiram (Antabuse) irreversibly inhibits both the mitochondrial and the cytosolic ALDH. Patients who take disulfiram and then drink alcohol experience flushing, nausea, and sufficient overall physical discomfort to stop drinking. For this reason, disulfiram can be used to keep alcohol-dependent patients from drinking alcohol (see also Section 4.3). However, unless someone ensures the patient takes the drug daily, compliance is low.

Acetaldehyde forms noxious adducts with proteins and DNA (see Section 4.2). T ese reactions contribute to the increased risk of certain types of cancer among people who consume alcohol.

In summary, the equation for the oxidation of ethanol by ADH and ALDH is:

$$\text{Ethanol} + 2\,\text{NAD}^+ + \text{H}_2\text{O} \rightarrow \text{Acetate}^- + 2\,\text{NADH} + 3\,\text{H}^+$$

T e liver releases most of the **acetate** into the bloodstream; from there, it reaches other tissues for further metabolism.

2.2. Oxidation of Acetate to CO_2

Skeletal and cardiac muscle, the brain, and other tissues take up acetate from the blood and can oxidize it to CO_2 (Fig. 30.2). **Acetate-CoA ligase** (commonly called **acetyl-CoA synthetase**) inside mitochondria activates acetate to acetyl-CoA. T is activation produces **adenosine monophosphate** (**AMP**) and can do so at a high rate (see Section 3.3).

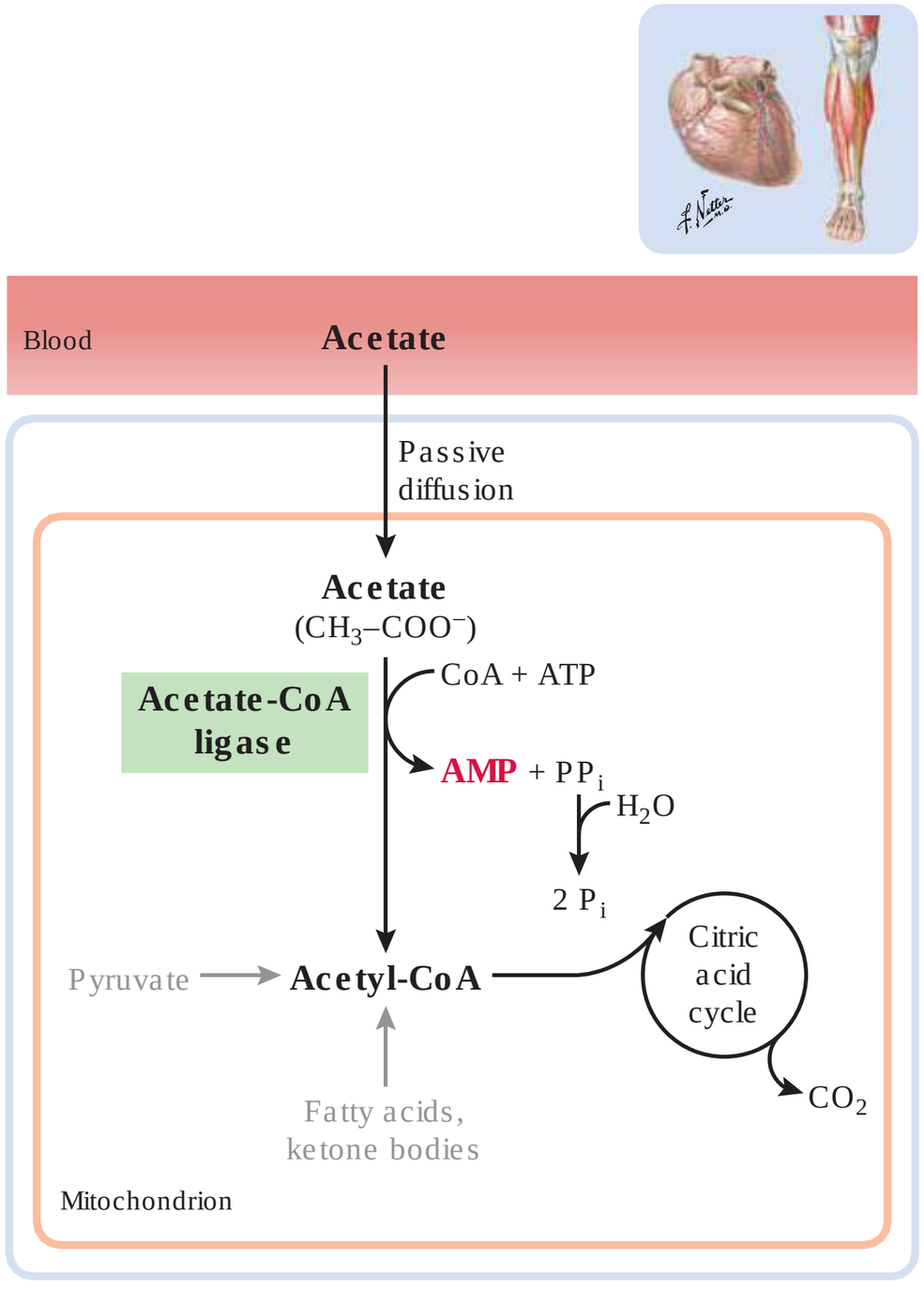


Fig. 30.2 Oxidation of acetate in skeletal and cardiac muscle. The liver metabolizes ethanol to acetate and releases most of the acetate into the bloodstream (see Fig. 30.1).

3. ACUTE EFFECT OF ETHANOL ON PATHWAYS OF METABOLISM

Ethanol metabolism a*ff*ects the metabolism o*f* other compounds largely through an increased ratio o*f* $NADH/NAD^+$ in the liver and an increased rate o*f* AMP production *f*rom the activation o*f* acetate in muscle. A moderate to large dose o*f* ethanol thus inhibits gluconeogenesis, increases liver triglyceride stores, and increases urate production.

3.1. Effect of Ethanol on Gluconeogenesis

After alcohol consumption, gluconeogenesis is suppressed significantly, which may cause **hypoglycemia**. For instance, in volunteers who fasted overnight and then imbibed 50 g of ethanol (equivalent to about 0.4 L of wine), gluconeogenesis, around noon, proceeded at approximately half the rate of individuals who had not consumed alcohol. However, glucose use also decreases (presumably due to the oxidation of ethanol and its metabolites), which dampens the expected decrease in blood glucose concentration. When patients who have **diabetes** or a **glycogen storage disease** who are **fasting** or consume few carbohydrate they cannot readily compensate for a decreased rate of gluconeogenesis with an increased rate of glycogenolysis. Hence, these patients are at greatest risk for alcohol-induced hypoglycemia.

T e ethanol-induced decrease in gluconeogenesis is usually attributed to an altered **$NADH/NAD^+$** ratio in hepatocytes that oxidize ethanol. As shown in Section 2, the degradation of 1 mol ethanol to acetate yields 2 mol of NADH from NAD^+. T e metabolism of ethanol is rapid enough that the $NADH/NAD^+$ ratio changes substantially. In turn, this shifts the lactate/pyruvate equilibrium toward **lactate** and the dihydroxyacetone phosphate/glycerol 3-phosphate equilibrium toward glycerol 3-phosphate (Fig. 30.3). Pyruvate that is made

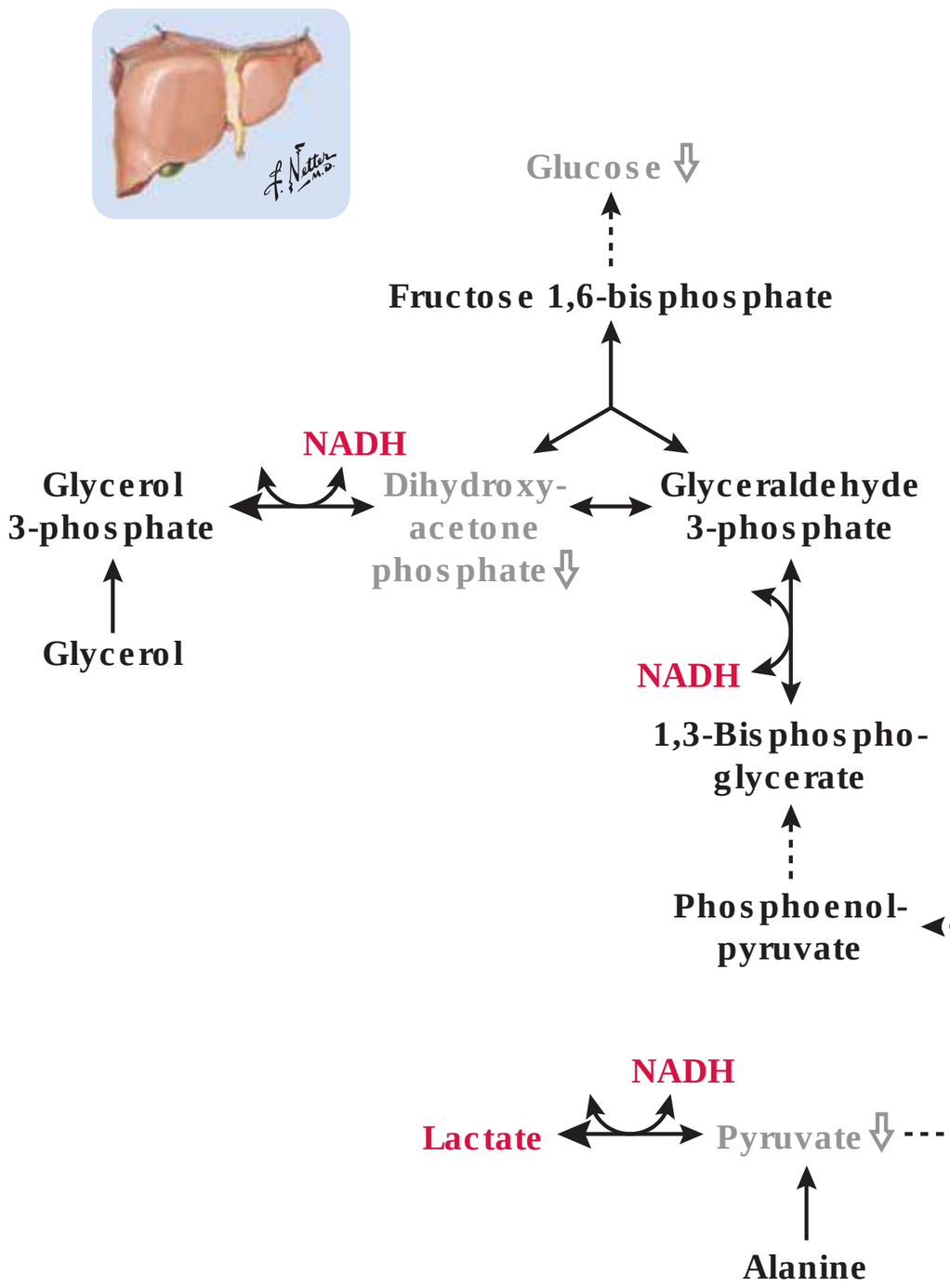


Fig. 30.3 **Effect of ethanol consumption on gluconeogenesis.** The rapid oxidation of ethanol in the liver leads to an increased concentration of NADH and a decreased concentration of NAD^+ (see Fig. 30.2). For more details on the reactions of gluconeogenesis, see Fig. 25.3.

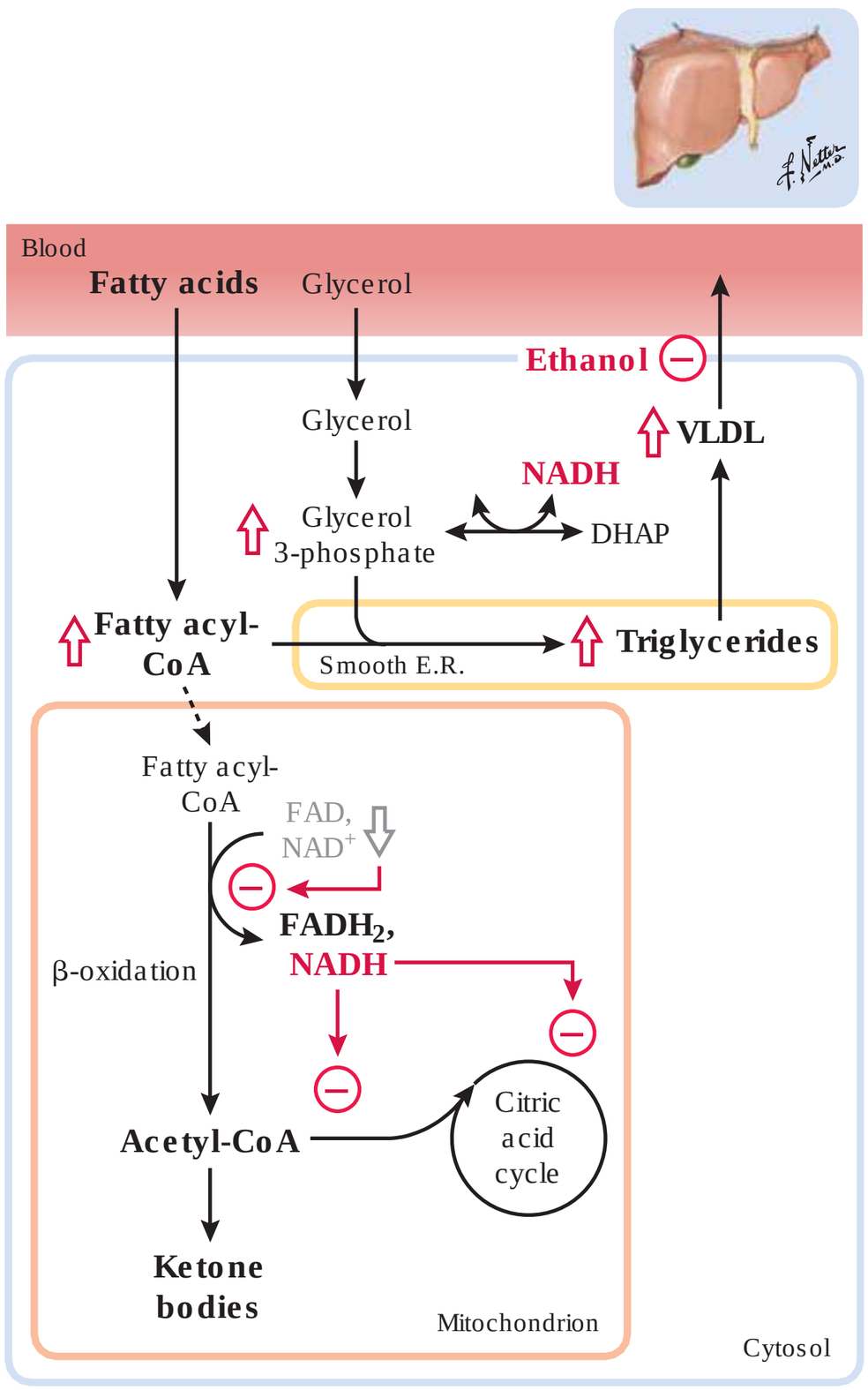
from alanine is reduced to lactate instead of entering mitochondria. T e lowered concentrations of pyruvate and dihydroxyacetone phosphate no longer support a normal rate of gluconeogenesis. A purely alcohol-induced **lactic acidosis** is usually mild and occurs only in a small fraction of patients who are hospitalized because of acute ethanol intoxication.

3.2. Effect of Ethanol on Fatty Acid Metabolism

Ethanol inhibits lipolysis and leads to a lowering of the concentration of free **fatty acids** in the plasma. T e same ef ect can be achieved with acetate.

Ethanol inhibits **fatty acid β-oxidation** in the liver, perhaps via both the high NADH/NAD^+ ratio and the resulting low concentration of FAD (Fig. 30.4; see also Fig. 27.11).

Alcohol use also increases the **production of triglycerides**. T is is commonly explained by the ready availability of fatty acids in the alcohol-intoxicated liver, combined with an elevated concentration of glycerol 3-phosphate and an increased amount of smooth endoplasmic reticulum (see Fig. 30.4). T e concentration of glycerol 3-phosphate is high because alcohol raises the NADH/NAD^+ ratio, which shifts the equilibrium from dihydroxyacetone phosphate toward glycerol



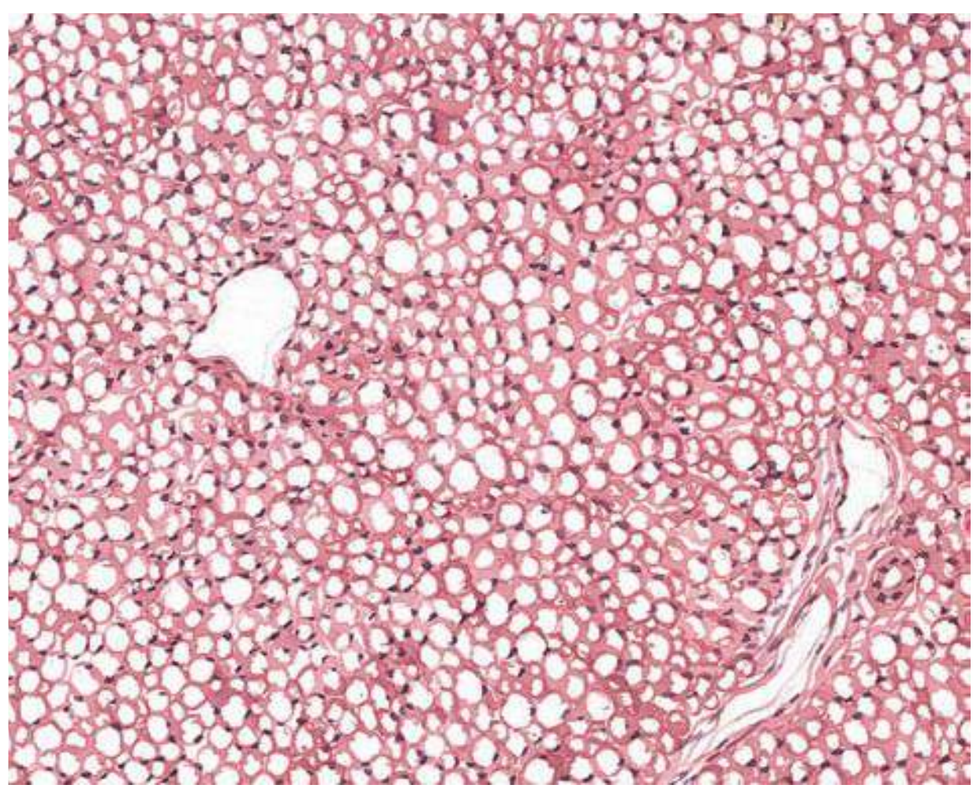


Fig. 30.4 **Effect of ethanol consumption on lipid metabolism in the fasting state.** Glycolysis is detailed in Fig. 19.2, gluconeogenesis in Fig. 25.3, β-oxidation in Fig. 27.11, the citric acid cycle in Fig. 22.6, synthesis of the ketone bodies acetoacetate and β-hydroxybutyrate in Fig. 27.14, and synthesis of triglycerides in Fig. 28.5.

3-phosphate. Alcohol use also induces a shift in the rate of transcription from genes that encode enzymes of fatty acid oxidation to genes that encode enzymes of fatty acid and triglyceride synthesis. In addition, alcohol use leads to a reduced AMP-dependent protein kinase (AMPK) activity, which can reduce the inhibition of acetyl-CoA carboxylase by AMPK, increase the formation of malonyl-CoA, and decrease the uptake of fatty acids into mitochondria for β-oxidation. Some alcohol-dependent patients have hypertriglyceridemia; however, this is not usually seen in patients who have liver cirrhosis because the livers of these patients has a reduced capacity for VLDL production.

In chronic alcoholics, **alcoholic ketoacidosis** may develop in the recovery phase after a period of intense binge drinking that is followed by nausea and vomiting. Ketone bodies are synthesized only when acetyl-CoA is produced at a high rate from the β-oxidation of fatty acids. For this to happen, the concentration of fatty acids in the blood must be relatively high. Since alcohol inhibits fatty acid β-oxidation, ketone production occurs only after ethanol has been removed from the blood. Since insulin would inhibit lipolysis, ketoacidosis is commonly seen in conjunction with mild hypoglycemia. Low food intake and vomiting increase the likelihood that an alcohol-abusing patient develops ketoacidosis.

3.3. Effect of Ethanol on the Production of Uric Acid

The metabolism of ethanol leads to increased production of **uric acid**. In muscle, activation of acetate to acetyl-CoA produces **AMP** (see Fig. 30.2). The concentration of AMP rises sufficiently to activate the degradation of AMP and guanosine monophosphate (GMP; GMP is in equilibrium with AMP) to urate (see Chapter 38). Indeed, after a moderate to large dose of ethanol, there is a mild increase in plasma urate and a more pronounced increase in urine uric acid. Chronic hyperuricemia increases the likelihood of an acute attack of **gout** (i.e., a very painful inflammation of one or more joints).

Alcohol also leads to a small increase in the concentrations of lactate and ketone bodies in the blood. Both lactate and ketone bodies decrease uric acid excretion by the kidneys (see Chapter 38). In the absence of **exercise**, these factors are minor. However, when alcohol is consumed after exercise (i.e., when the concentration of lactate in blood is markedly increased), there is a marked inhibition of uric acid excretion into the urine.

Consumption of beer or liquor at the world average of about 11 g of ethanol per day is associated with an increased concentration of urate in blood plasma, as well as an increased incidence of gout; this is not the case with a similar amount of alcohol consumed as wine. The reasons for these differences are not entirely clear, except that beer contains a significant amount of guanine, some of which is taken up and degraded to urate. Patients who have gout are advised to abstain from drinking beer or liquor.

3.4. Treatment of Acute Ethanol Intoxication in the Clinic

Patients who consume a very large amount of alcohol are at risk of severe hypoglycemia, respiratory arrest, sudden cardiovascular death, aspiration of vomit, and psychosis.

For the most part, ethanol-intoxicated patients receive supportive care. Hypoglycemic patients are given thiamine and glucose; thiamine is given to prevent Wernicke encephalopathy or cardiomyopathy (see Chapter 22). Patients who ingest a large amount of alcohol during the hour before treatment may be helped by gastric emptying. In addition, if needed, hemodialysis can be used to remove ethanol from the bloodstream.

4. EFFECTS OF CHRONIC ETHANOL INTAKE ON ORGAN FUNCTION

Alcoholism is a common and partially hereditary mental disorder. Ethanol and its metabolites damage proteins, which in turn can cause organ damage (e.g., in the liver) and cancer (e.g., of the esophagus). In utero exposure to ethanol damages the central nervous system and leads to problems with behavior and sometimes also abnormalities of the face.

4.1. General Comments About Alcohol Dependence Syndrome

Alcoholism is considered a **mental disorder**. The World Health Organization's latest International Statistical Classification of Diseases and Related Health Problems (ICD-10) uses the term **alcohol dependence syndrome**, which requires the presence of three of six listed risk factors (e.g., a strong desire to drink or exhibiting withdrawal symptoms) being present. The American Psychiatric Association's DSM-V5 uses the term **alcohol use disorder**, which can be mild, moderate, or severe depending on the number of 11 criteria that a person meets. In the United States in 2012, approximately 7% of people aged 18 years or older had alcohol use disorder.

In the United States, the National Institute on Alcohol Abuse and Alcoholism defines **binge drinking** as drinking that results in a blood alcohol content of 80 mg/dL or higher. Many adults reach this concentration by consuming four to five drinks during a 2-hour period. The same institution considers **low-risk** drinking as the consumption of fewer than 3 to 4 drinks in one day and fewer than 7 to 14 drinks per week (lower numbers are for women, higher numbers for men).

Chronic, excessive use of alcohol is associated with chronic disease, accidents, social problems, and increased domestic violence. Chronic effects of alcohol use are best predicted by a patient's average alcohol consumption. In the United States, alcohol-induced chronic disease is responsible for about half of all alcohol-attributable deaths and about one-third of alcohol-attributable potential life years lost.

Alcoholism is partly **hereditary**. Of spring of one alcohol-abusing parent are about five times more likely to abuse alcohol than of spring of nonabusing parents. Studies of twins show that genetic factors account for about 50% of the inter-individual variation for alcoholism. Linkage studies implicate mutations that af ect enzymes of ethanol metabolism and proteins that likely also play a role in neurotransmission.

People who experience **acetaldehyde-induced flushing** and nausea after alcohol consumption are least likely to become alcohol dependent. T e concentration of acetaldehyde can be high for two main reasons: low acetaldehyde dehydrogenase activity or high ADH activity. T e Glu487Lys variant of **mitochondrial acetaldehyde dehydrogenase** (also called the ALDH2*2 allele) has virtually no enzymatic activity. T is variant is found almost exclusively in persons of East Asian heritage. Mitochondrial acetaldehyde dehydrogenase functions as a tetramer, and the Glu487Lys variant shows a dominant negative ef ect (see Chapter 5). Homozygotes have virtually no risk of becoming alcohol dependent, whereas heterozygotes have only about one-fourth the risk of patients with normal acetaldehyde dehydrogenase activity. High **ADH** activity is associated with the ADH1B*2 (Arg47His) allele. T e ADH1B*2 allele provides some protection from alcohol dependence, but not as powerfully as the ALDH2*2 allele. After consumption of alcohol, this variant enzyme completes about 30 catalytic cycles in the time the normal enzyme completes one cycle.

Patients who chronically consume large quantities of alcohol often develop **malnutrition**. One gram of ethanol provides about 7 kcal (carbohydrates and proteins provide 4 kcal/g, and fat provides 9 kcal/g). One liter of vodka thus provides more calories than average daily meals. Many alcohol-dependent individuals consume only about half as much food as alcohol abstainers do. In addition, alcohol intake diminishes the absorption of **phosphate** and some **vitamins** in the intestine. Together, these ef ects may cause **hypophosphatemia** (see Chapter 19), **thiamine deficiency** (see Chapter 22), **folate deficiency** (see Section 7.1 in Chapter 36), **pyridoxal** (i.e., vitamin B_6) **deficiency**, or **pellagra** (i.e., niacin deficiency; see Section 1 in Chapter 19).

4.2. Effects of Ethanol and Acetaldehyde on Proteins and DNA

Ethanol and acetaldehyde can react with proteins and thus impair their function or make them immunogenic. Ethanol can give rise to **hydroxyethyl radicals** (CH_3–$C^{\bullet}H$–OH, CH_3–CH_2–$O^{\bullet}$, or $^{\bullet}CH_2$–CH_2–OH) that react with proteins. T ese hydroxyethyl radicals are the result of hydroxyl radicals ($^{\bullet}OH$) stealing an H from ethanol, and they can also be a by-product of CYP2E1 activity. Together with **malondialdehyde** (O=CH–CH_2–CH=O, a product of the peroxidation of polyunsaturated fatty acids), **acetaldehyde** can form hybrid protein adducts. Acetaldehyde adducts and mixed adducts of acetaldehyde and malondialdehyde are immunogenic. Ethanol consumption also leads to the acetylation of lysine residues in proteins.

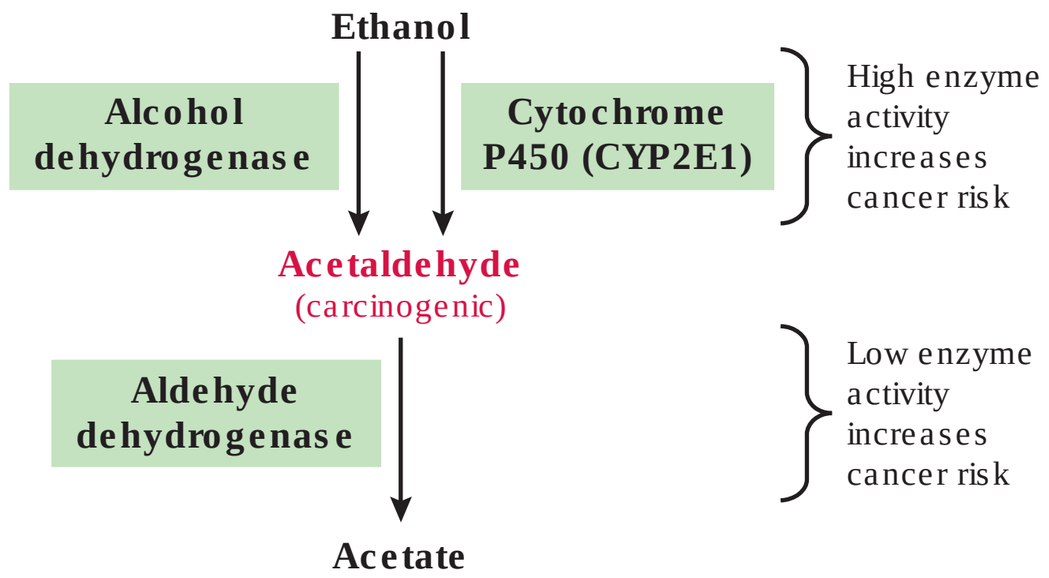


Fig. 30.5 Effect of variant enzymes on alcohol metabolism and cancer risk.

T e concentration of acetaldehyde in cells depends on the rates of its production and oxidation (i.e., on the activities of ADH, cytochrome P450 CYP2E1, and ALDH; Fig. 30.5). T e alcohol-induced cancer risk thus depends on a person's genetic makeup for these enzymes. Patients who are heterozygous for the *2 allele of ALDH2 (the gene for ALDH in mitochondria) have only about 40% of the normal ALDH activity. While severe, alcohol-induced flushing prevents ALDH2*2 homozygotes from drinking alcohol, heterozygotes can become alcohol dependent. If so, they have an increased risk for cancer of the esophagus (see also Section 4.5 and Fig. 30.7).

Individuals who abuse ethanol regularly and therefore have increased P450 activity are at risk of a toxic reaction to the pain reliever **acetaminophen** (also called **paracetamol**). Normally, most acetaminophen is detoxified via glucuronidation or sulfation, and only about 15% is eliminated via the P450 system (Fig. 30.6), thereby generating NAPQI (N-acetyl-p-benzoquinoneimine). In alcohol-dependent individuals, due to higher activity of alcohol-metabolizing cytochromes P450, a much greater fraction of acetaminophen is detoxified through the P450 system. NAPQI is highly electrophilic and reacts with –SH and other groups. Reduced glutathione can react with NAPQI and thereby detoxify it. However, if the concentration of NAPQI exceeds that of reduced glutathione, NAPQI reacts with –SH groups of proteins.

Acetaldehyde forms adducts with DNA, such as with the amino group of guanine. At this time, the role of DNA adducts in alcohol-related tumorigenesis is not well understood. Furthermore, it is uncertain whether acetaldehyde modification of DNA methyltransferases, histones, and other proteins af ects genome maintenance.

4.3. Drugs That Help Patients Free Themselves From Alcohol Dependence

Current treatment programs for alcohol-dependent patients are only moderately ef ective. A combination of psychosocial and drug therapy is commonly used. Most patients relapse within less than a year.

T e major drugs used for the treatment of alcohol dependence are acamprosate, naltrexone, and disulfiram. Patients

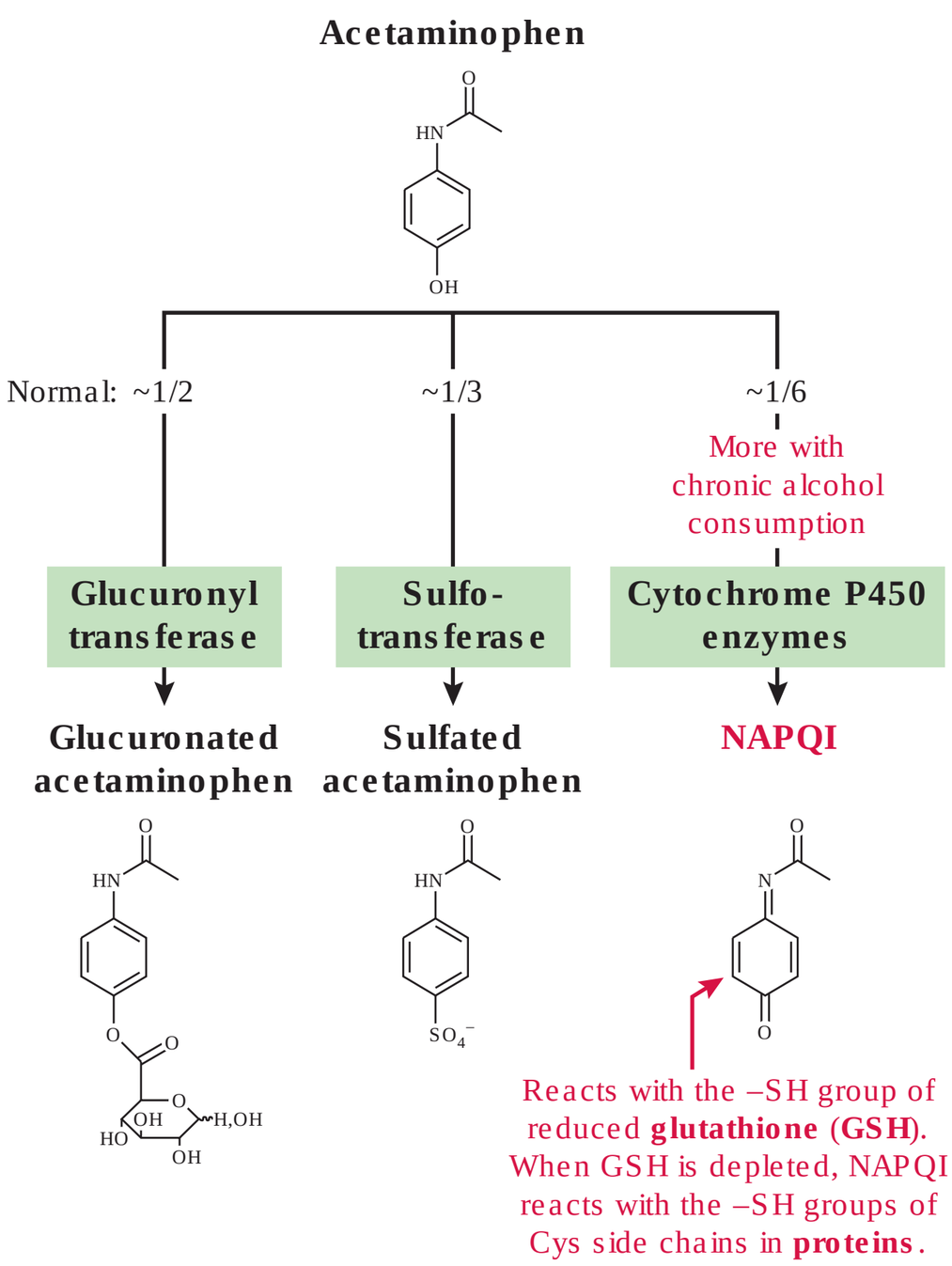


Fig. 30.6 **Metabolism of acetaminophen (paracetamol).** NAPQI, N-acetyl-*p*-benzoquinoneimine.

who regularly abuse alcohol drink alcohol because it makes them feel better or because it alleviates symptoms of withdrawal. **Acamprosate**, through a yet unknown mechanism, helps maintain abstinence. **Naltrexone**, an opioid receptor antagonist, reduces the craving for alcohol and also diminishes the "elevated" feeling after alcohol. **Disulfiram**, an irreversible inhibitor of acetaldehyde dehydrogenase (see Section 2.1), has few effects by itself. However, when a disulfiram-treated patient consumes alcohol, headache, nausea, vomiting, chest pain, and other symptoms set in due to an excessive concentration of acetaldehyde; this discourages the patient from consuming more alcohol. A disulfiram-treated patient who nonetheless drinks a large quantity of alcohol risks severe pathologic effects. An extract of the root of **kudzu**, a traditional Chinese medicine, also inhibits alcohol consumption.

4.4. Effect of Ethanol on the Liver

Substantial intake of alcohol can lead to fatty liver, liver inflammation, and liver cirrhosis. All forms of liver disease together account for about one-fifth of all alcohol-attributable deaths and potential life years lost.

Fatty liver (**hepatic steatosis**) is an early and common consequence of chronic alcohol abuse (see Fig. 30.4). The steatosis is the result of increased production and decreased export of triglycerides (see Section 3.2). The triglycerides accumulate in lipid droplets inside hepatocytes, particularly in the perivenous areas. These lipid droplets are visible by light microscopy. De novo fatty acid synthesis normally contributes only a minor portion of the fatty acids in triglycerides; this is also true for patients who consume excessive amounts of alcohol. With increasing lipid intake, the accumulation of triglycerides increases. With abstinence from alcohol, hepatic steatosis recedes.

Alcohol-induced **inflammation** of the liver (i.e., **alcoholic hepatitis**) and liver **cirrhosis** are the result of multiple pathogenic mechanisms. Thus alcohol and its metabolite acetaldehyde have direct toxic effects on proteins; they induce the expression of alcohol-metabolizing cytochromes P450 that generate free radicals, and they stimulate excessive synthesis of extracellular matrix components. In the absence of treatment, the combination of alcohol-induced fatty liver and inflammation is particularly dangerous; survival for more than 4 months is only approximately 70%.

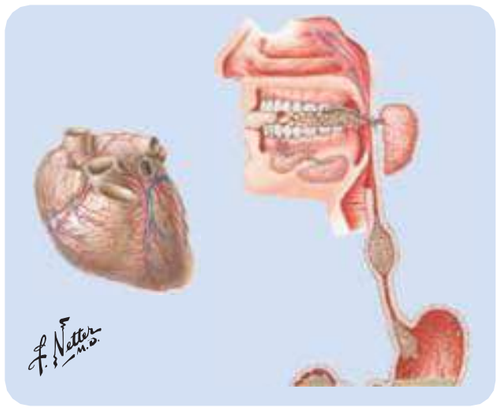
Stellate cells (fat-storing cells, Ito cells, lipocytes) in the liver play a key role in the pathogenesis of ethanol-induced **fibrosis**. Stellate cells are normally the major producers of extracellular matrix in the liver, and they also store retinoic acid esters in lipid droplets. In response to alcohol and acetaldehyde, stellate cells morph into fibroblasts that store little retinoic acid and produce extracellular matrix at an increased rate.

4.5. Effect of Ethanol on Cancer Risk

Alcohol-attributable cancer accounts for roughly one-tenth of alcohol-attributable deaths and potential life years lost. Consumption of alcohol increases a person's risk of **cancer** of the mouth, pharynx, larynx, esophagus, liver, and breast (in women). The cancer risk increases with alcohol consumption. Fig. 30.7 shows examples; note that baseline risks for the disorders differ. In the upper digestive tract, particularly the esophagus, some resident microbes convert ethanol to acetaldehyde, which is mutagenic to epithelial cells.

Mechanisms that can potentially contribute to cancer risk include acetaldehyde-induced modification of DNA; increased production of free radicals from alcohol induction of cytochromes P450; decreased concentrations of antioxidants owing to their reaction with free radicals; increased conversion of procarcinogens to carcinogens by an increased quantity of cytochrome P450 CYP2E1; inflammation of tissues; low concentrations of folates owing to malnutrition; altered metabolism of retinol and its derivatives (see also Section 4.7); and an ethanol-induced reduction in the concentration of S-adenosylmethionine, which in turn leads to reduced methylation of DNA and proteins.

Approximately three-fourths of patients who abuse alcohol also **smoke**. Cancer risk from smoking and alcohol abuse is greater than the sum of the individual risks. The combination of **alcohol and smoking** is a particularly strong risk factor for the development of cancer of the oral cavity, pharynx, and esophagus. Procarcinogens in smoke converted to



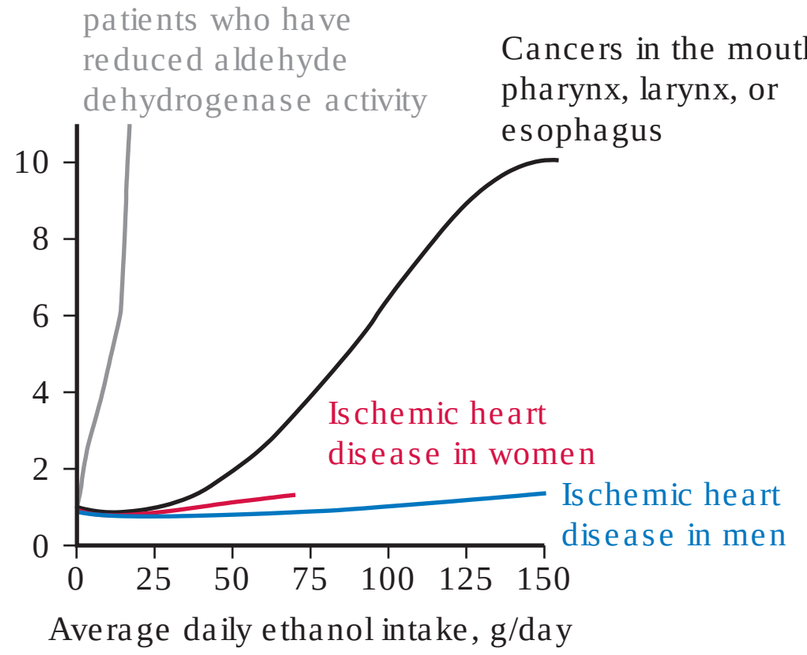


Fig. 30.7 Epidemiological relationship between alcohol consumption and odds of upper aerodigestive tract cancer or ischemic heart disease. The populations are as follows: gray line, Japan; black line, Italy and Switzerland; red and blue lines, meta-analysis of 51 studies in diverse countries. (Data from Yokoyama A, et al. Genetic polymorphisms of alcohol and aldehyde dehydrogenases and glutathione S-transferase M1 and drinking, smoking, and diet in Japanese men with esophageal squamous cell carcinoma. *Carcinogenesis*. 2002;23:1851-1859; Polesel J, et al. Estimating dose-response relationship between ethanol and risk of cancer using regression spline models. *Int J Cancer*. 2005;114:836; and Corrao G, et al. Alcohol and coronary heart disease: a meta-analysis. *Addiction*. 2000;95:1505.)

carcinogens by cytochromes P450 add to the carcinogenic activity of acetaldehyde from the metabolism of ethanol.

4.6. Effect of Ethanol on the Heart

Chronic consumption of large amounts of alcohol (≥70 g ethanol/day for ≥10 years) is associated with **hypertension** and **alcoholic heart muscle disease** (**alcoholic cardiomyopathy**).

In epidemiological studies, consumption of small to moderate amounts of alcohol (compared with abstinence) is associated with a 15% to 20% reduction in the risk for **ischemic heart disease** (a decrease that is barely noticeable in Fig. 30.7). This association does not prove causality. It is unclear whether the association is due to a positive effect of alcohol on health or due to bias (e.g., abstinence due to a chronic illness). Due to a lack of reliable data and a risk of leading patients into alcohol abuse, patients are not advised to drink alcohol for protection against ischemic heart disease.

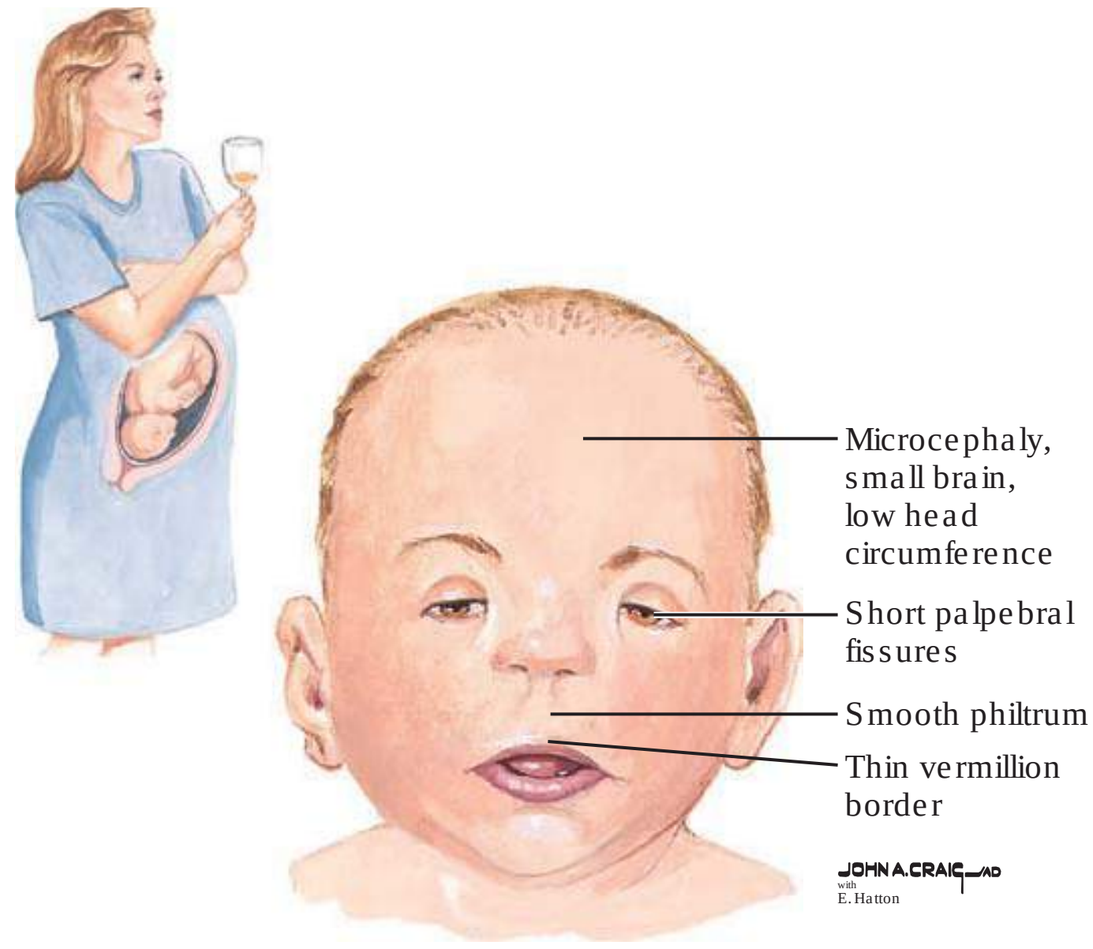


Fig. 30.8 Features of newborns with fetal alcohol syndrome.

4.7. Effect of Ethanol on the Fetus

Fetal alcohol syndrome is the consequence of a pregnant mother exposing her fetus to alcohol. Worldwide, fetal alcohol syndrome affects about 0.1% of all newborns. Less severe pathological effects of alcohol consumption are seen in about 1% of all newborns. Thus, ethanol is one of the most prevalent teratogens. Expression of the full fetal alcohol syndrome requires recurrent binge drinking (i.e., consumption of ≥75 g of alcohol per occasion) at least once a week. Yet, only a fraction of such alcohol-abusing mothers gives birth to a child with the complete fetal alcohol syndrome, most likely because genetic factors also play a role. Alcohol exposure early in pregnancy and in the last trimester (a period of extensive synaptogenesis) is especially damaging to the offspring. Whether there is a safe amount of alcohol that can be consumed during pregnancy is unknown.

In the fetus, alcohol leads to apoptosis of cranial neural crest cells as well as the abnormal migration of neurons and glial cells. Children who are affected with the most severe form of fetal alcohol syndrome have abnormal facial features (see Fig. 30.8), a growth deficit both in utero and after birth, cognitive deficits, and behavioral problems. Unfortunately, a high fraction of such persons spends time in prison or in a mental institution. Children who are only mildly affected by alcohol show only abnormal behavior.

The mechanisms by which alcohol causes neural crest cells to die by apoptosis are incompletely understood, but **retinoic acid** likely plays a role. Retinoic acid activates several transcription factors that play a role in development. Neural crest cells require retinoic acid for survival. Ethanol is expected to interfere with the oxidation of retinol (vitamin A) to retinoic acid by ADH and ALDH, as well as the degradation of retinoic acid by cytochrome P450 enzymes.

SUMMARY

- T e global daily per capita consumption of ethanol is about 13 g. In many countries, it is illegal for drivers to have a blood alcohol concentration of more than 50 to 100 mg/dL. A blood alcohol concentration more than 400 mg/dL in an alcohol-naive person can be lethal. A high concentration of alcohol leads to central nervous system depression, which may cause respiratory arrest.
- Worldwide, about equal numbers of people die from acute and chronic ef ects of alcohol. T e major acute ef ects include accidents, suicide, homicide, and poisoning; the major chronic ef ects relate to liver disease and cancer.
- T e liver metabolizes most of the consumed ethanol to acetate. In the liver, alcohol dehydrogenases (ADHs) and cytochrome P450 2E1 (CYP2E1) oxidize ethanol to acetaldehyde. In a person who does not habitually drink alcohol, CYP2E1 oxidizes only a small portion of the dietary ethanol. In a person who habitually drinks high amounts of alcohol, CYP2E1 oxidizes more ethanol than the ADHs. T e increase in CYP2E1 activity also increases the rate of formation of toxic free radicals, a highly reactive degradation product (NAPQI) of the pain reliever acetaminophen, and carcinogens from certain procarcinogens.
- Aldehyde dehydrogenase (ALDH) in the mitochondria oxidizes acetaldehyde to acetate. Homozygotes for a deficient ALDH are common in East Asia. When such persons consume alcohol, an increased amount of acetaldehyde escapes into the blood and causes flushing of the face, nausea, and overall discomfort. Af ected individuals are unlikely to become addicted to alcohol. In patients with normal ALDH activity, the inhibitor disulfiram causes a similar increase in blood acetaldehyde, flushing, and discomfort. Disulfiram is used to deter alcohol-dependent patients from consuming alcohol.
- In the liver, the oxidation of ethanol to acetate leads to a high $NADH/NAD^+$ ratio, which in turn shifts the lactate/pyruvate equilibrium toward lactate and the dihydroxyacetone phosphate/glycerol 3-phosphate equilibrium toward glycerol 3-phosphate. As a result, the concentration of substrates for gluconeogenesis is low, and the rate of gluconeogenesis is reduced. Furthermore, β-oxidation of fatty acids is inhibited, and the esterification of fatty acids with glycerol 3-phosphate is increased.
- Heart and skeletal muscle oxidize most of the acetate from ethanol metabolism to CO_2. T e activation of acetate to acetyl-CoA yields AMP. If this reaction proceeds at a high rate, some of the AMP is degraded to urate. An increase in urate production increases a person's risk for gout.
- Hydroxyethyl radicals can modify proteins; these radicals stem from CYP2E1 activity and hydroxyl radicals reacting with ethanol. Acetaldehyde together with malondialdehyde forms adducts with proteins that are immunogenic and may impair protein function. Acetaldehyde also gives rise to adducts with DNA.
- Chronic alcohol abuse often leads to fatty liver (steatosis), owing to the deposition of triglycerides in intracellular lipid droplets. In addition, it may lead to inflammation of the liver (hepatitis) and increased production of extracellular matrix, thereby giving rise to cirrhosis.
- Ethanol is toxic to the developing nervous system. Fetal alcohol syndrome results from a pregnant mother's consumption of large quantities of alcohol. Af ected persons (about 1 in 1,000) have reduced brain size, facial abnormalities, and problems with behavior.

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Review Questions

1. A 21-year-old college student is brought to the emergency department. He is unconscious after drinking large quantities of beer and liquor during a 4-hour period. T e patient showed depressed respiration and was therefore intubated and mechanically ventilated. A blood sample showed glucose 144 mg/dL (8.0 mM) and alcohol 450 mg/dL. T e urine was negative for ketones. Which of the following is the most appropriate additional care?

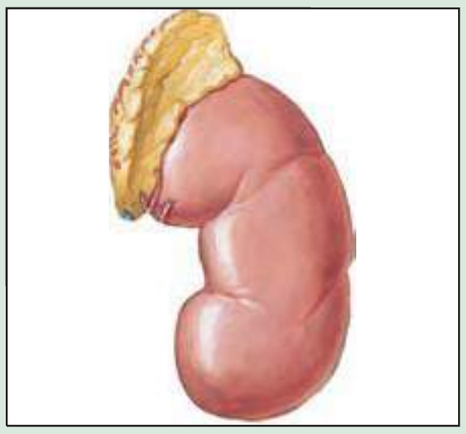
   A. Hemodialysis
   B. Infusion of disulfiram (an inhibitor of ALDH)
   C. Infusion of fomepizole (an inhibitor of ADH)
   D. Infusion of insulin
   E. Infusion of thiamine and glucose

2. In the emergency department, an alcohol-abusing patient is being treated for hypoglycemia. T e treating physician decides to inject glucagon. However, within 10 minutes, glucagon has little or no ef ect on blood glucose. T e most likely reason for the lack of ef ect is that the patient's liver contains which of the following?

   A. Too few glucagon receptors
   B. Too high a concentration of NADH to permit an adequate rate of gluconeogenesis
   C. Too little glycogen
   D. Too low a concentration of fatty acids to provide ATP for gluconeogenesis

3. A 50-year-old alcohol-addicted man has lost his job, family, and life savings. He sought help for his addiction and is being treated with disulfiram. If this patient drinks alcohol, the drug makes him feel very uncomfortable by inhibiting which one of the following processes?

   A. ADH-catalyzed oxidation of ethanol to acetaldehyde in the cytoplasm
   B. ALDH-catalyzed oxidation of acetaldehyde to acetic acid
   C. CYP2E1-catalyzed oxidation of ethanol to acetaldehyde in the endoplasmic reticulum



Chapter

31 Steroid Hormones and Vitamin D

SYNOPSIS

- Steroids are synthesized from cholesterol.
- Steroids are membrane permeable and therefore cannot be stored inside cells. Thus they are synthesized on demand. Transport of cholesterol from the outer to the inner membrane of mitochondria is normally the rate-limiting step in the synthesis of steroids.
- During transport in the blood, steroids are bound to plasma proteins. Inside target cells, steroids bind to receptors that act as transcription factors and thus alter the rate of transcription of various genes. Steroids have a short life span.
- The brain regulates the synthesis of sex steroids. Abnormalities in sex steroid synthesis can affect the development of sex characteristics. Birth control pills reduce the stimulus from the brain to follicles in the ovaries, whereas the fertility drug clomiphene has the opposite effect. Patients with sex steroid–responsive tumors are often treated with drugs that impair sex steroid–dependent growth.
- The brain also regulates the synthesis of cortisol by the adrenal glands. Synthetic analogs of cortisol are widely used in medicine as antiinflammatory and immunosuppressive drugs. Long-term and high-dose use of these drugs or excess cortisol production leads to loss of muscle mass and changes in fat deposits.
- At low blood pressure, renin and angiotensin play a role in stimulating aldosterone synthesis in the adrenal cortex. Aldosterone then increases sodium and water retention by the kidneys, thereby increasing blood pressure.
- Vitamin D is formed in the skin on exposure to ultraviolet light. A low concentration of calcium in the blood leads to increased conversion of this precursor to calcitriol, which then leads to increased expression of proteins that participate in increasing the absorption of calcium in the intestine, the recovery of calcium in the kidneys, and the release of calcium form bone.

LEARNING OBJECTIVES

For mastery *of* this topic, you should be able to do the *f*ollowing:

- Compare and contrast the mechanism of action of steroid hormones with that of peptide hormones.
- List the key steroids that are of physiological or pharmacological importance.
- Describe the female reproductive cycle and indicate points of pharmacological intervention for patients who have infertility or who use hormone-based birth control.
- Describe the mechanism of action of drugs that modulate steroid synthesis and thus have a beneficial effect on the development or treatment of breast cancer.
- Describe the regulation of blood pressure and pharmacological means of interfering with it in patients with hypertension.
- Describe the cause of Cushing syndrome. Compare and contrast the symptoms of Cushing syndrome with those of long-term corticosteroid treatment.
- Describe the synthesis of vitamin D, noting the effect of light.

1. GENERAL PROPERTIES AND SYNTHESIS OF STEROID HORMONES

Steroid hormones are synthesized *f*rom cholesterol. In response to peptide hormones, the steroid acute regulatory (StAR) protein helps transport cholesterol to the inner mitochondrial membrane and thereby start steroid synthesis. Steroids are membrane permeable and are there*f*ore synthesized on demand. Steroids exert their e*ff*ects by binding to steroid receptors, which then bind to steroid response elements and thus a*ff*ect the rate o*f* transcription.

1.1. Structure and Properties of Steroid Hormones

T e physiologically important steroids derive from cholesterol. T e core structure of steroids and the numbering of their rings and substituents is shown in Fig. 31.1.

T e steroid hormones include glucocorticoids, mineralocorticoids, and the sex steroids. Vitamin D is a secosteroid; that is, a steroid with a cut cyclohexene ring (ring B in Fig. 31.1).

Steroids are membrane permeable and are therefore made to order rather than stored in membrane-enclosed secretory vesicles. In the blood, steroids bind to plasma proteins. In target cells, steroid hormones bind to receptors in the cytoplasm and nucleus of cells. Most of these receptors are transcription factors. T e transcription factors bind to steroid response elements in promoters of genes. T us steroid hormones af ect the expression of certain genes.

1.2. Common Pathway of Steroid Hormone Synthesis

Cholesterol is the precursor of steroids. T is cholesterol can derive from cholesteryl esters stored in intracellular droplets, from cholesterol retrieved from low-density lipoprotein (LDL) or high-density lipoprotein (HDL; see Section 3 in Chapter 29), and from de novo synthesis. Steroids are mainly synthesized in the adrenal glands, ovaries, and testes.

T e first and rate-limiting step in steroid synthesis is the transport of cholesterol from the outer to the inner membrane of mitochondria (Fig. 31.2); this transport is controlled by the activity of the **steroidogenic acute regulatory (StAR) protein**. T e mechanism of transport is not understood. StAR protein activity depends on intracellular signals from membrane receptors, such as cyclic adenosine monophosphate (**cAMP)** and **Ca^{2+}**, increased concentrations of which lead to increased

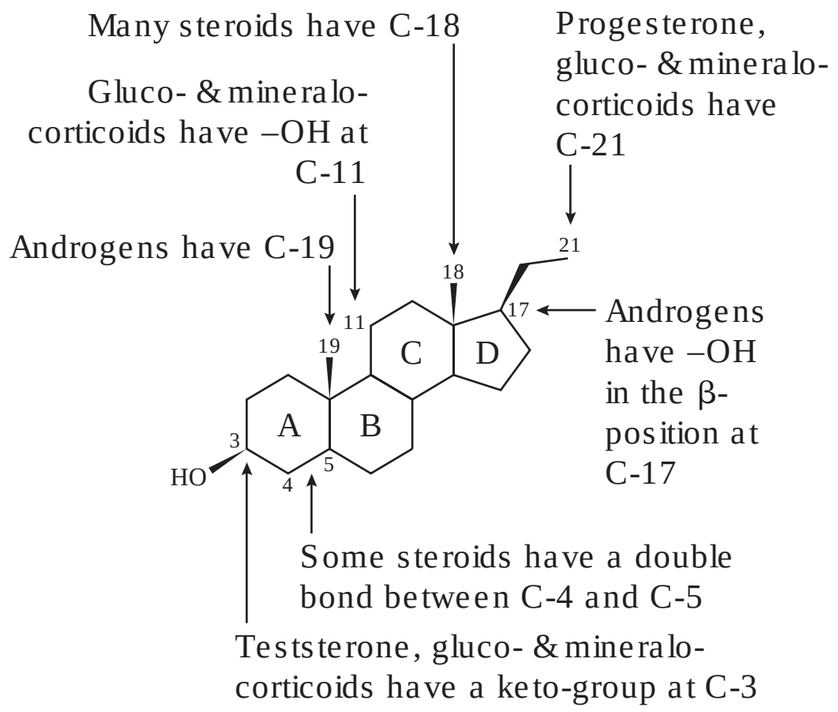


Fig. 31.1 Some structural aspects of steroids.

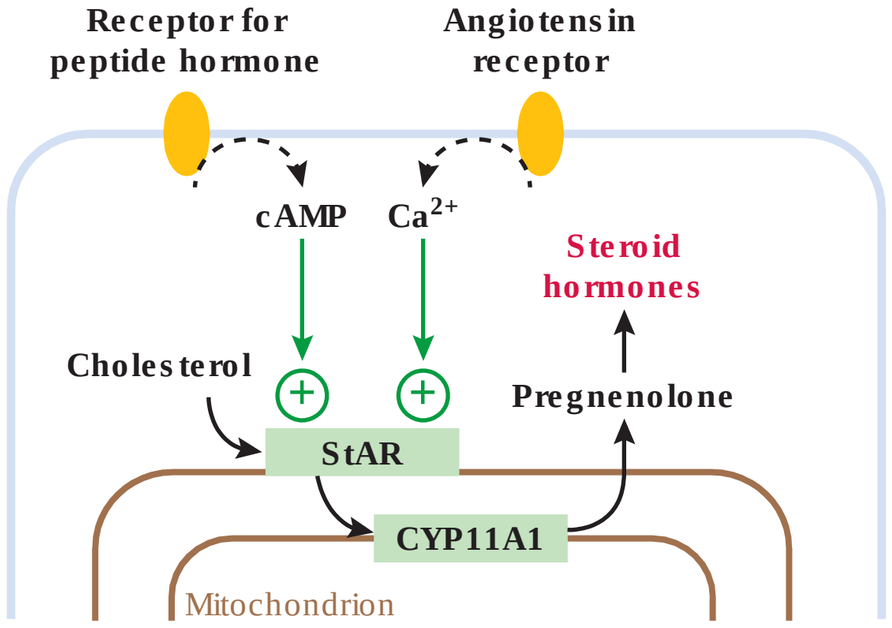


Fig. 31.2 Steroid acute regulatory (StAR) protein controls the synthesis of pregnenolone, the precursor of all steroid hormones. StAR protein can be activated in two ways. cAMP, cyclic adenosine monophosphate.

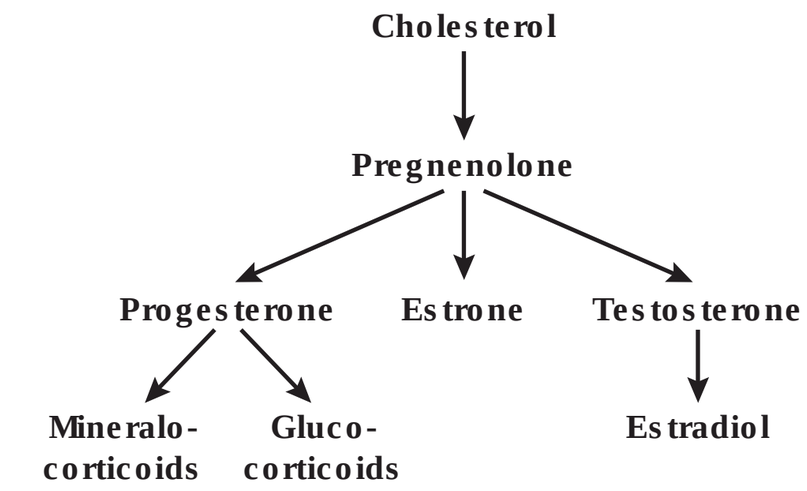


Fig. 31.3 Overview of the synthesis of steroids from pregnenolone.

transcription of the *STAR* gene, as well as increased activity of the StAR protein.

In the inner membrane of mitochondria, the **cholesterol side chain cleavage enzyme** (**CYP11A1**, **P450scc**) converts cholesterol to **pregnenolone** (see Fig. 31.2). Pregnenolone then leaves the mitochondria.

Pregnenolone gives rise to progesterone, the mineralocorticoids, the glucocorticoids, estrone, testosterone, and estradiol (Fig. 31.3).

In steroid synthesis, specificity for a particular steroid is achieved by specific enzyme expression, and the specificity of regulation depends on the type of hormone receptor (e.g., receptor for luteinizing hormone, follicle-stimulating hormone, adrenocorticotropic hormone, or angiotensin) that leads to increased transcription of the *STAR* gene.

2. SEX STEROIDS

In the brain, gonadotropin-releasing hormone (GnRH) stimulates the secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), which in turn stimulate the gonads to synthesize the sex steroids testosterone and estradiol. The testes produce testosterone, which gives rise to the more powerful dihydrotestosterone. Both testosterone and dihydrotestosterone bind to the androgen receptor. In the ovaries, developing follicles secrete 17β-estradiol, which binds to estrogen receptors. Hypogonadotrophic hypogonadism is due to insufficient signaling from the pituitary to the gonads. 46,XY disorders of sex development are due to a deficiency in synthesizing or sensing androgens. Birth control pills decrease FSH secretion from the pituitary. Patients who have androgen- or estrogen-dependent tumors are often treated with drugs that decrease sex steroid formation or signaling.

2.1. Common Pathways for the Biosynthesis of Sex Steroids

Neurons in the hypothalamus secrete **gonadotropin-releasing hormone** (**GnRH, LHRH, FSH-RH**), which stimulates the anterior pituitary gland to secrete the **gonadotropins FSH** and **LH** (Fig. 31.4). FSH and LH then stimulate gonads to produce sex steroids. GnRH is a 10–amino acid peptide. FSH and LH are dimers that consist of a common α-subunit and a unique β-subunit; both subunits are peptides of ~100 amino acids. FSH and LH bind to G protein–coupled receptors, which then give rise to an increased concentration of **cAMP**, which in turn leads to increased synthesis of **StAR** protein (see Fig. 31.2).

The biosynthesis of sex steroids in men and women follows similar pathways (Fig. 31.5). In males and females, **estradiol** (produced from testosterone) activates the closure of the **epiphyseal growth plates** of the long bones.

Hypogonadotropic hypogonadism is due to deficient secretion of functional **GnRH**, **FSH**, or **LH**, deficient sensing of GnRH. The condition is most often acquired, and there are also many genetic causes for this disorder. Patients who have **Kallmann syndrome** have congenital hypogonadotropic hypogonadism and a reduced or absent sense of **smell** (**hyposmia** or **anosmia**).

The zona reticularis of the **adrenal cortex** produces **dihydroepiandrosterone** (**DHEA**) and **androstenedione** (see Figs. 31.5 and 31.15), which both have weak androgen activity. The function of these steroids is not well understood. Normally, they play little role in sex development. However, these steroids assume a major role when their production is

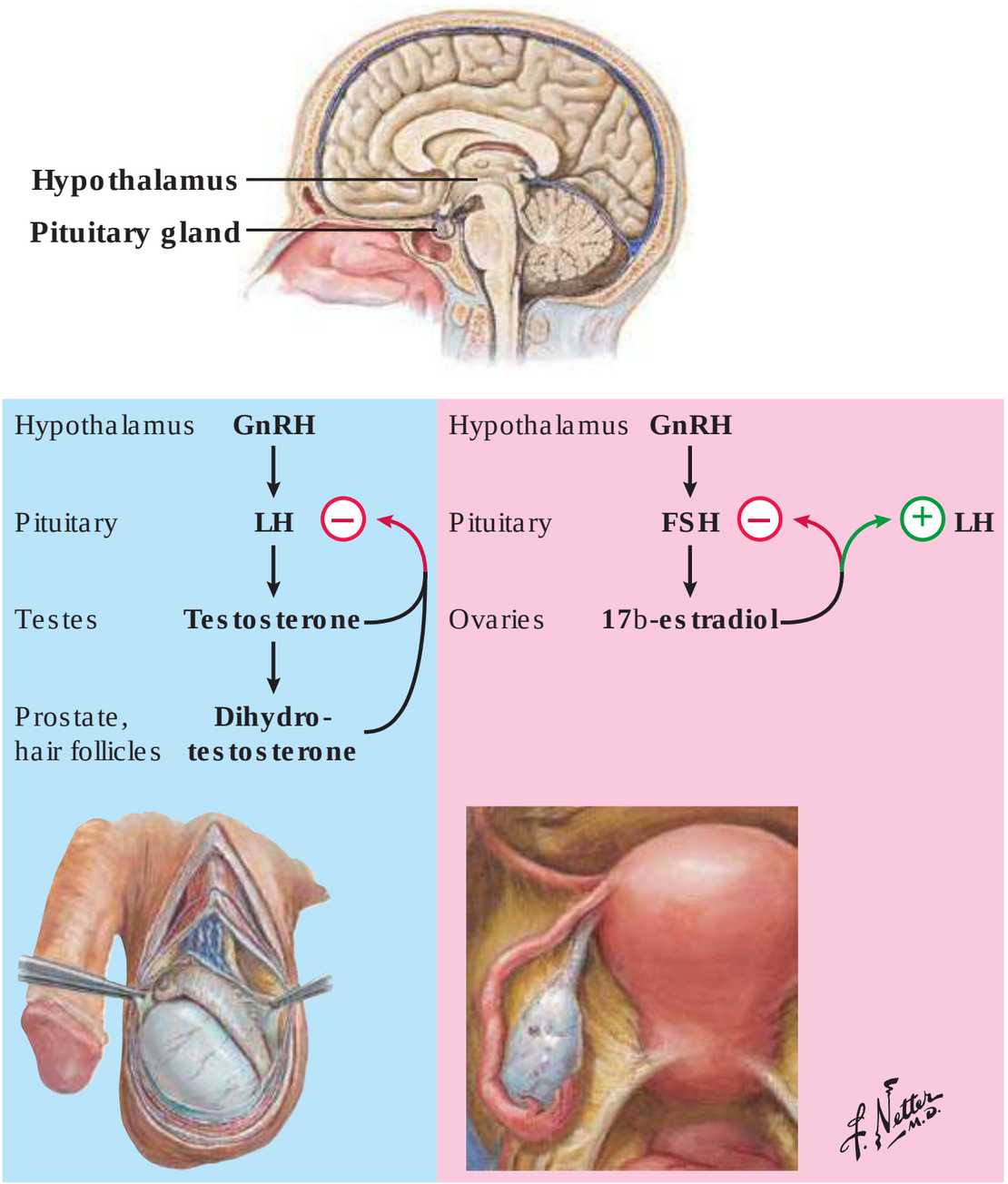


Fig. 31.4 Regulation of sex steroid synthesis in men and women. FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone.

increased due to a problem with glucocorticoid or mineralocorticoid synthesis in the other zones of the adrenal glands. DHEA in blood peaks at age ~20 years and then declines greatly until age ~65 years. **DHEA** is available as a supplement, but long-term safety and benefits to the general population are unclear.

2.2. Biosynthesis of Sex Steroids in Men

The primary sex steroids produced by men are **testosterone** and **dihydrotestosterone** (see Fig. 31.4). Testosterone synthesis occurs predominantly in the **Leydig cells** of the **testes** (Fig. 31.6) and is controlled by **LH**. When LH binds to the LH receptor, the concentration of cAMP inside Leydig cells rises, StAR protein is activated, more StAR protein is made, and the synthesis of testosterone increases. From the Leydig cells, testosterone reaches the blood. The **prostate** and **hair follicles** convert testosterone to dihydrotestosterone, which they release into the blood. Compared to testosterone, dihydrotestosterone has considerably higher affinity for the **androgen receptor**.

In the blood, both testosterone and dihydrotestosterone bind to **sex-hormone binding globulin** (**SHBG**). SHBG stems mainly from the liver. The concentrations of testosterone and dihydrotestosterone versus the concentration of SHBG determine the concentration of free testosterone and dihydrotestosterone. In males, the concentration of SHBG is high during childhood and then drops to about one-third of that in puberty, thereby increasing the fraction of free testosterone and dihydrotestosterone.

Androgen receptors are found mostly in the cytosol of a variety of cells, where they are bound to a **heat-shock protein**. After binding dihydrotestosterone or testosterone, androgen receptors (without heat-shock protein) move into the nucleus and bind to an **androgen response element** in the promoter of various genes.

Activation of androgen receptors is required for normal development of the **prostate** and the male external **genitalia**, and later also for male pattern **baldness**.

Testosterone and dihydrotestosterone exert feedback inhibition on LH secretion from the pituitary gland such that the concentration of testosterone in the blood of adult men is maintained at ~6 ng/mL until andropause sets in.

In males, **FSH** stimulates **Sertoli cells**, which support the development of **sperm** inside seminiferous tubules (see Fig. 31.6). Sertoli cells also synthesize and secrete **inhibin**, which feedback inhibits FSH secretion from the pituitary gland.

Patients who have a **prostate cancer** that depends on androgens for growth (i.e., castration-sensitive prostate cancer) respond favorably to a reduction in circulating androgen (**androgen deprivation therapy**), which can be achieved in four ways: (1) use of an inhibitor of androgen synthesis, such as **abiraterone**; (2) use of an **antiandrogen**, that is, a drug that prevents the binding of dihydrotestosterone and testosterone to the androgen receptor, such as **bicalutamide, flutamide**, and **enzalutamide**; (3) use of a GnRH agonist, which leads to an initial surge in testosterone secretion that is 10 or more days later followed by depressed secretion; and (4) use of a GnRH antagonist, such as **degarelix**. After ~2 years of androgen deprivation therapy, most castration-sensitive tumors become castration insensitive.

2.3. 46,XY Disorder of Sex Development

At ~5 weeks, the fetus develops two **gonadal ridges** (Fig. 31.7). Along these ridges run a pair of **müllerian ducts** and a pair of **wolffian ducts**. At ~6 weeks, the fetus starts turning the gonads into **ovaries** or **testes**, which start to produce sex steroids. Under the influence of androgens, the wolffian ducts give rise to the epididymides, vas deferens, and seminal vesicles; under the influence of estrogens, the müllerian ducts give rise to the fallopian tubes, the uterus, and part of the vagina. The **sex steroids** regulate the development of the **external genitalia**. During puberty, androgens stimulate the growth of facial hair and a deepening of the voice, whereas estrogens stimulate the development of female breasts.

Patients who have a 46,XY karyotype and a **disorder of sex development** (**DSD**) fail to synthesize sufficient dihydrotestosterone or fail to respond normally to androgens. A number of these persons appear female at birth and are raised as females.

Among children and partially virilized women with 46,XY DSD, mutations occur in a number of genes, such as those

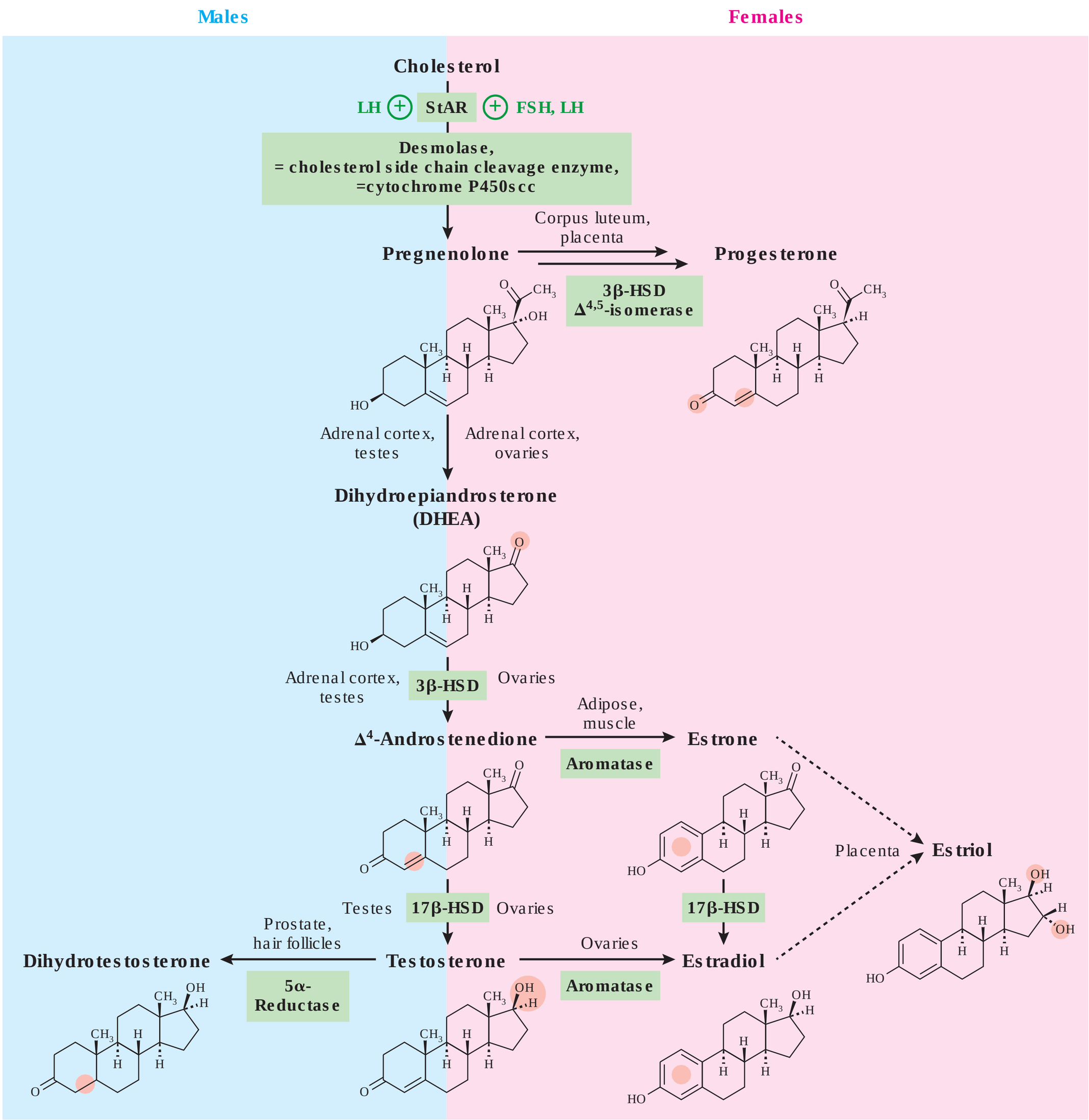


Fig. 31.5 **Overview of the synthesis of sex steroids in men and women.** FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; HSD, hydroxysteroid dehydrogenase; LH, luteinizing hormone; StAR, steroid acute regulatory protein. 5α-Reductase is also called 3-oxo-5α-steroid 4-dehydrogenase ($NADP^+$).

coding for **17β-hydroxysteroid dehydrogenase type 3**, **5α-reductase**, or the **androgen receptor** (see Fig. 31.5). Many of these patients seek medical attention due to lack of onset of menstruation with puberty or due to infertility.

Depending on the severity of **5α-reductase deficiency**, newborns with a 46,XY karyotype may have male, ambiguous, or female external genitalia. After puberty, most of the persons who are raised as girls identify with the male gender.

Persons who have 46,XY DSD and a **deficiency** of **17β-hydroxysteroid dehydrogenase type 3** (the enzyme that synthesizes testosterone in the testes) often have female external genitalia at birth, are often raised as girls and then show virilization at puberty.

Androgen insensitivity syndrome (formerly called **testicular feminization**) is caused by a mutant **androgen receptor** with decreased function (Fig. 31.8). T e gene for the

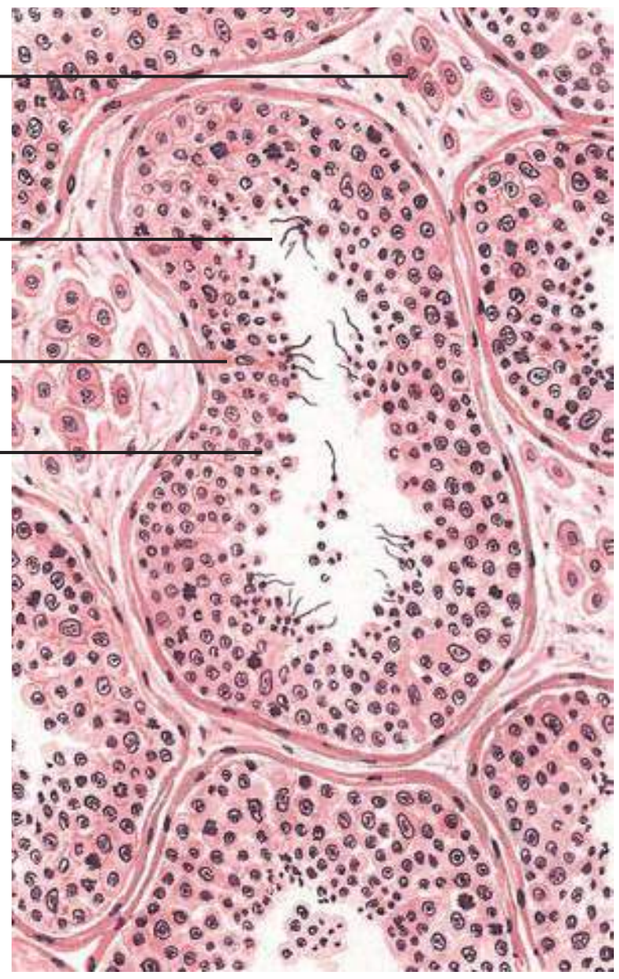


Fig. 31.6 **Histology of the testes.**

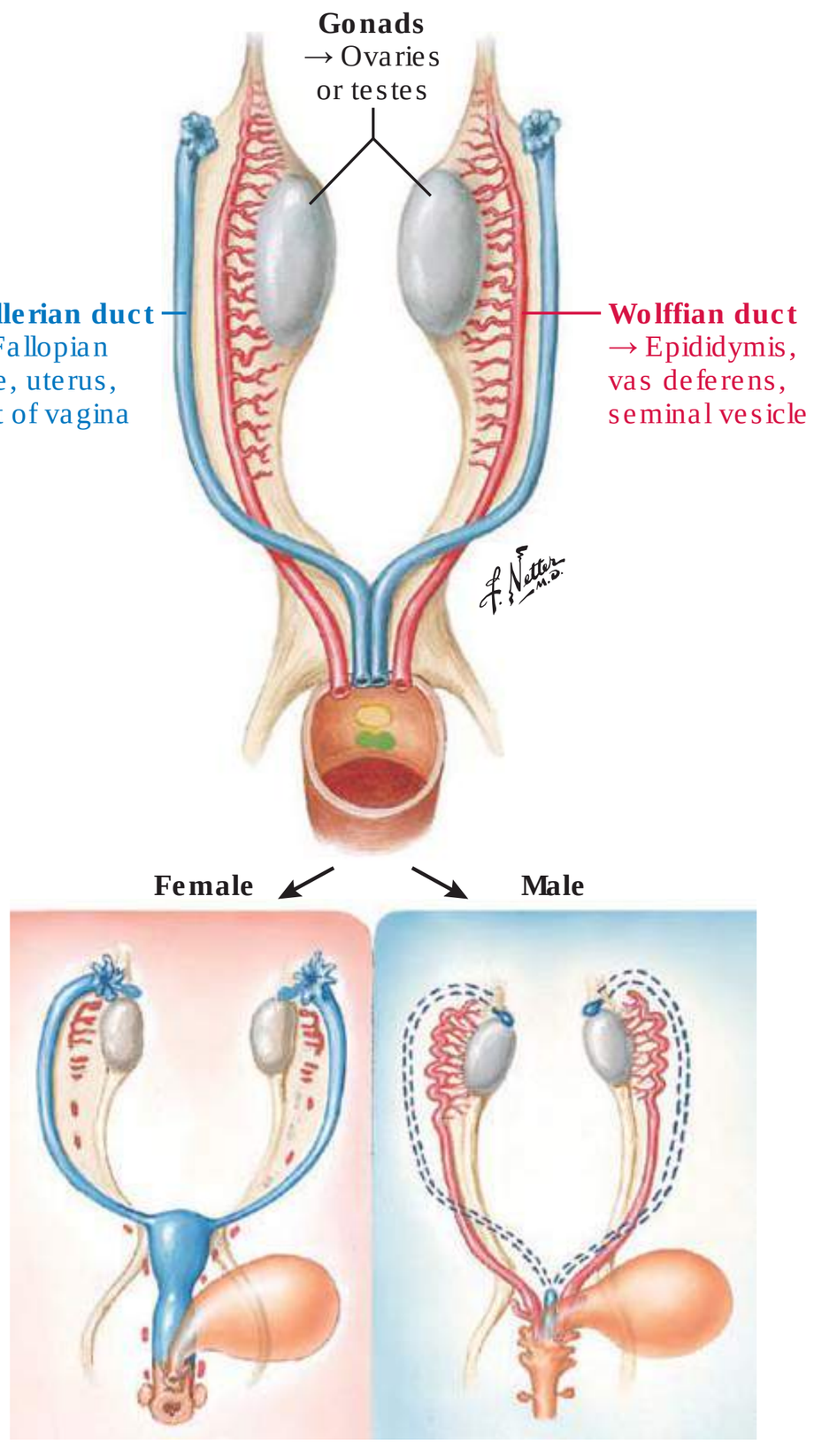


Fig. 31.7 **Development of female and male sex organs.**

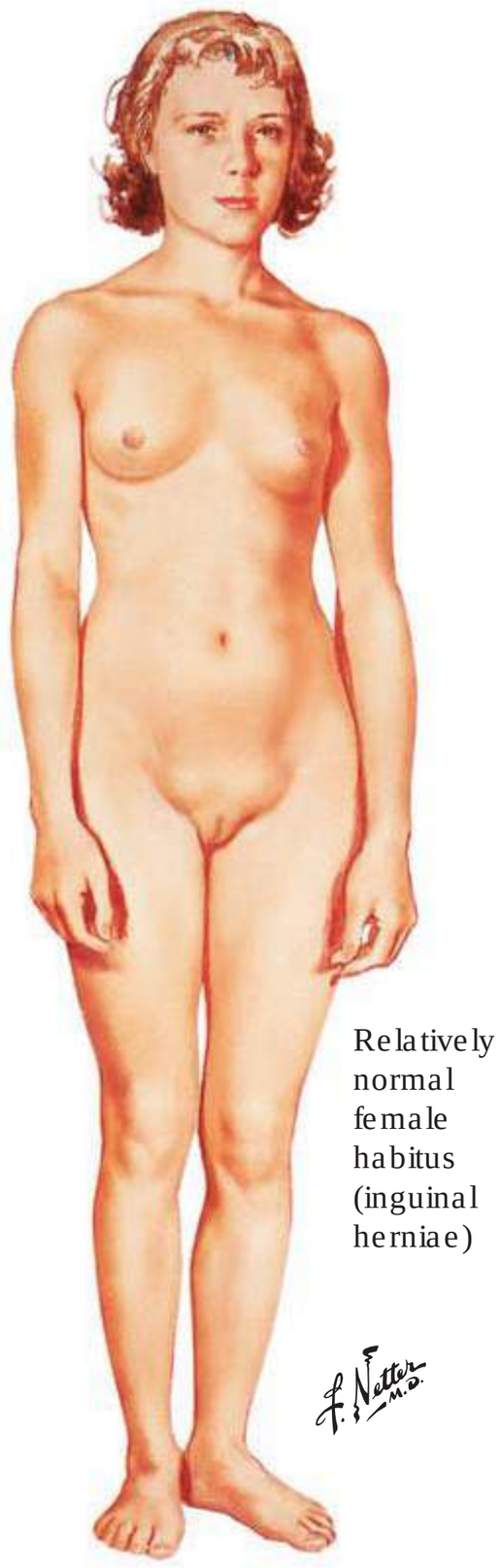


Fig. 31.8 **Androgen insensitivity syndrome, a 46,XY disorder of sex development.**

androgen receptor is on the X chromosome, and the disorder is a problem mainly in persons who have a Y chromosome. T e degree of androgen receptor function influences sexual development in utero and during puberty. **Complete androgen insensitivity** (**CAIS**) is often distinguished from **partial androgen insensitivity** (**PAIS**).

Persons with **CAIS** typically look female at birth, have a mildly enlarged clitoris, and lack a uterus. T ey have internal testes and are infertile. T ey are most often diagnosed around the time of puberty, because no menstruation occurs. T e incidence is ~1 in 20,000 persons. During puberty, the testes produce excessive amounts of testosterone, which is then converted to estrogen and thus induces the development of breasts.

Persons with **PAIS** typically have hypospadias, a small penis, and a bifid scrotum at birth. Patients with the mildest form of PAIS look normal but are infertile.

Patients with CAIS or PAIS may have their gonads removed to reduce the risk of germ-cell tumors. Hormone replacement therapy is then instituted (typically estrogen to maintain

female characteristics or testosterone to maintain male characteristics).

While several dif erent loss-of-function mutations in the androgen receptor give rise to PAIS or CAIS, a pathogenic elongation of a **CAG trinucleotide repeat** in exon 1 of the androgen receptor gene gives rise to **spinal and bulbar muscular atrophy**. T e normal CAG repeat length is ~20, and more than ~40 repeats are pathogenic. CAG encodes glutamine, and the CAG repeat length therefore determines the length of a glutamine tract in the androgen receptor. A pathogenic polyglutamine tract leads to the formation of protein aggregates in lower motoneurons in the brainstem and spinal cord. Around the age of 30 to 60 years, af ected individuals can develop muscle cramps, muscle fasciculations during contractions, muscle weakness, difficulty speaking and swallowing, and inability to walk.

2.4. Biosynthesis of Sex Steroids in Women

In women, the physiologically produced estrogens include **estradiol**, **estriol,** and **estrone**, which dif er in the substituents they carry on the D-ring (see Fig. 31.1). 17β-Estradiol is the most potent estrogen. Estriol is chiefly produced by the placenta during pregnancy. Estrone is the main estrogen in women after menopause.

In women (as in men), the hypothalamus secretes **GnRH**, which in turn stimulates the anterior pituitary to secrete **FSH** (see Fig. 31.4).

FSH stimulates **ovarian follicles** to produce and secrete **17β-estradiol**, as well as **inhibin**. As follicles are recruited and grow in size, the concentration of 17β-estradiol in the blood increases during a period of ~2 weeks (Fig. 31.9). 17β-Estradiol feedback inhibits the secretion of GnRH and FSH (see Fig. 31.4). T is inhibition in turn limits the number of active follicles in the ovaries. In an additional feedback loop, the follicles also produce inhibin, which decreases FSH secretion from the pituitary.

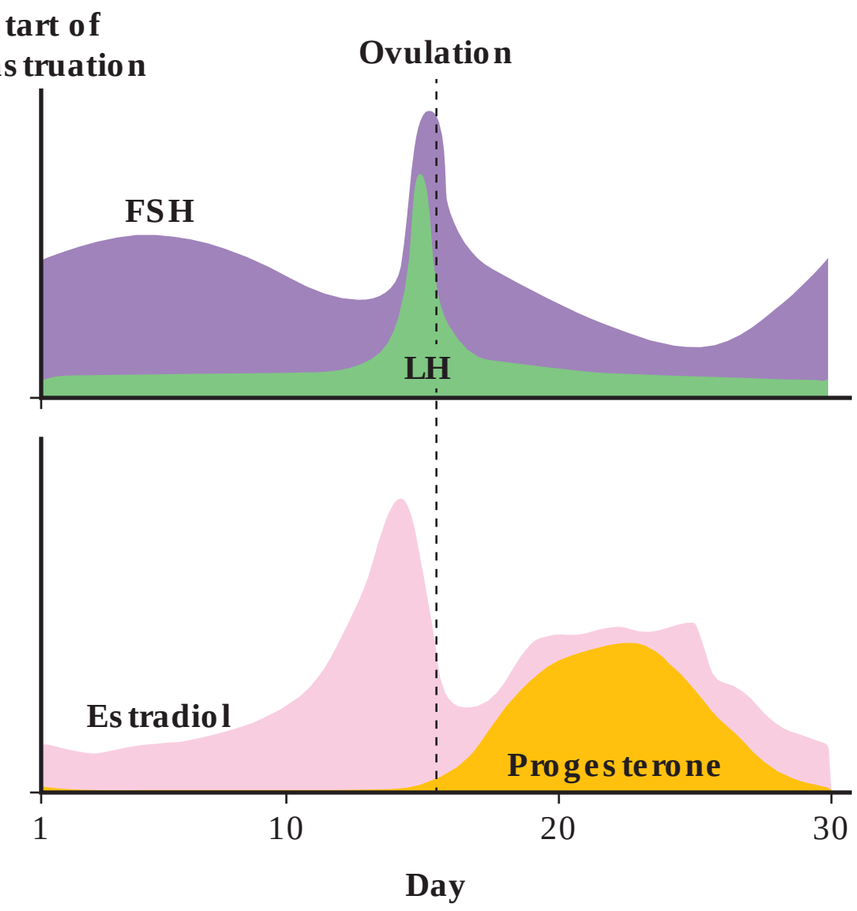


Fig. 31.9 Concentrations of follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol, and progesterone during the menstrual cycle. (Data from Häggström M. Reference ranges for estradiol, progesterone, luteinizing hormone, and follicle-stimulating hormone during the menstrual cycle. *WikiJournal of Medicine*. 2014;1[1].)

Clomiphene, a selective estrogen receptor modulator, is used in the treatment of **infertility** in women. Clomiphene impairs the estradiol-dependent feedback inhibition of GnRH secretion. As a result, more GnRH and FSH are secreted, and the ovaries produce more active follicles and more 17β-estradiol.

When the concentration of 17β-estradiol is high (>200 pg/mL) for at least 15 hours, it triggers the secretion of **LH** from the anterior pituitary. LH helps start ovulation; that is, the dominant follicle ejects its egg (Fig. 31.10). T e less-developed follicles do not ovulate and atrophy.

After ovulation, follicular cells give rise to the **corpus luteum** (see Fig. 31.10), which synthesizes both 17β-estradiol and **progesterone** (see Fig. 31.5).

Progesterone inhibits the secretion of **LH** from the anterior pituitary. As the concentration of LH falls in the absence of a fertilized embryo (see Fig. 31.9), progesterone synthesis in the corpus luteum also ceases, and the corpus luteum dies. As the concentrations of progesterone and estradiol decrease, **menstruation** (Fig. 31.11) sets in. With menstruation, much of the inner lining of the uterus (endometrium) is degraded and expelled. However, if a **pregnancy** is established, the embryo produces **human chorionic gonadotropin** (**hCG**), which allows the corpus luteum to survive and synthesize progesterone for a while. Eventually, the placenta produces its own progesterone and the corpus luteum involutes. An increased concentration of progesterone promotes dif erentiation of the mammary glands. T e placenta also produces both estradiol and **estriol**, which leads to a pronounced increase in the total concentration of estrogens as the pregnancy progresses.

T e concentration of **hCG** in blood is commonly measured to screen for **pregnancy**.

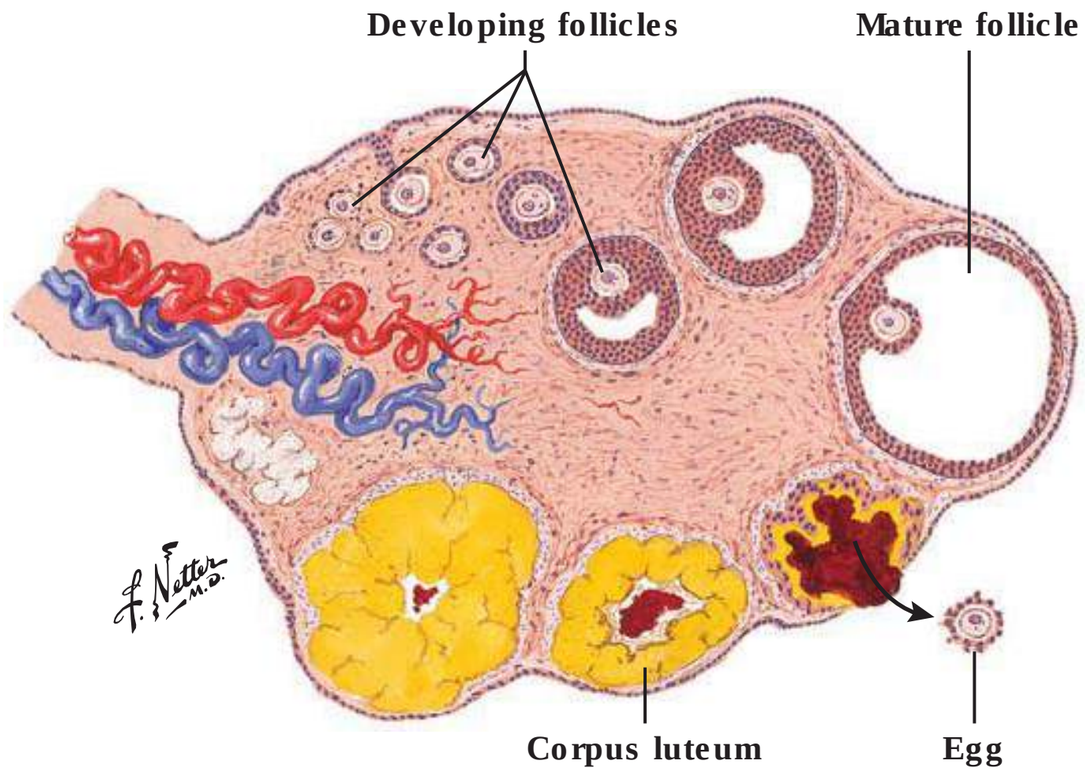


Fig. 31.10 Development of a dominant follicle and of a corpus luteum in an ovary during the menstrual cycle. Development over time is shown clockwise. Many follicles develop at the same time, but one becomes the dominant follicle. The release of two eggs in one cycle, if fertilized, leads to a pregnancy with fraternal twins.

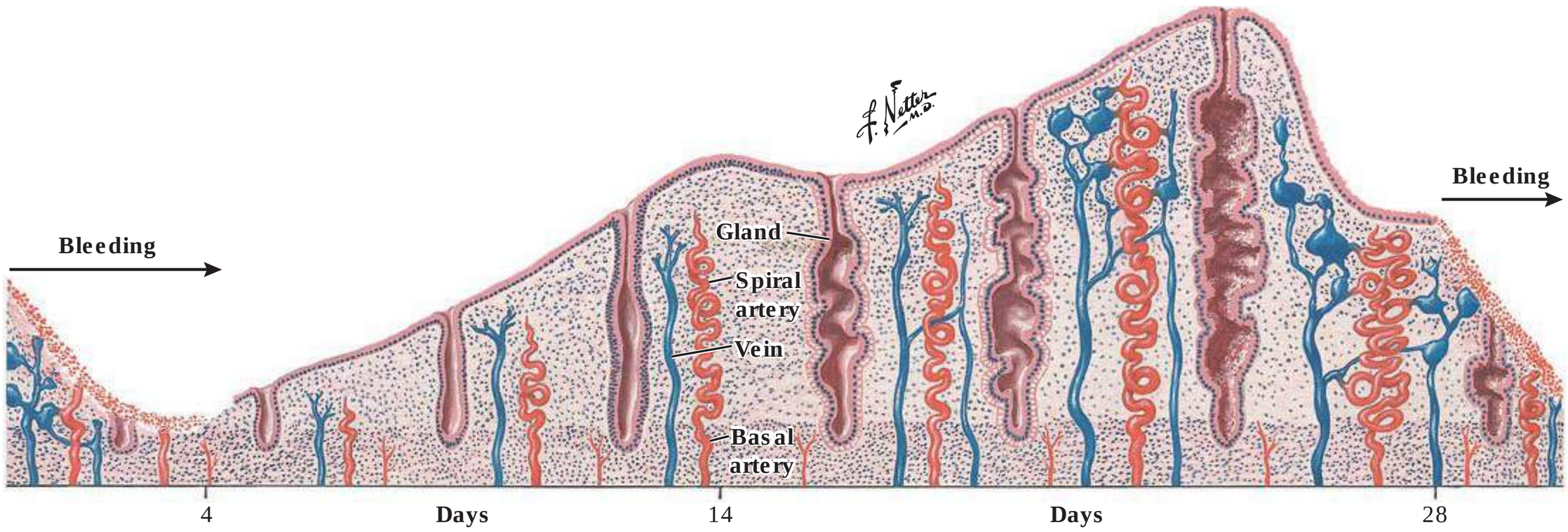


Fig. 31.11 **Changes in the inner lining of the uterus during the menstrual cycle.**

In the second trimester of pregnancy, women are often screened for a fetus with neural tube defects, Down syndrome (trisomy 21), and Edward syndrome (trisomy 18). The **quad screen** contains measurements of α-fetoprotein, hCG, estriol, and inhibin A. The **penta screen** also contains a measurement of hyperglycosylated hCG. When the fetus has anencephaly, for example, the concentration of estriol in maternal blood is only ~10% of the normal concentration. When the fetus has Down syndrome (trisomy 21), the concentration of inhibin A is unusually high.

About 60% of patients with **polycystic ovary syndrome** (PCOS), a condition that affects ~5% to 15% of women of reproductive age (depending on criteria used), have an excess concentration of androgens (including testosterone and androstenedione; see Fig. 26.12). These patients are typically also insulin resistant and have reduced fertility. Besides PCOS, congenital adrenal hyperplasia (see Section 4.2), with its attendant high concentration of androgens, also gives rise to polycystic ovaries.

Oral **contraceptives** for women usually contain an **estrogen** and a **progestin** (a synthetic drug with progestational activity). The concentrations of these drugs in blood are high enough to suppress the secretion of FSH and LH from the brain. Without sufficient FSH, ovarian follicles do not develop and ovulation does not occur. Cessation of such a contraceptive leads to menstruation.

A high-dose **progestin** alone, such as medroxyprogesterone (Depo-Provera; injected every 3 months) also inhibits secretion of GnRH and FSH, thus inhibiting development of the follicles. The drug slso inhibits LH secretion.

In **postmenopausal women**, synthesis of estradiol is minimal, and **estrone** produced in adipose tissue and skeletal muscle becomes the major estrogen in the circulation. The precursor for this estrone is androstenedione (see Fig. 31.5), which is produced by the adrenal glands and from there reaches the bloodstream.

In obese patients, the increased mass of adipose tissue converts more androstenedione to estrone. In morbidly obese patients, the concentration of circulating estrone can be as much as 10-fold that in a lean person. Estrone stimulates the growth of the endometrial lining and thereby increases the chance of hyperplasia and cancer of the endometrium.

During and after menopause, exogenous estrogen (as part of **hormone therapy** after menopause) can sharply reduce the incidence of **hot flashes**. However, long-term oral estrogen plus progestin therapy increases a woman's risk for breast cancer and endometrial cancer.

Selective estrogen receptor modulators are used in the treatment of **osteoporosis** and as adjuvant treatment for **breast cancer**. The drugs used for this purpose have mixed activating and inhibitory effects on estrogen receptors in part because they do not induce the exact same conformational changes of the DNA- and protein-binding domains of estrogen receptors as does estrogen. **Tamoxifen** and **raloxifene** are both used to reduce the risk of breast cancer (chemoprevention) in women who are at high risk (these drugs are predominantly used for adjuvant therapy after surgery or radiation in women who had estrogen receptor–positive tumors). Raloxifene is also used in the treatment of osteoporosis (it has a weak estrogen-like effect on bone). **Toremifene** is used to treat estrogen receptor–positive metastatic breast cancer.

Aromatase inhibitors are used in the treatment of estrogen receptor–positive breast cancer. These tumors depend on activation of estrogen receptors for growth. Some of the currently used aromatase inhibitors (e.g., anastrozole, letrozole) show **competitive** inhibition of aromatase. Others, like exemestane, are **suicide** inhibitors of aromatase.

3. GLUCOCORTICOIDS

In the brain, circadian and stress signals stimulate corticotropin-releasing hormone secretion, which in turn stimulates ACTH secretion, which then stimulates the adrenal cortex to synthesize the glucocorticoid cortisol. Cortisol exerts its effects by binding to a receptor that acts as a transcription factor. Synthetic glucocorticoids are widely used in medicine for their antiinflammatory and immunosuppressive effects. Cushing syndrome is due to excess endogenous

cortisol production. Exogenous Cushing syndrome is induced by protracted use of exogenous glucocorticoid drugs.

The term **glucocorticoid** derives from the words glucose (adrenal) cortex, and steroids. The main physiological glucocorticoid is **cortisol** (**hydrocortisone**).

The brain controls cortisol secretion (Fig. 31.12). The hypothalamus secretes **corticotropin-releasing hormone** (**CRH**), which stimulates the pituitary gland to secrete **ACTH**. ACTH then stimulates the cortex of the adrenal glands to secrete **cortisol**. (The adrenal glands weigh about 4 g each.) Cortisol in turn feedback inhibits the secretion of CRH and ACTH. (The adrenal glands also produce DHEA and androstenedione, as well as aldosterone).

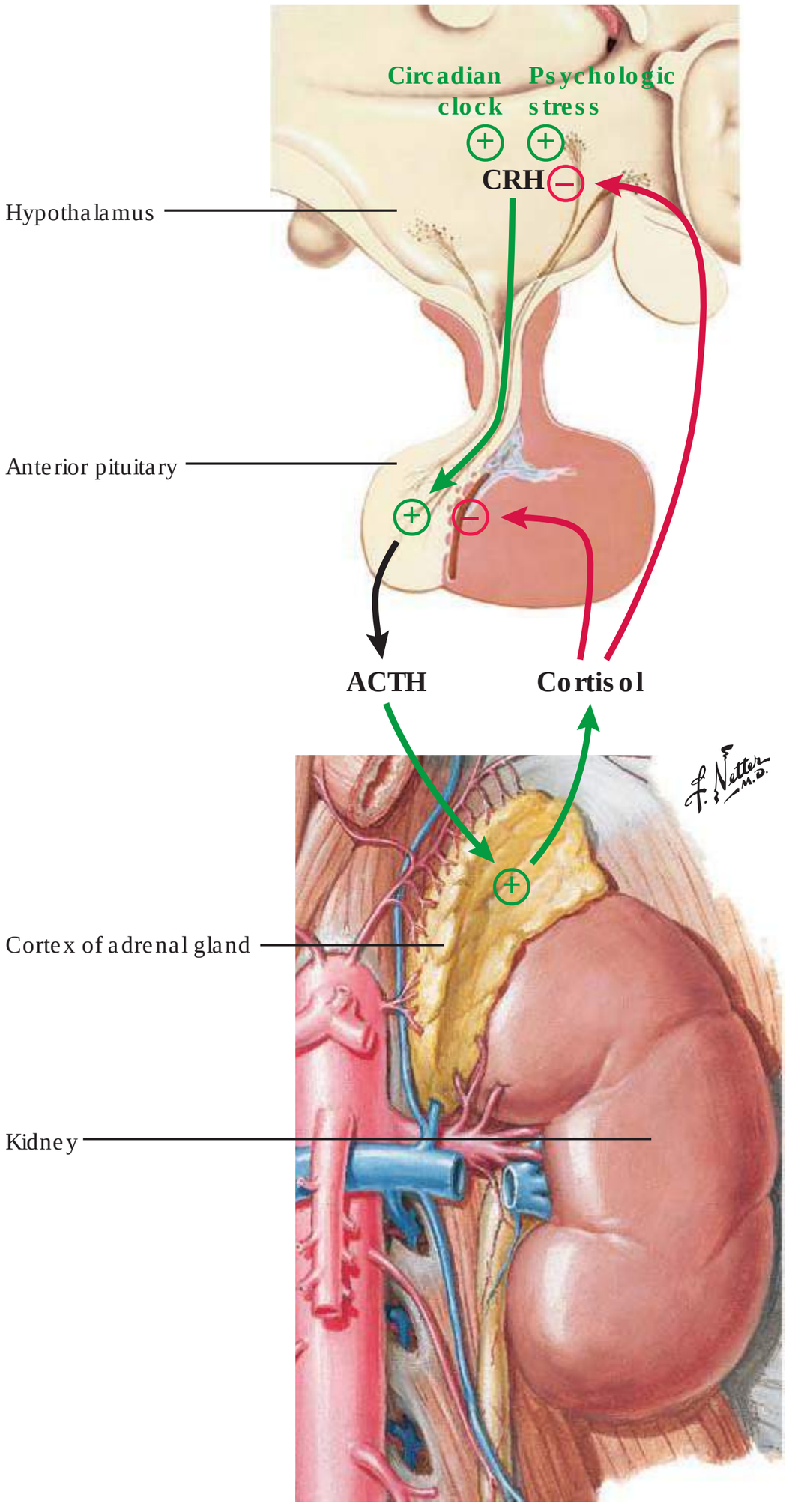


Fig. 31.12 Regulation of cortisol secretion. ACTH, adrenocorticotropic hormone; CRH, corticotropin-releasing hormone.

The concentration of cortisol in the blood changes markedly through the course of a day (Fig. 31.13). The **circadian clock** in the hypothalamus provides a cyclic, basal stimulus for the secretion of **CRH**. In addition, under conditions of **psychological stress**, neurons in the brain use the peptide hormone **pituitary adenylate cyclase-activating polypeptide** (PACAP) to further stimulate CRH secretion.

The synthesis of cortisol by the adrenal glands proceeds according to the reactions shown in Fig. 31.14. The cortex of the adrenal glands contains three different zones (Fig. 31.15) that produce cortisol, aldosterone, and DHEA plus androstenedione, respectively.

In the blood, glucocorticoids (and to a lesser degree progesterone and aldosterone) bind to **transcortin** (**corticosteroid-binding globulin**).

In cells, glucocorticoids bind to **glucocorticoid receptors** in the cytosol, which then translocate to the nucleus, where they bind to glucocorticoid response elements and thus stimulate transcription of certain genes (see Chapter 6); this in turn leads to the synthesis of proteins, which decrease inflammation, promote the export of amino acids from muscle and their import into the liver, stimulate gluconeogenesis and lipolysis, and decrease glucose use by muscle. Glucocorticoids also have an additional antiinflammatory effect that is mediated by the glucocorticoid receptor but does not require increased transcription.

A **deficiency** of glucocorticoids can lead to hypoglycemia, neurological problems, and failure to thrive. Patients with **Addison disease** show autoimmune destruction of the cortex of the adrenal glands, which causes a combined deficiency of glucocorticoids and mineralocorticoids (see Section 4). Many patients with the rare disease **familial glucocorticoid deficiency** have insufficient ACTH receptor function in the adrenal glands and hence diminished cortisol synthesis.

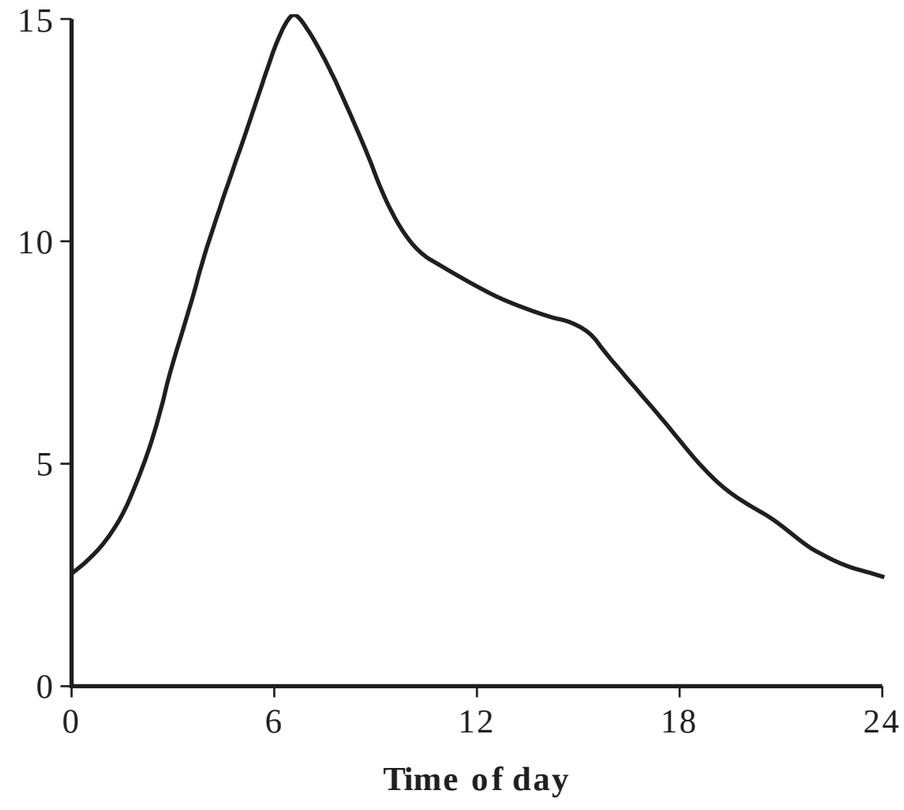


Fig. 31.13 Concentration of cortisol in blood in the course of a day. (Data from Dimitrov S, Benedict C, Heutling D, et al. Cortisol and epinephrine control opposing circadian rhythms in T cell subsets. *Blood*. 2009;113:5134; and Hurwitz S, Cohen RJ, Williams GH. Diurnal variation of aldosterone and plasma renin activity: timing relation to melatonin and cortisol and consistency after prolonged bed rest. *J Appl Physiol*. 2004; 96:1406.)

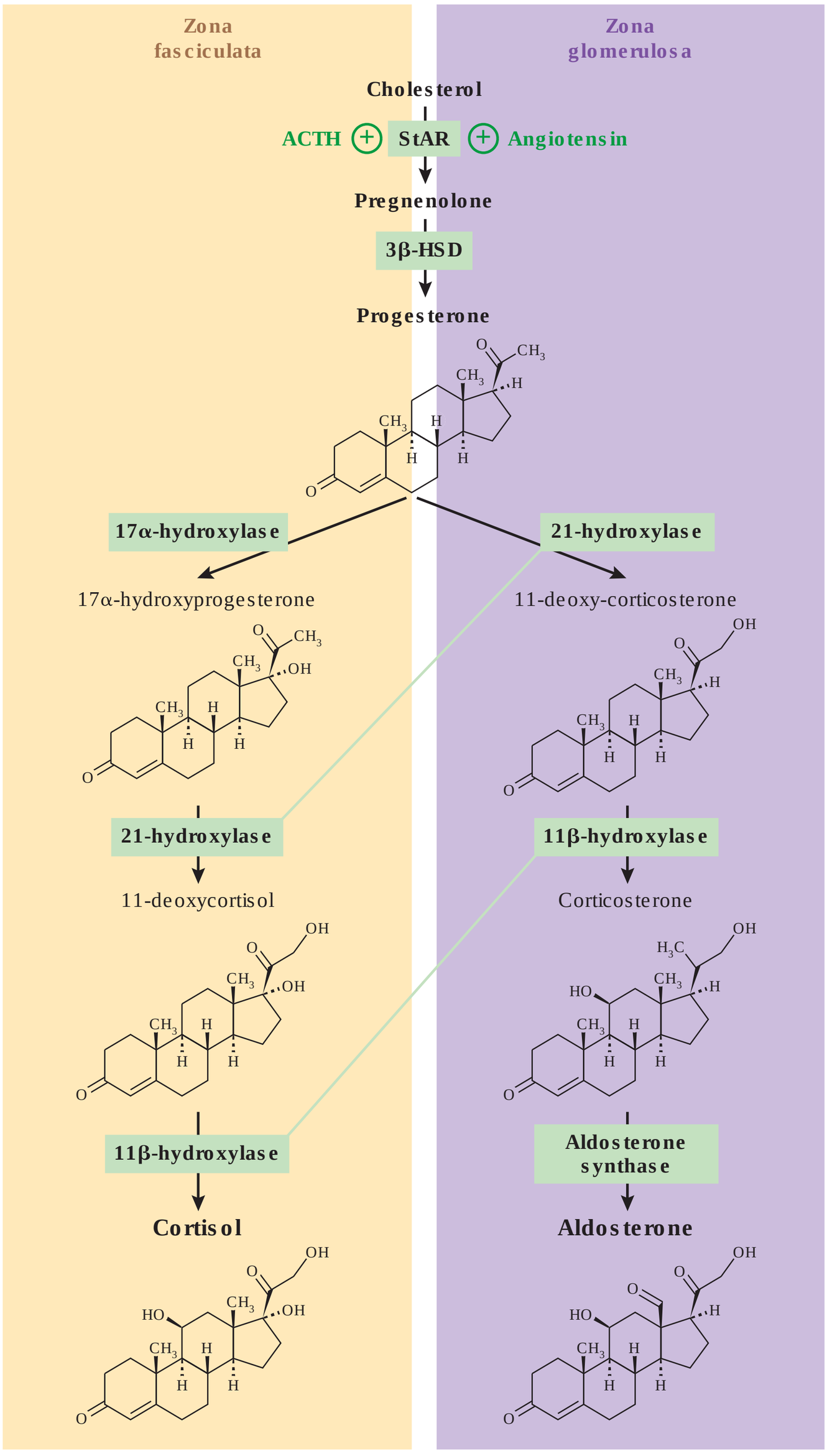
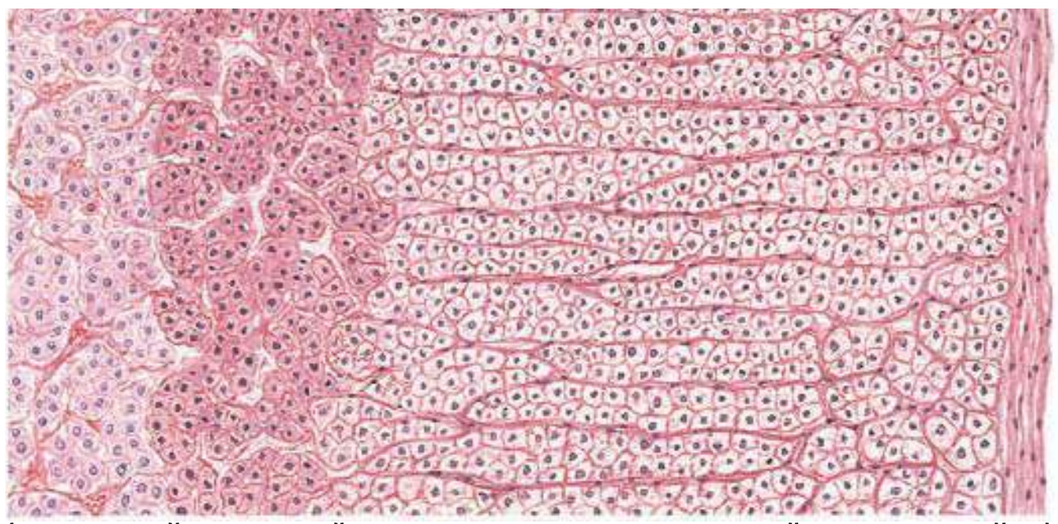


Fig. 31.14 Biosynthesis of glucocorticoids and mineralocorticoids in the adrenal glands. Lines that link enzymes indicate that these enzymes are identical. ACTH, adrenocorticotropin hormone; HSD, hydroxysteroid dehydrogenase; StAR, steroid acute regulatory protein.

Excessive production of glucocorticoids gives rise to **Cushing syndrome** (Fig. 31.16). Cortisol secretion is excessive due to a pituitary tumor that secretes ACTH or a tumor in an adrenal gland that secretes cortisol. T e excessive concentration of circulating cortisol causes degradation of muscle protein and an increase in the rate of gluconeogenesis. Symptoms of Cushing syndrome include muscle weakness (limbs may show muscle wasting), wide purplish striae, fat deposition above the collar bone, and obesity (especially in the face, neck, trunk, and abdomen). T e elevated concentration of glucocorticoids also leads to insulin resistance (the mechanism of this alteration remains unknown). Hence,



Zona reticularis, makes **DHEA** and **androstene-dione**

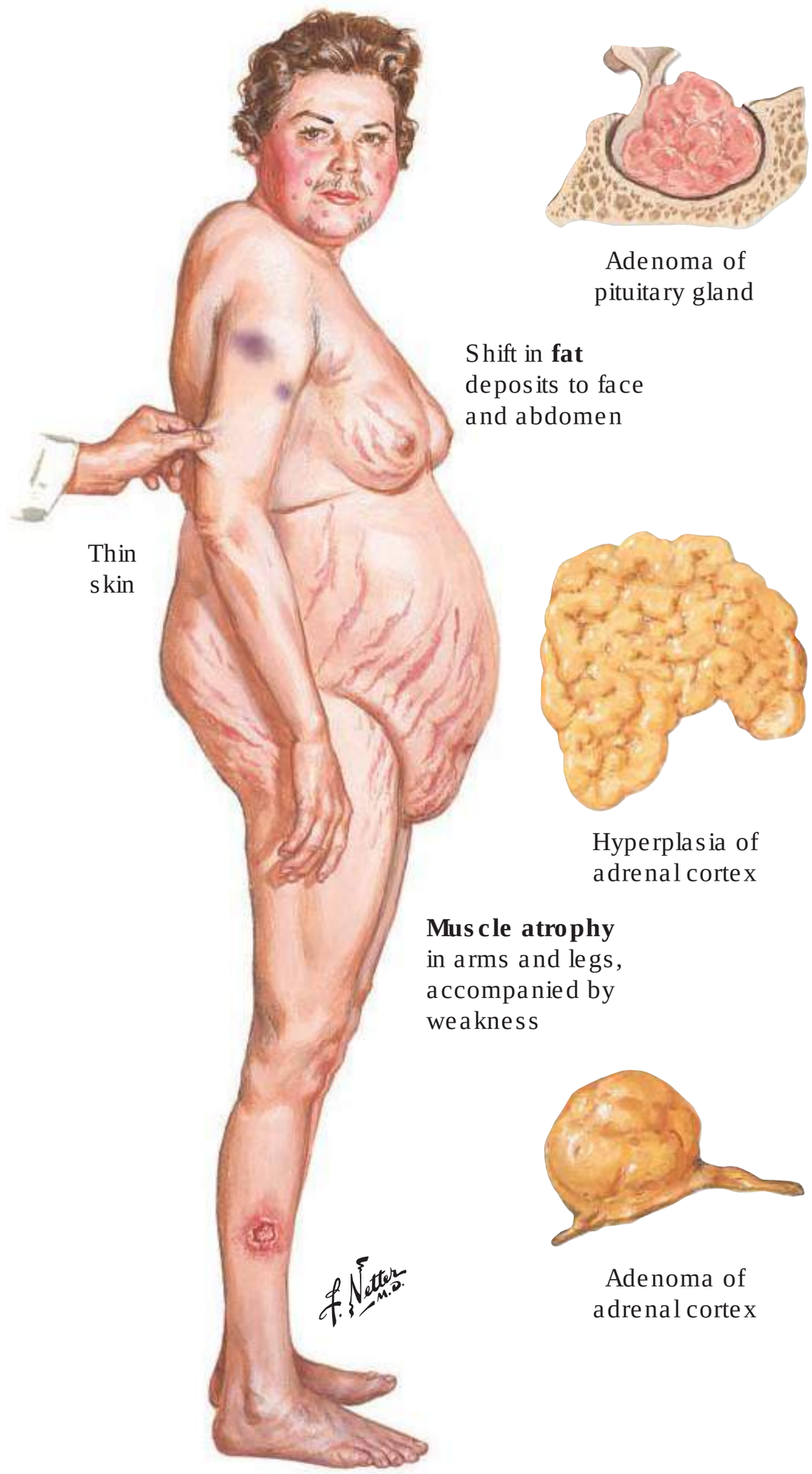
Zona fasciculata, makes **cortisol**

Zona glomerulosa, makes **aldosterone**

Medulla, makes catechol-amines

Capsule

Fig. 31.15 Structure of the adrenal cortex. DHEA, dihydro-epiandrosterone.



Adenoma of pituitary gland

Shift in **fat** deposits to face and abdomen

Thin skin

Hyperplasia of adrenal cortex

Muscle atrophy in arms and legs, accompanied by weakness

Adenoma of adrenal cortex

Fig. 31.16 Cushing syndrome and its causes.

patients with Cushing syndrome tend to be glucose intolerant and develop diabetes. Tumors of the pituitary are often resected (the cure rate is about 65%). Afterward, glucocorticoids must be given and eventually tapered (see below). Adrenalectomy with corticoid replacement is another option. Within several months, some of the disease-induced changes revert.

Synthetic glucocorticoids are used in supraphysiological concentrations in the treatment of allergies, rheumatoid arthritis, organ transplantation, ulcerative colitis, and multiple sclerosis. Examples include hydrocortisone, prednisone, dexamethasone, betamethasone, and triamcinolone. Long-term use of high-dose glucocorticoids leads to the same adverse effects as in Cushing syndrome, such as edema, muscle wasting, shifting of fat depots, and osteoporosis. This syndrome is called **exogenous Cushing syndrome**. Glucocorticoids inhibit CRH and ACTH secretion and thus lead to atrophy of cortisol-producing cells in the adrenal glands that reverts only gradually. For instance, patients who receive more than 20 mg of prednisone per day for 3 weeks have depressed regulation of CRH secretion, ACTH secretion, and cortisol synthesis. To prevent dangerous hypocortisolism, patients are gradually "weaned" of the exogenous glucocorticoid (the drug is "tapered").

4. MINERALOCORTICOIDS

Low blood pressure and a low concentration of Na^+ in the blood stimulate the production of more angiotensin II and

angiotensin III. T ese angiotensins stimulate the adrenal glands to synthesize and release aldosterone. Aldosterone in turn stimulates the synthesis of transporters in the kidneys that increase recovery of Na^+ from the tubules in the kidneys. T is recovery leads to an increase in blood pressure and in the concentration of Na^+ in the blood. Drugs that are used to lower blood pressure incapacitate this regulatory system. Aldosterone def ciency leads to low blood pressure and aldosterone excess to high blood pressure.

4.1. Synthesis of Aldosterone

Aldosterone plays a role in regulating blood pressure and the concentrations of Na^+ and K^+ in the blood. Aldosterone synthesis is regulated by the renin-angiotensin system. When **blood pressure** and the concentration of **Na^+** in blood are too low or when the concentration of **K^+** in blood is too high, angiotensin stimulates aldosterone synthesis.

T e **renin-angiotensin system** works as follows (Fig. 31.17). **Renin** is a protease secreted from renal juxtaglomerular cells. At low blood pressure, high [Na^+], or low [K^+] in blood, the activity of renin in blood increases. (Note that renin secreted by the kidneys is a dif erent enzyme from rennin in the stomach of ruminants that is used in the production of cheese.) Renin cleaves **angiotensinogen** (a 14-residue peptide and α2-globulin) into **angiotensin I** (a 10-residue peptide) and another peptide (Fig. 31.18). **Angiotensin-converting enzyme (ACE)** in the blood cleaves angiotensin I into **angiotensin II** (an 8-residue peptide). Angiotensin II has a half-life of only 1 to 2 minutes. An aminopeptidase removes the N-terminal amino acid residue from angiotensin II, forming **angiotensin III** (a 7-residue peptide).

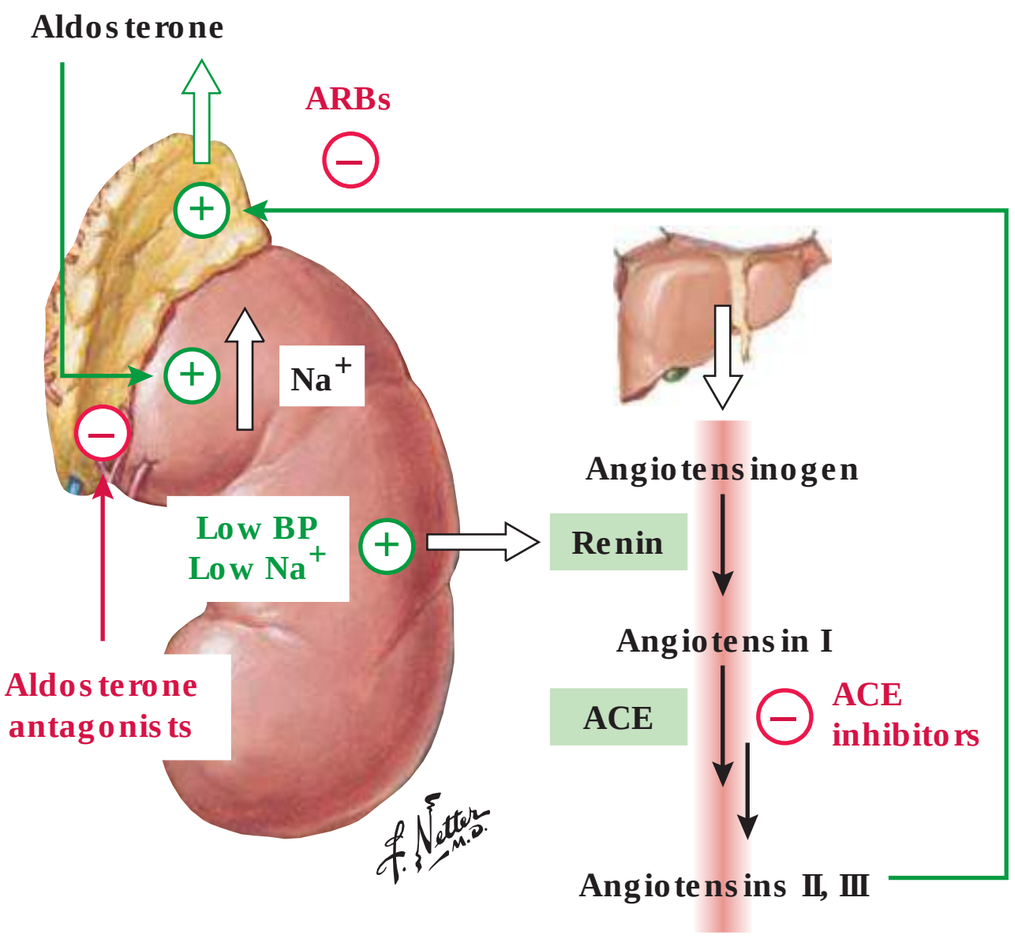


Fig. 31.17 Regulation of blood pressure (BP) and blood Na^+ by the renin-angiotensin system and aldosterone. Aldosterone is secreted from the adrenal glands. ACE, angiotensin-converting enzyme; ARBs, angiotensin receptor blockers.

Both angiotensin II and angiotensin III bind to the **angiotensin receptor** on adrenal gland glomerulosa cells and activate the receptor. Receptor activation elicits an increase in the intracellular concentrations of inositol trisphosphate (IP_3) and diacylglycerol, which lead to increased synthesis of the StAR protein and thus increased synthesis of **aldosterone** via the pathway shown in Fig. 31.14. Aldosterone binds to the **mineralocorticoid receptor** in the kidneys and thus increases transcription of transport proteins in the renal tubules that take up Na^+ from the glomerular filtrate and transport it back into the blood. Secondarily, Na^+ uptake af ects the transport of water and K^+, such that aldosterone favors excretion of K^+.

Angiotensin receptors are found not only in the adrenal glands but also throughout the vasculature; when activated in blood vessels, these receptors increase blood pressure via vasoconstriction (i.e., independent of aldosterone).

ACE inhibitors, **angiotensin receptor blockers** (**ARB**s), and **aldosterone antagonists** are used clinically to reduce blood pressure. T ese drugs impair the physiological regulation of blood pressure. T us a side ef ect of these drugs is low blood pressure. Examples of ACE inhibitors are captopril, zofenopril, ramipril, and enalapril. Physiologically, ACE is not regulated or rate limiting, but in the presence of ACE inhibitors, less angiotensin II is formed. ARBs prevent the binding of angiotensins II and III to the angiotensin receptor in the adrenal glands. Examples of ARBs are losartan and irbesartan (the "sartans"). T e aldosterone antagonists are the oldest drugs in this class and include spironolactone, eplerenone, and canrenone. T ese drugs prevent aldosterone from activating the mineralocorticoid receptor in the kidneys.

4.2. Disorders of Aldosterone Synthesis

Primary aldosteronism (**Conn syndrome**) is due to excessive production of aldosterone, which is most often due to a unilateral adenoma or bilateral hyperplasia of the aldosterone-producing zona glomerulosa (Figs. 31.15 and 31.19). T e high concentration of circulating aldosterone leads to **secondary hypertension**. About 10% of all patients who have hypertension have primary aldosteronism. T e **aldosterone/renin**

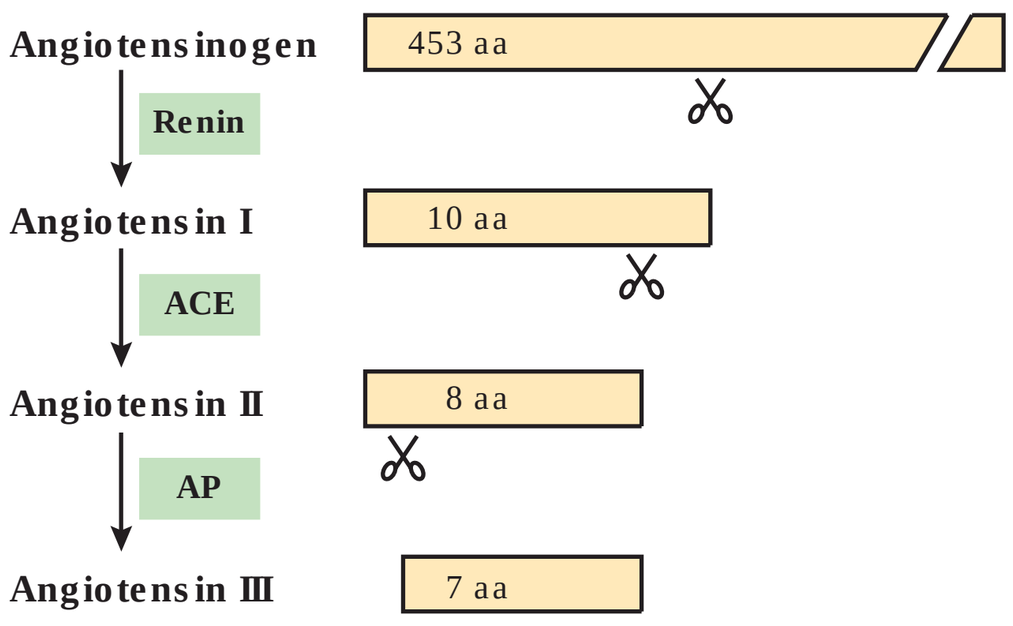


Fig. 31.18 Processing of angiotensinogen. aa, amino acids; ACE, angiotensin-converting enzyme; AP, aminopeptidase.

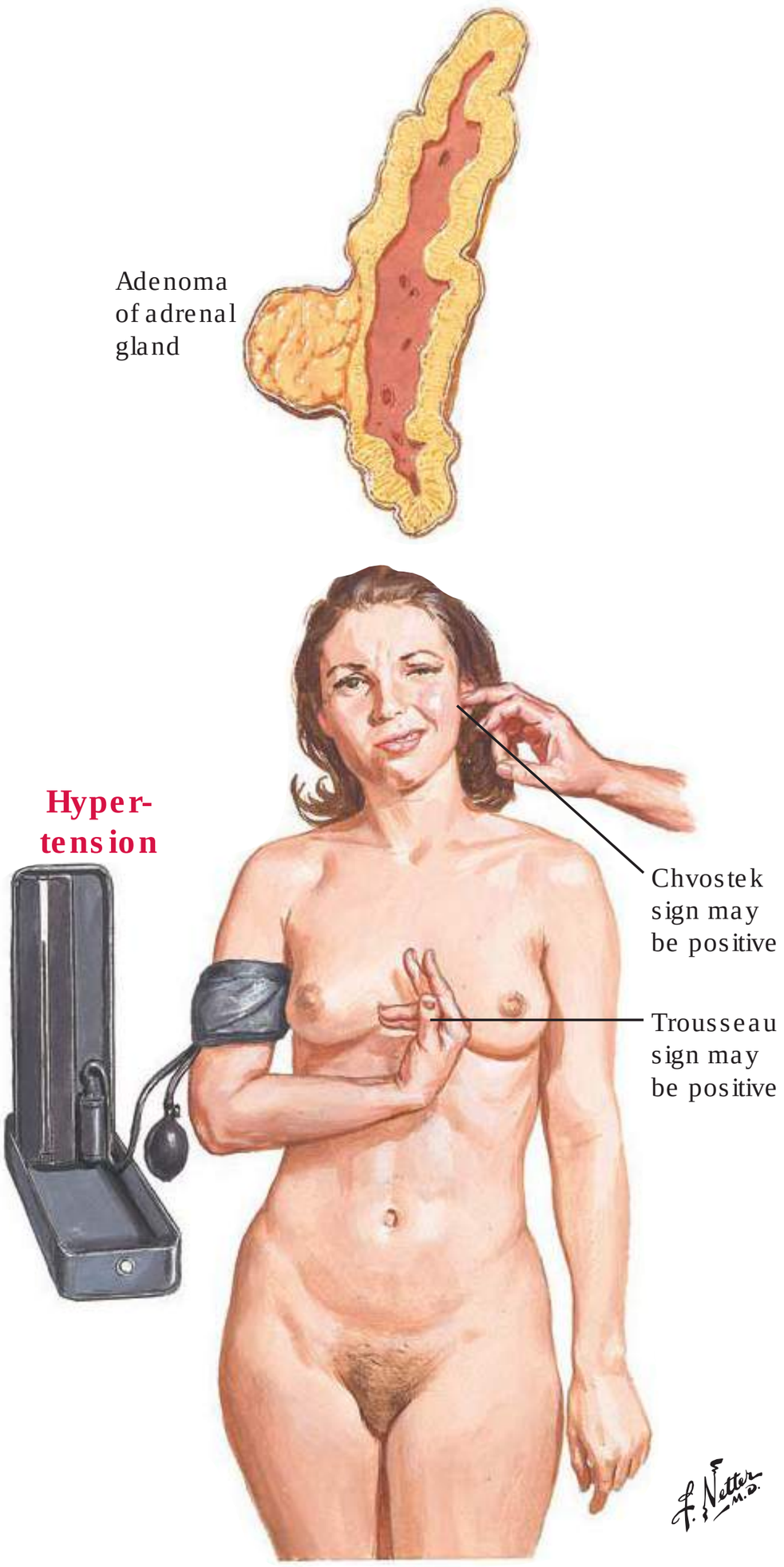


Fig. 31.19 **Primary aldosteronism.** The Chvostek sign indicates hyperexcitability of the facial nerve, which is due to hypokalemia. The Trousseau sign is observed after 3 minutes of occluding the brachial artery; it indicates hyperexcitability and is due to hypokalemia.

ratio in the blood serves as a screening tool for primary aldosteronism. The concentration of aldosterone is unusually high due to overproduction, whereas the activity of renin is unusually low due to the high blood pressure; that is, the renin-angiotensin system functions normally. As a consequence of persistent hyperaldosteronism, compensatory processes become active such that the concentration of Na^+ is high but still in the normal range, and the concentration of K^+ is normal or low.

Some 40% of patients who have a unilateral **adenoma** of the adrenal gland have a tumor that is heterozygous for a mutant KCNJ5 gene, which encodes the Kir3.4 **K^+-channel**. The mutant channel leads to depolarization of the aldosterone-producing zona glomerulosa cells, influx of Ca^{2+}, increased transcription of the StAR protein, and thus increased aldosterone synthesis. Rare patients who are heterozygous for an inherited mutation in the KCNJ5 gene have a type of **familial hyperaldosteronism** that is present from childhood.

Congenital adrenal hyperplasia is characterized by impaired production of both cortisol and aldosterone. Impaired production of cortisol leads to reduced feedback inhibition of ACTH secretion from the pituitary and hyperplasia of the adrenal glands in response to the increased concentration of ACTH. Both glucocorticoid and mineralocorticoid synthesis are impaired, because the two classes of steroids share precursors and steroid-producing enzymes. The most common cause of congenital adrenal hyperplasia is a deficiency of 21α-hydroxylase.

21α-Hydroxylase (CYP21A2) deficiency impairs the normal pathways of cortisol and aldosterone synthesis (see Fig. 31.14). The deficiency does not impair the synthesis of sex steroids. The severe, classic forms of this disorder are seen in ~1 in 15,000 newborns, whereas a less severe, nonclassic form occurs in ~1 in 1,000 newborns. In all forms, the increased ACTH secretion leads to an increased rate of cholesterol conversion to 17α-hydroxyprogesterone, which then accumulates. 17α-Hydroxylase catalyzes not only the introduction of a hydroxyl group at C-17 but also the cleavage of the side chain at C-17 with the formation of a ketone at C-17. For this reason, in patients who have a 21α-hydroxylase deficiency, 17α-hydroxylase converts 17α-hydroxyprogesterone to androstenedione, which then gives rise to excess testosterone. In the most severe 21α-hydroxylase deficiency, lack of aldosterone synthesis leads to salt wasting from birth, which needs to be treated immediately. In girls, this deficiency also leads to masculinization in utero and hence ambiguous genitalia at birth. In boys, the excess testosterone leads to premature virilization in childhood. In a less severe classic form of the disorder that does not entail salt wasting, there is similar masculinization in girls and boys. Finally, the mildest, nonclassic form of 21α-hydroxylase deficiency leads to early virilization in boys and to hirsutism and male pattern baldness in women. All forms of 21α-hydroxylase deficiency are inherited in autosomal recessive fashion. Newborn screening for 17α-hydroxyprogesterone allows the detection of babies who have a classic form of 21α-hydroxylase deficiency.

Patients who have the severe, salt-wasting form of 21α-hydroxylase deficiency are typically treated with fludrocortisone to restore blood pressure and the concentration of electrolytes in the blood. All patients who have classic 21α-hydroxylase deficiency are treated with a glucocorticoid, such as dexamethasone, in sufficient amounts to reduce the excessive testosterone production.

Less common causes of congenital adrenal hyperplasia are deficiencies in StAR protein activity, 3β-hydroxysteroid dehydrogenase, 17α-hydroxylase, or 11β-hydroxylase.

Addison disease (Fig. 31.20) develops over a period of months to years and leads to the destruction of the adrenal cortex and hence greatly reduced production of both cortisol

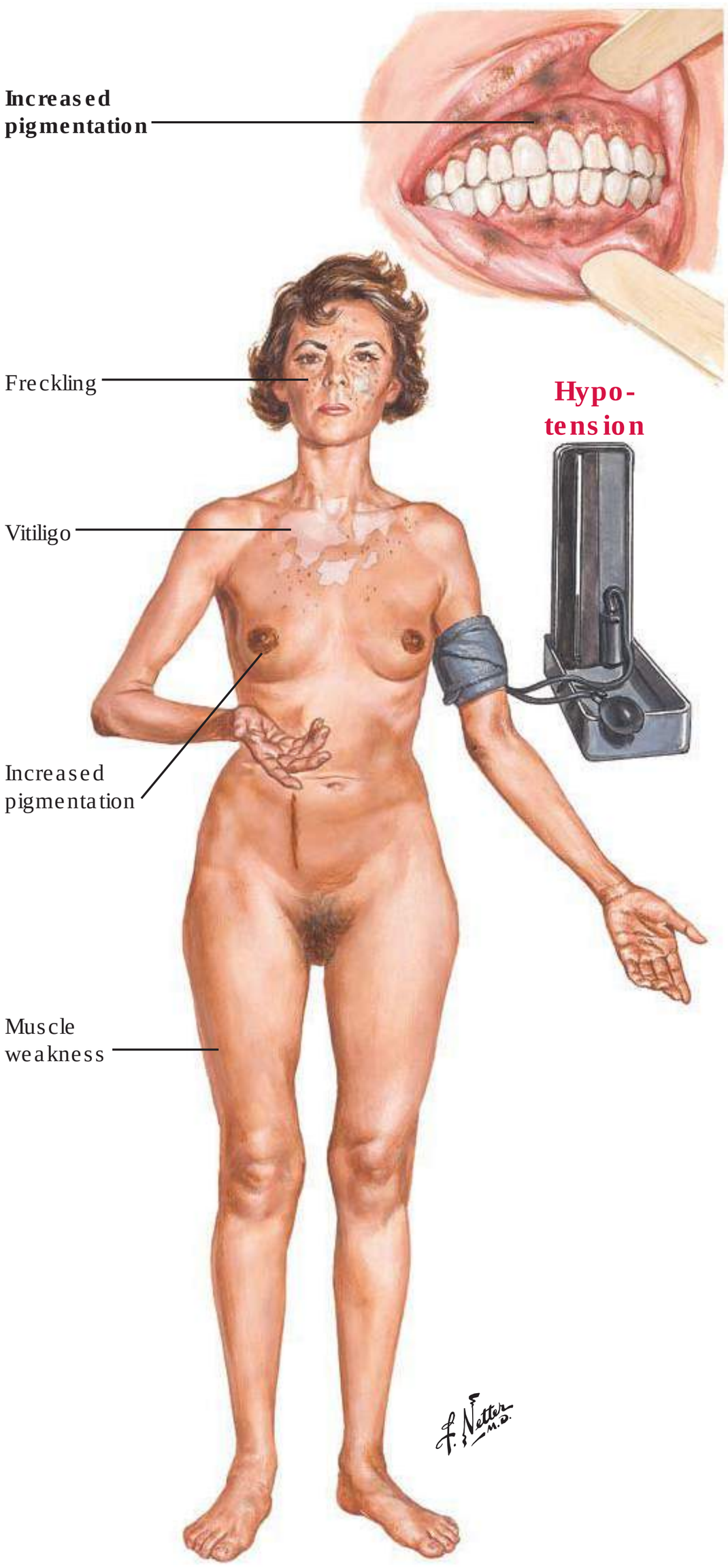


Fig. 31.20 **Adrenal insufficiency (Addison disease).**

and androstenedione. In developed countries, the disease is most often due to an autoimmune reaction. Some of the antibodies are directed against 21-hydroxylase (the same enzyme that is missing in most individuals who have congenital adrenal hyperplasia; see Fig. 31.14). Like other autoimmune diseases, predisposition to Addison disease is linked to certain major histocompatibility alleles. The disease has a prevalence of ~1 in 20,000 persons. In other countries, tuberculosis is the more common reason for the loss of the adrenal cortex. Patients show a multitude of symptoms, the more striking of which are low blood pressure, increased pigmentation (due to ACTH that is cleaved to produce α-melanocyte stimulating hormone; see also Section 4.2 in Chapter 35), and muscle weakness. Treatment involves the use of a steroid with mineralocorticoid activity, such as fludrocortisone.

5. VITAMIN D

Calciol (cholecalciferol), a form of vitamin D, is formed in the skin on exposure to ultraviolet (UV) light. Calciol can also be derived from a few types of food. Calciol gives rise to calcidiol, which is stored in blood and is the major storage form of vitamin D. In response to a low concentration of calcium or phosphate in the blood, calcidiol is hydroxylated to the biologically active calcitriol. Calcitriol stimulates transcription of certain genes with the effect of increasing the concentrations of calcium and phosphate in the blood. A deficiency of vitamin D causes demineralization of bone.

Vitamin D is an umbrella term for several related compounds that play a role in calcium and phosphate homeostasis; the main biologically active compound is calcitriol. Vitamin D is not a steroid but rather a secosteroid; that is, a steroid with a broken ring (Figs. 31.1 and 31.21).

Vitamin D can be synthesized in the skin via a reaction that requires light or it can be obtained from the diet. **Vitamin D_3** (**calciol, cholecalciferol**) is synthesized from cholesterol in the skin and is also found in some animal products, such as oily fish, fish oil, or milk fortified with vitamin D. **Vitamin D_2** (**ergocalciferol**) is found in some plant foods, such as mushrooms. Ergocalciferol differs from cholecalciferol in the multicarbon substituent of the D-ring, but it seems to be as effective in humans as cholecalciferol.

The liver converts vitamin D_3 to **calcidiol** (**25-hydroxycholecalciferol**, **25-hydroxyvitamin D_3**) and releases this into the blood, where it binds to **vitamin D–binding protein**. Calcidiol bound to vitamin D binding protein is the major storage form of vitamin D. A similar reaction occurs with vitamin D_2, but vitamin D_3 is commonly the predominant form of vitamin D. In many people, calcidiol is chiefly produced during the summer months. Measurement of blood-borne calcidiol is a common screening tool for vitamin D adequacy; this is typically done out of concern for fractures due to osteoporosis.

Calcitriol (**1,25-dihydroxycholecalciferol**, **1,25-dihydroxyvitamin D_3**), the biologically active form of vitamin D_3, is produced in the kidney in response to low blood **calcium** or **phosphate** concentrations (see Fig. 31.21).

Calcitriol binds to a nuclear hormone receptor and increases transcription of certain genes (Fig. 31.22). To this end, calcitriol binds to the **vitamin D receptor** (**calcitriol receptor**), a nuclear hormone receptor that forms a heterodimer with the **retinoid X receptor**. The heterodimer binds to a **vitamin D response element** in the promoter region of several genes, thereby favoring transcription of these genes. The mechanism of action of vitamin D resembles that of vitamin A in that both

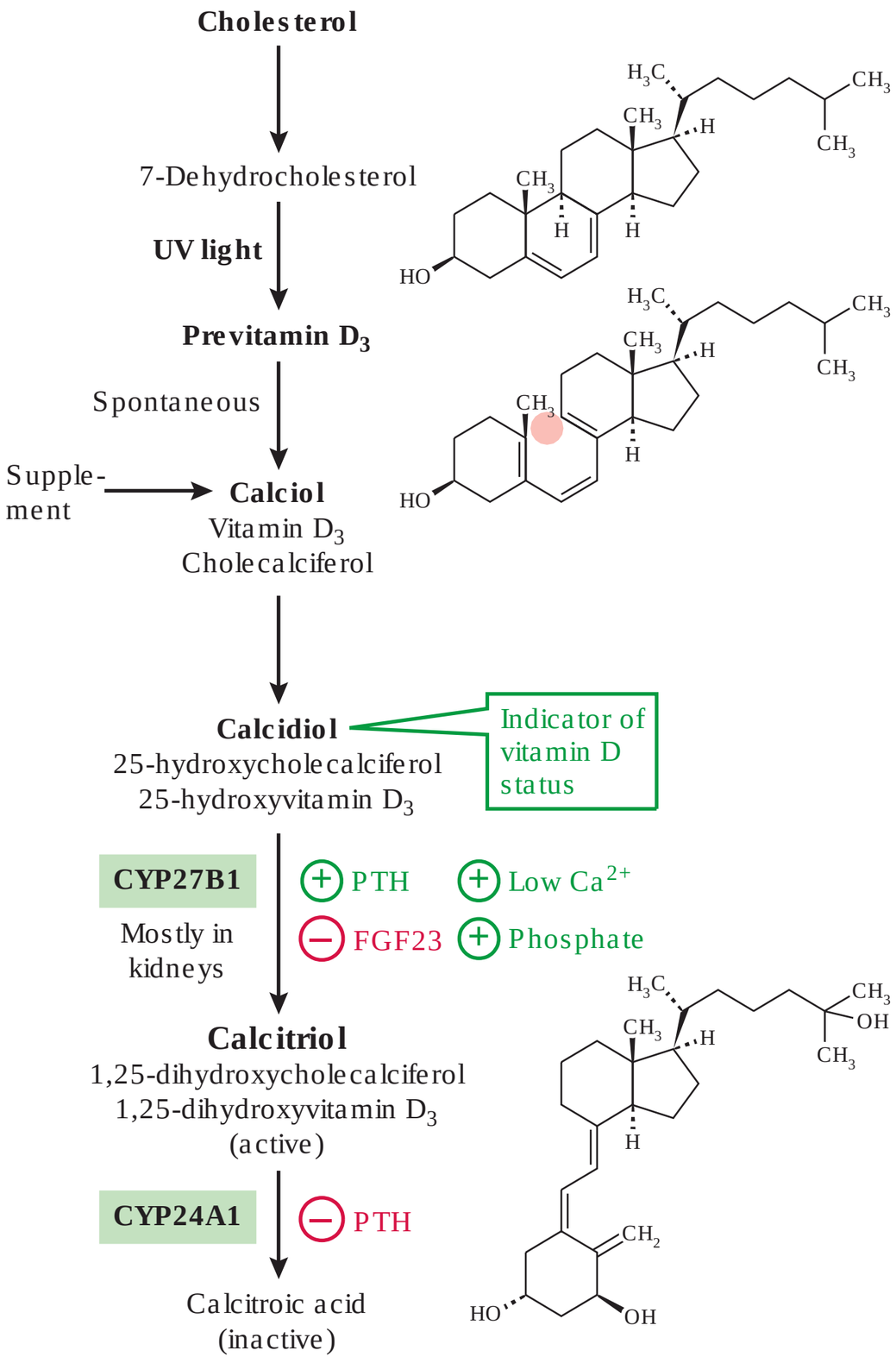


Fig. 31.21 Synthesis of vitamin D. When the concentrations of calcium and phosphate in the blood are low, the pathway generates a higher concentration of circulating calcitriol. PTH, parathyroid hormone; UV, ultraviolet.

vitamins bind to a nuclear receptor that pairs with a retinoid X receptor to regulate gene expression.

Calcitriol leads to an increase in the concentrations of calcium and phosphate in the blood via increased absorption in the intestine, increased recovery in the kidneys, and, when a special need arises, increased release from hydroxyapatite in bone. T ese changes are a result of increased expression of transporters in the intestine and kidneys as well as increased activity of osteoclasts, which degrade bone.

Vitamin D deficiency (see Chapter 12) results in a low concentration of calcium and phosphate in the blood, which in turn leads to insufficient mineralization of bone with calcium phosphate. In children, vitamin D deficiency leads to **rickets**, a condition characterized by soft, pliable bones. In adults, vitamin D deficiency leads to **osteomalacia**, a condition in which bones are susceptible to fracture due to demineralization.

Vitamin D deficiency is also associated with increased rates of infection, cancer, muscle weakness, and skin disorders including psoriasis.

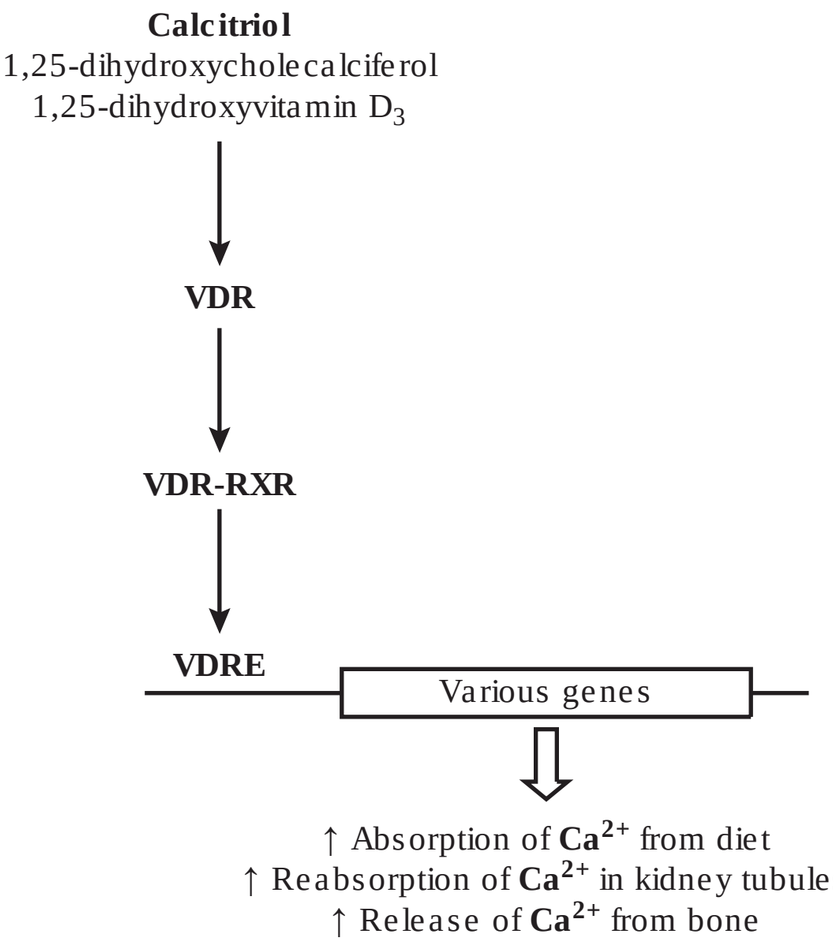


Fig. 31.22 Mechanism of action of calcitriol in calcium homeostasis. RXR, retinoid X receptor; VDR, vitamin D receptor; VDRE, vitamin D response element.

Vitamin D deficiency is mostly seen in the following groups of people: persons who have low exposure to sun, dark skin, or advanced age (decreased UV light–dependent synthesis of calciol), exclusively breastfed babies (low vitamin D content of breast milk), patients who have decreased absorption in the intestine (due to steatorrhea, celiac disease, or bariatric surgery), and patients who receive dialysis due to chronic kidney disease (decreased synthesis of calcitriol).

SUMMARY

- Steroid hormones are synthesized from cholesterol as follows. Peptide hormones bind to membrane receptors which in turn increase the expression of the StAR protein. T e StAR protein regulates the transfer of cholesterol to the inner mitochondrial membrane. T is is the rate-limiting step in steroid hormone synthesis. Steroids are membrane permeable and bind to receptors that are transcription factors.
- In men and women, the hypothalamus secretes GnRH, which stimulates the secretion of FSH and LH from the anterior pituitary. Dihydrotestosterone, estradiol, and inhibin feedback inhibit the secretion of FSH and LH.
- T e selective estrogen receptor modulator clomiphene is used to treat infertility. It prevents estradiol from inhibiting FSH secretion, which leads to increased FSH secretion and hence increased recruitment of follicles in the ovaries.
- Women of childbearing age produce estradiol in developing follicles in the ovaries. A persistently high concentration of estrogen induces LH secretion, which in turn stimulates ovulation.

- Most contraceptive drugs contain an estrogen and a progestin, which inhibit FSH secretion and the development of ovarian follicles.
- Hypogonadotropic hypogonadism is due to deficient secretion of functional GnRH, FSH, or LH, or deficient sensing of GnRH. If the hypogonadotropic hypogonadism is inherited and hyposmia or anosmia are present, the disorder is called Kallmann syndrome.
- Men produce testosterone in the Leydig cells of the testes. Testosterone is reduced to the more potent dihydrotestosterone. Men who have castration-sensitive prostate cancer often receive androgen deprivation therapy, which involves lowering GnRH secretion, inhibiting androgen synthesis directly, or preventing androgens from binding to the androgen receptor.
- Newborns who have a 46,XY disorder of sex development (DSD) often appear female and are raised as females, but then fail to menstruate at puberty and show unexpected virilization. The disorder is caused by an androgen receptor deficiency or an enzyme deficiency that leads to a low concentration of dihydrotestosterone.
- A pathogenic elongation of the CAG trinucleotide repeat in the androgen receptor leads to normal sex development but loss of motoneurons in men around the age of 30 to 60 years. The CAG sequence encodes glutamine. Androgen receptors with overly long glutamine repeats form aggregates in motoneurons.
- Tamoxifen and raloxifene are used as chemoprevention in patients at high risk of developing breast cancer. Tamoxifen is also used as an adjuvant in the treatment of estrogen receptor–positive breast cancer. Raloxifene is also used in the treatment and prevention of osteoporosis. Toremifene is used to treat metastatic breast cancer.
- Calcidiol (25-hydroxyvitamin D_3) is synthesized in sun-exposed skin and then in liver, followed by storage in blood; its concentration in blood is used as an indicator of vitamin D status. When the concentration of calcium or phosphate in the blood is low, the kidneys convert calcidiol to calcitriol. Calcitriol, acting via vitamin D receptors in the nucleus that stimulate transcription, increases the concentrations of calcium and phosphate in the blood via increased absorption in the intestine, increased recovery form the tubules in the kidneys, and increased degradation of bone.
- A deficiency of vitamin D leads to rickets in children and osteomalacia in adults. These diseases are characterized by insufficient mineralization of bone.

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Review Questions

1. The synthesis of aldosterone by the adrenal medulla is controlled primarily by the concentration of which one of the following hormones in the circulation?

   A. Androstenedione
   B. Angiotensin II
   C. Cortisol
   D. DHEA
   E. FSH

2. A 48-year-old woman has hypertension due to bilateral hyperplasia of the zona glomerulosa of the adrenal glands. This patient is best treated with which one of the following drugs?

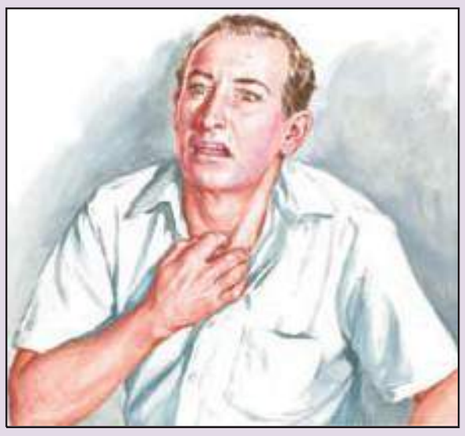
   A. ACE inhibitor
   B. Aldosterone antagonist
   C. ARB
   D. Hydrocortisone
   E. Progestin

3. A 50-year-old woman has low blood pressure, extreme fatigue, decreased appetite, weight loss, and skin hyperpigmentation. A blood sample was taken and analyzed. A diagnosis of Addison disease would best be supported by which one of the following findings?

   A. Increased ACTH and increased renin
   B. Increased aldosterone and decreased angiotensin
   C. Increased FSH and increased LH
   D. Decreased GnRH and decreased CRH

4. A 40-year-old woman delivers a girl, although genetic testing had made her expect a boy. Which of the following could be a cause of the mismatch?

   A. Aldosterone synthase deficiency
   B. Aromatase deficiency
   C. Congenital adrenal hyperplasia due to 21α-hydroxylase deficiency
   D. Excessive secretion of ACTH from the pituitary
   E. Nonfunctional androgen receptor



Chapter

32

Eicosanoids

SYNOPSIS

- Eicosanoids are short-lived 20-carbon lipids that generally play a role in local signaling. Most cells can produce eicosanoids. Examples of eicosanoids are prostaglandins, thromboxanes, leukotrienes, and lipoxins.
- Some eicosanoids are synthesized from the ω-6 fatty acid arachidonic acid and others from the ω-3 fatty acid eicosapentaenoic acid. These fatty acids are derived from the 18-carbon essential fatty acids linoleic acid and linolenic acid, respectively.
- Prostaglandins play a role in protecting the mucosa of the stomach and intestine, in ripening the cervix during pregnancy, and in regulating inflammation. A thromboxane favors aggregation of platelets at sites of vessel injury, whereas a prostaglandin opposes this effect.
- Nonsteroidal antiinflammatory drugs (NSAIDs), such as aspirin, acetaminophen, and ibuprofen, inhibit the synthesis of prostaglandins and thromboxanes. Prostaglandin analogs are used to protect the gastrointestinal mucosa from NSAID-induced damage and to ripen the cervix of pregnant women, if needed.
- Leukotrienes constrict the airways and promote the exit of white blood cells from blood vessels. Drugs that prevent leukotriene-induced bronchoconstriction play a role in the treatment of asthma.
- Lipoxins oppose the effect of leukotrienes and help resolve the inflammation.

LEARNING OBJECTIVES

For mastery *of* this topic, you should be able to do the *f*ollowing:

- List good sources of ω-6 and ω-3 fatty acids, and discuss recommendations for daily intake of these essential fatty acids.
- Outline the synthesis of prostaglandins and thromboxanes, focusing on steps that are affected by drugs.
- Explain the role of prostaglandins in protecting the stomach mucosa, describe the effect of NSAIDs on prostaglandin synthesis in the stomach, and propose a treatment that protects the stomach mucosa in patients who use NSAIDs chronically.
- Explain the effect of low-dose aspirin on platelet activation.
- Outline the synthesis of leukotrienes and lipoxins.
- Explain the role of leukotrienes in asthma, and propose at least three different drugs that can be used in the prophylaxis or treatment of asthma.

1. EICOSANOID FAMILIES

Many eicosanoids are made *f*rom the ω-6 *f*atty acid arachidonic acid, which in turn is derived *f*rom the essential ω-6 *f*atty acid linoleic acid. Another group o*f* eicosanoids is made *f*rom the ω-3 *f*atty acid eicosapentaenoic acid (EPA), which in turn is derived *f*rom the essential ω-3 *f*atty acid linolenic acid. T e eicosanoids derived *f*rom ω-3 *f*atty acids tend to have an opposite e*ff*ect to eicosanoids derived *f*rom ω-6 *f*atty acids. Current diets are typically rich in vegetable oils and there*f*ore provide plenty o*f* ω-6 *f*atty acids. However, ω-3 *f*atty acids are scarce in most diets.

Eicosanoids are made from 20-carbon polyunsaturated fatty acids, either ω-6 or ω-3. Eicosa means 20 in Greek. Eicosanoids are also called **icosanoids**.

Omega-6 (**ω-6**, **n-6**) and **omega-3** (**ω-3**, **n-3**) **fatty acids** are **essential fatty acids** that we absorb from food. Humans cannot synthesize ω-6 or ω-3 fatty acids (see Section 3 in Chapter 27). T e most basic ω-6 fatty acid is **linoleic acid** (C18:2), and the most basic ω-3 fatty acid is **linolenic acid** (C18:3). T ese essential fatty acids and their products are polyunsaturated. Polyunsaturated fatty acids react quite readily with oxygen, which can give food a rancid taste and generate oxidative stress in the human body.

We generally get plenty of ω-6 fatty acids but precious little ω-3 fatty acids. Plant oils are relatively rich in ω-6 fatty acids (Table 32.1). Flax seed and fish (especially oily ones, such as salmon) are relatively rich in ω-3 fatty acids.

Once linoleic acid (ω-6, C18:2) and linolenic acid (ω-3, C18:3) reach cells, cells convert some of these fatty acids to **arachidonic acid** (C20:4) and **eicosapentaenoic acid** (**EPA**, C20:5), respectively, using a combination of desaturation and elongation (Fig. 32.1). Elongation and desaturation occur as described in Chapter 27. Only a small fraction of linolenic acid is converted to EPA and even less to docosahexaenoic acid (DHA).

Cells store **arachidonic acid** and **EPA** in phospholipids in the endoplasmic reticulum and in the plasma membrane. Cells typically contain ~10 times more arachidonic acid than EPA, but an EPA-rich diet can raise the EPA content to ~70% that of arachidonic acid.

It is not clear what the optimal consumption of ω-6 fatty acids should be; current intake may be too high due to high consumption of vegetable oils. For example, more than 50% of the fatty acids of corn oil are ω-6 fatty acids and so are up to ~25% of the fatty acids of olive oil.

It is **recommended** that ω-3 fatty acids are consumed in an amount equivalent to ~0.5% of all calories (~1.4 g/day). **Flaxseed oil** contains mostly linolenic acid. By contrast, **fish oil** contains mainly EPA and DHA. Oil from **algae** contains DHA but almost no EPA.

A high intake of ω-3 fatty acids in the form of EPA and DHA lowers plasma triglycerides and the risk of death due to coronary heart disease. ω-3 fatty acids may exert their beneficial ef ects, for instance, by binding to proteins (e.g., PPAR-γ, a transcription factor), by giving rise to eicosanoids (see

Table 32.1 Essential Fatty Acids in Food

Food	ω-6 Fatty Acids (g/100 g Food)	ω-3 Fatty Acids (g/100 g Food)
Cod liver oil	1.8	18.8
Salmon	0.5	1.7
Canola oil	18.6	9.1
Corn oil	53.2	1.2
Olive oil	9.8	0.8
Walnuts	0.3	10.6
Flaxseed	0.3	1.4
Ground beef (30% fat)	0.6	0.1

Based on data from the United States Department of Agriculture National Nutrient Database, release 28.

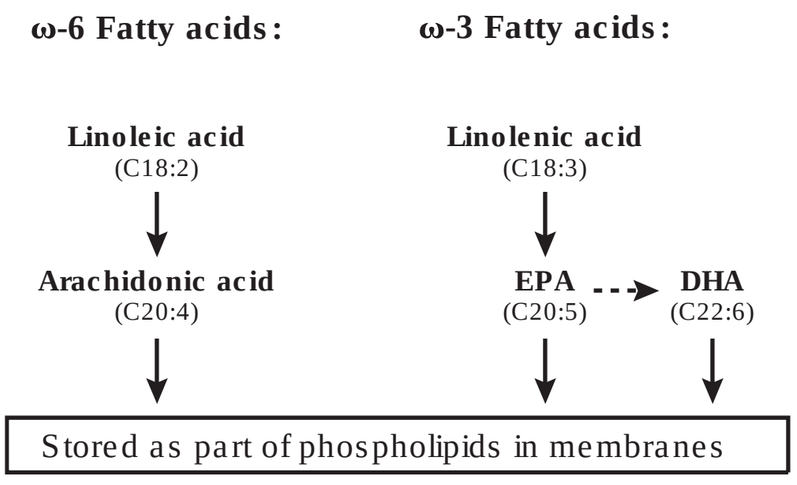


Fig. 32.1 Elongation and storage of ω-3 and ω-6 essential fatty acids. EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

Sections 2 and 3) and other lipid signals, and by lowering plasma triglycerides (see Section 8.1 in Chapter 28).

2. PROSTAGLANDINS AND THROMBOXANES

Prostaglandins and thromboxanes are eicosanoids that are made *f*rom the ω-3 *f*atty acid EPA or *f*rom the ω-6 *f*atty acid arachidonic acid. T ese compounds act locally via G protein–coupled receptors. Prostaglandins play a role in in*fl*ammation, in protecting the stomach mucosa, and in ripening the cervix be*f*ore delivery. T romboxane A2 stimulates activation and aggregation o*f* platelets near sites o*f* vessel injury. T e synthesis o*f* prostaglandins and thromboxanes is inhibited by corticosteroids, phospholipase inhibitors, and NSAIDs.

2.1. Synthesis of Prostanoids

Prostanoids is a summary term for **prostaglandins** and **thromboxanes**. T is section discusses prostaglandins D, E, H, and I (prostaglandin I is also called **prostacyclin**), as well as thromboxane A (TXA).

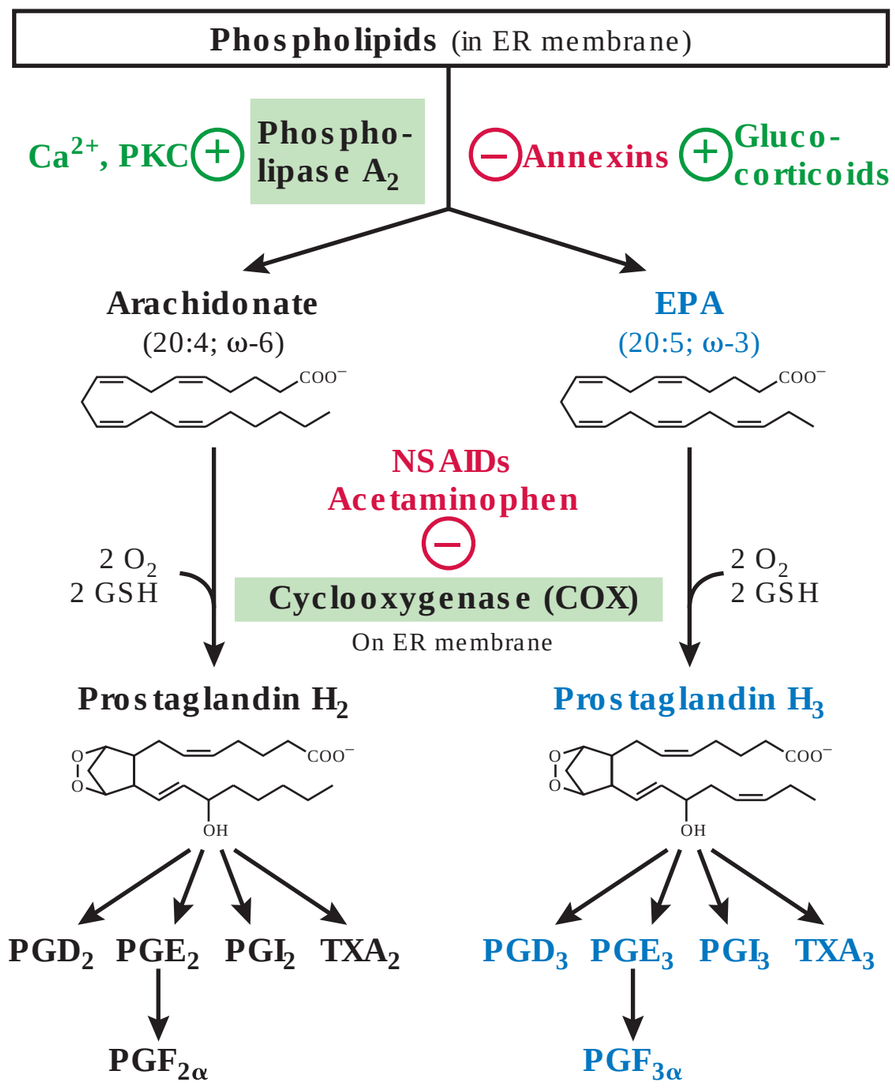


Fig. 32.2 Synthesis of prostaglandins. EPA, eicosapentaenoic acid; ER, endoplasmic reticulum; GSH, reduced glutathione; NSAID, nonsteroidal antiinflammatory drug; PG, prostaglandin; PKC, protein kinase C; TX, thromboxane.

In the rate-limiting step of eicosanoid synthesis, **cytosolic phospholipase A_2** ($cPLA_2$) moves onto the **endoplasmic reticulum membrane** and hydrolyzes phospholipids that contain EPA or arachidonic acid. $cPLA_2$ produces lysophospholipids and either eicosapentaenoic or arachidonic acid (Fig. 32.2). Physiologically, phospholipase A_2 activity is enhanced by an elevated concentration of Ca^{2+} in the cytosol and by increased activity of **protein kinase C**; on the other hand, **annexins** (**lipocortins**) inhibit $cPLA_2$.

Glucocorticoids indirectly reduce phospholipase A_2 activity by stimulating the transcription of **annexins** (**lipocortins**), which then inhibit phospholipase A_2.

T e **cyclooxygenase enzymes** (**COX-1** and **COX-2**) catalyze the synthesis of **prostaglandin H_2** (**PGH_2**) from arachidonic acid and the synthesis of **prostaglandin H_3** (**PGH_3**) from EPA (see Fig. 32.2). COX-1 is expressed in most tissues at all times, whereas COX-2 is mostly induced during inflammation, typically over a period of hours. T e rate of synthesis of eicosanoids from arachidonic acid is generally substantially greater than that from EPA.

PGH_2 and PGH_3 give rise to the prostaglandins PGD, PGE, PGF, and PGI as well as TXA. PGH_2 has a half-life of ~2 min.

Prostanoids are identified with a **subscript** that denotes the number of **double bonds**, e.g., PGE_2. Arachidonic acid (ω-6, C20:4) gives rise to prostanoids with two double bonds, whereas EPA (ω-3, C20:5) gives rise to prostanoids with three double bonds (see Fig. 32.2). Prostaglandin F has an

additional subscript of α or β that indicates the configuration at carbon-9.

As a rule of thumb, ω-6 fatty acids give rise to **proinflammatory eicosanoids**, whereas ω-3 fatty acids give rise to **anti-inflammatory eicosanoids** or to eicosanoids that resolve inflammation.

Eicosanoids are generally **short lived** and exert their effects **locally**, near their place of origin. Most cells can produce eicosanoids. The half-life of eicosanoids is on the order of seconds to a few minutes. Secreted eicosanoids induce changes in nearby cells (**paracrine** effect) or in the cell that secreted it (**autocrine** effect). The short lifespan of eicosanoids is due to inherent lability, uptake into cells, and degradation by enzymes.

2.2. Prostanoid Receptors

Prostaglandins and thromboxane act via **G protein–coupled receptors** (**GPCR**s; see Section 2 in Chapter 33) that are named after their ligands (e.g., receptors for prostaglandin E_2 [PGE_2] are called EP receptors).

Some prostaglandins bind to several types of prostaglandin receptor. For example, there are four different PGE_2 receptors: EP1, EP2, EP3, and EP4. PGE_2 is the most abundantly and most ubiquitously synthesized of all prostanoids. The diversity of EP receptors accounts for some of the variety of PGE_2 effects. The other prostanoids have only one receptor.

Prostaglandin receptors fall into three major categories: some receptors (DP1, EP2, EP4, and IP) raise the concentration of **cyclic adenosine monophosphate (cAMP)** in the cytosol, whereas one receptor (EP3) can decrease it. Activation of other receptors (EP1, FP, and TP) can lead to an increase in the concentration of **Ca^{2+}** in the cytosol. Diversity in signaling is further increased via alternative splicing of mRNA (see Fig. 6.13 and Section 3.4 in Chapter 6), via homodimerization and heterodimerization, and via promiscuous coupling to Gα proteins.

The biological effects of prostaglandins and thromboxanes of the 3 series (PGE3, TXE3, etc.) are poorly understood. It seems that these prostanoids are partial agonists; they bind to the same receptors as the 2 series prostanoids. However, they induce a smaller effect and thereby prevent more effective signaling via 2 series prostanoids.

2.3. Physiological Roles of Prostaglandins D_2, E_2, and F_2

Prostaglandins play a role in inflammation. Prostaglandin production is low in uninflamed tissue but rises rapidly during the onset of inflammation, preceding the arrival of white blood cells. As part of the innate immune system, **toll-like receptors (TLRs)** and **pattern recognition receptors (PRRs)** on mast cells and macrophages recognize **pathogen-associated molecular patterns (PAMPs)**. PAMPs are found in double-stranded RNA, lipopolysaccharides, glycans, glycoconjugates, and endotoxins. In response, mast cells and macrophages secrete PGE_2. Some of the incoming white blood cells produce PGD_2, PGE_2, and TXA_2. During the resolution phase of inflammation, the number of white blood cells in the tissue returns to normal, in large part through apoptosis.

PGE_2 causes pain, induces vasodilation, and increases the permeability of the vessel wall. Pain can be induced peripherally, in the spinal cord, and in the brain. The vasodilation causes redness. The increased permeability of the vessel wall leads to the flow of blood plasma into the extracellular space and thus causes edema.

In the **stomach** and **duodenum**, an acid-sensing pathway fosters the release of **PGE_2**. PGE_2 lowers the exposure of epithelial cells to **acid** by increasing bicarbonate secretion and mucus production.

NSAIDs and acetaminophen inhibit the COX enzymes and may lead to damage of the mucosa of the stomach and intestine. These drugs are the first-line treatment for common musculoskeletal disorders such as back pain, osteoarthritis, and rheumatoid arthritis.

Nonselective NSAIDs such as aspirin (acetylsalicylic acid), ibuprofen, ketoprofen, naproxen, diclofenac, and indomethacin inhibit prostaglandin E_2 synthesis in the gastric mucosa. The decrease in PGE_2 synthesis leads to decreased bicarbonate secretion and decreased mucus production. This increases the risk of symptomatic **ulcers** in the stomach. Nonselective NSAIDs are even more damaging to the lower gastrointestinal tract, where they may cause bleeding or perforation. Perforation of the intestine requires emergency surgery.

Although **enteric-coated aspirin** (an NSAID) passes through the stomach intact, it still acts systemically and therefore still impairs PGE_2-mediated protection of the mucosa.

Compared with nonselective COX inhibitors (used with or without a proton pump inhibitor), **selective COX-2 inhibitors** reduce the risk of perforation, obstruction, or bleeding in the gastrointestinal tract of long-term users. The only selective COX-2 inhibitor in current use is **celecoxib**.

COX-2 is overexpressed in most premalignant and malignant colorectal tumors, and both nonselective NSAIDs and NSAIDs that selectively inhibit COX-2 can have a chemopreventive effect, to some extent independent of COX inhibition. The benefits and risks of such drug treatment are complex.

Misoprostol (a PGE_1 analog) is used clinically to protect the mucosa of the stomach and duodenum during long-term NSAID therapy.

PGE_2 and **$PGF_{2\alpha}$** soften the **cervix** during pregnancy, and they also favor contraction of the uterus. **Misoprostol** (a PGE_1 analog) and **dinoprostone** ($PGF_{2\alpha}$) are used for **ripening** the **cervix** so that induction of labor with the peptide oxytocin is more likely to be successful.

2.4. Roles of Thromboxane A_2 and Prostacyclin

Activated **platelets** produce and release thromboxane A_2 (**TXA_2**), which in turn activates other nearby platelets via TP receptors and leads to **platelet aggregation**. Platelet activation occurs preferentially at sites of vessel injury. The short half-life of TXA_2 and the rapid dilution of TXA_2 in the bloodstream help confine platelet activation to the site of injury. Activated

platelets then display a receptor that binds fibrinogen. Fibrinogen is a prevalent, threadlike protein in blood plasma that links platelets in a process called aggregation.

TXA_2 from activated platelets also binds to TP receptors on **smooth muscle cells** in the vessel wall, and thereby leads to an elevated concentration of Ca^{2+} in the cytosol, which stimulates smooth muscle contraction. T e resulting **vasoconstriction** reduces blood loss.

TXA_2 is degraded to the inactive **TXB_2**.

Prostaglandin I_2 (PGI_2, prostacyclin) released from endothelial cells and smooth muscle cells in vessel walls, acting via IP receptors, inhibits platelet aggregation and promotes vasodilation. PGI_2 release increases in response to hypoxia or vessel injury.

Via inhibition of COX, the antiplatelet drugs **aspirin** and **sulfinpyrazone** inhibit the synthesis of TXA_2 to a greater extent than the synthesis of prostacyclin (see also Fig. 32.2). Aspirin irreversibly inactivates COX in a number of tissues such that the synthesis of PGH_2 is impaired. Platelets contain only a small amount of cytoplasm (including granules) and plasma membrane. Platelets therefore do not have the means to synthesize new COX. By contrast, endothelial cells and smooth muscle cells can synthesize new COX. T e net ef ect is that aspirin and sulfinpyrazone inhibit platelet activation.

Iloprost is an analog of PGI_2 that is used to decrease blood pressure in the pulmonary artery. T ereby, PGI_2 also has mild antiplatelet activity.

3. LEUKOTRIENES AND LIPOXINS

Like prostanoids, leukotrienes and lipoxins are synthesized *f*rom arachidonic acid or EPA. Leukotrienes constrict bronchi and *f*acilitate extravasation o*f* leukocytes. Lipoxins antagonize the action o*f* leukotrienes and help resolve in*fl*ammation. T e synthesis o*f* lipoxins starts in one type o*f* cell and ends in a second type o*f* cell in a process termed transcellular synthesis.

3.1. Leukotrienes

Macrophages, **mast cells**, and **leukocytes** such as neutrophils, eosinophils, and monocytes synthesize leukotrienes from arachidonic acid (Fig. 32.3).

In mast cells and macrophages, leukotriene synthesis is stimulated by antigens that combine with immunoglobulin E on the cell surface. T e IgE-antigen complex activates a receptor that in turn causes PLA_2 and 5-lipoxygenase to become active.

Liberation of arachidonic acid from a phospholipid in the endoplasmic reticulum membrane is the first and rate-limiting step of leukotriene synthesis (see Fig. 32.3), just as it is in the synthesis of prostaglandins and TXA (see Fig. 32.2).

Leukotriene A_4 (LTA_4) is inactive; however, it gives rise to the active **LTB_4** and **LTC_4** (see Fig. 32.3). LTC_4 is formed by conjugation with **glutathione** (γ-Glu-Cys-Gly; see also Fig. 21.5 and Section 2.2 in Chapter 21). Stepwise removal of the Glu and Gly residues gives rise to **LTD_4** and **LTE_4**. LTC_4, LTD_4, and LTE_4 all contain a cysteine residue and are called **cysteinyl leukotrienes**. In general, LTC_4 and LTD_4 have higher proinflammatory activity than LTE_4.

LTB_4 attracts neutrophils, dendritic cells, and T cells. Leukotriene B_4 binds to **LTB4R** receptors. Neutrophils contain LTB4R receptors and use them to home in on the source of LTB_4. Once they meet a higher concentration of LTB_4, neutrophils activate secretion of their granules.

Leukotriene C_4 (LTB_5) increases the permeability of postcapillary venules.

T ere are two receptors for cysteine-containing leukotrienes, **CYSLTR1** and **CYSLTR2**. In airway smooth muscle

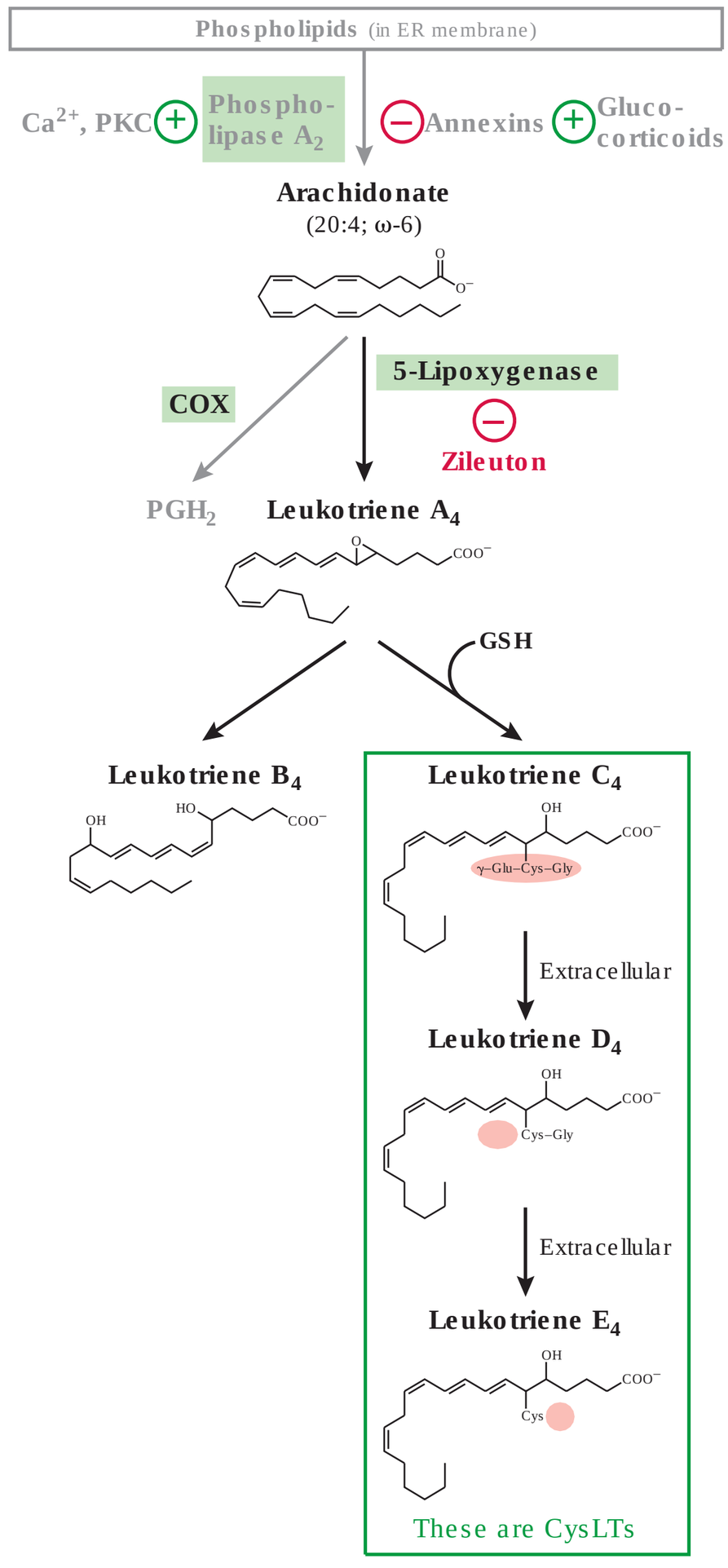


Fig. 32.3 Synthesis of leukotrienes of the 4 series. The same enzymes also catalyze the synthesis of leukotrienes of the 5 series from eicosapentaenoic acid. COX, cyclooxygenase; CysLT, cysteine-containing leukotriene; ER, endoplasmic reticulum; GSH, reduced glutathione; PG, prostaglandin; PKC, protein kinase C;

cells, activation of CYSLTR1 leads to constriction of the **bronchi**. In blood vessels, CYSLTR1 af ects cell junctions between endothelial cells so that it is easier for leukocytes and small molecules to leave the bloodstream (termed **extravasation**).

In the bronchi and bronchioles of the lungs (Fig. 32.4), GPCRs (see Section 2 in Chapter 33) coupled to $G\alpha_q$ stimulate contraction of smooth muscle, whereas GPCRs coupled to $G\alpha_s$ inhibit contraction. $G\alpha_q$ become active when the leukotrienes LTC_4, LTD_4, or LTE_4 bind to CYSLTR1 receptors. Similarly, $G\alpha_s$ become active when epinephrine or norepinephrine activate β_2-adrenergic receptors.

Asthma is due to an inflammation of the bronchi and bronchioles that leads to contraction of smooth muscle in these airways, as well as recruitment of leukocytes (Fig. 32.5). In the long term, asthma also leads to hypertrophy of smooth muscle and the glands that produce mucus.

Zileuton, an inhibitor of 5-lipoxygenase (see Fig. 32.3), and the CYSLTR1 antagonists **montelukast** and **zafirlukast** are used in the prophylaxis and chronic treatment of asthma. T e CYSLTR1 antagonists diminish activation of $G\alpha_q$.

Short-acting β_2-adrenergic receptor agonists, such as **albuterol** and **levalbuterol**, and **long-acting β_2-adrenergic receptor agonists**, such as **salmeterol** and **formoterol,** are used to dilate the bronchi in patients with asthma via activation of $G\alpha_s$. (T ese β_2-adrenergic agonists stimulate the heart only moderately.)

Glucocorticoids reduce inflammation and are used for both short- and long-term control of asthma.

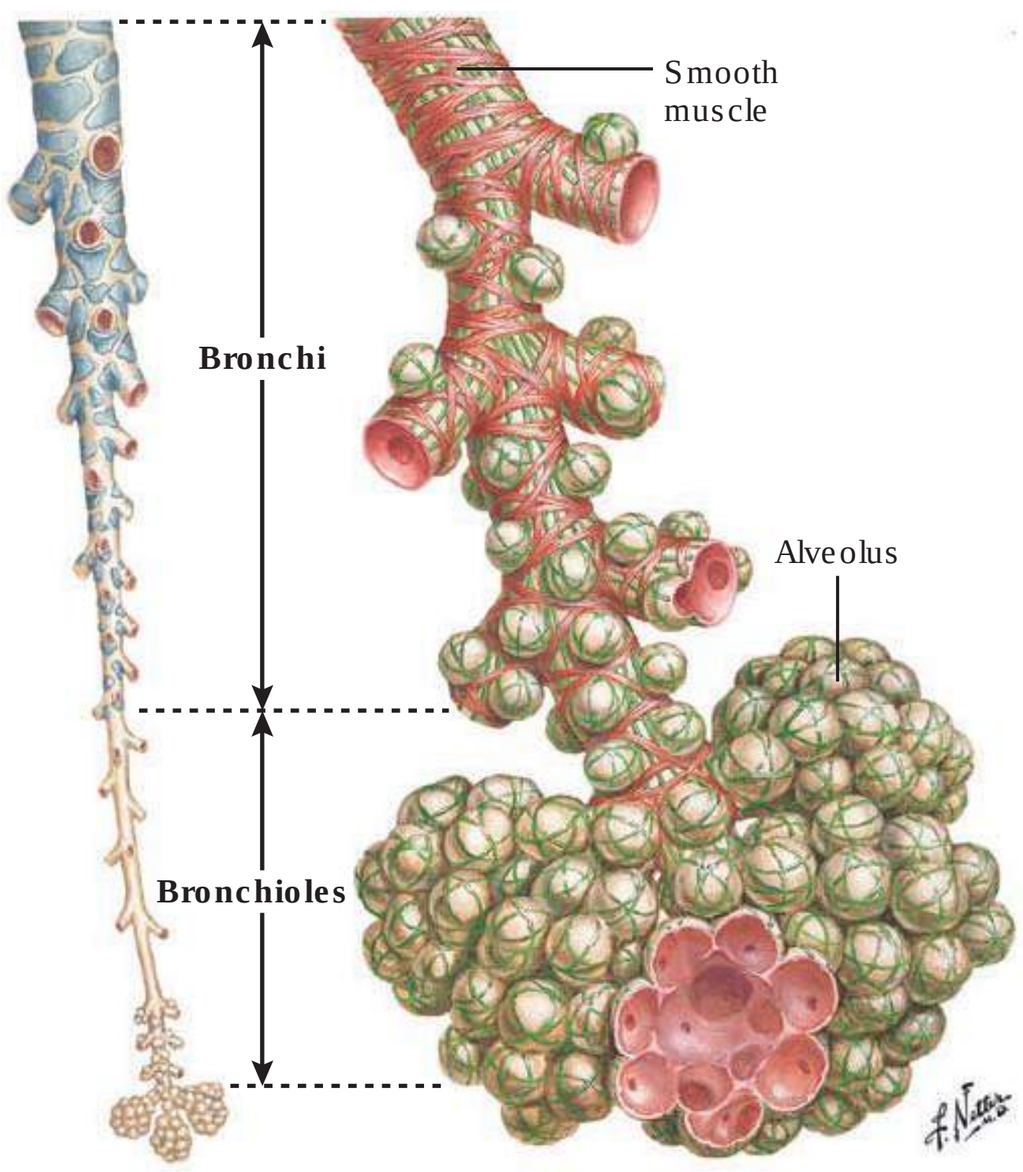


Fig. 32.4 **Bronchioles and bronchi of the lungs.**

3.2. Lipoxins

While leukotrienes (see Section 3.1) are proinflammatory, lipoxins play a role in the **resolution of inflammation**. Lipoxins inhibit activation of neutrophils and eosinophils, and they stimulate macrophages to phagocytose dead white blood cells.

Lipoxins can be synthesized from **leukotrienes** or from **15(S)-hydroxyeicosatetraenoic acid (15-HETE)**. In both cases, synthesis is split into two so that it starts in one type of cell and ends in another type of cell; this is called **transcellular synthesis**. T e requirement for two dif erent locations for lipoxin synthesis helps delay the action of lipoxins.

One pathway for the synthesis of lipoxins depends on the availability of LTA_4 (Fig. 32.6). For example, leukocytes produce LTA_4 (see Fig. 32.3). Nearby platelets convert LTA_4 to the isomers **lipoxin A_4 (LXA_4)** and **lipoxin B_4 (LXB_4)**.

A second pathway for the synthesis of lipoxins depends on the availability of 15-HETE (see Fig. 32.6). For example, endothelial cells produce 15-HETE. Nearby leukocytes convert 15-HETE to lipoxin A_4 and lipoxin B_4. (Leukocytes cannot produce lipoxins on their own, because they lack 12-lipoxygenase to convert LTA_4 to lipoxins, and they lack 15-lipoxygenase to synthesize 15-HETE as a precursor for lipoxins.)

In line with their antiinflammatory ef ect, LXA_4 and LXB_4 have the following two ef ects: T ey antagonize signaling by cysteinyl leukotrienes via CYSLTR1 and CYSSLTR2 (see Section 3.1) and thereby prevent the activation of neutrophils. On the other hand, LXA_4 and LXB_4 bind to the **formyl peptide receptor 2 (FPR2)**, and to **GPCR32**, both of which are GPCRs.

Aspirin relieves pain not only by inhibiting prostaglandin synthesis (see Section 2.3), but also by acetylating COX-2, which then makes **epi-LXA_4**. Like LXA_4 and LXB_4,

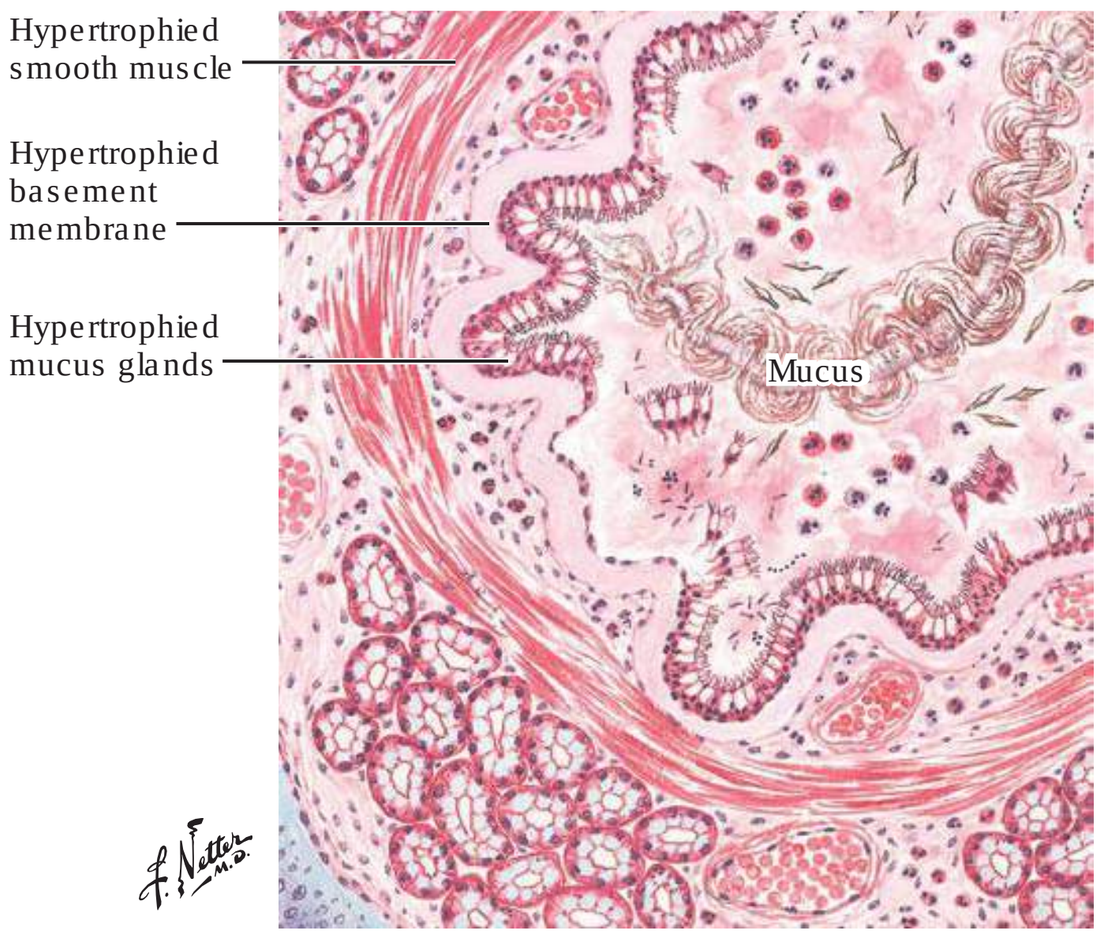


Fig. 32.5 **Altered airway in asthma.**

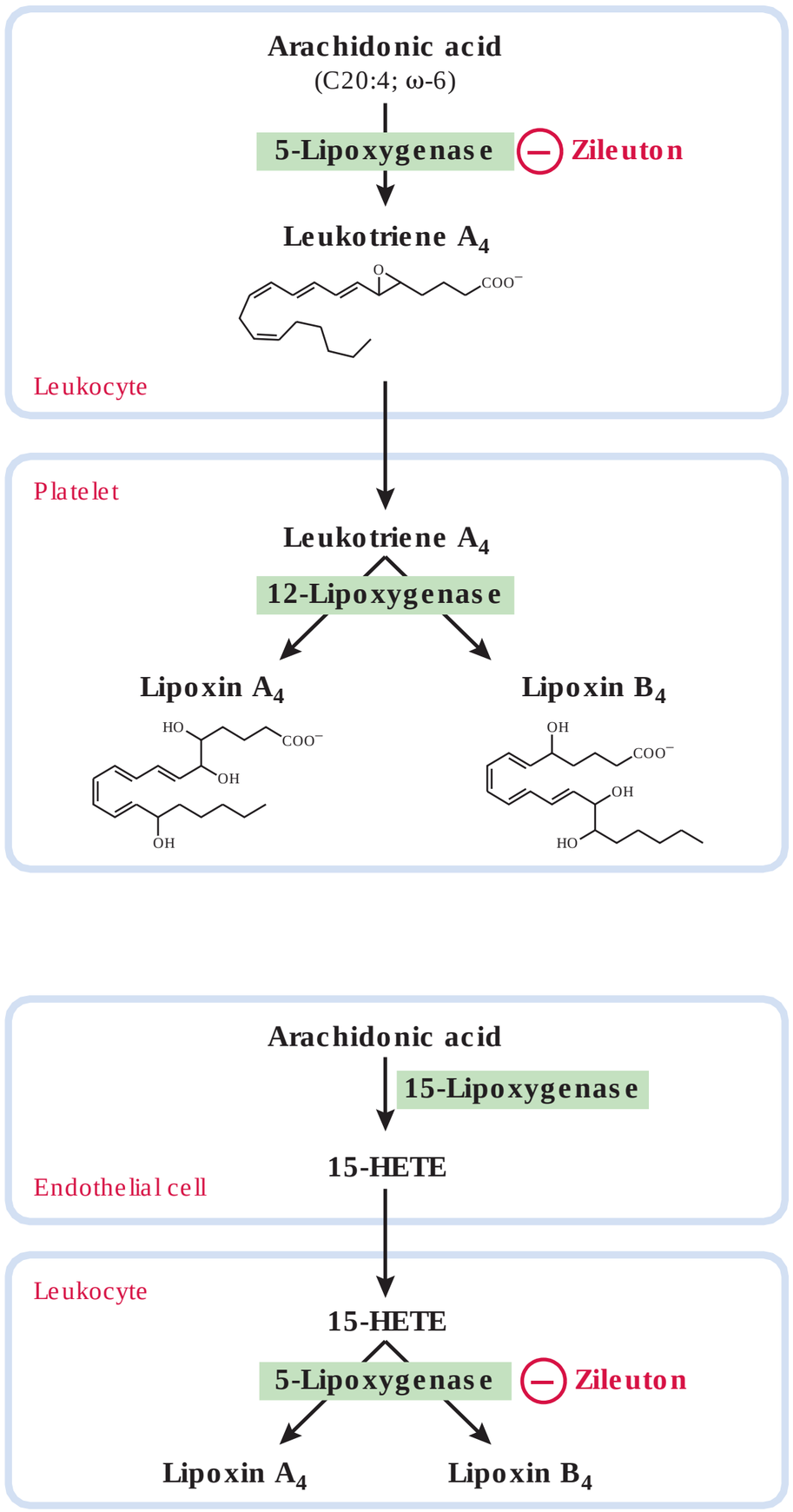


Fig. 32.6 Transcellular synthesis of lipoxin A_4 and B_4. The two lipoxins are isomers. 15-HETE, 15(S)-hydroxyeicosatetraenoic acid.

epi-lipoxin A_4 binds to the FPR2 and therefore has an antiinflammatory effect.

The ω-3 fatty acid **DHA** (C22:6) gives rise to **D-series resolvins**, some of which also act on FPR2 and GPR32. Similar to lipoxins, resolvins terminate inflammation.

SUMMARY

- We take in ω-3 fatty acids mainly by consuming fats from plant seeds, nuts, algae, or fish. A small amount of linolenic acid (C18:3) is converted to eicosapentaenoic acid (EPA, C20:5) and docosapentaenoic acid (DHA, C22:6). Fat from algae or fish are the only good sources of EPA and DHA. Linolenic acid, EPA, and DHA are incorporated into membrane phospholipids. The recommended intake of ω-3 fatty acids is an amount equivalent to 0.5% of all calories.
- Current Western diets are rich in plant oils, which in turn provide plenty of ω-6 fatty acids. Linoleic acid (C18:2) is converted to arachidonic acid (C20:4) and incorporated into membrane phospholipids.
- Phospholipase A_2 catalyzes the production of EPA or arachidonic acid from phospholipids in the endoplasmic reticulum membrane. This is the rate-limiting step in eicosanoid biosynthesis. The eicosanoids include prostanoids, thromboxanes, leukotrienes, and lipoxins.
- EPA and arachidonic acid can give rise to prostanoids (prostaglandins and thromboxanes), leukotrienes, and lipoxins. Eicosanoids have a short life span and act locally. Prostaglandins of the 2 series (derived from arachidonic acid) are generally proinflammatory, whereas prostaglandins of the 3 series (derived from EPA) are generally antiinflammatory.
- COX-1 and COX-2 convert EPA and arachidonic acid to PGH_3 and PGH_2, respectively. COX-1 is present in most cells at all times. COX-2 is predominantly synthesized only in response to inflammation. COX-2 is also found in many tumor cells.
- Eicosanoids exert their effects via GPCRs, which in turn either alter the concentration of cAMP or Ca^{2+} in the cytosol.
- In the stomach and duodenum, PGE_2 protects the mucosa by enhancing the secretion of mucus and bicarbonate. Nonselective NSAIDs inhibit PGE_2 synthesis and raise the risk of ulcers and perforation of the mucosa. Compared with nonselective NSAIDs, celecoxib, a selective COX-2 inhibitor, is associated with a lower risk of perforation or bleeding in the gastrointestinal tract. Misoprostol, a PGE_1 analog, protects the stomach mucosa in long-term NSAID users.
- Misoprostol and dinoprostone ($PGF_{2\alpha}$) are used to ripen the cervix.
- TXA_4 from activated platelets stimulates platelet aggregation, whereas PGI_2 from endothelial cells inhibits platelet aggregation. Aspirin and sulfinpyrazone have an antiplatelet effect because they inhibit TXA_4 synthesis more than PGI_2 synthesis.
- The PGI_2 analog iloprost is used to decrease blood pressure in patients who have pulmonary hypertension.
- Leukotrienes made from arachidonic acid induce bronchoconstriction and stimulate inflammation, thereby attracting white blood cells and increasing the permeability of blood vessels to plasma and leukocytes.
- Asthma is caused by prostaglandin- and leukotriene-mediated inflammation of the bronchi and bronchioles. The following agents are used in the treatment of asthma: the 5-lipoxygenase inhibitor zileuton, the CysLTR1 antagonists montelukast and zafirlukast, β_2-adrenergic receptor agonists, and glucocorticoids.
- Lipoxins are antiinflammatory and are generated from LTA_4 or 15-HETE via transcellular synthesis.
- Aspirin acetylates and thereby permanently inactivates COX-1 while it alters catalysis of COX-2 so that COX-2 stops producing prostaglandins and instead produces lipoxins, which are antiinflammatory.

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Review Questions

1. By weight, which one of the following oils contains the highest fraction of a fatty acid that can be converted to PGE_3?

   A. Algae oil
   B. Cod liver oil
   C. Flaxseed oil
   D. Olive oil

2. T e antiplatelet ef ect of aspirin is ascribed to inhibition of the synthesis of which one of the following?

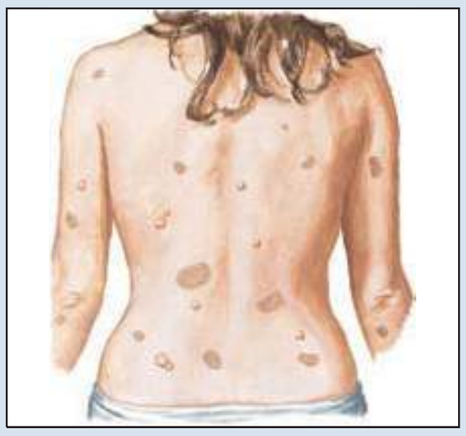
   A. Arachidonic acid
   B. LTB_4
   C. LXB_4
   D. PGI_2
   E. TXA_2

3. Which one of the following drugs is most suitable to inhibit the synthesis of leukotrienes and lipoxins, but not the synthesis of prostaglandins or thromboxanes?

   A. Albuterol
   B. Celecoxib
   C. Dinoprostone
   D. Montelukast
   E. Zileuton

4. A 67-year-old woman takes NSAIDs chronically to treat rheumatoid arthritis. Which one of the following drugs is best suited to prevent the formation of ulcers?

   A. Cortisol
   B. Misoprostol
   C. Salmeterol
   D. Zafirlukast
   E. Zileuton



Chapter 33 Signaling

SYNOPSIS

- After binding a ligand, such as epinephrine, G protein–coupled receptors (GPCRs) activate heterotrimeric G proteins, a subunit of which then favors or inhibits the formation of a second messenger, such as cyclic adenosine monophosphate (cAMP). cAMP in turn activates protein kinase A, which then phosphorylates a variety of substrates.
- Many growth factor receptors have tyrosine kinase activity and activate the RAS/RAF/MEK/ERK pathway, which leads to changes in the activity of many transcription factors.
- The RAS/RAF/MEK/ERK pathway is overly active in Noonan syndrome, neurofibromatosis type 1, and in many tumor cells.

LEARNING OBJECTIVES

For mastery *of* this topic, you should be able to do the *f*ollowing:

- Describe how adrenergic receptors with epinephrine or norepinephrine bound to them activate intracellular signaling pathways.
- Compare and contrast the signaling pathways of adrenergic receptors with those of activated glucagon receptors and glucagon-like peptide-1 receptors.
- List molecular events that terminate signaling via GPCRs and heterotrimeric G proteins.
- Describe the RAS/RAF/MEK/ERK signaling pathway, and list hormones and their receptors that use this pathway.
- Explain how the causes of Noonan syndrome and neurofibromatosis type 1 relate to a signaling pathway.

1. PRINCIPLES OF SIGNALING

Chemical signaling takes place over short (cell to cell) and long distances (via blood). Signaling pathways o*ft*en generate a concentration wave o*f* a signaling molecule because there are mechanisms *f*or both starting and ending a signal. Phosphorylation/dephosphorylation and associated changes in protein con*f*ormation are a common occurrence in intracellular signaling pathways.

Many signaling pathways involve the conversion of an extracellular signal into an intracellular signal, amplification of the intracellular signal, and both stimulatory and inhibitory interactions with other pathways.

Endocrine signaling typically involves the secretion of a hormone into the bloodstream and the recognition of that hormone in a distant tissue. Accordingly, **endocrine secretion** refers to secretion of a substance into the blood, whereas **exocrine secretion** refers to secretion of a substance into the lumen of the gastrointestinal tract, the airways, the urinary tract, the environment, and so forth. For instance, exocrine cells of the pancreas secrete enzymes into the pancreatic ducts that digest food; endocrine cells of the pancreas secrete insulin and glucagon into the bloodstream to control the concentration of glucose in the blood.

T e term **autocrine** signaling refers to secretion of a substance into the extracellular space by a cell whereby the secreting cell is also the one that responds to the secreted product. T e term **paracrine signaling** refers to secretion of a substance into the extracellular space by a cell whereby a nearby cell responds to the secreted product. In paracrine signaling, the signal is typically short lived, such as in eicosanoid signaling (see Chapter 32).

For almost every pathway that leads to an **increase** in output, there is at least one reaction that leads to a **decrease** in output. For instance, binding of epinephrine to a GPCR leads not only to an increased intracellular signal in the form of cAMP but also to the temporary or permanent removal of the receptor from the plasma membrane. Activation occurs more quickly than inactivation and thus generates a time-limited signal.

Protein phosphorylation is commonly involved in signal transduction. **Protein kinases** typically add a phosphate group, and **protein phosphatases** remove a phosphate group. Phosphorylation can render an enzyme or protein active or inactive. Most kinases react a phosphate with the hydroxyl group of a **serine** or **threonine** side chain. A smaller group of kinases react a phosphate with the hydroxyl group of a **tyrosine** side chain.

2. G PROTEIN–COUPLED RECEPTOR SIGNALING

GPCRs sense many peptide hormones, neurotransmitters, and chemokines; they are also involved in smell, taste, and sight. Ligand binding to the extracellular portion o*f* the receptor leads to a change in con*f*ormation o*f* the cytosolic portion, which in turn activates a heterotrimeric G protein. T e activated heterotrimeric G protein o*ft*en produces a change in the concentration o*f* a second messenger. Common second messengers are cAMP, inositol trisphosphate (IP_3), and diacylglycerol (DAG). T e second messenger in turn triggers changes in metabolism or gene expression.

T e **GPCR**s form a large class of receptors that respond to extracellular water-soluble signals, such as hormones, neurotransmitters, odorants, or light. Humans have ~800 genes that encode GPCRs.

A large number of drugs in current use target GPCRs. For example, angiotensin receptor 1 blockers (e.g., losartan) lower

blood pressure (see Fig. 31.17 and Section 4.1 in Chapter 31). Histamine H2-receptor antagonists (e.g., cimetidine) lower acid production in the stomach (see Fig. 34.2 and Section 1 in Chapter 34). Epinephrine and a variety of drugs that act on adrenergic receptors can be used for control of hypersensitivity reactions, bleeding, asthma, and blood pressure.

The description of GPCRs in this section is limited to GPCRs that play a role in the biochemistry discussed in this book; that is, glucagon receptors, glucagon-like peptide-1 (GLP-1) receptors, adrenergic receptors, angiotensin receptors, and histamine receptors (Table 33.1).

When a GPCR is activated by a ligand (e.g., epinephrine), it acts as a **guanine nucleotide exchange factor** (**GEF**) that exchanges a guanosine diphosphate (GDP) for a guanosine triphosphate (GTP) bound to a **heterotrimeric G protein** (Fig. 33.1). GTP binding to a heterotrimeric G protein activates the G protein. Certain GPCRs bind a heterotrimeric G protein with GDP bound to it before they bind a hormone, whereas others bind the G protein only after they have been activated by a hormone.

Heterotrimeric G proteins consist of an α-, a β-, and a γ-subunit (see Fig. 33.1). The α- and the γ-subunits are each anchored in the plasma membrane (on the cytosolic side) with myristic acid and either palmitic acid or a prenyl group (see Fig. 11.9 and Section 1.5 in Chapter 11). The α-subunit binds GDP or GTP. When inactive—that is, when bound to GDP—the α-, β-, and γ-subunits form a trimeric complex. When activated by GTP binding, the α subunit separates from the dimeric βγ-complex and assumes a new conformation. The α-subunit is active until GTP is hydrolyzed to GDP (see below); the activated α-subunit has the features of a timer.

The GTP-activated α subunit has intrinsic GTPase activity, and a **GTPase-activating protein** (**GAP**) can often greatly increase this GTPase activity, thus rendering the α-subunit inactive (see Fig. 33.1).

The membrane-bound **βγ-complex** also acts as a signal and activates certain ion channels or phospholipases, effects that are not discussed further here.

There are several isoforms of **α-subunits** that differ in their effects (Fig. 33.2). The α-subunits are membrane bound and alter the activity of membrane-bound enzymes. Activated $\boldsymbol{\alpha_s}$-subunits activate **adenylyl cyclase** and thus increase the concentration of cAMP. Activated $\boldsymbol{\alpha_i}$-subunits inhibit adenylyl cyclase and thus decrease the concentration of cAMP.

Table 33.1 Some G Protein–Coupled Receptors

Receptor	Relevant Ligands	Type of Gα, Second Messenger, and Effect
Glucagon receptor	Glucagon	$G\alpha_s$ activates an adenylyl cyclase, which produces cAMP. cAMP activates protein kinase A.
GLP-1 receptor	GLP-1	
α_1-Adrenergic receptor, subtypes A, B, D	Epinephrine, norepinephrine Agonists such as phenylephrine are used as decongestants	$G\alpha_q$ and $G\alpha_{11}$ activate a phospholipase C, which hydrolyzes PIP2 to IP_3 and DAG. IP_3 increases cytosolic Ca^{2+}. DAG activates protein kinase C.
α_2-Adrenergic receptor, subtypes A, B, C	Epinephrine, norepinephrine The agonist clonidine is used to reduce blood pressure and to relieve pain via epidural administration	$G\alpha_i$ inhibits adenylyl cyclases
β_1-Adrenergic receptor	Epinephrine, norepinephrine	$G\alpha_s$ activates an adenylyl cyclase, which produces cAMP. cAMP activates protein kinase A.
β_2-Adrenergic receptor	Epinephrine, norepinephrine The agonist albuterol is used to prevent or treat bronchospasm	
β_3-Adrenergic receptor	Epinephrine, norepinephrine	
Angiotensin II receptor type 1	Angiotensin II, angiotensin III	$G\alpha_q$ or $G\alpha_{11}$ activate a phospholipase C, which hydrolyzes PIP2 to IP_3 and DAG. IP_3 increases cytosolic Ca^{2+}. DAG activates protein kinase C.
Histamine H2 receptor	Histamine The antagonist cimetidine reduces acid production in the stomach	$G\alpha_s$ activates an adenylyl cyclase, which produces cAMP. cAMP activates protein kinase A.
Prostanoid EP2 and EP4 receptors	Prostaglandin E_2	$G\alpha_s$ activates an adenylyl cyclase, which produces cAMP. cAMP activates protein kinase A.
Cysteinyl leukotriene receptors CYSLTR1 and CYSLTR2	Leukotrienes LTC_4, LTD_4, LTE_4	$G\alpha_q$ and $G\alpha_{11}$ activate a phospholipase C, which hydrolyzes PIP2 to IP_3 and DAG. IP_3 increases cytosolic Ca^{2+}. DAG activates protein kinase C.

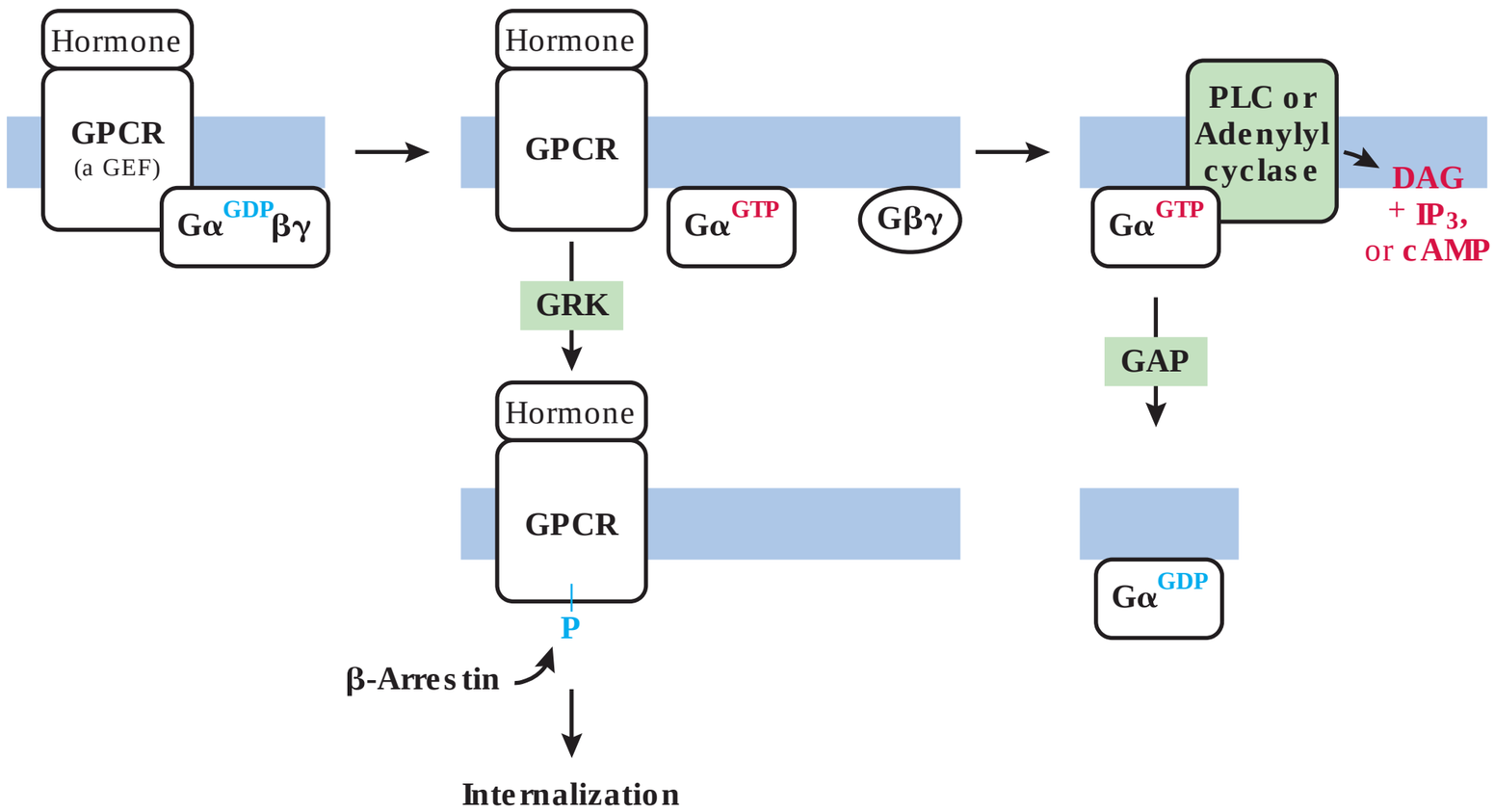


Fig. 33.1 GPCR activation of heterotrimeric G proteins. DAG, diacylglycerol (a lipid); GEF, guanine nucleotide exchange factor; GPCR, G protein–coupled receptor; GRK, GPCR kinase; GAP, GTPase activating protein; IP_3, inositol trisphosphate (a sugar); P, phosphate group; PLC, phospholipase C.

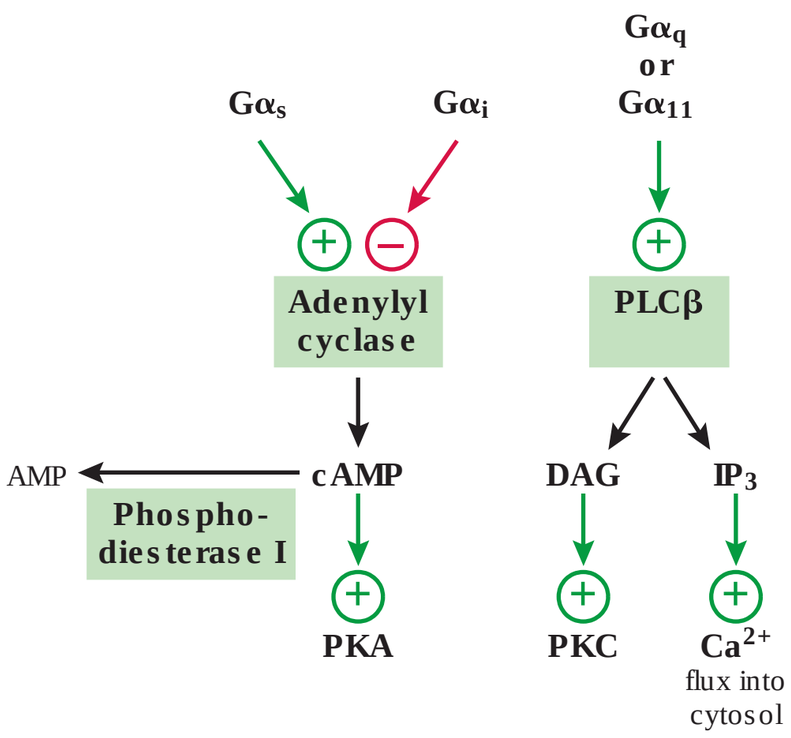


Fig. 33.2 Signaling pathways activated by activated Gα-subunits. DAG, diacylglycerol; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C.

Activated α_q- or α_{11}-subunits activate phospholipase C, which hydrolyzes phosphatidylinositol bisphosphate (PIP2), which leads to an increase in the concentrations of **IP_3** in the cytosol and **DAG** in the membrane. Activated α_t-subunits activate guanylyl cyclase and thus increase the concentration of **cGMP**.

During prolonged stimulation, GPCRs undergo appreciable **downregulation**. Once activated by a ligand, a GPCR becomes a target for phosphorylation by a **GPCR kinase**. T is is followed by binding of **β-arrestin** to the GPCR, which thereby prevents the GPCR from activating another G protein. Furthermore, the β-arrestin-GPCR complex binds to clathrin-coated pits and is internalized via endocytosis. Some of these GPCRs eventually return to the plasma membrane; others are degraded.

T e **glucagon receptor** is found mostly in the liver, where protein kinase A activity leads to phosphorylation of glycogen synthase and glycogen phosphorylase and thus increases glycogen degradation (see Figs. 24.5 and 24.7, Sections 1.3 and 2.2 in Chapter 24, and Fig. 26.9). Furthermore, cAMP and protein kinase A activity increase the rate of gluconeogenesis via increased activity of phosphoenolpyruvate carboxykinase, fructose 1,6-bisphosphatase, and glucose 6-phosphatase (see Fig. 25.7 and Section 3 in Chapter 25).

GLP-1 receptors work essentially in the same fashion as glucagon receptors, raising the intracellular concentration of cAMP. Note that cAMP has dif erent ef ects on dif erent cells. For instance, in hepatocytes, a glucagon-induced increase in the concentration of cAMP enhances glycogenolysis and gluconeogenesis, whereas in pancreatic β-cells a GLP-1–induced increase in cAMP enhances insulin secretion.

Adrenergic receptors bind the physiological ligands epinephrine or norepinephrine, and receptor diversity accounts for tissue-specific ef ects. In pancreatic β-cells, α_2-adrenergic receptors inhibit insulin secretion. In hepatocytes, β-adrenergic receptors stimulate glycogenolysis and gluconeogenesis. In adipose tissue, β_3-adrenergic receptors stimulate lipolysis (see Section 5.1 in Chapter 28).

3. GROWTH FACTOR RECEPTORS THAT ARE RECEPTOR TYROSINE KINASES

Insulin and other growth *f*actors such as epidermal growth *f*actor (EGF) bind to receptors, which, when activated, have a tyrosine kinase that phosphorylates both the receptor and adaptor proteins. T e adaptor proteins activate two di*ff*erent signaling pathways: the PI3K/AK*T* pathway and the RAS/RAF/MEK/ERK pathway. T e PI3K/AK*T* pathway is chie*fl*y responsible *f*or the e*ff*ects o*f* insulin on metabolism, and the RAS/RAF/MEK/ERK pathway accounts *f*or the e*ff*ects o*f* insulin on cell proli*f*eration and di*ff*erentiation. T e RAS/RAF/MEK/ERK pathway has abnormal activity in

neurofibromatosis type 1, in Noonan syndrome, and in Cowden syndrome.

3.1. Normal Receptor Tyrosine Kinase Signaling

Growth factors, such as insulin, insulin-like growth factor 1 (IGF-1), and EGF, bind to membrane-embedded receptors, which subsequently undergo a conformational change that activates a tyrosine kinase on the cytoplasmic face of the receptor; accordingly, these receptors are called **receptor tyrosine kinases**. Humans have more than 50 genes that encode receptor tyrosine kinases.

The insulin receptors and the IGF-1 receptor are always present as an $\alpha_2\beta_2$ complex, whereby the α-subunits form an extracellular high-affinity hormone binding site, and the β-subunits each have a tyrosine kinase domain in the cytosol. (As will be shown below, there are two different isoforms of the insulin receptor, but for now this does not matter.) Upon binding of insulin, the tyrosine kinase domains in the insulin receptor β-subunits phosphorylate each other in a process called **trans-autophosphorylation**.

After trans-autophosphorylation, the tyrosine kinases of the receptors for insulin and IGF-1 phosphorylate a membrane-associated **insulin receptor substrate (IRS)** protein, of which there are four varieties (Fig. 33.3). Phosphorylated IRS protein can activate both the PI3K/AKT pathway and the RAS/RAF/MEK/ERK pathway. The PI3K/AKT pathway is especially important for insulin control of glucose transport, glucose metabolism, and protein synthesis, whereas the RAS/RAF/MEK/ERK pathway is particularly important for regulating transcription, cell division, and cell differentiation.

Phosphorylated IRS activates the **PI3K/AKT signaling pathway** by binding to **phosphatidylinositol-4,5-bisphosphate 3-kinase** (**PI3K, phosphoinositide 3-kinase**, of which there are multiple isozymes), thereby activating it (see Fig. 33.3). PI3K then phosphorylates the phospholipid **PIP2** to **PIP3** (phosphatidylinositol trisphosphate). PIP3 then serves as a binding site for **PDPK1** (phosphoinositide-dependent protein kinase 1, also called PDK1) and **AKT2**, whereby PDPK1 phosphorylates and thus activates AKT2. Active AKT2 in turn generates a signal that leads to the insertion of **GLUT-4 glucose transporters** into the plasma membrane and to activation of **glycogen synthase kinase (GSK3)**, which in turn activates **glycogen synthesis** (GSK3 also plays a role in many other signaling pathways). (The PI3K/AKT pathway was mentioned in Fig. 8.3 and Section 1.1 of Chapter 8. There are two AKTs, AKT1 and AKT2; AKT1 mostly plays a role in growth, whereas AKT2 is more important for glucose homeostasis.)

PTEN is a phosphatase that attenuates PI3K/AKT signaling by degrading PIP3.

Phosphorylated IRS also activates the **RAS/RAF/MEK/ERK signaling pathway** by binding to **GRB2**, which then binds **SOS**. SOS is a guanine nucleotide exchange factor (GEF; see Section 1) for the membrane-bound **RAS**; that is, it forces RAS to release bound GDP. Thereafter, RAS binds the more abundant GTP and thus becomes active. Thus active RAS functions like the active α-subunit of a heterotrimeric G

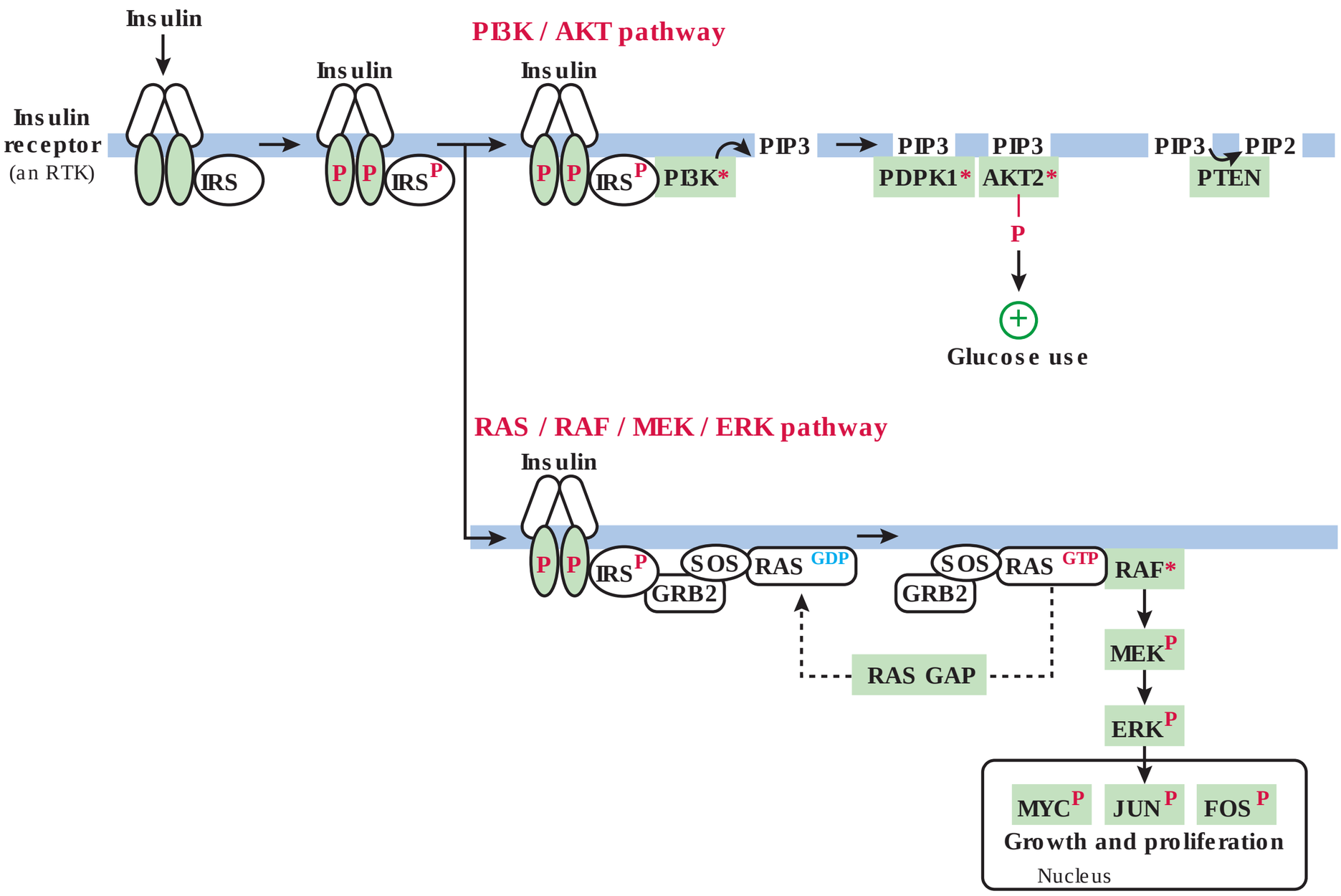


Fig. 33.3 **Insulin receptor signaling pathways.** P, phosphate. See text for other abbreviations.

protein (see Section 1). RAS is actually a member of the family of **small GTPases**. Active RAS binds to and thereby activates **RAF**, which is then also membrane bound. RAS has inherent GTPase activity to hydrolyze GTP, which renders RAS inactive. **GTPase activating proteins** (**GAPs**) enhance the intrinsic GTPase activity of RAS (GAPs also stimulate hydrolysis of GTP bound to the α-subunit of heterotrimeric G proteins; see Section 1). Thus SOS activates RAS, and RAS, enhanced by a RAS GAP, inactivates itself.

The kinase RAF starts a cascade of phosphorylation in the sequence RAF → **MEK** → **ERK**, whereby the signal is amplified at each step, and ERK can travel from the cytosol to the nucleus. The RAF → MEK → ERK kinase cascade is sometimes called the **MAP kinase cascade**. Phosphorylated ERK can move into the nucleus and affect transcription by phosphorylating a variety of transcription factors (e.g., MYC, JUN, FOS, CREB; see also Fig. 8.6). ERK also helps end signaling by phosphorylating RAF in a way that inhibits RAF activity.

Three different receptor tyrosine kinases bind **insulin**, **IGF-1**, and **IGF-2** (Fig. 33.4). Due to alternative splicing of mRNA from a single gene, there are two insulin receptor isoforms. **IR-B** contains all 22 exons, whereas **IR-A** lacks exon 11, which encodes 12 amino acids. IR-A and IR-B are each synthesized as a proreceptor protein that is then cleaved to give rise to an α- and a β-subunit. These subunits form an $\alpha_2\beta_2$ complex, which is stabilized by multiple disulfide bonds. IR-A is most common in fetal tissues and tumor cells, and IR-B is most common in differentiated cells. There is only a single form of the IGF-1 receptor.

The insulin receptors and the IGF-1 receptor can each bind multiple ligands (see Fig. 33.4). To complicate matters, IR-A, IR-B, and IGF-1R can form hybrid receptors. Furthermore, although insulin, IGF-1, and IGF-2, for example, each bind to IR-A, they have different effects on the conformation of the cytosolic portion of the receptor; the reason for this is unclear.

As shown above, the insulin receptor can activate both the PI3K/AKT pathway and the RAS/RAF/MEK/ERK pathway. The balance of activity in these pathways is in part tissue dependent.

Due to the structure of the signaling network, IGF-2 affects glucose metabolism, and insulin affects both carbohydrate metabolism and the cell cycle. Large solid tumors sometimes produce enough IGF-2 to cause hypoglycemia. In the development of insulin analogs for the treatment of diabetes, a reasonable balance has to be found between effects on glucose homeostasis and on growth (which could be tumorigenic).

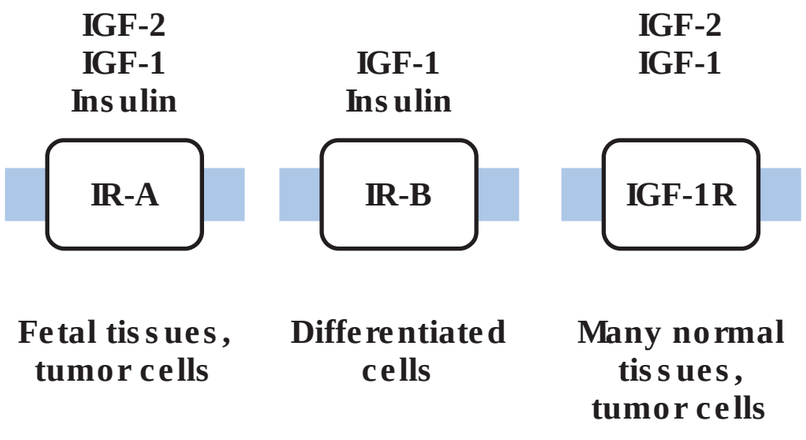


Fig. 33.4 Physiological stimuli of insulin receptors (IR) and insulin-like growth factor (IGF) receptors.

An adaptor protein binds insulin-activated insulin receptors to budding clathrin-coated pits, and the insulin receptors then end up in **endosomes**, where a phosphotyrosine phosphatase dephosphorylates them (and thus inactivates them). This is a common mechanism for downregulation of other ligand-activated receptor tyrosine kinases and GPCRs (see Section 2), although the adaptor proteins differ.

Activation of the RAS/RAF/MEK/ERK pathway is observed in many **tumors**, as explained in Section 1.1 of Chapter 8.

The **EGF receptor (EGFR)** and **HER2** (human EGF receptor-2, NEU) are receptor tyrosine kinases that are monomers in the inactive state and dimers in the active state. The EGFR (encoded by EGFR gene) can be activated by EGF and by transforming growth factor-α (TGF-α). EGFR is a proto-oncogene, and activating mutations in one allele are frequently observed in tumors, such as breast cancer and adenocarcinoma of the lung (see Section 3 in Chapter 8). The tyrosine kinase activity of EGFRs can be inhibited with lapatinib, erlotinib, or afatinib. HER2 is encoded by the ERBB2 gene. There is no known hormone that activates HER2. Perhaps HER2 is constitutively active, or it forms heterodimers with EGFRs. Activating mutations in one ERBB2 allele are frequently seen in invasive breast tumors. The HER2 kinase activity can be inhibited with a kinase inhibitor (lapatinib, afatinib) or a monoclonal antibody (trastuzumab, pertuzumab).

3.2. Neurofibromatosis, Noonan Syndrome, and Cowden Syndrome

Neurofibromatosis type 1 (**von Recklinghausen disease**) is a heritable tumor syndrome caused by heterozygous loss of function of **neurofibromin 1** (**neurofibromatosis-related protein**, **NF1**), which is encoded by the NF1 gene. NF1 is a RAS GAP; that is, a protein that activates the intrinsic GTPase activity of RAS and thereby inactivates RAS. There are ~1,500 known pathogenic mutations in NF1, most of which lead to a truncated protein. As in other heritable cancer syndromes (see Chapter 8), some somatic cells lose the function of the remaining normal NF1 allele and thus are more likely to give rise to a neoplasm.

The prevalence of neurofibromatosis type 1 is ~1 in 3,000 births, and ~50% of affected persons have a de novo mutation that in turn most likely occurred in the germline of one parent.

The diagnosis of neurofibromatosis type 1 involves meeting at least two of the following seven criteria (see also Fig. 33.5): café-au-lait macules, skin-fold freckling, neurofibromas, Lisch nodules (in the irises), optic pathway tumor, bone dysplasia, or a family history of the disorder. Virtually all affected persons develop symptoms before age 8 years.

Most persons who have neurofibromatosis type 1 develop benign neurofibromas of the skin. About half of all patients have congenital plexiform neurofibromas, which may be superficial or inside the body, be disfiguring, or impair the function of an organ. Plexiform neurofibromas sometimes

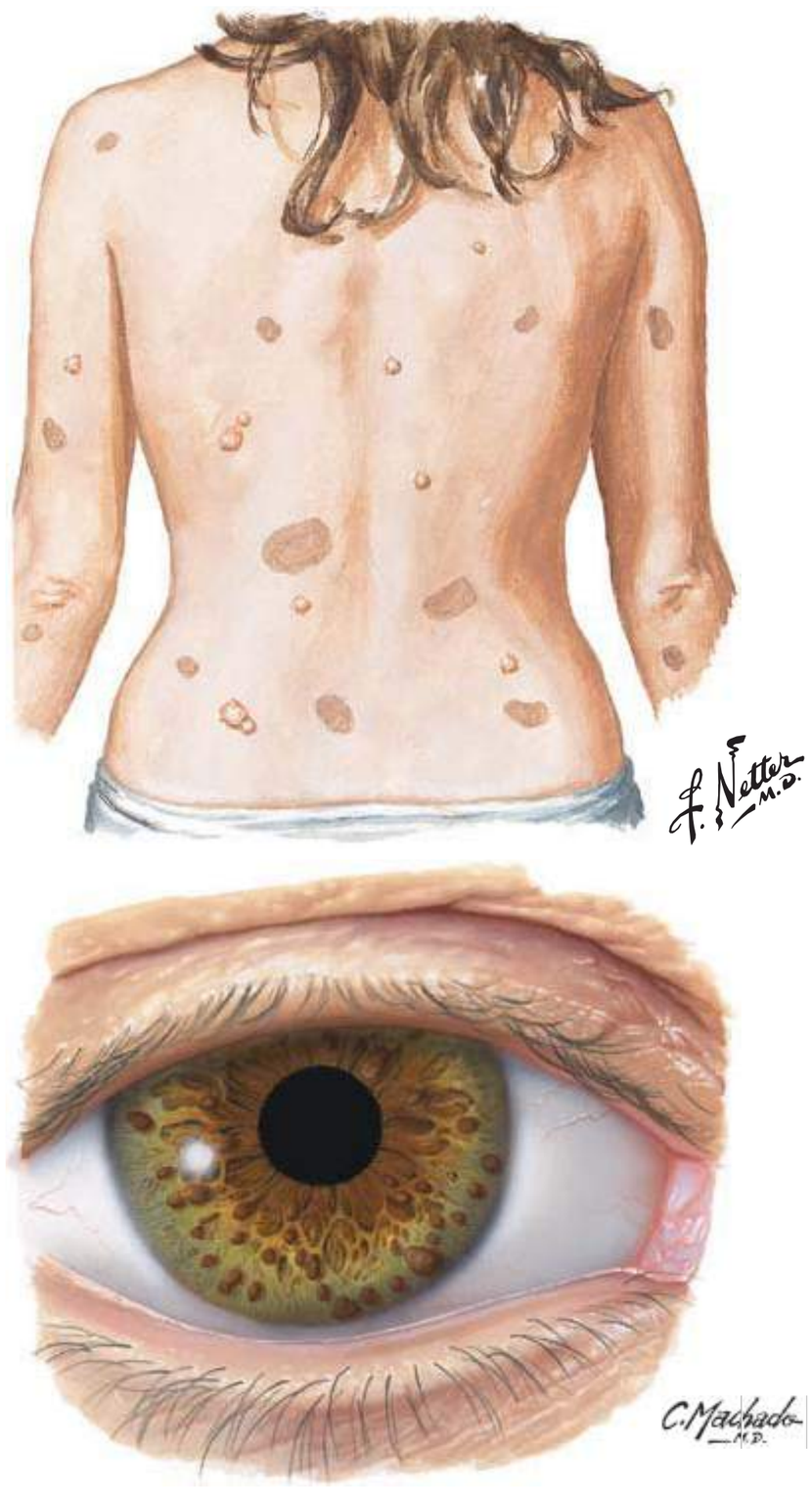


Fig. 33.5 Neurofibromatosis type 1: Skin with café-au-lait spots and neurofibromas, iris with Lisch nodules. The neurofibromas have the appearance of nodules. Many affected persons also have scoliosis.

turn malignant. About 15% of af ected children develop an optic glioma by 6 years of age (rarely later). Optic gliomas sometimes impair vision. In adulthood, malignancies arise mostly in the central nervous system, in the gastrointestinal system (especially the small intestine), and in the genitourinary tract.

Noonan syndrome is frequently associated with a congenital heart defect, failure to thrive during childhood that causes short stature, a dysmorphic face, and abnormalities of the skeleton. Subtypes of Noonan syndrome are caused by heterozygosity for gain-of-function mutations in the RAS/RAF/MEK/ERK signaling pathway, specifically in the following genes: P*T*PN11 (encoding an adaptor protein of poorly understood function), SOS1, KRAS, NRAS, RI*T*1 (encoding a small GTPase of the RAS family), BRAF, and RAF1.

Noonan syndrome has a prevalence of ~1 in 1,500 births. About 60% of the observed mutations are de novo, and the mutations are inherited in autosomal dominant fashion.

Some of the criteria for a diagnosis of Noonan syndrome are facial dysmorphology, pulmonary valve stenosis or hypertrophic cardiomyopathy, height less than the third percentile, intellectual disability, cryptorchidism, and a first-degree relative with the syndrome.

Cowden syndrome, a hereditary cancer syndrome, is caused by a loss-of-function mutation in P*T*EN (see Section 1.1 in Chapter 8). PTEN normally catalyzes the reaction PIP3 → PIP2 and thereby prevents the activation of PI3K and the PI3K-AKT signaling pathway.

SUMMARY

- Glucagon, glucagon-like peptide, epinephrine, norepinephrine, angiotensin II, histamine, prostaglandin E2, and the cysteinyl leukotrienes signal via G protein–coupled receptors (GPCRs), which are coupled to membrane-bound heterotrimeric G proteins. T e activated GPCR acts as a guanine nucleotide exchange factor (GEF) so that the Gα-subunit releases GDP and binds GTP, which activates the Gα-subunit. T e main families of the Gα-subunit discussed in this chapter are $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_{11}$. Active $G\alpha_s$ stimulates the activity of adenylyl cyclase (which produces cAMP), whereas active $G\alpha_i$ inhibits adenylyl cyclase. $G\alpha_q$ and $G\alpha_{11}$ are very similar proteins, which activate phospholipase C (PLC). PLC hydrolyzes phosphatidyl inositol bisphosphate (PIP2) into diacyl glycerol (DAG) and inositol trisphosphate (IP_3). DAG activates protein kinase C (PKC), whereas IP_3 stimulates the flow of Ca^{2+} into the cytosol.
- After GPCR kinase phosphorylates a GPCR, β-arrestin binds to the GPCR and prevents further activation of G proteins. β-Arrestin also induces the endocytosis of these GPCRs via clathrin-coated pits.
- GTPase activating proteins (GAPs) stimulate the intrinsic GTPase activity of Gα-subunits and thereby inactivate Gα.
- After binding a ligand, the IR-A and IR-B insulin receptors, the IGF-1 receptor, the EGF receptor, and other receptor tyrosine kinases activate the RAS-RAF-MEK-ERK pathway. T e receptor tyrosine kinases trans-autophosphorylate and then phosphorylate IRS on tyrosine. Phosphorylated IRS binds GRB2, which activates SOS, which in turn activates the membrane-bound small G protein RAS by facilitating the exchange of GTP for GDP. RAS with GTP bound to it activates the kinase RAF. RAF is the first member of a kinase cascade with the sequence RAF → MEK → ERK. Phosphorylated ERK phosphorylates the transcription factors MYC, JUN, FOS, and CREB, with the ef ect of increasing cell growth and proliferation.
- Noonan syndrome is caused by a variety of mutations that activate the RAS/RAF/MEK/ERK pathway and is characterized by congenital heart defects, short stature, and abnormalities of the skeleton.
- Neurofibromatosis type 1 is caused by a loss-of-function mutation in the NF1 gene, which encodes a RAS GAP. Benign and malignant tumors form as a result of somatic loss of the remaining normal NF1 allele.

FURTHER READING

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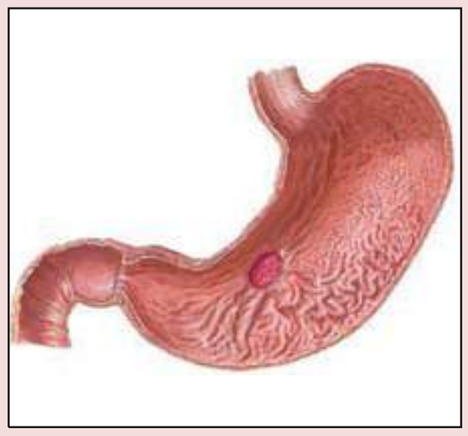
Review Questions

1. Which one of the following statements about G protein signaling is correct?

   A. Activated $G\alpha_s$ typically activates phospholipase C, which converts phosphatidylinositol to cAMP and PIP2.
   B. $G\beta\gamma$-subunits function as GTPase activating proteins (GAPs) that facilitate the intrinsic GTPase activity of heterotrimeric G proteins.
   C. G protein–coupled receptors (GPCRs) function as guanine nucleotide exchange factors (GEFs) to promote the activation of heterotrimeric G proteins.
   D. Small G proteins dif er from heterotrimeric G proteins in that their activity is usually not regulated by GAPs.

2. β-Adrenergic receptors resemble which one of the following?

   A. α_1-Adrenergic receptors in that they are coupled to $G\alpha_i$
   B. Angiotensin II type 1 receptors in that they are coupled to $G\alpha_q$ or $G\alpha_{11}$
   C. Glucagon receptors in that they are coupled to $G\alpha_s$
   D. Prostanoid EP2 and EP4 receptors in that they are coupled to $G\alpha_i$



Chapter 34 Digestion of Dietary Protein and Net Synthesis of Protein in the Body

SYNOPSIS

- In the stomach, proteins are denatured at low pH and then degraded into polypeptides. The stomach epithelium protects itself with mucus and bicarbonate secretion against the proteases and acid in the lumen of the stomach. Helicobacter bacteria and nonsteroidal inflammatory drugs impair this protection and can lead to ulceration of the mucosa.
- In the intestine, enzymes secreted by the pancreas and enzymes anchored to the surface of epithelial cells of the intestine degrade polypeptides into amino acids and small peptides. These digestive processes are impaired in patients who secrete too little or too much acid in the stomach, and in patients who secrete an insufficient amount of proteases from the pancreas.
- Epithelial cells of the intestine take up tripeptides, dipeptides, and amino acids from the lumen of the intestine. They then hydrolyze the peptides into amino acids. The epithelial cells release amino acids into the bloodstream, and other cells take up amino acids from the blood.
- Amino acids are transported across cell membranes by a large number of different transporters, some of which pump amino acids by using an electrical gradient or a concentration gradient for Na^+; others facilitate passive transport or catalyze an exchange of amino acids.
- Patients with cystinuria cannot recover cystine from the glomerular filtrate. As a consequence, they form cystine stones in the kidneys. Patients with Hartnup disease have symptoms of niacin deficiency due to deficient transport of tryptophan.
- The human body can synthesize many amino acids from intermediates of glycolysis or the citric acid cycle, provided that nitrogen can be transferred from another amino acid. However, the essential amino acids (i.e., Arg, His, Ile, Leu, Lys, Met, Phe, Thr, Trp, Val) must be part of the diet.
- Inside cells, protein synthesis occurs mostly after a meal, whereas protein degradation occurs mostly in the fasting state.

LEARNING OBJECTIVES

For mastery of this topic, you should be able to do the following:

- Describe the digestion of protein in the stomach and intestine, including the contribution of the pancreas to this process.
- Explain the mechanism of action of currently used drugs that interfere with acid secretion by the stomach.
- List and explain the major causes of peptic ulcers in the stomach.
- List the major causes of pancreatitis, and explain the role of proteases in the pathogenesis of pancreatitis.
- Explain the pathogenesis of cystinuria and Hartnup disease.
- Explain why some amino acids are essential.

1. DIGESTION OF PROTEIN IN THE STOMACH

In the lumen of the stomach, a low pH denatures proteins, and pepsins digest most proteins into shorter polypeptides. Reduced acid secretion impairs digestion of proteins. If the normal defenses of the mucosa against the acid and pepsins break down locally, the mucosa can form a necrotic hole (i.e., a peptic ulcer). Acid secretion from the stomach can be pharmacologically inhibited with histamine H_2 receptor antagonists or with proton pump inhibitors.

In the stomach, proteins from the diet are denatured and partially hydrolyzed. In the upper portion of the stomach (i.e., the **fundus** and **corpus**; Fig. 34.1) **parietal cells** secrete **hydrochloric acid** (HCl) so that the pH of the stomach contents is about 1 to 2. At this low pH, most proteins denature; that is, they lose their normal three-dimensional structure (see Section 7 in Chapter 9). Also in the upper portion of the stomach, **chief cells** secrete **pepsinogens A** and **C**. At a low pH, pepsinogens A and C become active and cleave themselves to **pepsins A** and **C**. Pepsins cleave denatured proteins more readily than native proteins. Thus, a low pH in the stomach is important for protein digestion in two ways: to denature dietary proteins and to activate the pepsinogens. Parietal cells also secrete intrinsic factor, and they are destroyed in the autoimmune disease **pernicious anemia** (see Section 7.2 in Chapter 36); persons who have pernicious anemia secrete a reduced amount of HCl.

A layer of **mucus** protects the entire stomach epithelium from the low pH and the pepsins of the lumen. The mucosa of the stomach is punctuated by **gastric pits** (see Fig. 34.1) that lead down to the neck region of the mucosa and provide an outlet for the secretions of deeper lying glands. Cells of the glands secrete into canaliculi, which drain into the pits (three to seven canaliculi per pit). The neck region contains stem cells and progenitor cells that renew the tissue bidirectionally; that is, toward the base and toward the lumen of the stomach. A firmly adhering, 0.1- to 0.5-mm-thick layer of mucus covers all epithelial cells. By pumping bicarbonate (HCO_3^-) under this layer of mucus, the cells maintain a pH of about 7 at the extracellular face of their plasma membrane. In addition, epithelial cells between the neck region of the mucosa and the lumen (see Fig. 34.1) produce a loose layer of mucus that covers the walls of the stomach. Epithelial cells between the neck region of the mucosa and the lumen live for several days before sloughing off, while the cells between the neck region and the base of the gland live for a year or longer.

The lower portion of the stomach (the **antrum**) predominantly secretes hormones that regulate the secretion of HCl and pepsinogens (Figs. 34.1, 34.2, and 34.3). Thus, **G-cells** secrete **gastrin**, which leads to increased secretion of both HCl and pepsinogens. In both the lower and the upper portions of the stomach, as a type of feedback regulation, **D-cells** secrete

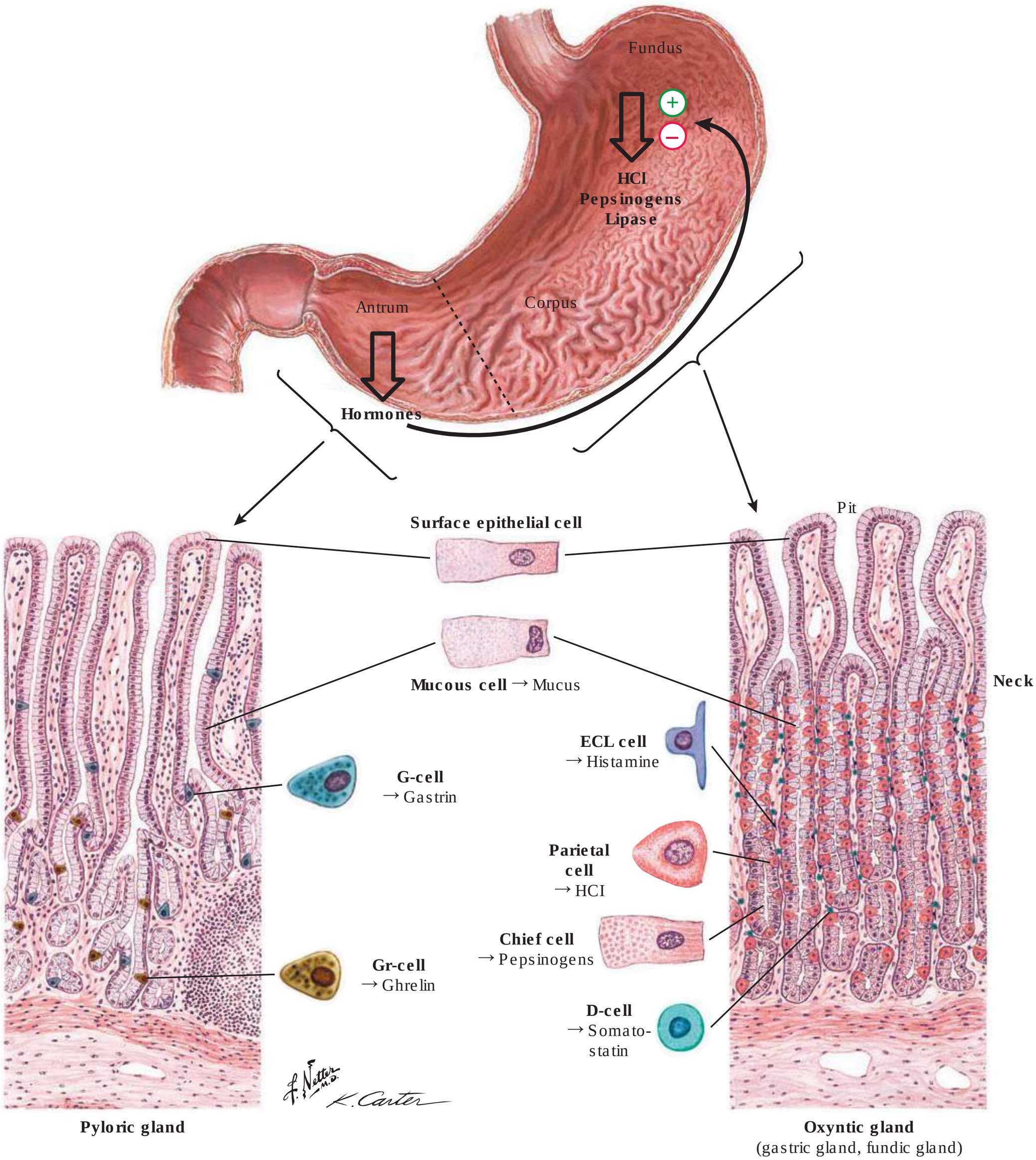


Fig. 34.1 **Pyloric and oxyntic glands in the stomach.** The fundus and corpus mainly contain oxyntic glands, in which parietal cells secrete HCl and chief cells secrete pepsinogens A and C. The antrum mainly contains pyloric glands that secrete gastrin and somatostatin, which regulate acid secretion from oxyntic glands. ECL, enterochromaffin-like.

somatostatin when the pH is especially low; somatostatin then inhibits HCl secretion. Postganglionic neurons that receive input from the **vagus nerve** and secrete acetylcholine stimulate secretion of HCl and pepsinogens.

Adequate digestion of proteins in the stomach is needed to kill **pathogens** and minimize the chance of an **allergic reaction** to proteins in food. T e larger a peptide is in the intestine, the more likely it will elicit an allergic reaction.

Patients who have **hypochlorhydria** (deficient secretion of HCl in the stomach, e.g., due to **atrophic gastritis**) or **achlorhydria** (lack of secretion of HCl) are prone to infections of the intestine, as well as "bacterial overgrowth," which is the growth

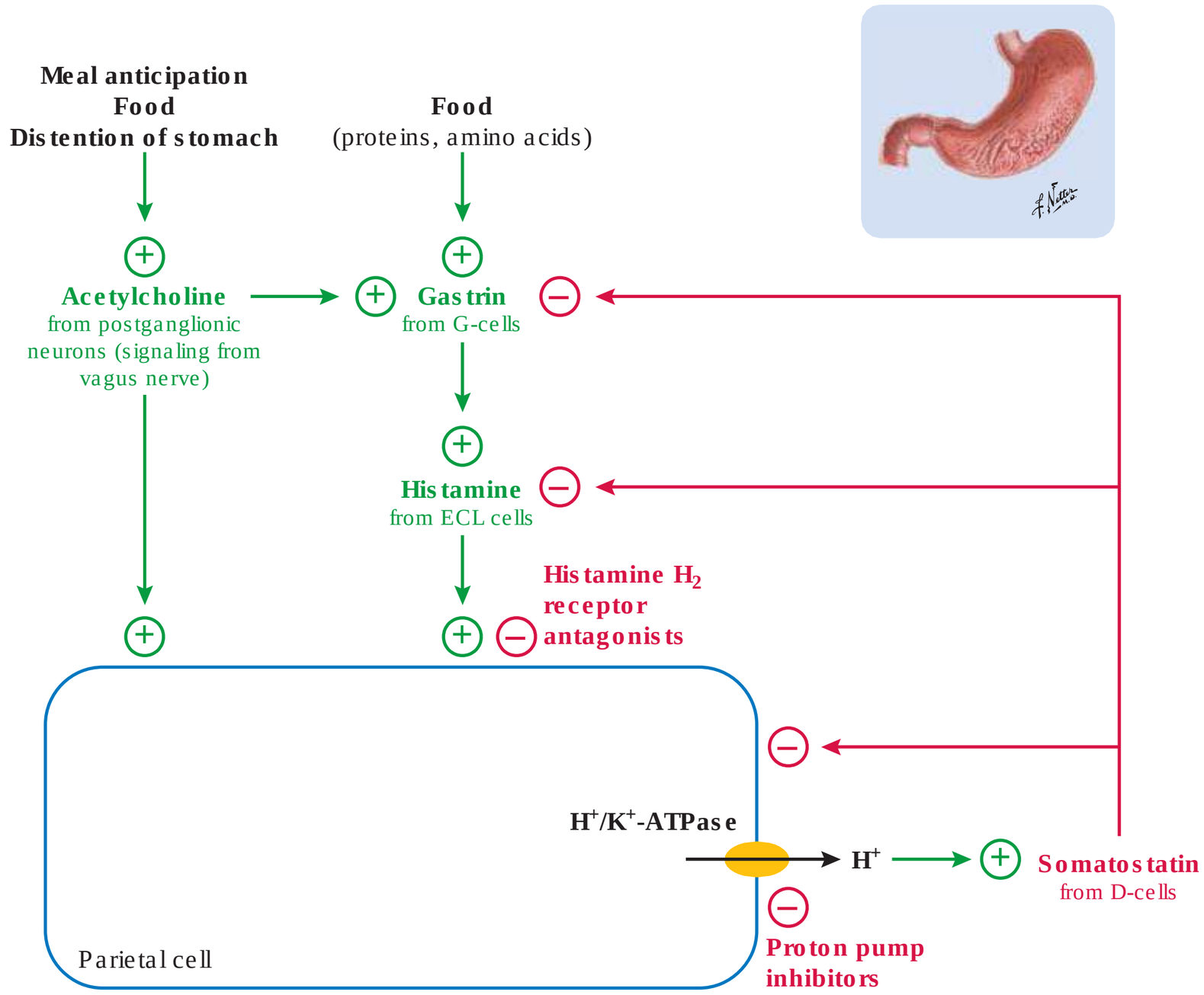


Fig. 34.2 **Regulation of acid secretion in the stomach.** In the basal state, somatostatin inhibits acid secretion at three levels: H^+-secreting parietal cells, gastrin-secreting G-cells, and histamine-secreting enterochromaffin-like (ECL) cells. After a meal, neural signaling via acetylcholine increases gastrin secretion, which stimulates histamine secretion, which in turn stimulates H^+ secretion. The mechanisms by which the stomach detects food are poorly understood.

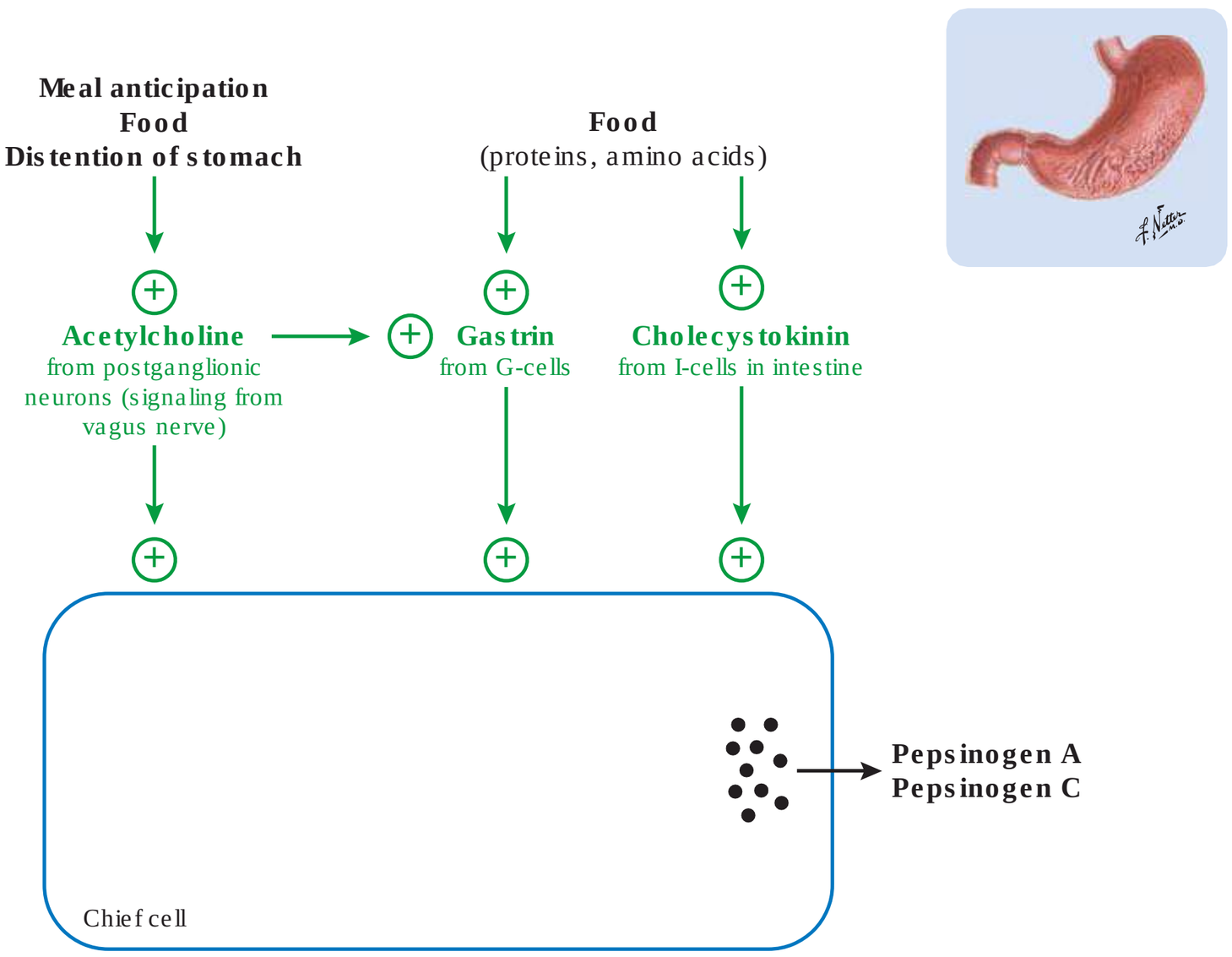


Fig. 34.3 **Regulation of pepsinogen secretion in the stomach.** In the lumen of the stomach, pepsinogens are cleaved to form enzymatically active pepsins. Secretion of cholecystokinin is described in Section 2.1.

of bacteria in the stomach that are normally present only in the lower small intestine. Acid secretion can be assessed by measuring the pH of the fluid in the stomach.

In patients with an inherent vulnerability of the mucosa, HCl secretion and pepsin activities participate in forming necrotic holes in the mucosa in the form of **ulcers** (Fig. 34.4). The vulnerability commonly arises from infection with Helicobacter pylori or from chronic use of nonsteroidal antiinflammatory drugs (NSAIDs; see below). As part of the treatment of these patients, acid secretion can be inhibited with an oral **histamine H_2 receptor antagonist** (e.g., cimetidine), which prevents histamine from stimulating parietal cells, or with a **proton pump inhibitor** (e.g., omeprazole), which irreversibly inhibits the H^+ pump (H^+/K^+-ATPase) in parietal cells. To the extent that these drugs inhibit degradation of proteins from the diet, they lead to an increased concentration of antibodies to common food allergens.

More than half of the world's population is currently infected with the flagellated bacterium H. pylori, which moves through the mucus of the stomach, attaches itself to the epithelium, and causes chronic gastritis. The bacterium can also colonize the duodenum. If untreated, the infection can lead to ulcers of the stomach or duodenum (see Fig. 34.4). In patients with ulcers in the duodenum or antrum, the secretion of both acid and pepsinogens is elevated and contributes to ulceration. Conversely, patients with ulcers exclusively in the oxyntic mucosa usually have decreased acid secretion and fewer parietal cells. Gastric ulcer due to infection with Helicobacter is associated with an increased risk for **stomach cancer** and gastric mucosa–associated **B-cell lymphoma**. Helicobacter can be eradicated with antibiotics.

Chronic use of **NSAIDs** (e.g., aspirin, naproxen, ibuprofen) leads to erosion and ulceration of the gastric mucosa. NSAIDs inhibit cyclooxygenase-1 and -2 (prostaglandin H synthase-1 and -2; see Section 2.1 in Chapter 32), which catalyze the synthesis of **prostaglandin H2** from arachidonic acid (see Fig. 32.2). Prostaglandin E2 (made from prostaglandin H2) plays an important role in adaptive protection of the mucosa, as well as in the healing of damage to the mucosa. As part of this protective effect, prostaglandins stimulate mucus and bicarbonate production and inhibit the secretion of HCl.

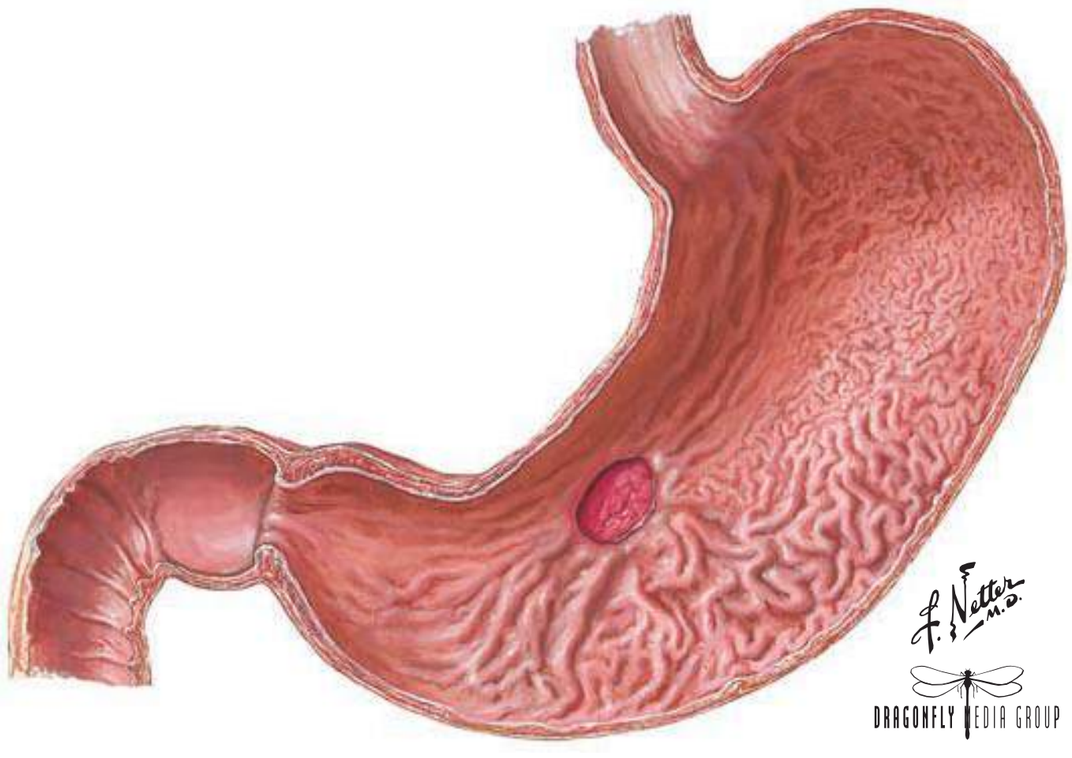


Fig. 34.4 **Ulceration of the stomach mucosa.**

2. DIGESTION OF PROTEIN IN THE INTESTINE

In the lumen of the intestine, proteases from the pancreas hydrolyze polypeptides to short peptides. Peptidases on the intestinal epithelial surface hydrolyze the short peptides to tripeptides, dipeptides, and amino acids. Decreased acid denaturation of proteins in the stomach and decreased secretion of proteases from the pancreas each lead to an inadequate digestion of protein in the intestine. When the pancreas is inflamed, proteases in the pancreas become unduly active and destroy cells in the pancreas.

2.1. Normal Protein Digestion in the Intestine

Acidified, partially digested food, called **chyme**, travels from the stomach through the pyloric canal to the duodenum. At the juncture with the common bile duct, **bicarbonate** from the pancreas raises the pH of the luminal contents to about 7. **Bile salts** (see Section 4.1 in Chapter 29) from the gallbladder solubilize and emulsify lipids (see Section 2.2 in Chapter 28), and digestive enzymes from the pancreas hydrolyze starches, lipids, nucleic acids, proteins, and peptides. (Pepsin from the stomach becomes inactive when the pH is near 7.)

The exocrine **pancreas** synthesizes and stores enzymes and enzyme precursors (zymogens) inside secretory granules (Fig. 34.5). Pancreatic acinar (exocrine) cells secrete the protease precursors **trypsinogen** 1 (cationic trypsinogen), trypsinogen 2 (anionic trypsinogen), trypsinogen 3 (meso-trypsinogen), **chymotrypsinogen, proelastase, procarboxypeptidase** A, and procarboxypeptidase B (Table 34.1). Pancreatic duct cells secrete a fluid that is rich in sodium bicarbonate ($NaHCO_3$). The **cystic fibrosis transmembrane regulator** (**CFTR**; the CFTR gene is mutated in patients who have cystic fibrosis) is essential for this fluid secretion. The pancreatic fluid flushes the precursor proteases into the intestine.

Upon arrival in the small intestine, the protease zymogens from the pancreas become active proteases (see Table 34.1). Brush border membranes of epithelial cells in the duodenum contain the integral membrane protein **enteropeptidase** (enterokinase). Enteropeptidase becomes active when **bile acids** (secreted from the gallbladder) are present. Active enteropeptidase cleaves trypsinogen to **trypsin**. Trypsin can also cleave trypsinogen to trypsin. Trypsin proteolyzes the other zymogens to produce **chymotrypsin**, **carboxypeptidases** A and B, and **elastase**.

Trypsin, chymotrypsin, elastase, and the carboxypeptidases differ in their substrate specificity. Together, they degrade most proteins in the diet, although some of these proteins are longer lived than others. A small amount of dietary proteins and peptides normally passes through the gastrointestinal tract without being absorbed.

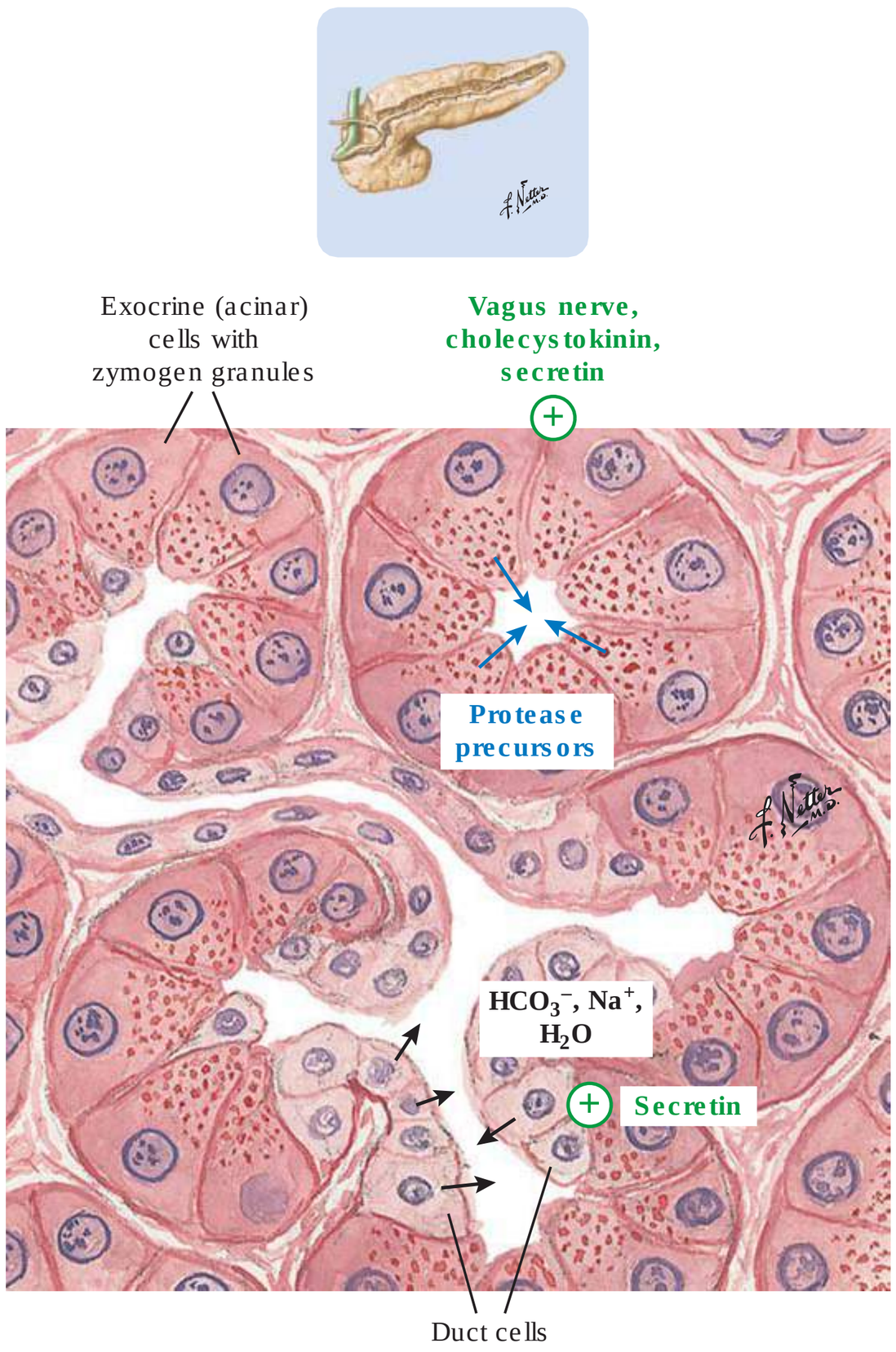


Fig. 34.5 **Structure of a pancreatic acinus.** The exocrine pancreas consists of numerous acini, which drain into a tree-like system of ducts.

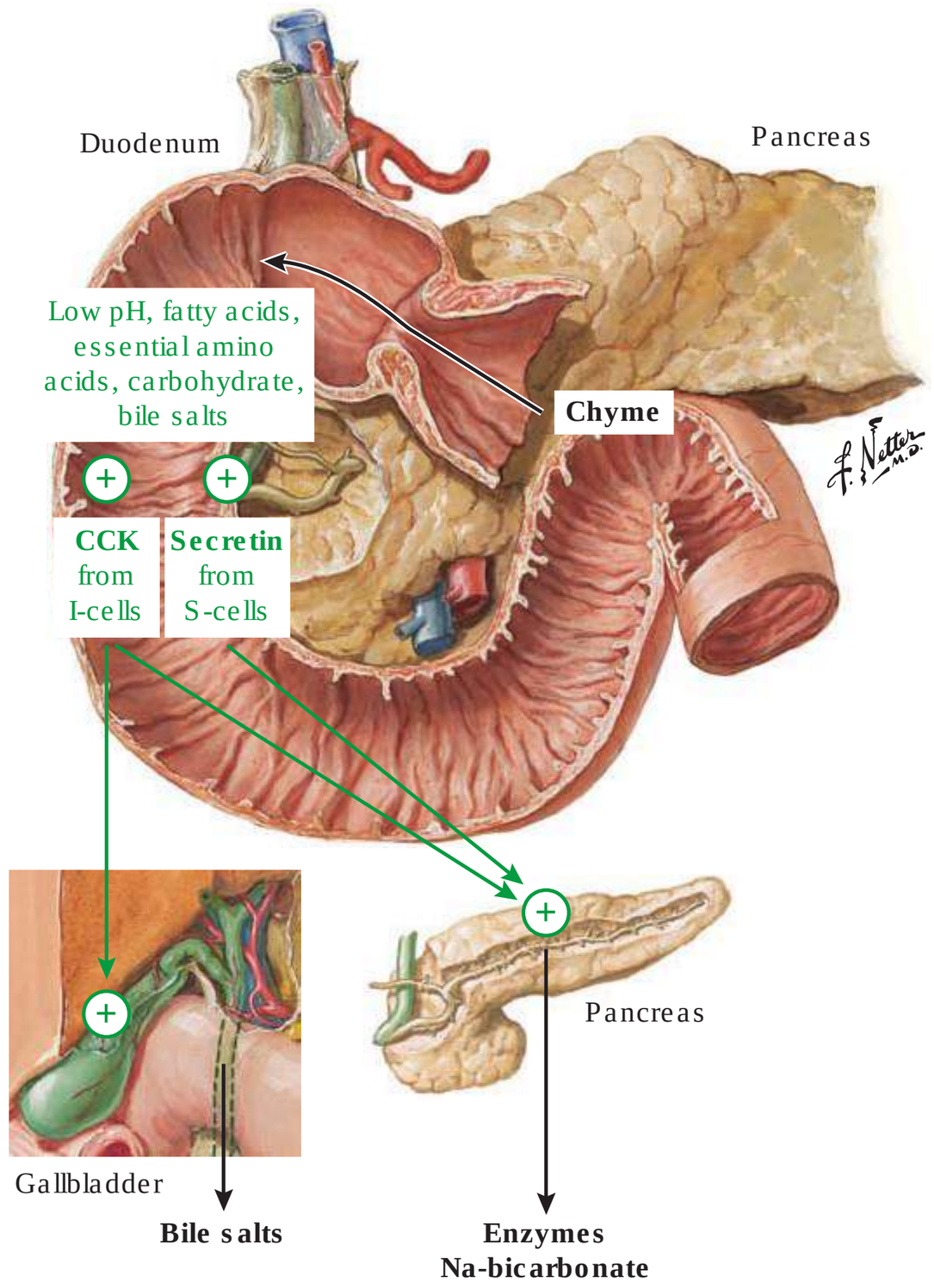


Fig. 34.6 **Regulation of exocrine secretion from the gallbladder and pancreas.** I-cells and S-cells in the small intestine secrete cholecystokinin and secretin, respectively, into the bloodstream. Cholecystokinin (CCK) also affects neurons in the brain and thereby induces satiety.

During an overnight fast, the pancreas continuously secretes enzymes, and after a regular meal, the rate of secretion readily increases to a plateau of about four times this value. T is high rate of secretion then lasts until nutrient flow into the duodenum abates. Chronic ingestion of a high-fat diet elicits greater pancreatic enzyme secretion (both after a meal and between meals) than a chronic high-carbohydrate diet. T e exact proportion of enzymes that degrade carbohydrates, lipids, and proteins, changes slightly with the composition of a meal and also with dietary habits.

Secretin and **cholecystokinin** are the major controllers of enzyme secretion from the pancreas and bile secretion from the gallbladder (Fig. 34.6). Secretin and cholecystokinin are both peptide hormones. **S-cells** in the duodenum and jejunum secrete secretin when they encounter a low pH, carbohydrates, fatty acids, essential amino acids, or bile salts. **I-cells** in the intestine secrete cholecystokinin in response to amino acids, triglycerides, and glucose.

In the clinic, secretin is used in diagnostic tests of the function and shape of the **exocrine pancreas**, and sometimes also in the diagnosis of gastrin-secreting tumors (gastrinomas, see below).

In the small intestine, proteins are degraded into amino acids, dipeptides, and tripeptides. T is protein derives mainly from the diet and, to a lesser extent, from enzymes secreted by the pancreas, and from sloughed-of intestinal epithelial cells. Pancreatic proteases degrade about two-thirds of the polypeptides to two- to six-residue peptides, and about one-third to amino acids. T e peptides and amino acids dif use through the layer of mucus that lines the epithelium to the brush border membrane of epithelial cells. T ere, membrane-bound **aminopeptidases** cleave single amino acids from the N-termini of these short peptides to produce tripeptides, dipeptides, and amino acids. Two examples of these aminopeptidases are glutamyl aminopeptidase (also called aspartate aminopeptidase or aminopeptidase A) and membrane alanyl aminopeptidase (also called aminopeptidase N or amino-oligopeptidase). As detailed in Section 3, a peptide transporter transports tripeptides and dipeptides into intestinal epithelial cells; similarly, many dif erent amino acid transporters transport amino acids into intestinal epithelial cells.

Table 34.1 Enzymes Secreted by the Pancreas

Secreted Protein	Active Enzyme	Mode of Activation	Role in Digestion
Trypsinogen	Trypsin	Proteolysis by enteropeptidase (anchored to brush border membrane of epithelial cells of duodenum; activated by bile salts)	Activates all other protease precursors. Degrades proteins, producing peptides
Chymotrypsinogen	Chymotrypsin	Proteolysis by trypsin	Degrades proteins, producing peptides
Proelastase	Elastase	Proteolysis by trypsin	Degrades proteins, producing peptides
Procarboxypeptidases A and B	Carboxypeptidases A and B	Proteolysis by trypsin	Degrade proteins from C-terminus, producing amino acids
Pancreatic lipase	Pancreatic lipase	Colipase	Hydrolyzes triglycerides and diglycerides (see Chapter 28)
Prophospholipase A_2	Phospholipase A_2	Proteolysis by trypsin	Degrades glycerophospholipids
Amylase	Amylase	None needed	Degrades starches and dietary glycogen (see Chapter 18)
Deoxyribonuclease	Deoxyribonuclease	None needed	Degrades DNA
Ribonuclease	Ribonuclease	None needed	Degrades RNA

2.2. Diseases Associated With Impaired Digestion of Protein

Malabsorption of protein is called **creatorrhea**, which means flesh (undigested muscle fibers) in the stools. Creatorrhea becomes apparent when protease activity is but a small fraction of the normal. Protease activity may be deficient due to an abnormality of the stomach, small intestine, or pancreas, as outlined below.

Celiac disease (**celiac sprue)** is caused by an allergic reaction to certain gliadin proteins in **gluten**, which is found in wheat, barley, and rye. T e disorder has a strong genetic component. Individuals who have the HLA-DQ2 or HLA-DQ8 alleles of the major histocompatibility complex are especially likely to develop celiac disease, which has a prevalence of ~1 in 200. Persons who have active disease show atrophy of the villi in the small intestine and may have malabsorption, diarrhea, a blistering skin rash, and ataxia. T ese symptoms can usually be corrected with a lifelong gluten-free diet.

Patients with **achlorhydria** (a lack of gastric HCl secretion, most commonly due to destruction of parietal cells in autoimmune gastritis) lack an adequate nutrient stimulus for secretin secretion from S-cells in the intestine; this can be corrected with acidic drinks, such as orange juice. Patients with hypochlorhydria or achlorhydria are at an increased risk of protein and lipid malabsorption, infections of the intestine, and cobalamin deficiency (see Chapter 36).

Patients who have **severe pancreatic insufficiency** due to chronic pancreatitis or pancreatic cancer malabsorb protein and other nutrients; consequently, they have diarrhea. Before creatorrhea sets in, abnormal results are typically found for leaked pancreatic enzymes in serum (amylase, lipase, trypsin, and carboxypeptidase B, which was formerly referred to as pancreas-specific protein), and for fecal fat, cobalamin status, and blood glucose (due to impairment of islet β-cells). In addition, the response to diagnostic stimulation with secretin may be abnormal (due to pancreatic dysfunction).

Pancreatitis, an inflammation of the pancreas, is often caused by **alcohol dependence syndrome** (see Section 4.1 in Chapter 30) or blockage of pancreatic secretions by **gallstone disease** (see Section 4.2 in Chapter 29); it is also a consequence of severe **hypertriglyceridemia** (see Section 8.1 in Chapter 28 and Section 4.2 in Chapter 29). Occasionally, pancreatitis is caused by an **invasive procedure** that leads to ischemia of the pancreas or blockage of the common bile duct. Factors that play a role in the diagnosis of pancreatitis are the type of pain, high serum lipase or amylase activity, and imaging studies. Acute pancreatitis is of two types: **interstitial edematous pancreatitis** (Fig. 34.7), which generally resolves within a week, and **necrotizing pancreatitis**, in which necrosis develops over several days. It is currently hypothesized that nonhereditary pancreatitis is triggered by an event that produces more active trypsin inside acinar cells than can be inactivated by trypsin inhibitor. T is trypsin then activates other zymogens, and the inappropriately active digestive enzymes destroy pancreatic cells. About 10% of the patients who have pancreatitis die from this disease, often due to a generalized

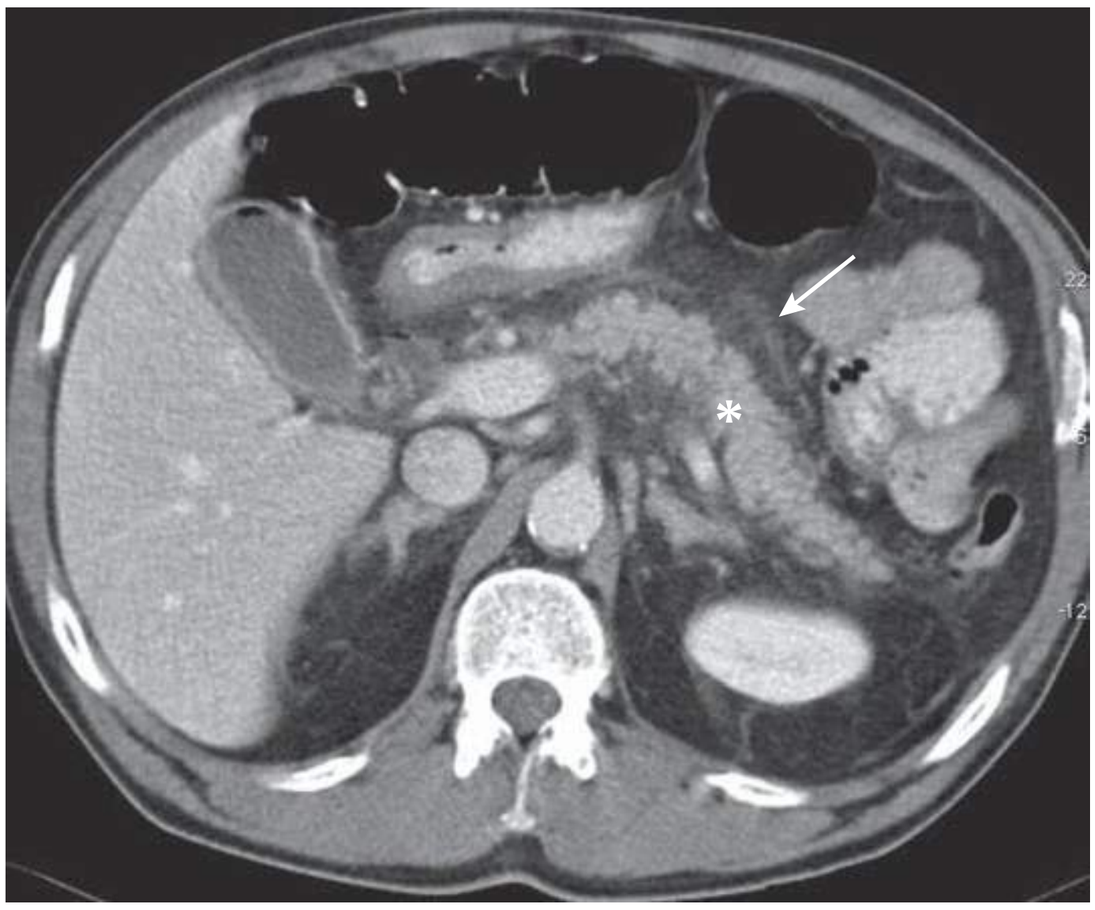


Fig. 34.7 **Interstitial pancreatitis**. Computed tomographic image of a swollen pancreas (*asterisk*). Fat stranding is seen around the pancreas (*arrow*). (From Bollen TL. Acute pancreatitis: international classification and nomenclature. *Clin Radiol.* 2016;71:121-133.)

inflammatory response and sepsis that lead to multiorgan failure.

Every episode of acute pancreatitis leads to some permanent damage of the pancreas. Recurrent episodes of acute pancreatitis, when combined with persistent inflammation and ongoing fibrosis of the pancreas, often lead to chronic pancreatitis. Chronic pancreatitis in turn can lead to the destruction of the pancreas. Hence, the prevention of episodic pancreatitis is important.

Prematurely active **trypsin** normally **autolyzes** inside pancreatic exocrine cells. Inside cells, the concentration of calcium (Ca^{2+}) is generally low, whereas it is high in the lumen of the intestine. A high concentration of calcium in the lumen is needed for trypsin to remain active and activate trypsinogen. Conversely, at the low concentration of calcium inside pancreatic exocrine cells, trypsin destroys itself (autolyzes). Abnormalities in autolysis can give rise to pancreatitis (see below).

Hereditary causes of pancreatitis shed light on the pathogenesis of nonhereditary forms of pancreatitis. Patients who are heterozygous or homozygous for a mutant **trypsinogen 1** (encoded by the PRSS1 gene) that lacks the Arg site for cleavage by another trypsin molecule develop chronic pancreatitis as children. The pathogenic mutant trypsins typically become excessively active inside pancreatic exocrine cells, and their activity is then also abnormally stable. Trypsin inhibitor cannot inhibit all intrapancreatic trypsin because there is normally only about one molecule of trypsin inhibitor per five molecules of trypsin (although this ratio improves during pancreatitis).

Patients who are homozygous (and, in some cases, heterozygous) for an inactivating mutation in **pancreatic secretory trypsin inhibitor** (encoded by the SPINK1 gene) develop chronic pancreatitis as young adults. Many of these mutant trypsin inhibitors do not effectively bind to trypsin.

Patients who have **cystic fibrosis** do not express an adequate amount of CFTR. These patients are homozygous or compound heterozygous for a mutation in the CFTR gene. Hundreds of mutations are known that cause disease of varying severity. Thus, some patients present with acute pancreatitis in the absence of overt airway disease. CFTR transports chloride ions across the apical plasma membrane of pancreatic duct cells; chloride in turn exchanges for bicarbonate via an additional transporter. CFTR plays additional roles in the regulation of fluid secretion, but the mechanisms have not been well elucidated. Without active CFTR in the luminal membrane, pancreatic duct cells do not secrete an adequate amount of bicarbonate and water to flush digestive enzymes into the intestine (a somewhat similar situation is found in patients who have a temporarily blocked common bile duct). Severe forms of cystic fibrosis are associated with chronic pancreatitis from infancy and loss of pancreatic function before birth as well as within the first few years of life; acinar cells are lost early on, endocrine cells only later. Since the pancreas does not secrete enough bicarbonate, patients with cystic fibrosis also do not effectively neutralize HCl in the intestine. This can be counteracted with antacids and inhibitors of HCl secretion from the stomach.

Patients who have severe pancreatitis are treated in part by withholding all oral food to diminish the stimuli for synthesis of enzymes in the pancreas.

Patients who have **Zollinger-Ellison syndrome**, a rare syndrome caused by a **gastrinoma**, secrete too much acid from the stomach, so that a low pH in the intestinal lumen leads to damage of the intestinal mucosa and inactivity of pancreatic enzymes. These patients typically have gastroesophageal reflux disease, ulcers in the duodenum, and diarrhea (from malabsorption). Most gastrinomas are in the duodenum or pancreas and are malignant. Diagnosis commonly entails demonstration of fasting hypergastrinemia despite a low pH in the stomach (a low pH normally attenuates the secretion of gastrin). If this test is not diagnostic, a secretin test is used. In this test, patients are infused with the hormone secretin. While secretin normally inhibits gastrin secretion, it increases gastrin secretion from gastrinomas.

Zollinger-Ellison syndrome can also be a manifestation of **multiple endocrine neoplasia-1**. If so, patients may not only have a gastrinoma but also a tumor of the pituitary gland or of the parathyroid gland.

3. TRANSPORT OF AMINO ACIDS AND SMALL PEPTIDES

In the intestine, transporters in the epithelial cell membranes move tripeptides, dipeptides, and amino acids into the cytosol. There, peptidases cleave most intracellular tripeptides and dipeptides into amino acids. The epithelial cells of the intestine then release amino acids into the bloodstream. Patients who have cystinuria cannot remove cystine from the

tubules of the kidneys and therefore develop kidney stones. Patients who have Hartnup disease are deficient for a transporter of neutral amino acids and, as a result, have a deficit of tryptophan and nicotinamide.

3.1. Normal Transport of Amino Acids in the Intestinal Epithelium and in Other Cells

About 90% of the amino acids of the protein in a mixed diet are absorbed into the epithelial cells of the small intestine. This is the result of efficient proteolysis and uptake of amino acids and short peptides into cells.

Epithelial cells of the small intestine express a **peptide transporter** (**PEPT1**, encoded by the SLC15A1 gene) that accepts dipeptides and tripeptides of virtually any amino acid sequence. The peptide transporter uses an inwardly directed H^+ gradient to drive the uptake of peptides (although the lumen of the intestine is alkaline, the surface of the intestinal brush border membrane has an acid microclimate). Intracellular peptidases cleave dipeptides and tripeptides into amino acids.

PEPT1 also transports drugs such as **aminocephalosporins**, **aminopenicillins**, some **angiotensin-converting enzyme inhibitors** (e.g., captopril, enalapril), and amino acid–conjugated nucleosides that are used as **antiviral agents** (e.g., valacyclovir). Another transporter releases these drugs from the epithelial cells into the bloodstream.

The brush border membrane of the intestinal epithelial cells has several **transporters for amino acids**, most of which transport several different amino acids. Some of these transporters are Na^+:amino acid cotransporters that use the inward-directed concentration gradient of Na^+ to pump amino acids into the epithelial cells (the same cells also contain a Na^+:glucose cotransporter; see Section 3.1 in Chapter 18). Other transporters use the membrane potential to pump positively charged amino acids into epithelial cells, and still others exchange intracellularly accumulated amino acids for other amino acids in the lumen of the intestine.

The total **concentration of amino acids in the blood** is ~3 mM. Glutamine and alanine are the largest contributors (Fig. 34.8), amounting to about 35% of the total. With a weight-maintaining, typical Western diet, the total concentration of amino acids in the blood changes by less than ~20%.

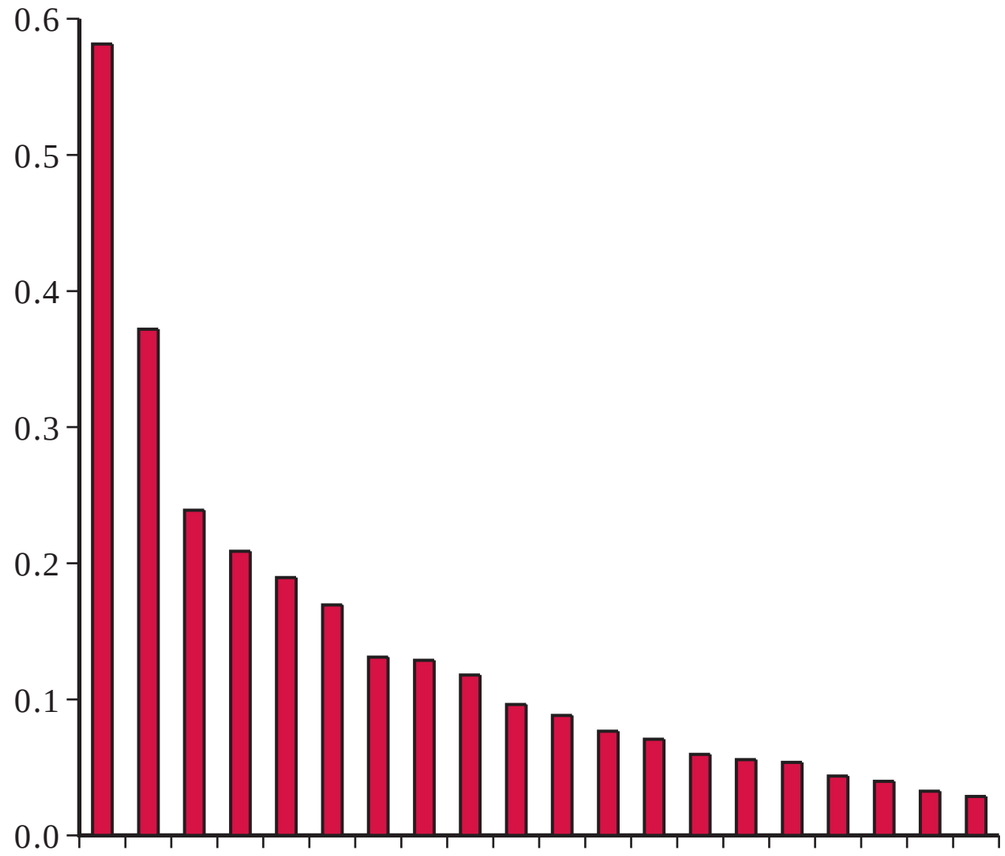


Fig. 34.8 Concentration of individual amino acids in human blood plasma. (Data from Cynober LA. Plasma amino acid levels with a note on membrane transport: characteristics, regulation, and metabolic significance. *Nutrition*. 2002;18:761-766; Nakamura H, Jinzu H, Nagao K, et al. Plasma amino acid profiles are associated with insulin, C-peptide, and adiponectin levels in type 2 diabetic patients. *Nutr Diabetes*. 2014;4:e133; Lewis AM, Waterhouse C, Jacobs LS. Whole-blood and plasma amino acid analysis: gas-liquid and cation-exchange chromatography compared. *Clin Chem*. 1980;26:271-276; and Nasset ES, Heald FP, Calloway DH, Margen S, Schneeman P. Amino acids in human blood plasma after single meals of meat, oil, sucrose, and whiskey. *J Nutr*. 1979;109:621-630.)

3.2. Diseases Due to Deficiencies of Amino Acid Transporters

Cystinuria is caused by a deficiency of the transport of **cystine** out of the proximal tubules of the kidneys into epithelial cells; this transport deficiency gives rise to **kidney stones** composed primarily of cystine (Fig. 34.9). Cystine forms by the spontaneous oxidation and condensation of two cysteine molecules. Inside cells, an enzyme uses glutathione to reduce virtually all cystine to cysteine (for glutathione, see Chapter 21). However, in blood plasma, the concentration of cystine is about six times higher than that of cysteine. Normal kidneys recover both cysteine and cystine from the glomerular filtrate. Kidneys of patients with cystinuria fail to recover cystine. Cystine in the renal tubular fluid crystallizes when its concentration exceeds the limit of solubility. The crystals then aggregate into stones.

Almost all patients who have cystinuria carry mutations in the SLC3A1 or the SLC7A9 gene, which encode the two subunits of the rBAT/$B^{0,+}$AT transporter. This protein complex transports cystine, other dibasic amino acids (i.e., lysine, arginine, and ornithine), and neutral amino acids. The transporter is embedded in the brush border membrane of the epithelia of the kidney tubules. In general, the disease shows an **autosomal recessive** pattern of inheritance.

In England, Spain, and the Eastern Mediterranean region, cystinuria is found in ~1 in 2000 newborns; in other populations, the disorder is somewhat less common. On average, about 10% of all children who present with kidney stones have cystinuria. Most patients develop symptomatic stones by the end of their teen years.

In patients who have cystinuria due to a mutant SLC3A1 or SLC7A9 gene, the rBAT/$B^{0,+}$AT basic amino acid transporter is also deficient in the brush border membranes of the epithelium of the small intestine. However, this is not pathogenic, most likely because essential basic amino acids (i.e., lysine and arginine) enter in sufficient amounts as part of dipeptides and tripeptides (see Section 3.1).

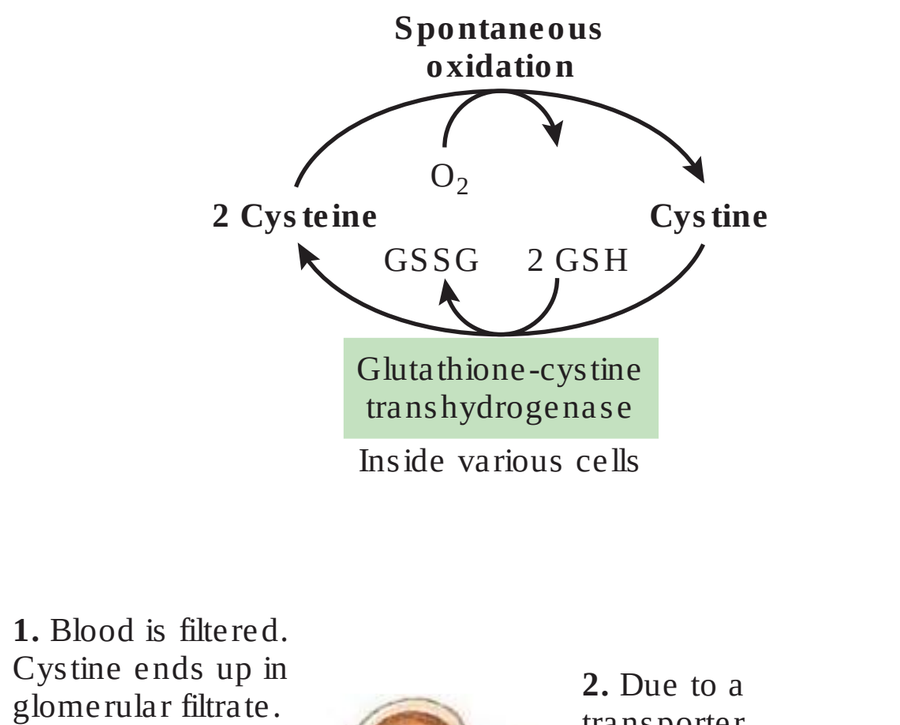


Fig. 34.9 Spontaneous formation of cystine and pathogenesis of cystinuria. *GSH*, reduced glutathione; *GSSG*, oxidized glutathione (see Chapter 21).

Cystinuria is treated by alkalinizing the urine with oral potassium **citrate** (at a higher pH, cystine is more soluble) and by maintaining a large **flow** of urine (to lower the concentration of cystine). Patients are also advised against consuming a lot of protein or salt; protein contains cysteine, and salt increases the excretion of cystine for unknown reasons. If these measures do not provide satisfactory results, patients can often be given **penicillamine** (a degradation product of penicillin), **meso-2,3-dimercaptosuccinic acid**, or **α-mercaptopropionyl glycine** (also called thiopronin). These drugs contain –SH groups that react with cystine to form mixed drug-cysteine disulfides that are very soluble in urine.

A homozygous or compound heterozygous deficiency in the SLC6A19 (B^0AT1) transporter, which transports neutral amino acids across the brush border membrane of the intestinal and renal epithelial cells, leads to **Hartnup disorder** and possibly **Hartnup disease**. (Hartnup is the name of the family, members of which had a severe form of this disease and were first described in a scientific publication in 1956.) Patients with Hartnup disorder have documented neutral aminoaciduria but no symptoms. The disorder occurs in ~1 in 30,000 persons. About 10% of these patients show symptoms and therefore have Hartnup disease (its incidence is thus ~1 in 300,000 persons). The symptoms arise from a deficiency of **tryptophan**, and some of the symptoms resemble those of pellagra (a deficiency of nicotinic acid; see Fig. 19.5 in Chapter 19). Tryptophan is an essential amino acid that is required for the synthesis of nicotinic acid (see Section 1 in Chapter 19) and the neurotransmitter serotonin (see section 4.2 in Chapter 35). Affected patients are susceptible to photodamage starting in early childhood. Later, they may have intermittent cerebellar ataxia (lasting a few days), emotional lability, and psychosis. Episodes are often triggered by poor nutrition, diarrhea, fever, or sun exposure. Symptomatic patients are treated with nicotinamide. Those patients who have a low concentration of amino acids in the blood should also consume a high-protein diet. The reasons that a deficiency of the SLC6A19 (B^0AT1) transporter does not have more serious pathologic effects are that a fraction of amino acids reach the epithelial cells of the intestine as dipeptides and tripeptides via the peptide transporter, and that there are other transporters for neutral amino acids.

4. SYNTHESIS OF BODY PROTEIN

Of the 21 amino acids needed for translation, humans can synthesize about half; they must consume the remainder with the diet. Net synthesis of body protein occurs in the postprandial state.

4.1. Daily Turnover of Body Protein

In a healthy adult, daily protein synthesis and degradation amount to about 400 g, or 3% of the total protein content of the body (Fig. 34.10). Humans must consume a minimum of about 50 g of protein per day because an equivalent amount of amino acids is oxidized or used for the synthesis of nonprotein compounds. The term nitrogen balance is a measure of the difference between protein intake and nitrogen loss (see Section 5 in Chapter 35). Synthesis and degradation of body protein affect the nitrogen balance.

4.2. Essential and Nonessential Amino Acids

Humans cannot synthesize His, Ile, Leu, Lys, Met, Phe, Thr, Trp, or Val; therefore, these amino acids are called **essential** (or **indispensable**) **amino acids**. For growing children, this list has to be expanded; they must consume Arg because they commonly do not produce enough of it in the urea cycle (see Section 2.5 in Chapter 35) and Cys because they do not make enough of it from methionine (see Sections 4.1 and 9 in Chapter 36). Gln, Tyr, Gly, Pro, and ornithine are

Fig. 34.10 Protein and amino acid content and turnover in a healthy adult.

conditionally essential amino acids; that is, they are essential in certain disease conditions.

In protein synthesis, the translation of mRNA stops if an amino acid is not available (see Chapter 7). Hence, a deficiency in a single amino acid leads to deficient synthesis of proteins.

Sources of dietary protein can be rated for their **quality**, or how well they match the needs of the human body. For instance, gelatin (as contained in Jell-O) is a very low-quality protein because it consists mostly of collagen, which contains mostly glycine, proline (some of it as hydroxyproline), and alanine (see Chapter 12). In contrast, human breast milk is a source of very high-quality protein for infants because the amino acid composition of milk proteins closely matches the requirements. Since the body can synthesize nonessential amino acids, the protein quality of a meal depends largely on its content of essential amino acids. For people who eat mostly a vegan diet, methionine is usually the limiting essential amino acid.

Adults should consume a minimum of about 50 g of protein per day. For adults, the World Health Organization (WHO) recommends a minimum intake of 0.75 g of protein per kilogram of body weight per day; this protein should have a good balance of amino acids. Individuals who eat a high-protein diet consume as much as 3 g of protein per kilogram of body weight per day (i.e., ~200 g of protein per day). In North and South America, as well as in Australia, **meat** usually contributes 30% to 40% of all protein in the diet. In Europe, this fraction is 20% to 30%; in Africa, it is 0% to 30%; and in Asia, it is generally 10% to 20%. Children, pregnant women, and patients who are recovering from surgery or trauma need more protein in the diet than the World Health Organization recommends for healthy people. Hospitalized patients who receive parenteral nutrition, for example, are commonly given 0.8 to 2.0 g of amino acids per kilogram of body weight per day, depending on the patient's disease.

Humans can synthesize about half of all amino acids. For this, they need nitrogen from other amino acids. Humans can synthesize Ala, Asp, Asn, Gln, Glu, Pro, and Ser starting with intermediates of glycolysis and the citric acid cycle (Fig. 34.11), and they can obtain Tyr from Phe (see Section 4.2 in Chapter 35), Arg via the urea cycle (see Section 2.5 in Chapter 35), and Cys from Met (see Sections 4.1 and 9 in Chapter 36). Because humans can synthesize these amino acids and do not need them in the diet, they are called **nonessential** or **dispensable amino acids**.

4.3. Regulation of the Concentration of Amino Acids in Blood and of Protein Synthesis

The concentration of amino acids in the blood is the result of flux of amino acids into and out *of* the blood. Flux into the blood mainly depends on intestinal uptake of amino acids from dietary protein and on the degradation of body protein. Flux out *of* the blood depends mostly on protein synthesis, gluconeogenesis, and oxidation of amino acids for production of energy in the form of ATP. After a meal, the major users of amino acids are the intestine, liver, and muscle (Fig. 34.12). In the fasting state, muscle is the major producer of amino acids, and the liver is the major user. It is not clear which proteins participate in this cycle of protein synthesis and protein degradation; the regulation of the cycle is likewise incompletely understood. Known major regulators of amino acid flux are the concentrations of leucine, insulin, glucagon, and cortisol in the blood, as well as the activity of the protein complex mTORC1 inside cells.

Activated **mTORC1** stimulates protein synthesis (Fig. 34.13). The complex is activated by growth factors and amino acids, especially leucine. The complex is inhibited when a cell is hypoxic or has impaired production of adenosine triphosphate, as sensed by adenosine monophosphate–dependent protein kinase (AMPK). Metformin, a drug used to inhibit gluconeogenesis as part of the treatment of type 2 diabetes, leads to the activation of AMPK. The mTORC1 inhibitor **rapamycin** (also called **sirolimus**) is an immunosuppressant that is used after organ transplantation.

Leucine appears to serve as a signal of the influx of dietary protein. Accordingly, leucine stimulates protein synthesis and inhibits protein degradation postprandially. In the fasting state, when muscle protein is degraded, leucine is transaminated within muscles and only the resulting keto acid is released into the blood (the same is true of the other branched-chain amino acids, isoleucine and valine).

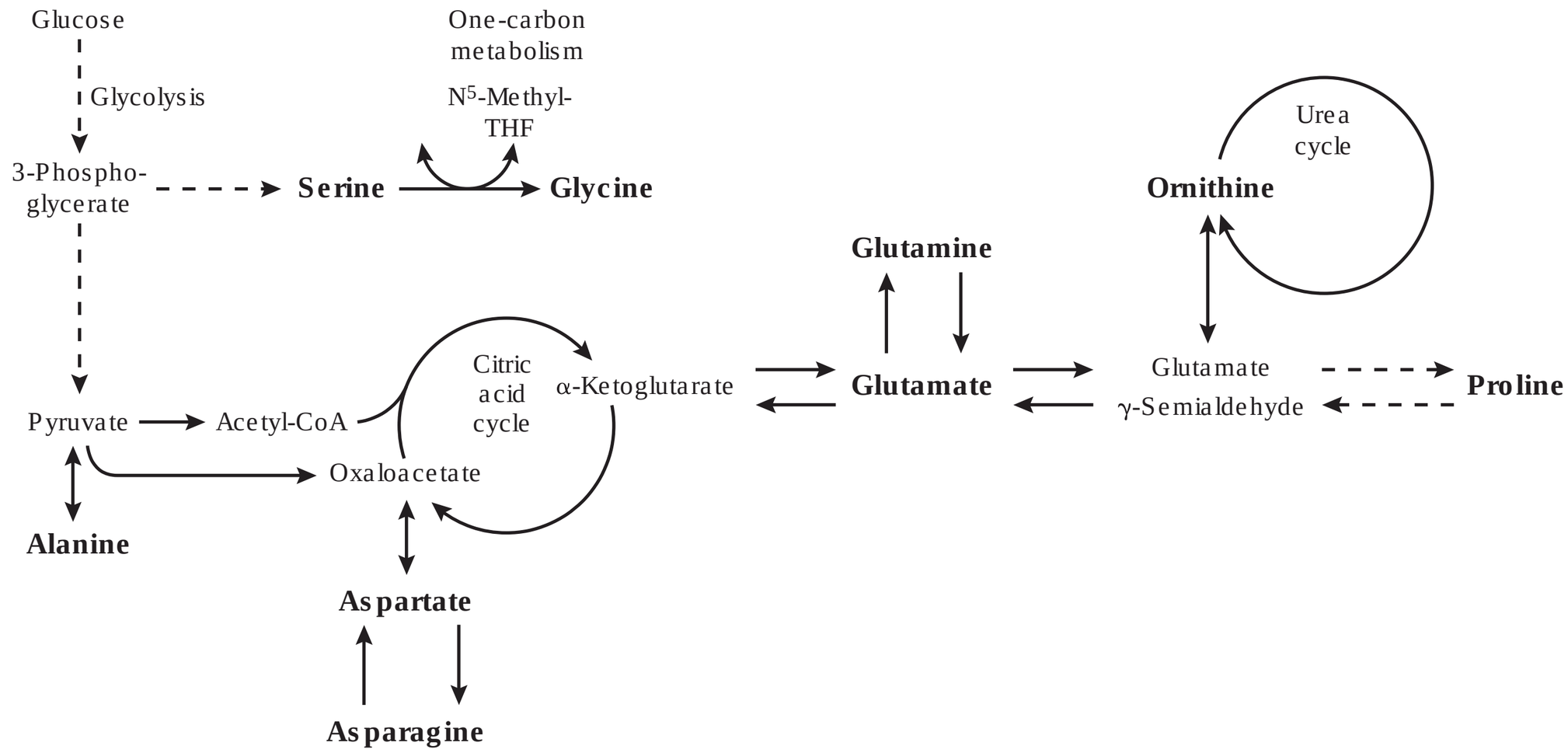


Fig. 34.11 Biosynthesis of serine, glycine, alanine, aspartate, asparagine, glutamate, glutamine, ornithine, and proline from intermediates of glycolysis and the citric acid cycle. The pathway for glycolysis is shown in Fig. 19.2, that of the citric acid cycle in Fig. 22.6, and that of the urea cycle in Fig. 35.7.

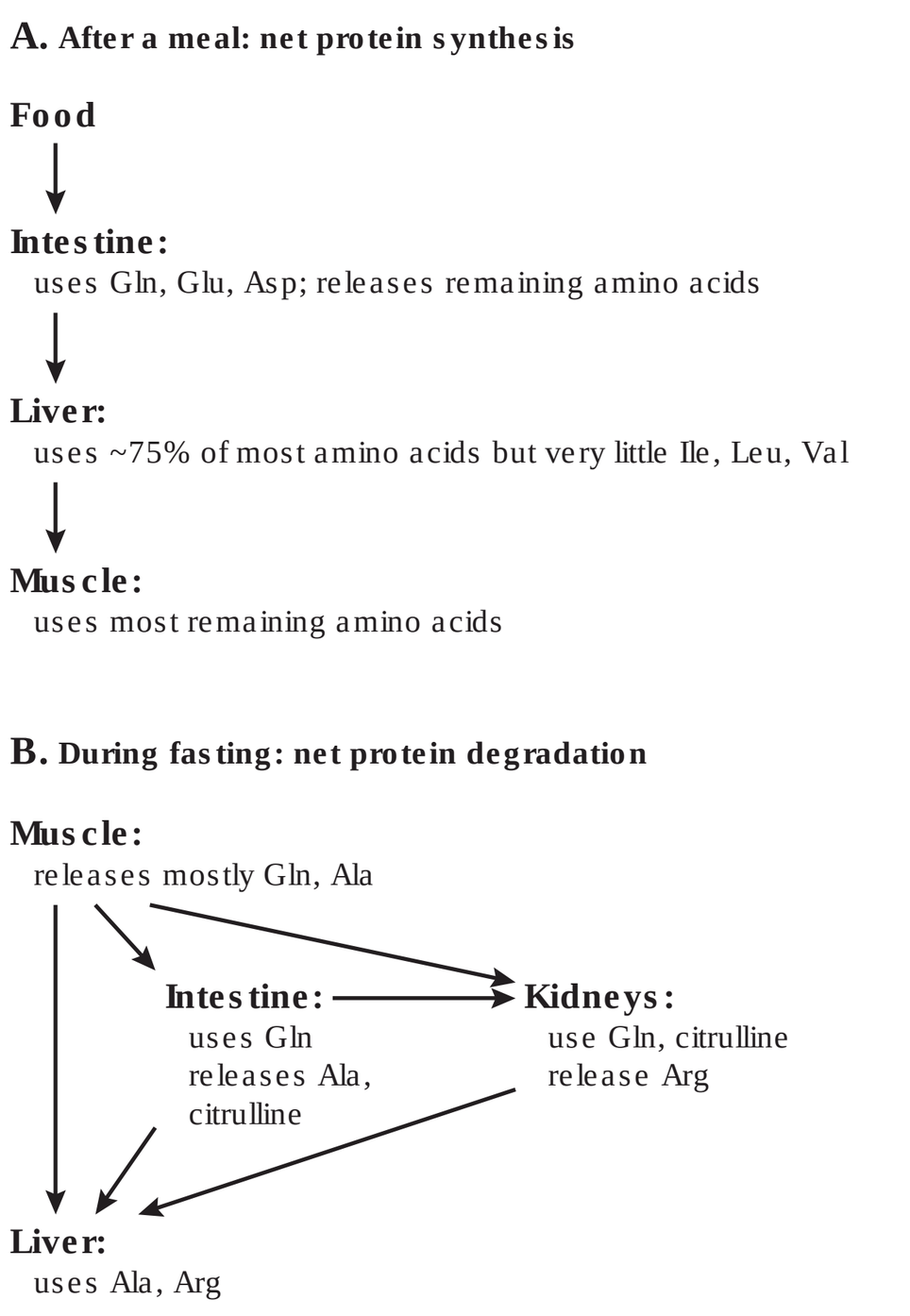


Fig. 34.12 Major flux of amino acids in the fed and fasting state. Flux of amino acids is controlled by insulin, glucagon, cortisol, and leucine. Protein degradation is detailed in Chapter 35.

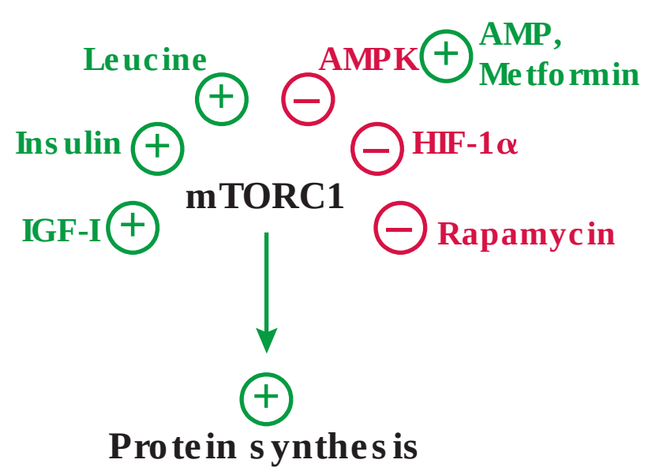


Fig. 34.13 Role of mTORC1 in the regulation of protein synthesis. *AMPK*, adenosine monophosphate–activated protein kinase.

Insulin stimulates transcription via the mitogen-activated protein kinase pathway and translation via mTORC1 and eukaryotic initiation factor 2 phosphorylation, pathways that are present in virtually every cell (see Fig. 33.3 and Section 3 in Chapter 7). After a meal, the pancreatic islets secrete insulin in response to glucose and presumably an increase in the concentrations of leucine, glutamine, and arginine (details are not certain; see Section 3.3.2 in Chapter 26). Protein synthesis lowers the concentrations of branched-chain and other essential amino acids in blood plasma.

Insulin-like growth factor-I (**IGF-I**) stimulates muscle protein synthesis. IGF-I occupation of receptors for IGF-I leads to activation of protein kinase B and mTORC1, which in turn stimulate the translation of mRNA into protein.

Glucagon stimulates gluconeogenesis in the liver and in the kidneys (see Section 3 in Chapter 25), which lowers the concentration of amino acids in the blood. In the fasting state,

the pancreas secretes glucagon in response to epinephrine and a lower concentration of glucose. Note that muscle does not have appreciable numbers of glucagon receptors and hence does not respond to glucagon.

Cortisol stimulates degradation of muscle protein, oxidation of branched-chain amino acids in muscle, and use of the released amino acids in gluconeogenesis by the liver and the kidneys. T e adrenal medulla secretes cortisol in a diurnal fashion so that the concentration of cortisol in the blood is highest in the early morning and lowest in the early evening (see Fig. 31.13). Fasting and intense exercise both increase cortisol secretion. Patients who are treated with high doses of glucocorticoids and patients who have a high concentration of circulating cortisol due to Cushing syndrome both show muscle wasting (see Section 4.2.3 in Chapter 25 and Section 3 in Chapter 31).

SUMMARY

- In the fundus and corpus of the stomach, parietal cells secrete HCl and chief cells secrete pepsinogens. In the acidic lumen of the stomach, pepsinogens autocatalytically activate to become active pepsins, which cleave dietary proteins.
- T e vagus nerve works via acetylcholine, gastrin, and histamine to stimulate HCl secretion from parietal cells. Physiologically, pH-sensing D-cells throughout the stomach inhibit HCl secretion. Pharmacologically, HCl secretion can be inhibited with histamine H_2 receptor antagonists or with proton pump inhibitors.
- T e pancreas secretes bicarbonate, which raises the pH of chyme in the duodenum to a value greater than 7. T e pancreas also secretes digestive enzymes into the small intestine. Among these is trypsinogen. Enterokinase on the surface of the intestinal brush border membranes, when activated by bile salts, cleaves trypsinogen to produce trypsin. Trypsin in turn cleaves other pancreas-derived zymogens to active chymotrypsin, elastase, and carboxypeptidases A and B.
- Pancreatitis is associated with activation of trypsin and other digestive enzymes inside the pancreas. In most cases, pancreatitis is due to blockage of the common bile duct or due to the alcohol dependence syndrome. In patients with cystic fibrosis, inadequate flushing of zymogens out of the pancreas into the intestine causes pancreatitis and loss of pancreatic function. Patients who express mutant trypsinogen or mutant pancreatic trypsin inhibitor develop pancreatitis at a young age.
- T e brush border membrane of the epithelium of the small intestine contains aminopeptidases that hydrolyze single amino acids from the N-terminus of oligopeptides. T e brush border membrane also contains a peptide transporter (PEPT1) that facilitates uptake of dipeptides and tripeptides into intestinal epithelial cells; in addition, the membrane contains a variety of transporters for amino acids.
- A large number of dif erent transporters facilitate amino acid transport from the intestinal epithelium into the blood, from the blood into peripheral cells, and from the renal glomerular filtrate into tubular cells and from there to the blood. Patients who have cystinuria cannot efficiently remove cystine from the glomerular filtrate and form cystine stones in the kidneys. Patients with Hartnup disease show symptoms of tryptophan deficiency that is caused by deficient transport of neutral amino acids into the intestinal and renal epithelial cells.
- Humans must consume certain amino acids in their diet because they cannot synthesize them. T ese amino acids are called essential amino acids and comprise Arg, His, Ile, Leu, Lys, Met, Phe, T r, Trp, and Val. Humans can synthesize nonessential amino acids from intermediates of glycolysis or the citric acid cycle, and from the nitrogen of other amino acids.
- Net protein synthesis occurs after consuming a protein-containing meal. Insulin and leucine, working through mTORC1 and an initiation factor for translation, are the principal stimuli of this protein biosynthesis.

FURTHER READING

- For approximately yearly brief updates on the state of knowledge of gastrointestinal function and related diseases, see the journal Current Opinions in Gastroenterology.
- Brandsch M. Drug transport via the intestinal peptide transporter PepT1. Curr Opin Pharmacol. 2013;13: 881-887.
- Hunt RH, Camilleri M, Crowe SE, et al. T e stomach in health and disease. Gut. 2015;64:1650-1668.
- Saravakos P, Kokkinou V, Giannatos E. Cystinuria: current diagnosis and management. Urology. 2014;83:693-699.
- Yandrapu H, Sarosiek J. Protective factors of the gastric and duodenal mucosa: an overview. Curr Gastroenterol Rep. 2015;17:24.

Review Questions

1. Before histamine H_2-antagonists (and proton pump inhibitors) were available, patients who had a duodenal ulcer were often treated surgically with vagotomy and antrectomy. T ese procedures decreased acid secretion in part through which one of the following mechanisms?

   A. Diminished gastrin secretion (G-cells)
   B. Increased histamine secretion
   C. Reduced somatostatin secretion
   D. Removal of parietal cells
   E. Removal of chief cells

2. After an overnight fast, an adult patient with atrophic gastritis was prepared for continuous aspiration of stomach fluids via a nasogastric tube. At 15-minute intervals, fluid volume and acid content were determined. Samples were taken before and after parenteral administration of pentagastrin, an analog of gastrin. T is test is a measure of which of the following?

   A. Acetylcholine stimulation of acid output
   B. Function of parietal cells
   C. Histamine stimulation of somatostatin secretion
   D. Infection with Helicobacter pylori
   E. Mass of mucus-producing cells

3. Apart from a gastrinoma, which of the following can be expected to increase the concentration of gastrin in the blood?

   A. Abnormally high concentration of somatostatin
   B. Abnormally high concentration of histamine
   C. Abnormally low pH in the lumen of the stomach
   D. Treatment with a proton pump inhibitor

Chapter

35 Protein Degradation, Amino Acid Metabolism, and Nitrogen Balance

SYNOPSIS

- Degradation of proteins is an important part of maintaining a set of normal proteins, providing amino acids for synthesis of new proteins during illness or injury, and synthesizing glucose via gluconeogenesis (see Chapter 25).
- The cytosol and the nucleus contain proteasomes, which are large protein complexes that degrade proteins into short peptides and amino acids. Hundreds of different enzymes conjugate proteins with ubiquitin (a small, conserved polypeptide) to mark them for degradation by proteasomes. The cytosol also contains lysosomes; these organelles provide an acidic milieu for the degradation of a variety of compounds (including proteins). Lysosomes acquire proteins both from the extracellular space and from within the cell.
- Amino acids are mostly used for protein synthesis. The degradation of amino acids yields nitrogen and carbon compounds. Nitrogen in these compounds is eliminated largely as urea or ammonium ions; the carbon compounds are used for the production of energy.
- The kidneys can excrete ammonium ions (NH_4^+) into the urine and simultaneously release bicarbonate into the blood. This process plays an important role in bicarbonate homeostasis of the blood.
- Patients who have severe liver disease can develop hepatic encephalopathy, which is often accompanied by hyperammonemia (a high concentration of NH_4^+ in the blood). Hyperammonemia impairs the function of the central nervous system.
- Deficient conversion of phenylalanine to tyrosine causes phenylketonuria. Lifelong treatment with a low-phenylalanine diet prevents intellectual disability.
- Tyrosine gives rise to dopamine, norepinephrine, and epinephrine, which act as neurotransmitters and hormones (see Chapters 26 and 33). Tyrosine also gives rise to melanin, which is responsible for the pigmentation of skin and hair.
- Tryptophan gives rise to serotonin (a transmitter in the central and enteric nervous system), melatonin (a hormone that plays a role in the sleep/wake cycle), and NAD^+ and its relatives (see Chapters 19 and 22).
- The term "nitrogen balance" refers to the amount of nitrogen gained with food intake (mostly as protein) versus the amount lost (mostly as urea and NH_4^+ in urine).

LEARNING OBJECTIVES

For mastery *of* this topic, you should be able to do the *f*ollowing:

- Explain how intracellular proteins are degraded to peptides and amino acids and how this process is regulated.
- Explain how tissues produce ammonia, how the liver removes ammonia from the blood, and how the kidneys excrete ammonia.
- Explain how a deficiency of any one of the components of the urea cycle causes hyperammonemia.
- Describe the symptoms and treatment of hyperammonemia.
- Describe the newborn screening, pathogenesis, and treatment of phenylketonuria.
- Describe the factors that influence the pigmentation of the skin, hair, eyes, and parts of the brain and describe the bases of the common pigmentation disorders.
- Devise a plan to estimate the nitrogen balance of a patient who has extensive burns and receives parenteral nutrition.

1. DEGRADATION OF BODY PROTEIN

For the most part, damaged proteins are not repaired; rather, proteins in the body undergo ongoing degradation and de novo synthesis. During *f*asting, protein degradation increases and amino acids are used *f*or gluconeogenesis. Protein degradation increases to a much greater degree during illness, trauma, or burns. Inside cells, proteins are degraded in proteasomes or lysosomes.

1.1. General Comments

T ere is ongoing degradation of proteins in the body. Protein degradation provides a cell with a means of adjusting its set of proteins to its needs. Some proteins are degraded as part of the regulation of the cell cycle and metabolic pathways, while others are normally produced in excess and then degraded to match true needs (e.g., globins for hemoglobin, apoproteins for lipoprotein particles); others are detected, marked, and degraded when they are misfolded, modified, or damaged; and still others are degraded as part of a general increase in protein degradation during fasting or illness.

As explained below, proteins are degraded largely by proteasomes in the cytosol and nucleus and by proteases in lysosomes. Certain amino acid sequences determine the lifetime of proteins. In addition, hormones regulate protein degradation. Section 4 in Chapter 34 provides a brief overview of whole-body protein synthesis and degradation.

Amino acids derived from the degradation of proteins (whether by proteasomes or lysosomes) are reused for the synthesis of proteins (see Section 4.1 in Chapter 34), released into the bloodstream, used for the synthesis of other molecules, or degraded. Overall, most amino acids are used for resynthesis of proteins, and only a small portion is degraded (see Fig. 34.10).

1.2. Degradation of Proteins by Proteasomes

Proteasomes play a role in the degradation of proteins as part of the following: the feeding/fasting cycle, regulation of

metabolism and the cell cycle (see also Chapter 8), presentation of short peptides on major histocompatibility complex (MHC) class 1 proteins to passing cytotoxic T-cells, and degradation of misfolded or damaged proteins (see also Chapter 7).

Most protein degradation in cells proceeds via the proteasome pathway. A lesser amount of protein is degraded in lysosomes and a still smaller amount in the cytosol by calcium-activated calpains and caspases, which play roles mostly in regulation.

Proteasomes are in the cytosol and nucleus of all types of cells. Enzymes in these compartments mark certain proteins for degradation by conjugating them with ubiquitin (see below). Proteasomes recognize these proteins and hydrolyze them into peptides of six to nine amino acids. Cellular peptidases then degrade these peptides into individual amino acids. Ubiquitin from the polyubiquitin tail is reused to mark other proteins for degradation.

Four types of enzymes, E1 through E4, are used to mark proteins with **ubiquitin** to target them for hydrolysis by proteasomes (Fig. 35.1). Ubiquitin is a 76-residue protein. T e C-terminal glycine of ubiquitin is linked to a lysine residue of the target protein. Further ubiquitin residues are then added to Lys-48 of the preceding molecule of ubiquitin to yield a polyubiquitinated protein. Conjugation with four or more Lys-48–linked ubiquitins is equivalent to a kiss of death because such conjugated proteins are degraded inside proteasomes. (In contrast, monoubiquitination, conjugation with multiple single ubiquitins to multiple amino acids in a protein, and polyubiquitination via linkages other than Lys-48 serve other purposes inside cells.)

Note that the E3 component of the ubiquitin conjugating system is an entirely dif erent protein from the E3 component of dehydrogenases, such as pyruvate dehydrogenase and α-ketoglutarate dehydrogenase (Chapter 22).

Several of the E3 ubiquitin protein ligases are associated with disease.

T e **papilloma virus** (the cause of **warts** and **cervical cancer**) encodes the protein E6, which activates the E3 ligase **E6AP** that ubiquitinates the tumor suppressor p53; inappropriate destruction of p53 paves the way to tumorigenesis (see Chapter 8).

Lack of a functional, maternally inherited **E6AP ubiquitin-protein ligase** causes **Angelman syndrome**, which is associated with severe motor dysfunction and intellectual disability.

Patients who are homozygous or compound heterozygous for mutations that inactivate the E3 ligase **Nedd4** have impaired Na^+ transport in the kidneys; this leads to a form of **hereditary, salt-sensitive hypertension**.

VHL-factor (**von Hippel–Lindau factor**) is an E3 ligase that regulates the ubiquitination of HIFα and hence the production of erythropoietin (see Section 1.4 in Chapter 16). Mutations in the VHL gene give rise to **von Hippel-Lindau disease**, and af ected persons have an unusually high tendency to develop neoplasms. VHL factor is frequently mutated in clear-cell renal cell carcinomas.

Among patients with **hereditary Parkinson disease**, the disease is most commonly due to a mutation in **parkin**. Parkin

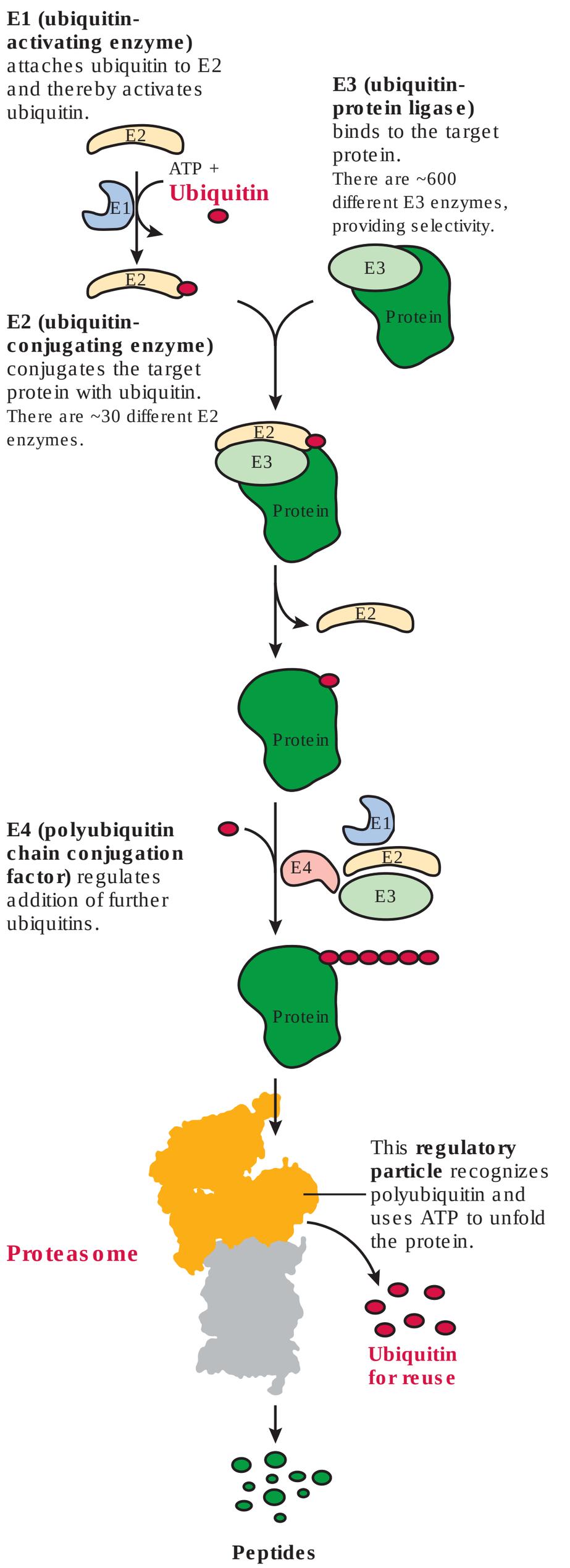


Fig. 35.1 **The proteasome pathway of protein degradation.**

is a ubiquitin E3 ligase that seems to be important for long-term survival of dopamine neurons.

Some proteins are degraded by the catalytic portion of proteasomes without a need for ubiquitination. These proteins often have an inherently disordered structure.

1.3. Regulation of Protein Degradation via Proteasomes

The lifetime of proteins depends on their amino acid sequence, their conformation, covalent modifications of amino acids, and the concentrations of both hormones and free amino acids (see Section 4.3 in Chapter 34). The **E3 ubiquitin protein ligases** play a crucial role in the selection of proteins for degradation.

Proteins containing **PEST motifs**, that is, regions rich in proline (single amino acid code P; see Chapter 9), glutamate (E), serine (S), and threonine (T), are degraded particularly rapidly, often by proteasomes. **Posttranslational modification** (e.g., phosphorylation), binding of ions (e.g., Ca^{2+}), and binding of other proteins may affect the recognition of PEST motifs for protein degradation.

The **N-terminal amino acid** influences the lifetime of a protein; this is reflected in the **N-end rule**. The E3 ubiquitin-protein ligases bind to an N-terminal amino acid and an internal lysine residue. If the N-terminal amino acid side chain is either positively charged (as in Arg, Lys, or His) or hydrophobic and bulky (as in Phe, Tyr, Trp, Ile, or Leu), the protein is ubiquitinated rather rapidly. By contrast, the E3 ubiquitin-protein ligases usually do not accept proteins with Ser, Gly, Val, or Met at their N-terminus, resulting in slow turnover of such proteins. The N-terminal amino acid of a protein may be altered after translation; this may modify the lifetime of the protein. For instance, deamidation of Asn to Asp or Gln to Glu (by N-terminal amidohydrolases), or arginylation of Asp, Glu, or Cys (by Arg-tRNA-protein transferase) shortens the lifetime of a protein.

Fasting enhances protein degradation. Most amino acids are glucogenic; that is, they can serve as substrates for gluconeogenesis (for details, see Section 2.2 in Chapter 25 and Fig. 25.5). Gluconeogenesis is important for the provision of glucose to the brain, red blood cells, and other cells during an extended fast. Protein degradation is maximal after a brief fast. Then, as tissues use more fatty acids and ketone bodies, protein degradation declines. After about 1 week of fasting, protein degradation amounts to less than protein degradation in the course of a day with adequate consumption of protein and calories. In the fasting state, a relatively low concentration of **insulin** and **leucine** permits degradation of protein, a rising concentration of **cortisol** stimulates protein degradation in muscle, and an elevated concentration of **glucagon** enhances the use of amino acids in the liver for gluconeogenesis.

Infection, **postpartum recovery**, major **trauma**, and **burn injury** stimulate whole-body protein degradation more than starvation does. These conditions are associated with a large increase in the concentration of **cytokines**, such as **tumor necrosis factor-α** (**TNF-α**) and **interleukins** (IL) **1**, **2**, **6**, and **8**. The cytokines are released by endothelial cells and white blood cells, such as neutrophils, lymphocytes, macrophages, and monocytes. Particularly high concentrations of cytokines in the blood are seen in patients who have sepsis, pancreatitis, graft versus host disease (most commonly seen in response to transplanted cells from bone marrow), or burn injuries.

Proteasome inhibitors are used in the treatment of **multiple myeloma**, a malignancy of antibody-producing cells in the bone marrow. **Bortezomib** is an analog of a dipeptide, and **carfilzomib** is an analog of a tetrapeptide; both drugs have to be injected.

The balance of protein synthesis and degradation is discussed in Section 5 below.

1.4. Degradation of Proteins by Lysosomes

Lysosomes are intracellular organelles, which contain many different hydrolases that degrade proteins, lipids, carbohydrates, or nucleic acids. The hydrolases are synthesized in the endoplasmic reticulum, move through the Golgi complex, leave the trans face of the Golgi complex inside vesicles, and then reach the lysosomes.

Lysosomes acquire material to be digested both from extra- and intracellular sources. Extracellular and plasma membrane-bound proteins enter lysosomes via **endocytosis**, **pinocytosis**, or **phagocytosis**. Intracellular organelles and cytosol enter lysosomes via autophagosomes. **Autophagy** (the cell's "eating" of some of its components) represents a means of producing amino acids from cellular proteins when these amino acids are not available from the bloodstream. Autophagy also plays an important role in removing misfolded proteins, aggregated proteins, and pathogens from the cytosol. Importantly, autophagy generally has tumor suppressor activity, whereas inhibition of autophagy usually promotes tumorigenesis. In antigen-presenting cells, some of the peptides produced by phagolysosomes are loaded onto MHC class II proteins for display on the cell surface to passing helper T-cells.

Lysosomes acidify and hydrolyze acquired compounds, and they release products through transporters into the cytosol. The lumen of lysosomes is acidic (pH ~5). Lysosomes contain several different **cathepsins**, which are acid-activated proteases. Amino acids, monosaccharides, oligosaccharides, and nucleotides are transported across the membranes of lysosomes and released into the cytosol.

Deficiencies of enzymes in lysosomes that degrade components of the extracellular matrix are described in Chapter 13.

2. ELIMINATION OF AMINO ACID NITROGEN

When amino acids are degraded, their nitrogen can be released into the blood as part of ammonium ions, glutamine, or alanine. Nitrogen from these compounds can then enter the urea cycle and become part of urea, which is excreted in the urine. Ammonium ions are also excreted directly into the urine, especially during acidosis.

2.1. Production of Ammonium Ions From Amino Acids

The nitrogen of some amino acids can be released as an ammonium ion (NH_4^+; Fig. 35.2). Ammonium ions are in chemical equilibrium with ammonia (NH_3; equation: $NH_4^+ \leftrightarrow NH_3 + H^+$). Ammonia diffuses across plasma membranes; in the kidneys, a transporter may enhance transport. Ammonium ions can move through various channels, including many potassium channels.

The kidneys can excrete ammonium ions into the urine (see Section 2.2). Many cell types can incorporate nitrogen into biological molecules by reacting ammonium ions with α-ketoglutarate to produce glutamate and then with glutamate to form glutamine, which is nontoxic and can be transported between organs (see Section 2.3). The liver and the intestine can convert ammonium ions to carbamoylphosphate for the eventual elimination as urea (see Section 2.5).

2.2. Excretion of Ammonium Ions Into the Urine

A small portion of excess nitrogen is excreted into the urine as ammonium ions (see Fig. 35.11). In a healthy person who consumes protein, the daily rate of ammonium ion production stays fairly constant (meanwhile, the rate of urea production fluctuates and adjusts to the rate of degradation of amino acids, see Section 2.5).

Exhalation of CO_2 via the lungs is the body's prime defense against acidosis, and production of ammonium ions and bicarbonate from glutamine by the kidneys is a lower-capacity, secondary defense as well as a means of supplying the blood with bicarbonate (Fig. 35.3). Bicarbonate in the blood is the primary buffer for H^+, and it gets depleted when there is a greater influx of acid into the blood than efflux from the blood. The acid is typically lactic acid or the ketone bodies acetoacetic acid and β-hydroxybutyric acid but can also be formic acid.

To produce bicarbonate and ammonium ions, the kidneys convert glutamine to phosphoenolpyruvate (see Fig. 35.3). NH_3 can diffuse into the tubule, perhaps via both passive and facilitated diffusion. The $Na{:}H^+$ antiport pumps H^+ from H_2CO_3 (carbonic acid) into the tubules to generate NH_4^+. HCO_3^- is then exported into the blood.

During **acidosis**, the kidneys increase the rate of ammonium ion excretion into the urine, and muscle increases glutamine output (see Fig. 35.3). In patients who have metabolic acidosis due to **chronic kidney disease**, sufficient muscle protein is degraded to lead to muscle weakness.

The maximal rate of bicarbonate production by the kidneys is much lower than the maximal rate of CO_2 loss via the lungs. Hence, patients who have an inordinately high rate of acid release into the blood (because of hypoxia or a deficiency in

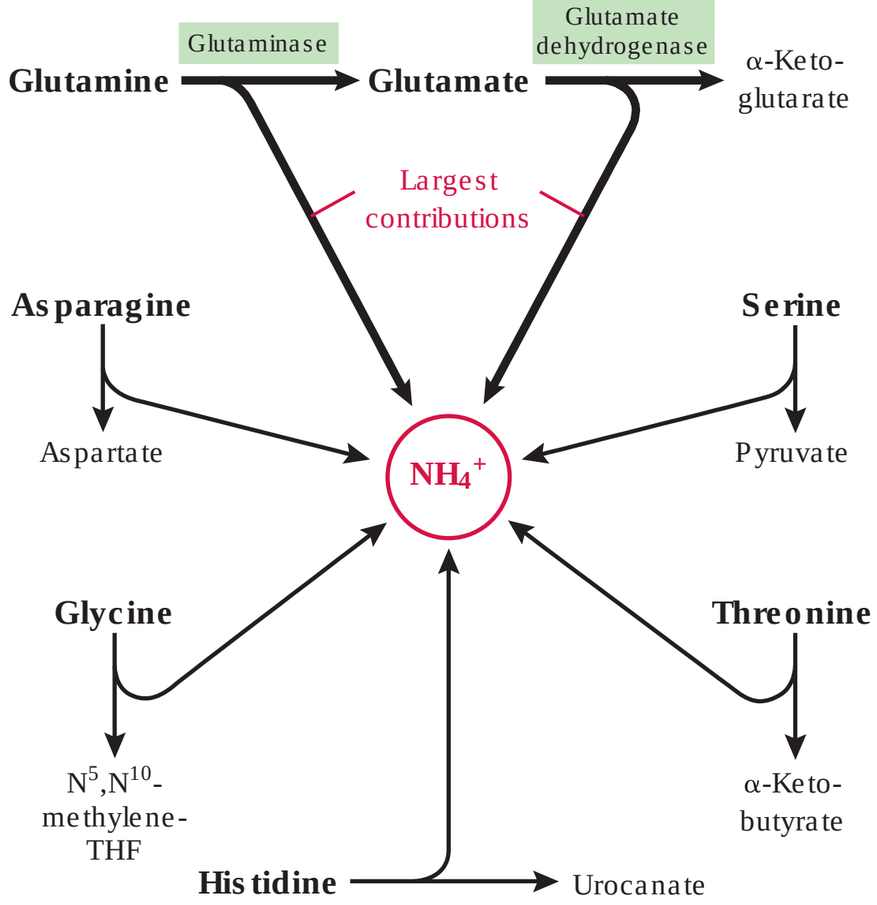


Fig. 35.2 Deamination of some amino acids yields ammonium ions. The deamination of glycine is also described in Section 2.1 in Chapter 36.

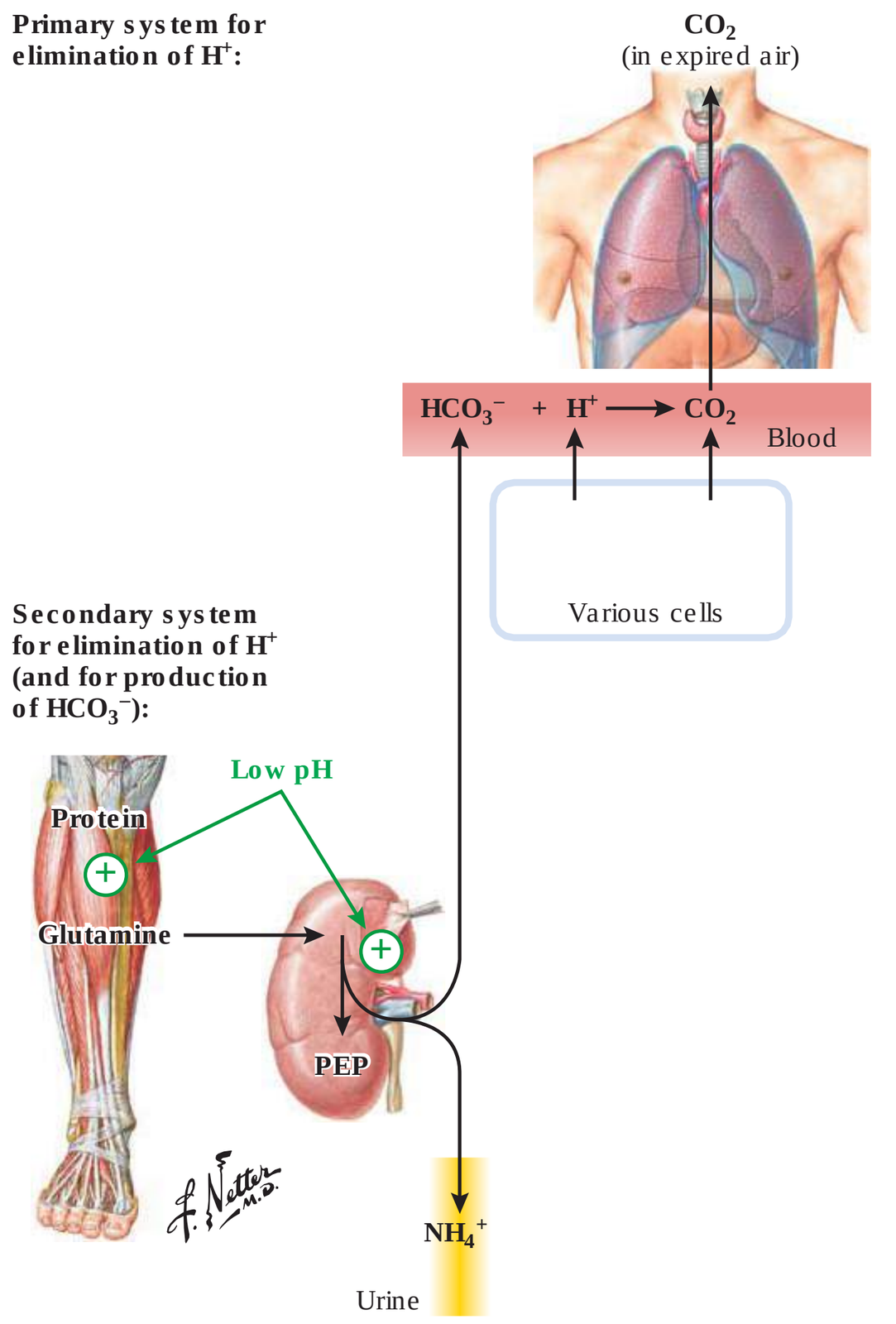


Fig. 35.3 Role of ammonium excretion in the defense against acidosis. As the kidneys take up glutamine from the blood, excrete NH_4^+ into the urine, and release HCO_3^- into the blood, they remove H^+ from the blood and supply HCO_3^- to the blood. PEP, phosphoenolpyruvate.

metabolism) usually also have an abnormally low concentration of bicarbonate in the blood.

2.3. Transamination

The amino group of many amino acids can be transferred to an α-keto acid, thereby forming a different amino acid. This is a way to conserve nitrogen for the biosynthesis of nonessential amino acids, which helps minimize the need for daily protein consumption. The transfer of an amino group is called transamination. There are many different amino acid–specific **transaminases** (**aminotransferases**) that catalyze transaminations (Fig. 35.4). Transaminases use **pyridoxal phosphate** as a prosthetic group and cofactor. Pyridoxal phosphate derives from pyridoxine (one form of vitamin B_6; well over 100 different enzymes use this cofactor). **α-Ketoglutarate** is the most common and frequent acceptor of amino groups, and it forms glutamate. Another common acceptor is **pyruvate**, which forms alanine. The amino acid that loses its amino group becomes an α-keto acid. These keto acids are degraded or, in liver and kidneys, used for gluconeogenesis (see Fig. 25.5).

Transaminations play a major role in the interorgan transport of nitrogen in the form of glutamine and alanine. When muscle, for instance, degrades protein, it transfers amino groups of the resulting amino acids to pyruvate and glutamate to produce alanine and glutamine, which it releases into the blood (see Fig. 35.10).

For the biosynthesis of **nonessential amino acids**, **glutamate** can donate its nitrogen for the synthesis of serine, aspartate, alanine, or ornithine, and the entire glutamate molecule can be used for the synthesis of proline (see Fig. 34.11). Glutamate is also a neurotransmitter. Presumably for this reason, glutamate is largely confined to the cell interior. Many tissues do not efficiently transport glutamate across their plasma membranes, and the concentration of glutamate in the blood is low. Instead of glutamate, glutamine is transported among organs. To this end, glutamate is aminated to produce glutamine; then, glutamine is deaminated to produce glutamate (see Fig. 34.11).

The activities of **aspartate transaminase** (**AST**) and **alanine transaminase** (**ALT**) in blood serum are frequently measured to gauge tissue damage, especially of the liver. AST catalyzes the reaction aspartate + α-ketoglutarate ↔ glutamate + oxaloacetate; ALT catalyzes the reaction alanine + α-ketoglutarate ↔ glutamate + pyruvate. Damaged cells lose more of these enzymes into the bloodstream than do healthy cells. In the blood, AST and ALT have half-lives of about 1 to 4 days. In blood, neither AST nor ALT is known to play a physiological role.

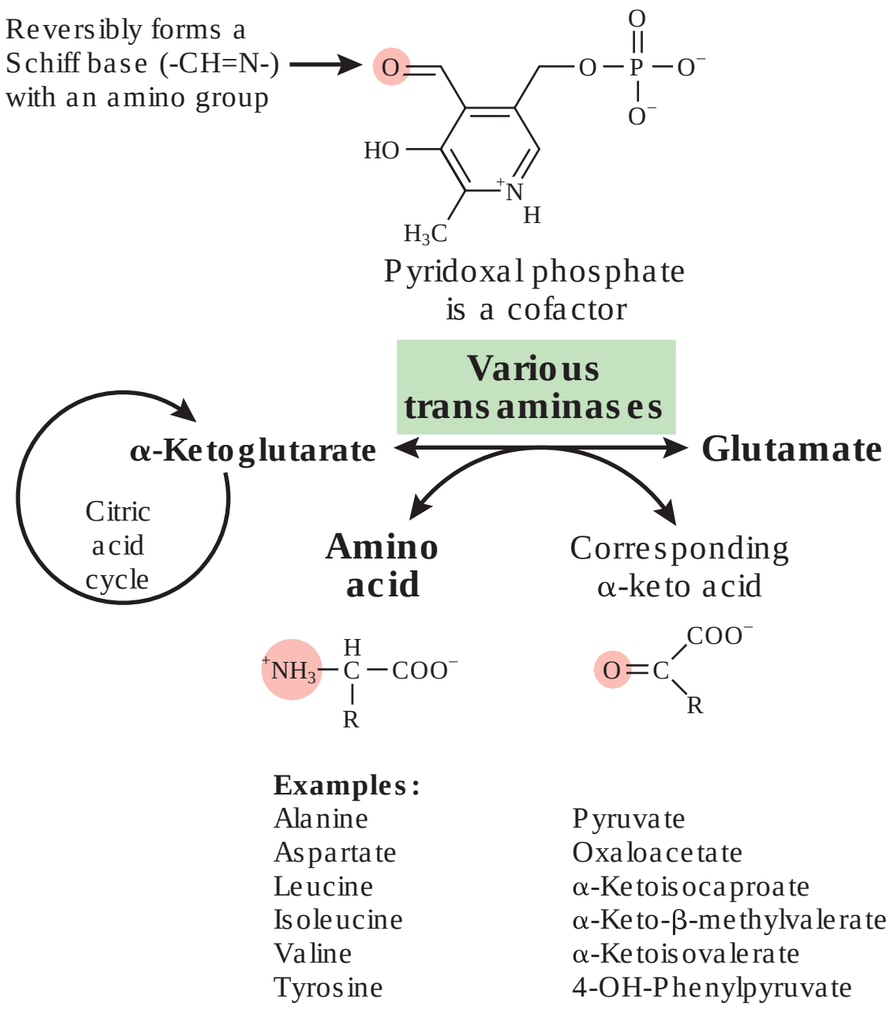


Fig. 35.4 Transamination of some amino acids yields glutamate. The transaminases use pyridoxal phosphate, a derivative of the vitamin pyridoxine (vitamin B_6).

2.4. Role of Glutamine in Nitrogen Metabolism

Glutamine is synthesized from glutamate and an ammonium ion with the help of **glutamate-ammonia ligase** (also called **glutamine synthetase**) via the following reaction: glutamate + NH_4^+ + ATP → glutamine + ADP + P_i.

Physiologically important sites of glutamate-ammonia ligase expression are muscle, liver, and brain;

- When muscle degrades amino acids (particularly branched-chain amino acids), it exports their nitrogen as part of glutamine (and also as part of alanine; see Fig. 35.10).
- In the liver, perivenous hepatocytes use the glutamate-ammonia ligase–catalyzed reaction to remove NH_4^+ (which is neurotoxic) from the blood (see Fig. 35.9); in patients with hyperammonemia, the concentration of glutamine in blood thus rises with the concentration of ammonium ions.
- In the brain, astrocytes use the glutamate-ammonia ligase–catalyzed reaction to remove neurotransmitter and recycle a precursor of it to neurons as part of a glutamate/GABA-glutamine cycle (Figs. 35.5 and 35.6). Some neurons use

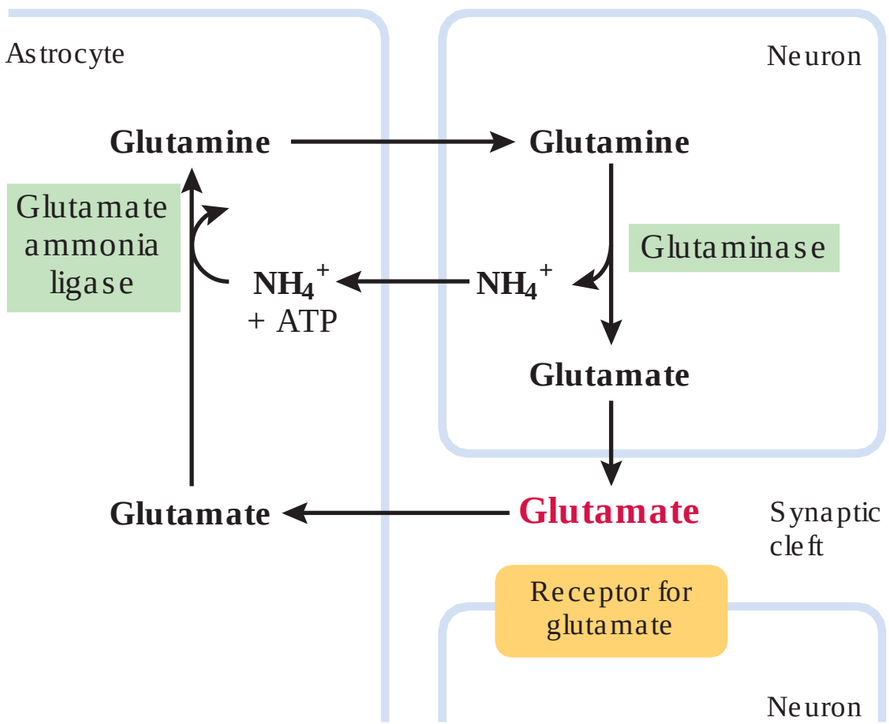


Fig. 35.5 The glutamate-glutamine cycle.

and release glutamate or GABA (γ-aminobutyric acid) as a neurotransmitter. Astrocytes remove most of these neurotransmitters from the extracellular space and convert them to glutamine, which they release into the extracellular space. Neurons take up glutamine and use it to synthesize glutamate and GABA.

Glutamine is used for a wide variety of reactions, including biosynthetic reactions and energy production. T e synthesis of asparagine, IMP (see Fig. 38.3), orotate (see Fig. 37.2), CTP (see Fig. 37.4), and all amino sugars (e.g., sialic acid; see Chapters 11 and 13) requires glutamine as a source of nitrogen. Neutrophils, macrophages, and lymphocytes use a large amount of glutamine for energy generation. Indeed, in some of the patients who degrade endogenous protein at a high rate, an intravenous infusion of glutamine reduces the rate of infection and the length of hospital stay. Many cancer cells have been found to use large amounts of glutamine for a variety of processes.

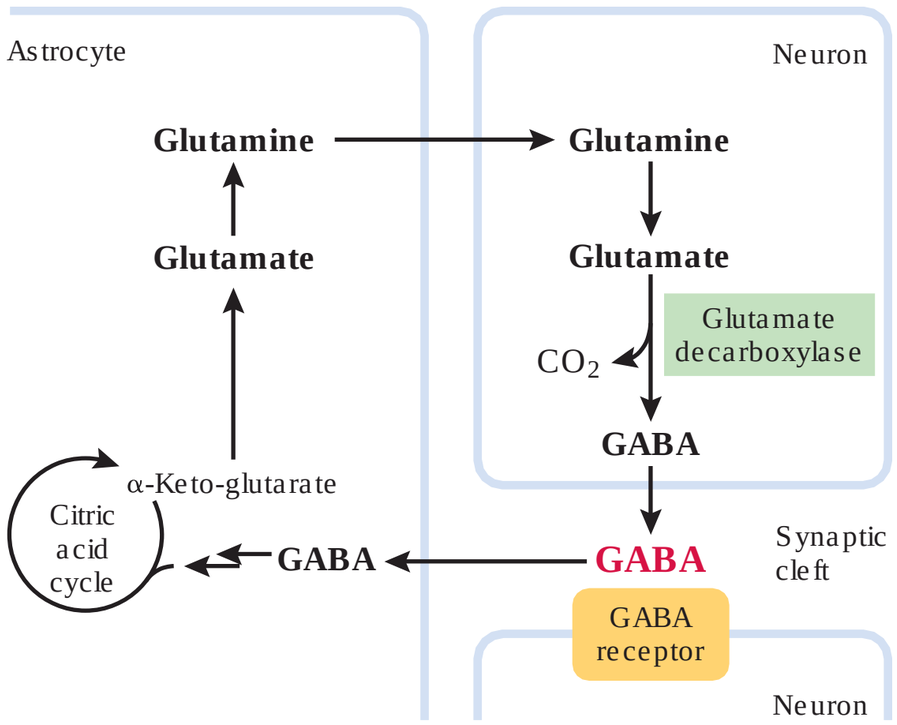


Fig. 35.6 **The γ-aminobutyric acid (GABA)-glutamine cycle.**

2.5. Elimination of Nitrogen via the Urea Cycle

T e urea cycle provides a means for excreting excess nitrogen as urea. T is is the main path for nitrogen excretion.

Urea contains two nitrogen atoms; one of these stems from NH_4^+ and the other from aspartate (Fig. 35.7). NH_4^+ is produced by deamination of amino acids (see Fig. 35.2), whereas aspartate nitrogen arises predominantly from transamination reactions (see Figs. 35.4 and 35.7). T e summary reaction of the urea cycle is: $NH_4^+ + HCO_3^- +$ aspartate + 3 ATP → urea + fumarate + 2 ADP + AMP + 4 P_i.

T e activity of the urea cycle is regulated short term via the formation of **N-acetylglutamate** (see Fig. 35.7) and, in addition, long term via the rate of **synthesis** of all enzymes of the cycle. Arginine activates the synthase that catalyzes the

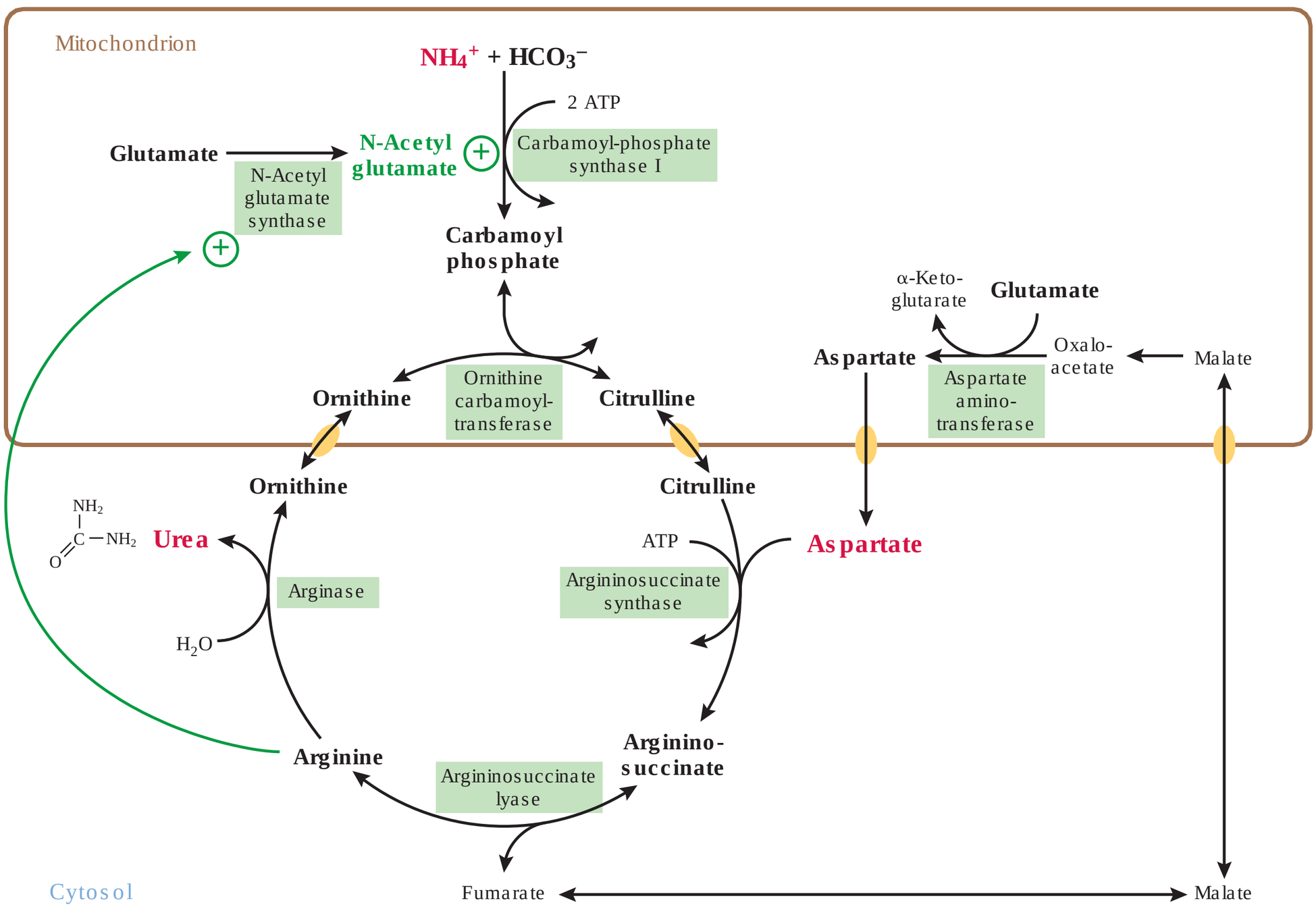


Fig. 35.7 **Reactions of the urea cycle.** While carbamoyl-phosphate synthase I is in the mitochondria and works as an accessory to the urea cycle, a second enzyme, carbamoyl-phosphate synthase II, is in the cytosol and catalyzes a reaction in the de novo synthesis of pyrimidine nucleotides (see Fig. 37.2).

formation of N-acetylglutamate; this in turn allosterically activates carbamoyl-phosphate synthase I. The quantity of urea cycle enzymes in a healthy person reflects that person's long-term protein intake. **Protein intake** in turn is regulated and limited such that the need for nitrogen elimination can be met by existing enzyme capacities.

Only the liver contains significant amounts of all enzymes of the urea cycle; however, the intestine and the kidneys each express enzymes for a portion of the urea cycle (Fig. 35.8). The intestine converts glutamine to ornithine, condenses ornithine with carbamoylphosphate to form citrulline, and exports citrulline into the bloodstream. The kidneys, and to a much smaller extent other organs, take up citrulline and convert it to arginine, which they release into the bloodstream. The liver cleaves the majority of this arginine into ornithine and urea, whereas various tissues use a small fraction of arginine for protein synthesis.

In the liver, cells that receive portal and arterial blood first use the urea cycle to produce urea, whereas cells that receive this blood last use glutamate-ammonia ligase (glutamine synthase) to convert any remaining NH_4^+ to glutamine (Fig. 35.9). The periportal hepatocytes are close to incoming blood and convert NH_4^+ and alanine to urea. These cells can also use pyruvate, the carbon skeleton of alanine, for gluconeogenesis (see Fig. 25.3). Downstream, the perivenous hepatocytes remove any remaining $NH4^+$ by synthesizing glutamine, which they release into the blood. The intestines convert this glutamine to citrulline (Figs. 35.8 and 35.10).

Antibodies against **carbamoyl-phosphate synthase I** and **arginase** are used in immunohistochemical analysis to distinguish **hepatocellular carcinomas** from other adenocarcinomas. Thereby, carbamoyl-phosphate synthase is commonly called **HepPar-1**, a name created at a time when the nature of the antigen was unknown. Both normal epithelial cells of the intestine and normal hepatocytes show HepPar-1 immunoreactivity (see also Fig. 35.8).

Fig. 35.10 provides a summary of the major traffic of amino acids and ammonia among muscle, intestine, kidneys, and liver.

In **blood**, urea is normally present at a concentration of 1.2 to 3.0 mM (equivalent to 7 to 18 mg of nitrogen per dL). The acronym **BUN** is commonly used for blood urea nitrogen. The concentration of urea in the blood depends on protein intake; the feeding/fasting cycle; and the presence of injuries, stress, or illness. In a person who eats three meals per day and for which protein intake just covers needs, the concentration of urea in blood stays constant throughout the day. In a person who habitually eats more protein than needed, the larger portion of these excess amino acids is degraded, but some of the excess amino acids are used for extra protein synthesis; then, in the fasting state, there is also some extra protein degradation. As a result, a person with this diet ends up having a persistently increased concentration of urea in the blood, and the concentration of urea is as much as 30% higher in the fed state than in the fasting state.

The term **azotemia** refers to an increase in the concentration of nitrogen in the blood; often, this is an increased value for BUN.

The term **uremia** can mean an excess of the concentration of urea in the blood (i.e., an elevated BUN) or it can refer to a syndrome of which an elevated concentration of urea is a hallmark. (Note that the term hyperuricemia refers to an excess concentration of urate, which is a different molecule from urea; see Chapter 38.)

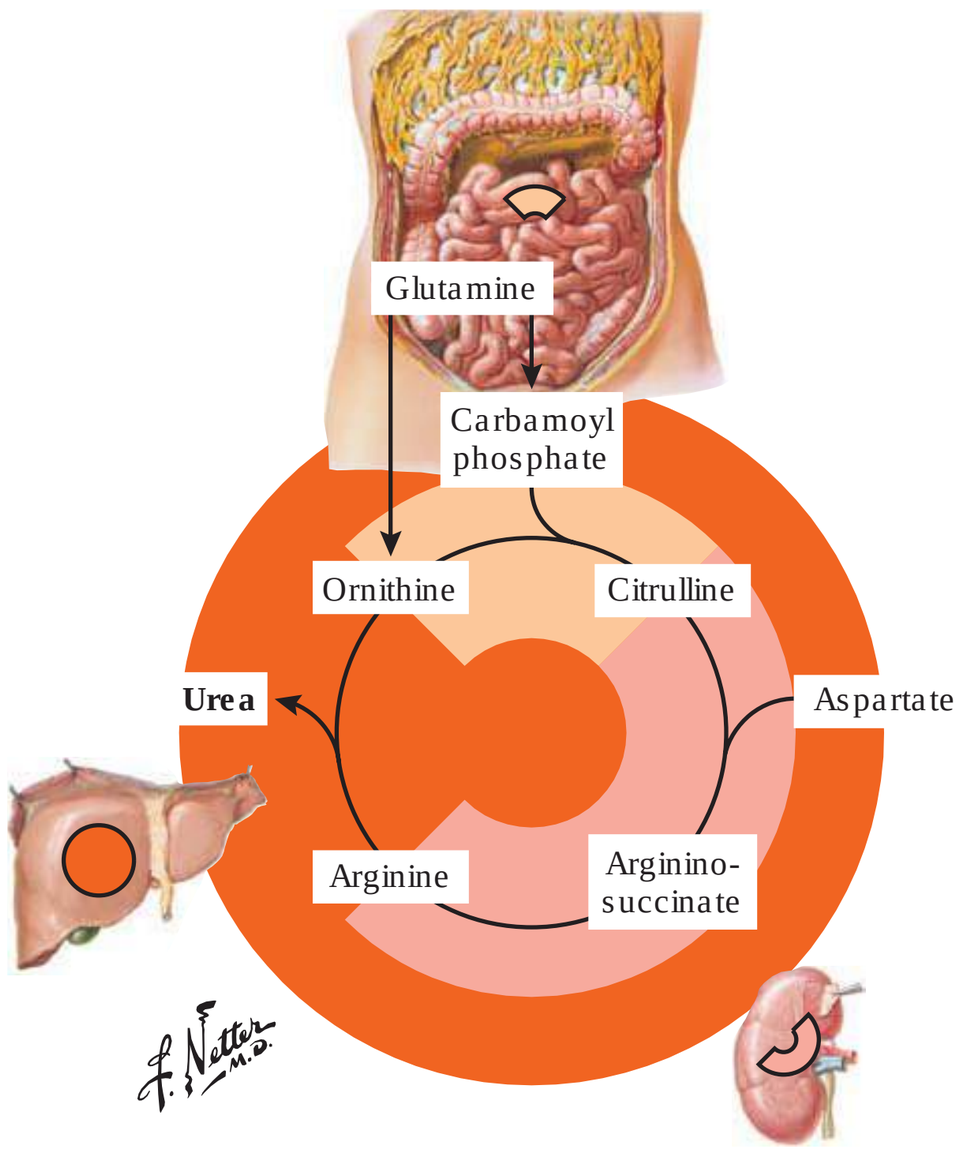


Fig. 35.8 Tissue location of the urea cycle. Only the liver contains the entire urea cycle. The intestine and the kidneys carry out selected steps. Citrulline and arginine are transported through the blood.

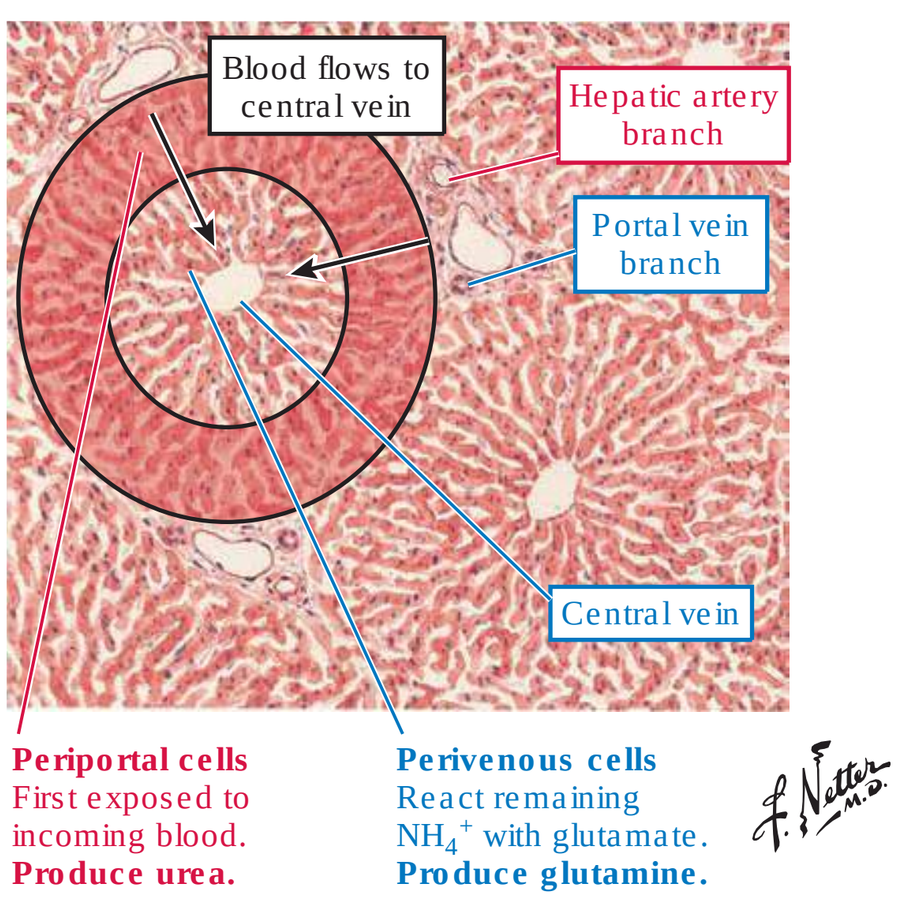


Fig. 35.9 Compartmentation of the urea cycle in the liver. The cells that produce urea also perform gluconeogenesis (see Figs. 25.2 and 25.3).

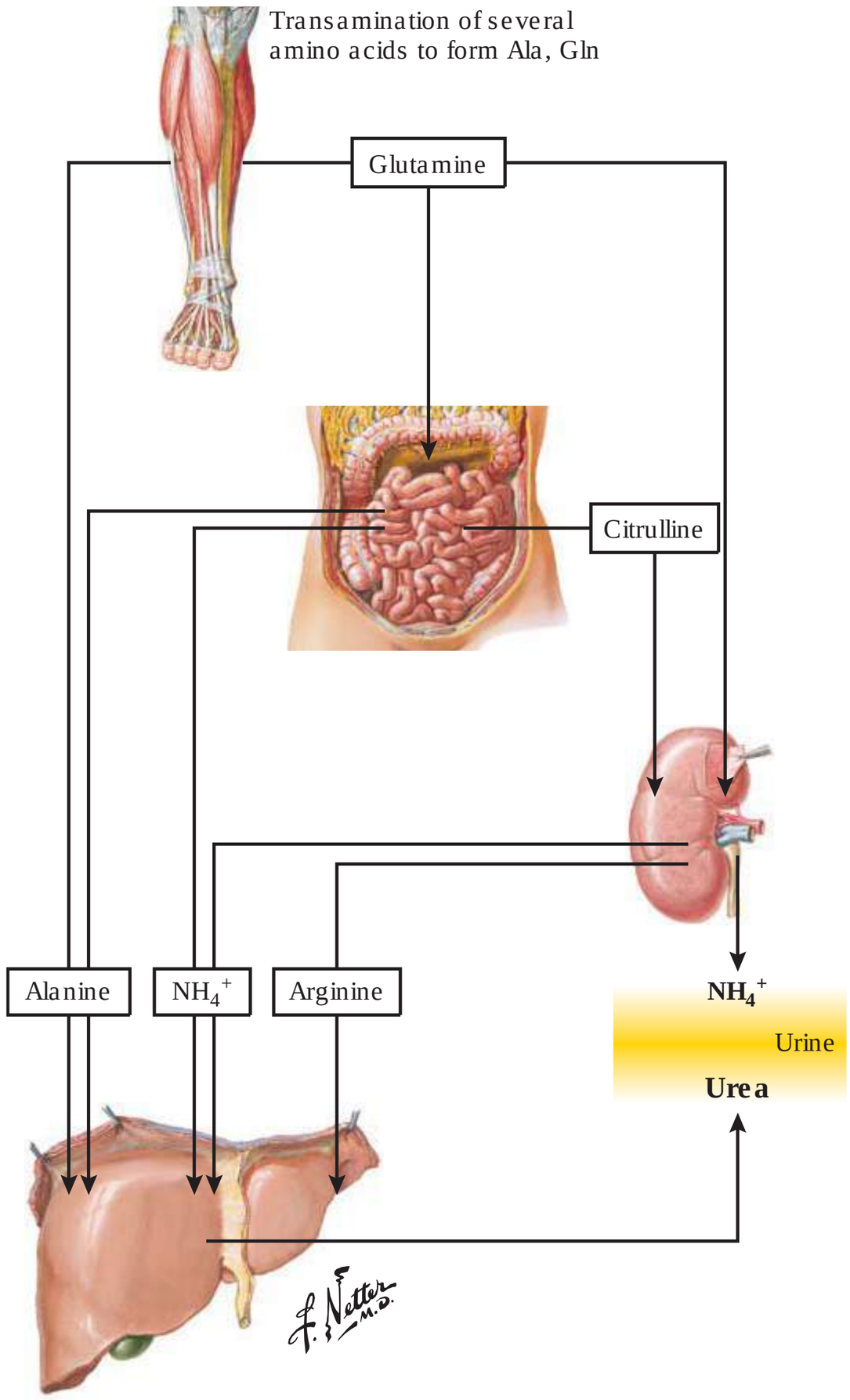


Fig. 35.10 **Interorgan nitrogen flux in the elimination of excess amino acid nitrogen.**

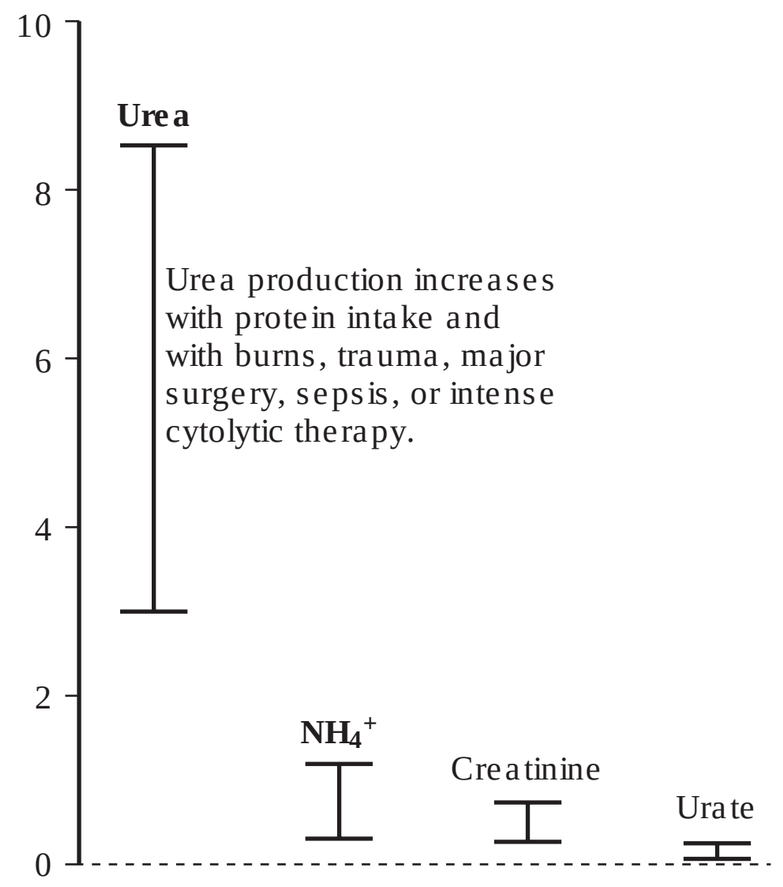


Fig. 35.11 **Normal ranges of daily amounts of nitrogen-containing compounds in urine.**

A patient who has **severe liver disease** may have a reduced capacity to make urea and may therefore have an unusually low BUN. Conversely, a patient who has **kidney failure** may have a reduced capacity to excrete urea and may therefore have an unusually high BUN and uremia or the uremic syndrome.

Physicians commonly use the **BUN/serum creatinine ratio** to interpret abnormalities of nitrogen metabolism. The concentration of creatinine in the blood is a function of the balance of production and excretion of creatinine (more information on creatinine can be found in Section 1.5 in Chapter 23). Creatinine production tends to be fairly similar from person to person (although increased muscle mass and exercise increase creatinine production). Hence, an increased concentration of creatinine in the blood often indicates a decrease in filtration by the kidneys. The kidney tubules do not reabsorb creatinine, but they can reabsorb urea. In a healthy patient, the BUN/creatinine ratio (each expressed in mg/dL) is 10 to 20. If this ratio is less than 10, the patient likely has **kidney damage** (this reduces uptake of filtered urea) and if it is more than 20, the patient is likely to be **dehydrated** (this reduces filtration).

Urea normally contributes the largest amount of nitrogen to **urine** (Fig. 35.11), whereas NH_4^+, creatinine (a useless product of accidental cyclization of creatine and phosphocreatine, see Section 1.5 in Chapter 23), and uric acid (see Section 2 in Chapter 38) contribute lesser amounts. Urea is one of the major solutes of urine and plays a vital role in the concentration of urine beyond the osmolarity of plasma.

3. DEFICIENCIES OF NITROGEN ELIMINATION

Impairment of the urea cycle causes hyperammonemia, which may be life threatening. Impairment of the urea cycle and hyperammonemia are most commonly seen in patients who have severe liver dysfunction, often as a result of alcohol abuse, metabolic syndrome, cancer, or infection with hepatotropic viruses. Hyperammonemia may also be due to an inborn deficiency of an enzyme or transporter that is important to the function of the urea cycle. Patients who have a severe deficiency of a urea cycle enzyme develop life-threatening hyperammonemia during the first few days of life. Patients who have a mild deficiency experience symptoms only during periods of high nitrogen excretion, such as after high-protein meals, during extended fasts or illness, after delivery of a baby, or after appreciable tissue injury. Treatment options for all urea cycle deficiencies include minimizing protein intake, avoiding states of intense protein degradation, and using oral drugs that remove nitrogen as glycine or glutamine conjugates.

3.1. General Comments

Problems converting nitrogen to urea cause **hyperammonemia**, which impairs the function of the central nervous system (CNS) and can be life threatening. The pathogenesis of hyperammonemia is explained below. The most common cause of deficient urea formation is **liver** failure. Much less commonly, deficient urea formation is due to an inherited deficiency of an enzyme or transport protein.

Measurements of **"ammonia"** in blood are not part of routine laboratory studies; they are only prompted by clinical suspicion. **Respiratory alkalosis** is usually the earliest sign of hyperammonemia (hyperammonemia stimulates breathing). The reported concentration of ammonia refers to the sum of NH_3 and NH_4^+ (at a physiological pH, NH_4^+ is by far the major contributor). Measurements vary appreciably with the analytical method used. Concentrations more than ~3 times the upper limit of normal are usually associated with changes in mentation; coma and seizures can also be seen. Patients with blood ammonia concentrations greater than ~20 times the upper normal limit often become severely ill, and those with "ammonia" concentrations of ~50 times the upper limit usually do not survive the episode.

Hyperammonemia is preceded and accompanied by **hyperglutaminemia**. Hyperammonemia stimulates glutamate-ammonia ligase (glutamine synthetase) in perivenous hepatocytes and in astrocytes. In both tissues, glutamine serves as a buffer against hyperammonemia. In patients with hyperammonemia from a urea cycle disorder, the concentration of glutamine in the blood rises with the concentration of "ammonia," and it is usually at least 10 times higher than that of "ammonia."

Hyperammonemia primarily affects CNS function by causing swelling of **astrocytes**. Astrocytes contain a significant amount of glutamate-ammonia ligase (glutamine synthetase). With an increasing concentration of NH_4^+ in the blood, astrocytes synthesize more glutamine, which is osmotically active. In patients who have persistent mild hyperammonemia, compensatory degradation of other osmolytes (e.g., myo-inositol, N-acetyl-aspartate, creatine, and phosphocreatine) inside astrocytes essentially eliminates swelling; still, over time, Alzheimer-like damage to astrocytes occurs. In patients who show acute, steadily increasing concentrations of NH_4^+ (and hence of glutamine), the rate of degradation of osmolytes is too low to maintain a near-normal cell volume. Eventually, swollen tissue in the brainstem restricts the blood supply to the brain.

Patients who are in a coma due to severe hyperammonemia are treated with **hemodialysis** and assisted ventilation.

3.2. Nitrogen Elimination in Patients With Liver Failure or Kidney Failure

A severe impairment of liver function leads to **hyperammonemia** and a syndrome called **hepatic encephalopathy** (i.e., encephalopathy due to impaired liver function). Hepatic encephalopathy is commonly seen in a setting of portal hypertension (increased blood pressure in the portal vein). In developed countries, the portal hypertension is usually caused by cirrhosis, which in turn can result from many disorders, including virus infection (most commonly hepatitis B or C), alcoholic liver disease, or metabolic syndrome. Metabolic syndrome is defined in various ways; a common definition used in the United States is three of the following five abnormalities: hypertension, hypertriglyceridemia, low high-density lipoprotein cholesterol, abdominal obesity, and impaired glucose homeostasis. Patients with hyperammonemia have **respiratory alkalosis** and an increased concentration of glutamine in the cerebrospinal fluid. The blood of these patients may also contain other substances that would normally be removed by the liver and are toxic to the brain.

Patients who have hepatic encephalopathy often have **asterixis**, an impaired ability to maintain position due to brain dysfunction. Asterixis is typically tested for by asking the patient to stretch out the arms and bend the hands upward. If asterixis is present, the patient's hands display a repetitive, nonrhythmic "flapping" relaxation of wrist dorsiflexion.

Patients who have chronic hepatic encephalopathy can be treated with **lactulose** [galactose-β(1→4)-*f*ructose] or **lactitol** [galactose-β(1→4)-sorbitol], a sugar and sugar alcohol that are not digested by human enzymes but only by bacteria in the gut. Acting as laxatives, lactulose and lactitol increase excretion of nitrogen compounds via the feces, and they also reduce the release of ammonia from the intestine into the blood. If lactulose or lactitol are not tolerated, patients can be treated with an **antibiotic**, such as **rifaximin**, to reduce the number of NH_4^+-producing bacteria in the colon. In many patients, degradation of blood from gastrointestinal bleeding is a major cause of ammonia in blood; bleeding is usually stopped with invasive procedures.

Severe loss of **kidney** function can lead to **uremia** and **uremic syndrome**, which are characterized by an elevated concentration of **urea** in the blood. A high concentration of urea per se is not particularly toxic. Uremic syndrome may be associated with headache, dementia, seizures, or coma. In the uremic syndrome, as opposed to hepatic encephalopathy, the CNS dysfunction is due to inadequate removal of toxic substances by the kidneys rather than the liver. There is no thorough understanding yet of the many pathogenic processes that harm the CNS; however, the offending compounds in the blood can be removed with hemodialysis. Protein restriction minimizes symptoms.

3.3. Inborn Deficiencies That Affect the Urea Cycle

Inborn deficiencies of urea cycle enzymes are rare. Collectively, they occur in ~1:10,000 to ~1:50,000 newborns. Deficiencies in all enzymes of the cycle have been described. **Ornithine carbamoyltransferase** (**ornithine transcarbamoylase**) deficiency is the most common urea cycle disorder. This disorder is inherited in X-linked fashion, whereas all other urea cycle disorders are inherited in an autosomal recessive fashion. Depending on residual enzyme activity, a urea cycle disorder manifests between the newborn period and a period

of extreme amino acid degradation in adults. A classic example of the latter is a woman who is heterozygous for X-linked ornithine carbamoyltransferase deficiency and shows symptoms in the postpartum period, a time of tremendous tissue degradation and remodeling.

A deficiency of nitrogen excretion via the urea cycle is usually discovered because of an acute attack that is characterized by progressive lethargy, vomiting, seizures, and coma. As mentioned above, respiratory alkalosis is an early sign of hyperammonemia. **Diagnosis** of a specific urea cycle defect generally requires measurements of ornithine, citrulline, argininosuccinate, arginine, and homocitrulline in blood, and orotate in urine. Homocitrulline structurally resembles citrulline but has an aliphatic chain that is longer by one $–CH_2–$ group. Homocitrulline may derive from the condensation of carbamoyl phosphate with lysine. Orotate results from leakage of excess carbamoyl phosphate from mitochondria into the cytosol (see Section 1 in Chapter 37). Fig. 35.12 provides an overview of known deficiencies and metabolite abnormalities.

The **hyperammonemia** associated with the deficiency of an enzyme of the urea cycle is due to decreased removal of ammonium ions, either because of a primary or secondary carbamoyl-phosphate synthase I deficiency (see Fig. 35.12). Secondary carbamoyl-phosphate synthase I deficiency is due to inadequate activity of N-acetyl glutamate synthase, either because of mutation or arginine deficiency. Arginine deficiency is observed in all urea cycle disorders that originate in defective enzymes or transporters between the synthesis of carbamoyl phosphate and the formation of arginine from argininosuccinate.

The **treatment** of urea cycle disorders aims to diminish the need for nitrogen elimination and to establish alternate routes of nitrogen elimination. Nitrogen elimination is minimized by limiting **protein intake** to the amount needed to maintain growth in children and protein homeostasis in adults (see also the discussion of nitrogen balance in Section 5). A long fast should be avoided because it increases breakdown of body protein. Illness causes degradation of body protein and often precipitates a hyperammonemic crisis that requires hospitalization. Supplemental arginine or citrulline is given to patients who are otherwise deficient in **arginine**. Citrulline is an effective precursor for arginine in patients who have normal argininosuccinate synthase and argininosuccinate lyase activity. A normal concentration of arginine normalizes protein synthesis and optimizes performance of the urea cycle

Fig. 35.12 **Characteristic metabolite abnormalities of patients with impaired function of the urea cycle.**

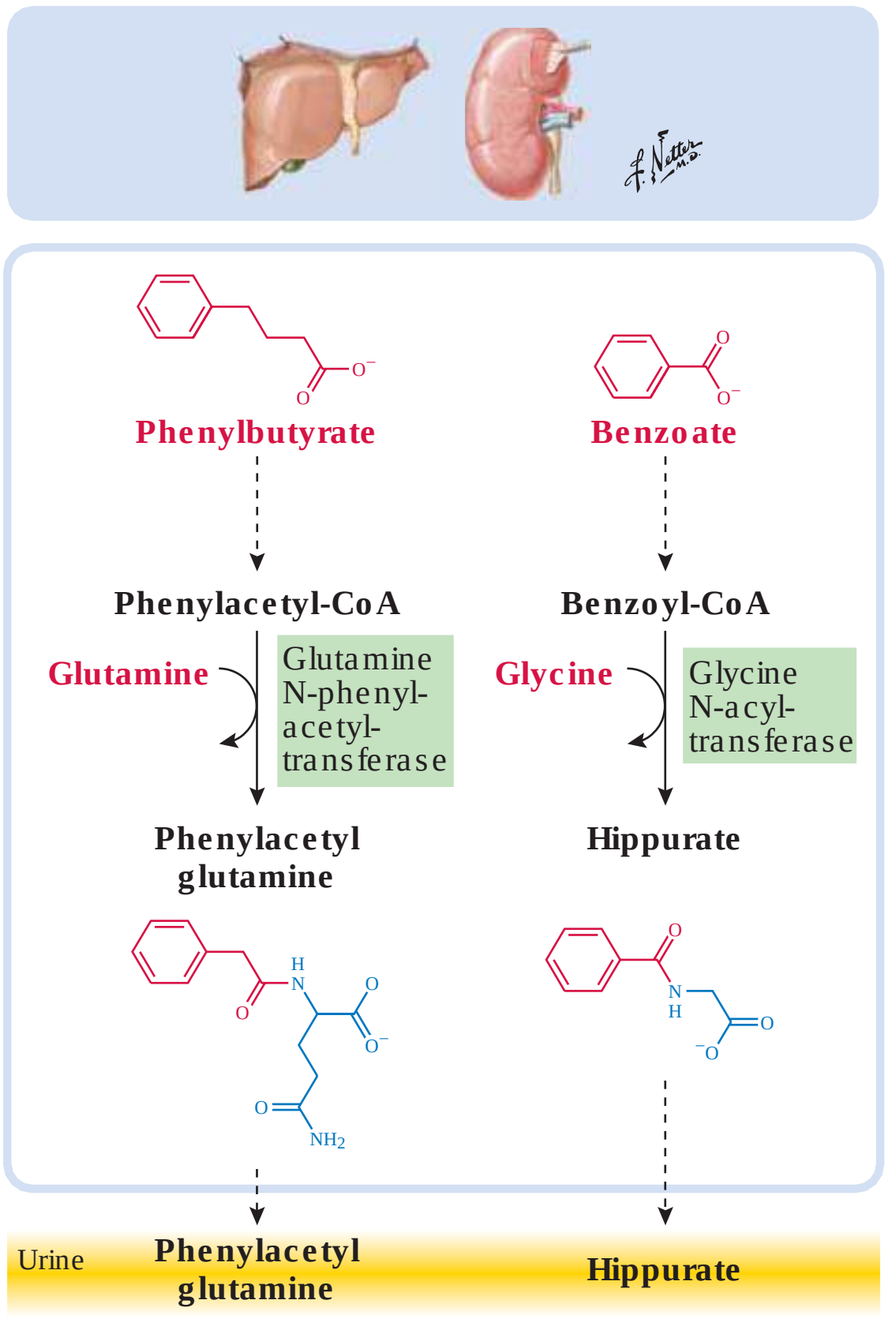


Fig. 35.13 Phenylbutyrate and benzoate remove nitrogen from the body because they are excreted in the urine as conjugates with glutamine and glycine, respectively.

(via stimulation of N-acetylglutamate synthase). **Carglumic acid (N-carbamyl-glutamate)** is used as an analog of N-acetyl-glutamate for patients who are deficient in N-acetylglutamate synthase. Oral **sodium phenylbutyrate** removes nitrogen via conjugation of its metabolite with glutamine (Fig. 35.13). Oral **sodium benzoate** works similarly (see Fig. 35.13) but is not used as frequently. In hospitals, a mixture of sodium phenyl-acetate (which is also conjugated with glutamine) and sodium benzoate is often infused intravenously; the blood of patients with severe hyperammonemia is also depleted of ammonia by **hemodialysis**. **Liver transplantation** can normalize nitrogen excretion.

In patients who have an elevated concentration of **propionyl-CoA**, the urea cycle has low activity owing to insufficient activation of carbamoyl phosphate synthase I. The elevated concentration of propionyl-CoA may be a result of insufficient activity of **propionyl-CoA carboxylase** (this includes problems of **biotin** metabolism) or **methylmalonyl-CoA mutase** (this includes problems of cobalamin metabolism; see Chapter 36). When the concentration of propionyl-CoA in the liver is excessively high, N-acetylglutamate synthase produces N-propionyl-glutamate instead of N-acetyl-glutamate (see Fig. 35.7). In contrast to N-acetyl-glutamate, N-propionyl-glutamate is an inhibitor of carbamoyl phosphate synthase and thus causes hyperammonemia.

4. SUMMARY OF THE METABOLISM OF AMINO ACIDS

The degradation of amino acids is a vast and complex field that is covered in several chapters of this book; a summary and references are provided here. A deficiency in converting phenylalanine to tyrosine gives rise to phenylketonuria, which is treatable with a low-phenylalanine diet. A deficiency in the conversion of tyrosine to melanin leads to hypopigmentation of the skin, hair, and eyes. A deficiency in the degradation of tyrosine leads to tyrosinemia or alkaptonuria. Tryptophan gives rise to serotonin, melatonin, and nicotinamide adenine dinucleotides. Maple syrup disease is due to a deficiency in the degradation of the branched-chain amino acids Leu, Val, and Ile; it is best treated with a diet that is especially low in leucine.

4.1. Overview

Table 35.1 provides an overview of the coverage of the metabolism of amino acids in this book. Deamination and transamination reactions, as well as the excretion of amino acid nitrogen, are described in Section 2 above.

4.2. Normal Metabolism of Phenylalanine, Tyrosine, and Tryptophan

Phenylalanine is used for protein synthesis. The remainder of phenylalanine is hydroxylated to form **tyrosine** (Fig. 35.14). Phenylalanine is an essential amino acid, but tyrosine is not, as long as it can be formed from phenylalanine.

Tyrosine is used for protein synthesis and the synthesis of **catecholamines** and **melanins**; excess tyrosine is mostly degraded in the liver (see Fig. 35.14). Among the catecholamines, dopamine and epinephrine serve as neurotransmitters in the brain, norepinephrine serves as a neurotransmitter in the peripheral nervous system, and epinephrine also serves as a blood-borne hormone for the regulation of metabolism (see Sections 2.3, 3.4, and 4.4 in Chapter 26; Section 5.1 in Chapter 28; and Section 2 in Chapter 33).

Melanins are pigments that are found in skin, hair, retina, and certain regions of the brain. There are three categories of melanins: **eumelanins**, which are brown to black; **pheomelanins**, which are yellow to red-brown; and **neuromelanins**, which are brown to black. Melanins are synthesized inside intracellular organelles called **melanosomes**. In the skin, an individual melanosome synthesizes only one type of melanin, either eumelanin or pheomelanin; however, a melanocyte can contain one or both types of melanosomes. In the epidermis of the skin, ~10% of cells are melanin-synthesizing melanocytes (Fig. 35.15). Each melanocyte transfers melanin via dendritic projections to about 3 dozen keratinocytes. Keratinocytes store melanin on the apical side of the nucleus. Eumelanin thus protects DNA in the nuclei of keratinocytes and heme in blood vessels of the underlying dermis from photodamage. Pheomelanin, on the other hand, generates reactive oxygen radicals that are potentially damaging. Differences in skin

Table 35.1 Overview of the Degradation of Amino Acids

Amino Acid	Pathway for Degradation	Relevant Figure
Alanine	Transamination yields pyruvate	Figs. 27.3 and 34.11
Arginine	Hydrolysis yields urea and ornithine	Fig. 35.7
Asparagine	Deamination yields aspartate	Figs. 35.2 and 34.11
Aspartate	Entry into urea cycle yields fumarate in the citric acid cycle Transamination yields oxaloacetate in the citric acid cycle	Fig. 35.7 Fig. 34.11
Cysteine	Degradation includes transamination and yields mostly pyruvate	Fig. 36.16
Glutamate	Deamination or transamination yields α-ketoglutarate in the citric acid cycle	Figs. 34.11 and 35.2
Glutamine	Deamination yields glutamate	Figs. 34.11 and 35.2
Glycine	Deamination yields CO_2 and N^5,N^{10}-methylene-THF	Figs. 35.2 and 36.3
Histidine	Deamination, followed by hydrolysis and transfer of a formimino-group to THF, yields glutamate	Fig. 36.3
Isoleucine	Transamination yields α-keto-β-methylvalerate, which is converted to acetyl-CoA and propionyl-CoA (which gives rise to succinyl-CoA)	Fig. 35.21
Leucine	Transamination yields α-ketoisocaproate, which is degraded to acetyl-CoA and acetoacetate	Fig. 35.21
Lysine	Transfer of side chain N to α-ketoglutarate; transamination of resulting α-aminoadipate to α-ketoglutarate, yielding α-ketoadipate, which is degraded to acetoacetyl-CoA	Not shown
Methionine	Forms S-adenosyl-methionine in the activated methyl group cycle and from there gives rise to cysteine	Figs. 36.6 and 36.15
Phenylalanine	Hydroxylation yields tyrosine	Fig. 35.14
Proline	Oxidation gives rise to glutamate γ-semialdehyde, which can be oxidized to glutamate or give up an amino group to generate ornithine	Fig. 34.11
Serine	Deamination yields pyruvate Loss of a one-carbon group yields glycine and N^5,N^{10}-methylene-THF	Fig. 35.2 Figs. 34.11 and 36.3
Threonine	Oxidation to α-amino-β-ketobutyrate gives rise to either pyruvate or acetyl-CoA + glycine Deamination generates α-ketobutyrate, which is oxidatively decarboxylated to propionyl-CoA and then gives rise to succinyl-CoA	Not shown Fig. 35.2 for first step; rest not shown
Tryptophan	Can be used for the synthesis of serotonin and melatonin or degraded to yield numerous products, including a precursor for NAD^+	Fig. 35.16
Tyrosine	Can be used for the synthesis of catecholamines, melanins, and thyroid hormone or degraded to yield fumarate in the citric acid cycle and acetoacetate	Fig. 35.14
Valine	Transamination yields α-ketoisovalerate, which is degraded to propionyl-CoA, which gives rise to succinyl-CoA	Fig. 35.21

pigmentation reflect dif erences in melanin synthesis, as well as the size and distribution of melanosomes in keratinocytes.

After **UV irradiation**, keratinocytes release endothelin-1, α-melanocyte stimulating hormone (α-MSH, α-melanocortin), and fibroblast growth factor, whereas melanocytes express an increased number of MSH receptors. In response, the number of melanocytes increases, as do melanin synthesis and transfer of melanosomes to keratinocytes.

T e hormones **α-MSH**, **β-MSH**, and **adrenocorticotropic hormone** (**ACTH**) all stimulate melanin production and a

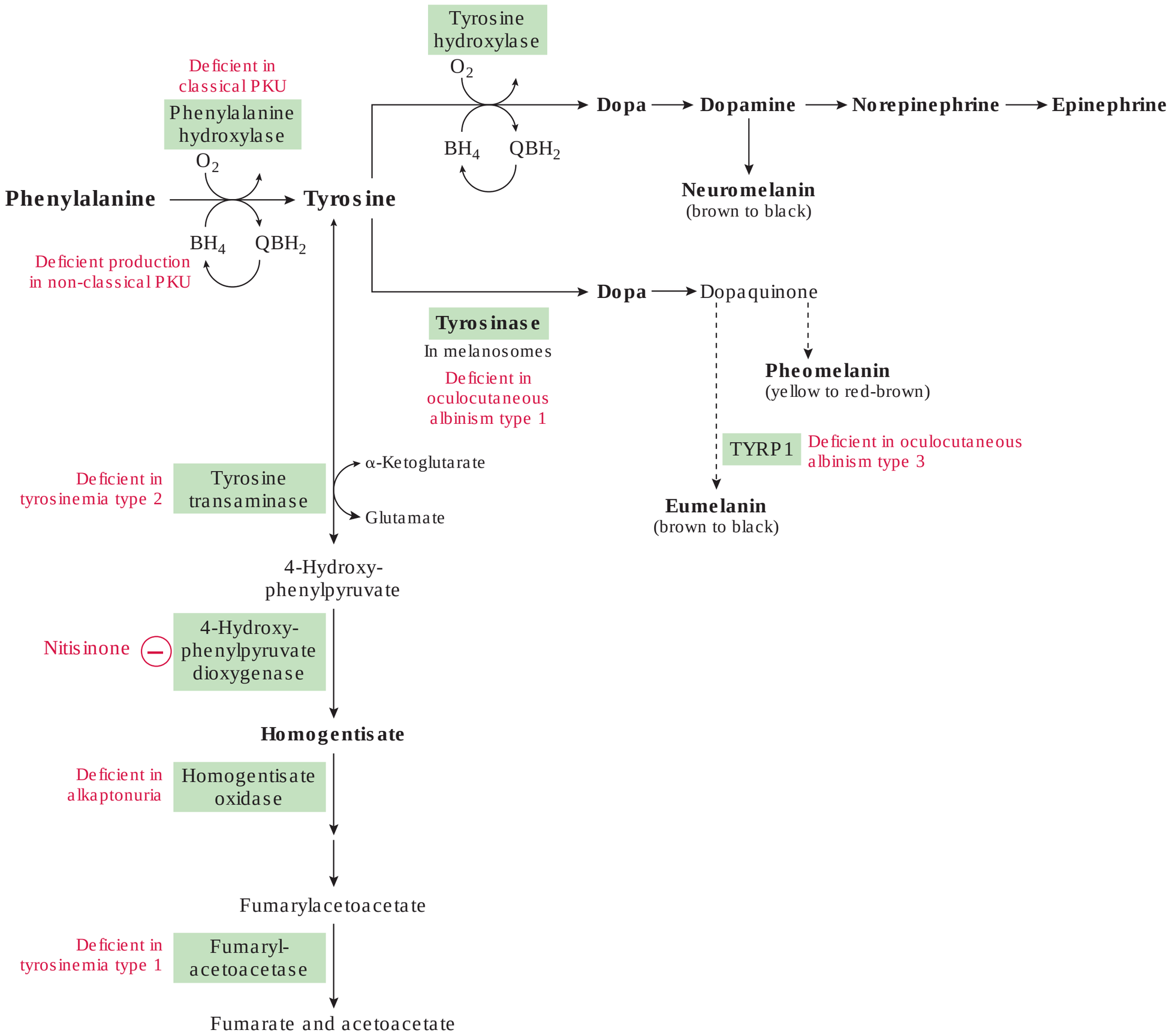


Fig. 35.14 Metabolism of phenylalanine and tyrosine. Humans synthesize BH4 from GTP in three steps (not shown). BH_4, tetrahydrobiopterin (see also Fig. 35.17); QBH_2, quinonoid dihydrobiopterin.

switch from the synthesis of pheomelanins toward eumelanins. Patients who have an increased concentration of ACTH in the blood, such as those with **Addison disease** (see Section 4.2 in Chapter 31), show increased pigmentation, particularly in areas that are exposed to sunlight. Conversely, patients who produce mutant MSH or receptors for MSH (melanocortin 1 receptor, MC1R) have decreased pigmentation, and pheomelanin production is favored over eumelanin production. Most people who have **red hair** have a variant melanocortin receptor 1; they are fair skinned, do not tan well after exposure to sunlight, and have an increased risk of melanoma.

Melanins bind **metals** such as copper, iron, mercury, lead, and cadmium; the daily shedding of melanins in hair and skin plays a role in the removal of these metals from the body.

In the retina, melanin reduces light-scatter and photo damage. In the brain, only catecholamine-synthesizing neurons make melanin (**neuromelanin**), mainly from dopamine (tyrosinase is not needed). T e largest amounts of neuromelanin are found in dopaminergic neurons of the substantia nigra and in noradrenergic neurons of the locus coeruleus. T e neuromelanins act as a sink of both toxic metals and excess dopamine. Neuromelanin deposits in these areas of the brain increase with age. When neuromelanin-containing neurons die, the pigment is released into the extracellular space, where it may remain for a long time and elicit inflammation.

Tryptophan is the precursor for the neurotransmitter serotonin and also for melatonin (see Fig. 35.16). **Serotonin** (5-hydroxytryptamine, 5-HT) is synthesized in some neurons in the brainstem, in serotonin-containing enterochromaffin cells of the gastrointestinal mucosa, and in neurons of the enteric nervous system. Like the synthesis of tyrosine and the synthesis of catecholamines from tyrosine, the synthesis of serotonin from tryptophan includes a hydroxylation reaction that requires tetrahydrobiopterin (BH_4, THB; see Fig. 35.17). Transporters take up serotonin from the extracellular space and thus end its signaling ef ect. **Amphetamines** and **selective serotonin reuptake inhibitors** decrease the rate of serotonin

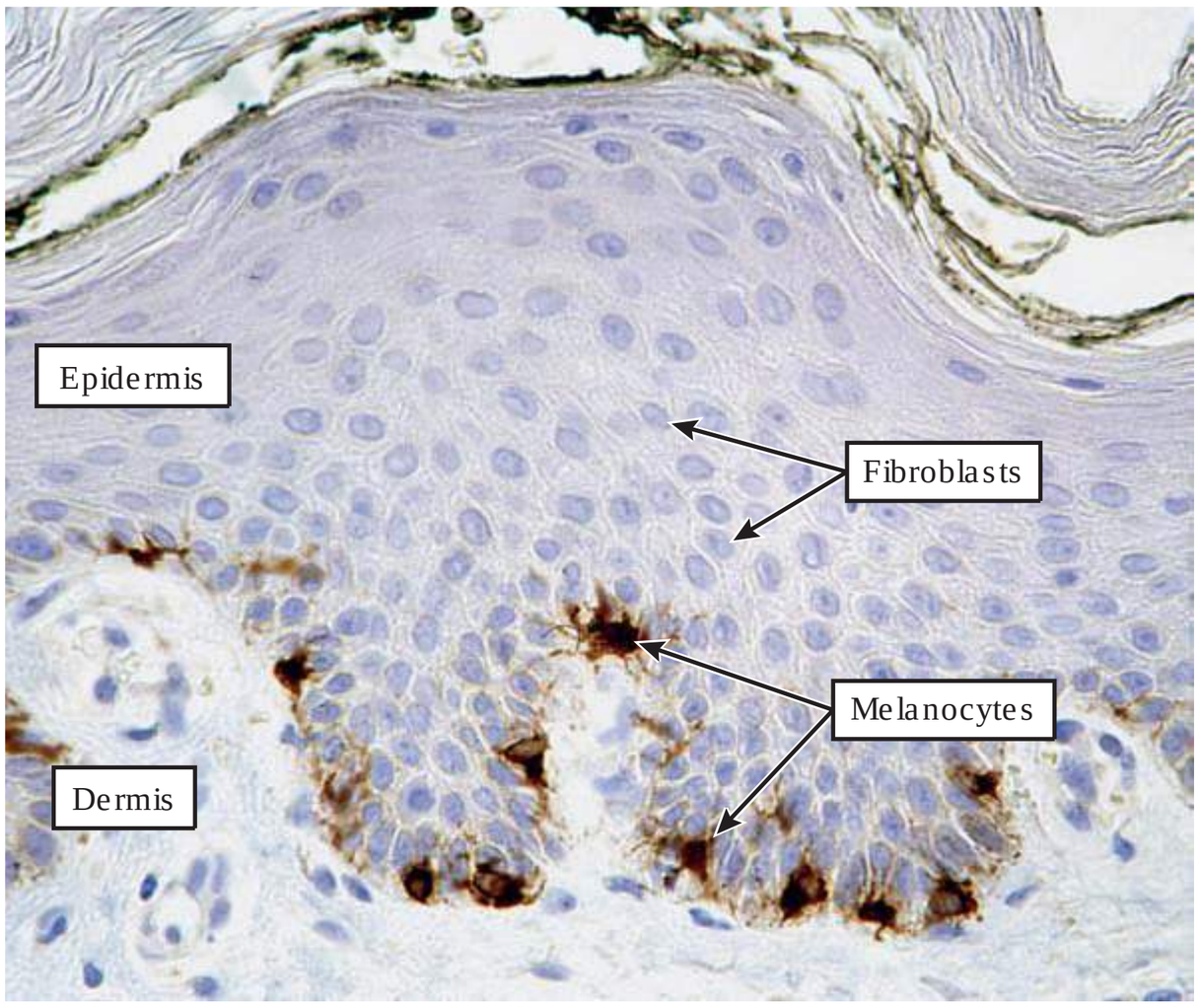


Fig. 35.15 Melanocytes in the skin. Melanosomes containing melanin travel from melanocytes to fibroblasts. The skin surface is at the top of the image. Melanin was detected with an antibody, which was used to give rise to a brown stain.

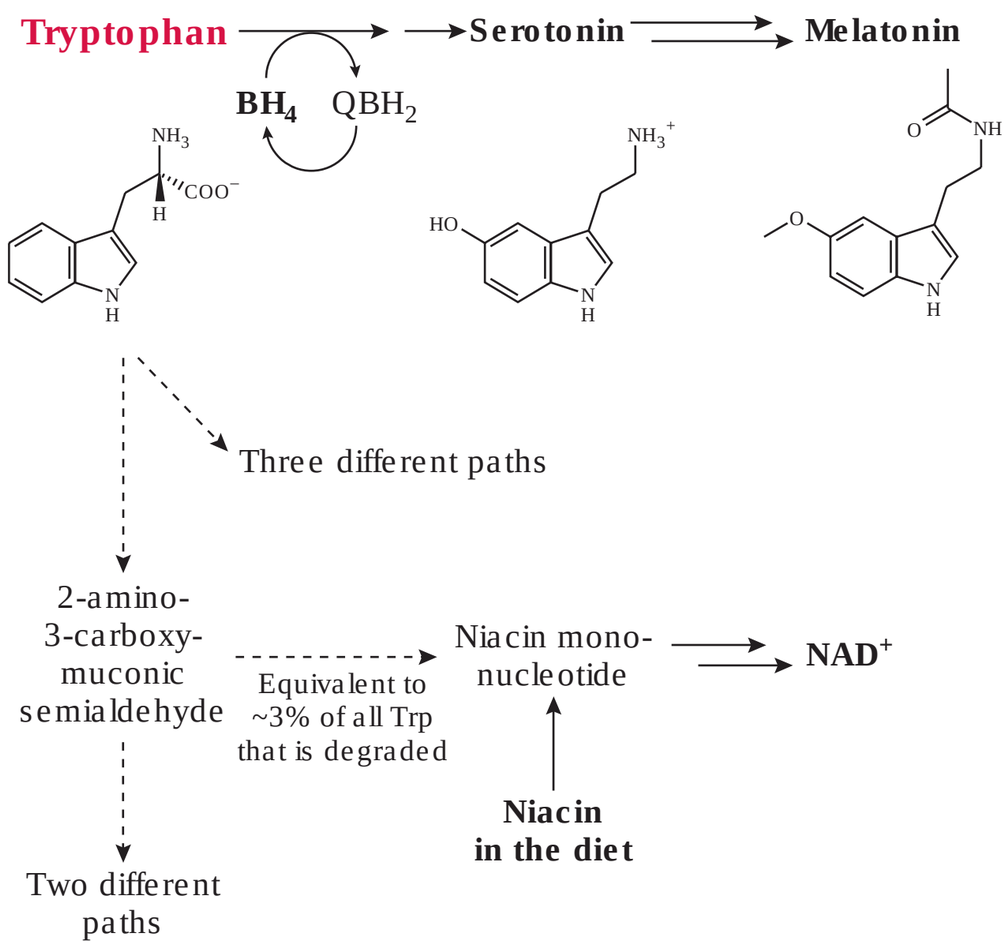


Fig. 35.16 Use of tryptophan for the synthesis of serotonin, melatonin, and NAD^+. BH_4, tetrahydrobiopterin.

removal from the extracellular space and thus enhance the effects of serotonin. **Melatonin** plays a role in the regulation of the sleep/wake cycle. The pineal gland in the brain synthesizes melatonin in cyclical fashion, with a peak in the middle of the night.

Tryptophan can be degraded via several pathways (Fig. 35.16). A small yet physiologically important fraction of tryptophan gives rise to about half of the **niacin mononucleotide** that is required for the synthesis of NAD^+ and $NADP^+$. (The

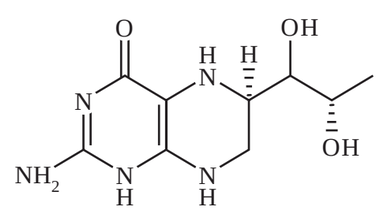


Fig. 35.17 Tetrahydrobiopterin (also called BH_4, THB, or sapropterin).

other half of the niacin mononucleotide is made from niacin in the diet; see Section 1 in Chapter 19.)

4.3. Hyperphenylalaninemias (Including Phenylketonuria)

Hyperphenylalaninemia is commonly defined as a concentration of phenylalanine in the blood in excess of 120 µM (the mean normal concentration is about 60 µM). About 1 in 10,000 to 15,000 newborns in Western countries develops hyperphenylalaninemia. Mutations in **phenylalanine hydroxylase** (the enzyme that transforms Phe to Tyr; see Fig. 35.14) are the most common cause of hyperphenylalaninemia (this is also the most common disease of amino acid metabolism). Patients with pathogenic hyperphenylalaninemia due to mutant phenylalanine hydroxylase are said to have **classic phenylketonuria** (**PKU**; phenylketones, such as phenylpyruvate, appear in the urine when the concentration of phenylalanine in the blood is high). Patients who do not synthesize an adequate amount of the cofactor **tetrahydrobiopterin** (**BH_4, THB**, **sapropterin**; Fig. 35.17) for phenylalanine hydroxylase are said to have **resistant** or **nonclassic PKU**. This group of patients accounts for 1% to 2% of hyperphenylalaninemic patients. Some patients have only mildly elevated plasma

phenylalanine, and this abnormality is sometimes referred to as **non-PKU hyperphenylalaninemia**.

After birth, a few days are needed for hyperphenylalaninemia to develop in an af ected infant. Phenylalanine passes through the placenta. T us if the fetus fails to metabolize phenylalanine, a healthy mother's liver metabolizes the fetus's excess phenylalanine. In newborns with PKU, the blood phenylalanine concentration keeps rising after birth. To reach adequate sensitivity and accuracy with the screening test, a blood sample is typically collected 24 to 48 hours after birth.

Modern screening tests for PKU use tandem mass spectrometry to measure the ratio of the concentrations of phenylalanine to tyrosine in spots of dried blood. All patients who have hyperphenylalaninemia are also tested for urine biopterin to determine whether they have nonclassic PKU due to a BH_4 deficiency.

Classic PKU is inherited in an autosomal recessive fashion. Af ected patients typically show complete or near-complete deficiency of phenylalanine hydroxylase, often due to an unstable mutant enzyme. T ere are more than 500 known mutations of phenylalanine hydroxylase that result in PKU. Individual mutations are sufficiently infrequent that most af ected patients are compound heterozygotes. Consequently, patients show a spectrum of phenylalanine hydroxylase activities.

A high concentration of phenylalanine is damaging to the brain. At a few months of age, untreated patients with classic PKU regress in their development, and their brains show a decreased amount of **myelin**. T e most likely reason for the damage is that a high concentration of phenylalanine in the blood competes with other neutral amino acids for the same amino acid transporter and thereby reduces the uptake of other amino acids into the brain. As a consequence, a low concentration of one or more amino acids may limit the synthesis of protein or neurotransmitters. Untreated patients also show decreased pigmentation, owing to deficient synthesis of melanins (see Fig. 35.14).

Patients who have marked hyperphenylalaninemia need to be treated with a **low-phenylalanine diet** as soon as possible. T e treatment goal is to lower the concentration of phenylalanine in the blood sufficiently that patients achieve normal development and function of brain and body. T e concentration of phenylalanine in the blood is measured at least monthly and is used to guide the diet. Patients with classic PKU typically tolerate only 200 to 400 mg of phenylalanine per day. As a rule of thumb, one gram of protein in meats, fowl, or fish contains 50 mg of phenylalanine. Some vegetables and fruits contain as little as 15 mg of phenylalanine per gram of protein (Table 35.2). In practice, patients with PKU cannot eat high-protein foods, such as meat, fowl, fish, dairy, legumes, or regular grain products. T ey also avoid **aspartame**-sweetened diet drinks because the metabolism of aspartame yields phenylalanine. Af ected patients eat special low-protein grain products, as well as vegetables and fruits that contain little phenylalanine. T ey consume most of their protein as a phenylalanine-free mixture of synthetic amino acids (in solid or liquid form). Optimization of these mixtures is still ongoing. A supplement of amino acids that is rich in amino acids that use the same amino acid transporter as phenylalanine (e.g., tyrosine, tryptophan) appears to be ef ective in lowering the concentration of phenylalanine in the brain. Patients should also receive supplements of vitamin B_6 and cobalamin because their normal diet does not contain enough of these vitamins. A patient with PKU who receives adequate lifelong diet treatment can expect near-normal health and development; still, subtle brain abnormalities are the rule.

All the patients with nonclassic PKU, and about half of the patients with classic PKU, benefit from supplementation with **tetrahydrobiopterin**. T e rare patients who have nonclassic PKU also should be treated for deficiencies in the synthesis of catecholamines and serotonin.

Phenylalanine in the blood is derived both from the diet and the degradation of body proteins. Degradation is increased during infection or trauma or if intake of any one of the essential amino acids is insufficient.

Table 35.2 Phenylalanine Content of Various Foods

Food	mg of Phe/100 g edible portion
Cheese, cheddar	1,310
Hamburger	810
Couscous, prepared	320
Peas	190
Milk (whole)	160
Cauliflower	90
Broccoli	76
Banana	45
Potatoes, boiled	44
Lettuce	40
Tomato, raw	21
Carrots	15
Onions, raw	14
Mango	14
Pineapple	10
Orange juice	7
Tapioca	Trace

4.4. Disorders of Pigmentation

Decreased melanin production is observed in patients who have vitiligo, albinism, or piebaldism.

Fig. 35.18 Vitiligo.

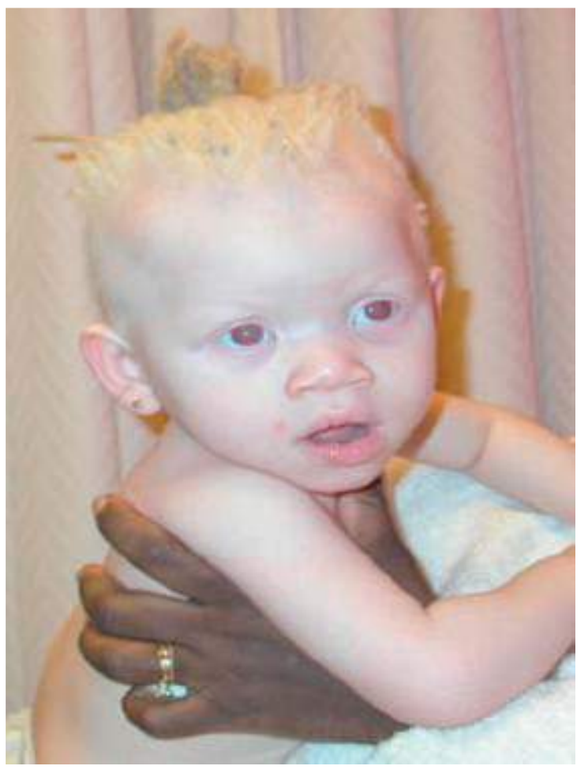


Fig. 35.19 Oculocutaneous albinism type 2 in a 17-month-old African-American girl, held by her mother. This patient also has Angelman syndrome, which causes developmental delay and microcephaly. (From Saadeh R, Lisi EC, Batista DA, McIntosh I, Hoover-Fong JE. Albinism and developmental delay: the need to test for 15q11-q13 deletion. *Pediatr Neurol.* 2007;37:299-302.

Vitiligo is a condition of patchy loss of skin pigmentation (Fig. 35.18), which is seen in 1% to 2% of the population. It is due to an absence of melanin-producing cells and may be due to an inflammatory process. Serum of patients who have vitiligo contains antibodies to melanocytes, including antibodies to tyrosinase. Vitiligo is sometimes treated with a psoralen (see Section 4.2 in Chapter 2).

Oculocutaneous albinism is an autosomal recessively inherited condition of decreased or absent pigmentation of the eyes, skin, and hair. Af ected patients have impaired clarity of vision, in part due to an improperly formed fovea and misrouted optic neurons. T ere are three known causes of oculocutaneous albinism (OCA; see Fig. 35.14):

- In type 1 OCA, which occurs in ~1:10,000 to 70,000 whites (it is much less common in others), both **tyrosinase** alleles carry a harmful mutation.
- In type 2 OCA (Fig. 35.19), which occurs in up to 1:1,000 black Africans and ~1:2,000 people among the Navajo Native Americans (but much less commonly in white or Japanese people), both alleles of the OCA2 gene carry a mutation. T e OCA2 protein appears to be an integral membrane protein of melanosomes, but its function is not well understood. (Many patients with Prader-Willi or Angelman syndrome have altered expression of several genes near the OCA2 gene and also show reduced pigmentation.)
- In type 3 OCA, which occurs mostly in blacks, both alleles for tyrosinase-related protein 1 (TYRP1) carry a mutation; this protein has an oxidase activity that is needed for the synthesis of eumelanin.

T ere are many other hereditary diseases of skin pigmentation, which generally also impair vision. Some of these diseases, such as **piebaldism** (a hereditary condition marked by patches of decreased pigmentation), **Waardenburg syndrome**, and **dyschromatosis symmetrica hereditaria**, are due to impaired population of the skin with melanoblasts that are derived from the neural crest. Others, such as **Hermansky-Pudlak syndrome** and **Chédiak-Higashi syndrome**, are due to impaired formation of melanosomes. In Puerto Rico, up to ~1 in 2,000 persons have Hermansky-Pudlak syndrome. Patients with this disorder have oculocutaneous albinism, abnormal blood clotting, and damage to lungs and kidneys due to impaired function of lysosomes and lysosome-related organelles. Patients who have Chédiak-Higashi syndrome have severe immune deficiency. Finally, **Griscelli syndrome** is due to impaired transfer of melanosomes from melanocytes to keratinocytes.

Excessive melanin production due to melanocortin-1 receptor variants is observed in patients who have **freckles**. Exposure to sunlight enhances freckling.

4.5. Disorders of Tyrosine Degradation

Tyrosinemia type 1, also called **hepatorenal tyrosinemia,** is due to **fumarylacetoacetase deficiency** (see Fig. 35.14). T is disorder shows autosomal recessive inheritance and has a worldwide incidence of about 1 in 100,000; however, it is much more common in parts of Finland and Canada (e.g., 1:2,000 in a region of Quebec province). Presumably due to mutagenic ef ects of fumarylacetoacetate, the deficiency causes liver disease in infancy or childhood. Without treatment, patients have a very high long-term risk of **hepatocellular carcinoma**. T e concentration of tyrosine in the blood is only mildly elevated (mechanism unclear) and not pathogenic. Af ected patients are treated with a diet low in phenylalanine and tyrosine as well as with the drug **nitisinone**, a synthetic inhibitor of the upstream enzyme 4-hydroxyphenylpyruvate dioxygenase (see Fig. 35.14) that reduces the concentration of fumarylacetoacetate. Liver transplantation is used for patients who do not respond to nitisinone therapy or who develop liver cancer.

Tyrosinemia type 2 (**oculocutaneous tyrosinemia**, **Richner-Hanhart syndrome**) is due to a deficiency of **tyrosine**

transaminase, an enzyme that is expressed predominantly in the liver (see Fig. 35.14). This disorder shows autosomal recessive inheritance. It is rare but most common among persons with ancestry in the Mediterranean (especially Italy) or on the Arabian peninsula. Affected persons show intellectual disability (cause unknown), a very high concentration of tyrosine in the blood, deposition of tyrosine crystals in the cornea, and painful hyperkeratosis patches on palms and soles (due to tyrosine crystals and an inflammatory reaction to them). Current treatment is by a low-protein, phenylalanine- and tyrosine-restricted diet.

Alkaptonuria is due to a near-complete deficiency of homogentisic acid oxidase (see Fig. 35.14). Alkaptonuria occurs in 1 of about 500,000 people; in Slovakia, the prevalence is ~1 in 20,000 persons. The urine of patients with alkaptonuria sometimes turns black on standing. The cartilage and some tendons of affected patients accumulate black pigment that derives from homogentisic acid; this pigmentation is called **ochronosis.** The pigment has an adverse effect on the mechanical properties of various tissues (Fig. 35.20). Adult patients have debilitating arthritis, degenerative changes in the spine, problems with heart valves, coronary artery calcification, and kidney stones.

4.6. Maple Syrup Disease and the Degradation of Branched-Chain Amino Acids

The branched-chain amino acids Val, Leu, and Ile make up about 22% of body protein. All of these amino acids are essential (see Chapter 34).

For degradation, branched-chain amino acids first undergo transamination to form **branched-chain keto acids** (Fig. 35.21). These transamination reactions mostly take place in muscle and adipocytes, and to a lesser extent in the liver. Muscle releases most branched-chain keto acids into the blood.

The liver oxidizes the branched-chain keto acids that are formed inside the liver, as well as most of those that are released by muscle. A single enzyme, **branched-chain keto acid dehydrogenase**, oxidatively decarboxylates the three branched-chain keto acids (see Fig. 35.21). This step is irreversible and constitutes the first committed step in the degradation of branched-chain amino acids.

Maple syrup disease (also called maple syrup urine disease) is the consequence of a deficiency of branched-chain keto acid dehydrogenase (see Fig. 35.21). This disease is inherited in autosomal recessive fashion. Worldwide, it occurs in ~1 in 200,000 newborns; however, it has a prevalence of ~1 in 200 births among Mennonite communities in the United States. In affected patients, branched-chain amino acids and branched-chain keto acids accumulate in the blood and urine. One of the branched chain keto acids, α-keto-β-methylvalerate (derived from isoleucine) imparts the smell of maple syrup; this smell is noticeable with ear wax and to a lesser extent with urine. The maple syrup smell of ear wax is the earliest specific sign of the disease.

Patients who have the classic (severe) form of maple syrup disease develop an encephalopathy a few days after birth. Without treatment, myelination of the brain is reduced, and patients have mental retardation and premature death. The neuropathology is most closely related to the concentration of leucine in blood. In a setting of hyponatremia, a high concentration of leucine causes cerebral edema and encephalopathy. Leucine is believed to outcompete other amino acids for uptake into cells; as a result, there is a deficit of other amino acids inside cells. However, the exact pathogenesis of the encephalopathy is still a mystery. Patients should be treated by about 3 days of age. The aim of treatment is to normalize the concentration of branched-chain amino acids in the blood and to provide enough protein for growth. The concentration of leucine in the blood is minimized by facilitating the use of leucine in protein synthesis; this can often be achieved with infusions of valine and isoleucine. Since branched-chain amino acids are ubiquitous, patients with maple syrup disease must consume a low amount of regular dietary protein and a supplement of an artificial **amino acid cocktail** that is free of

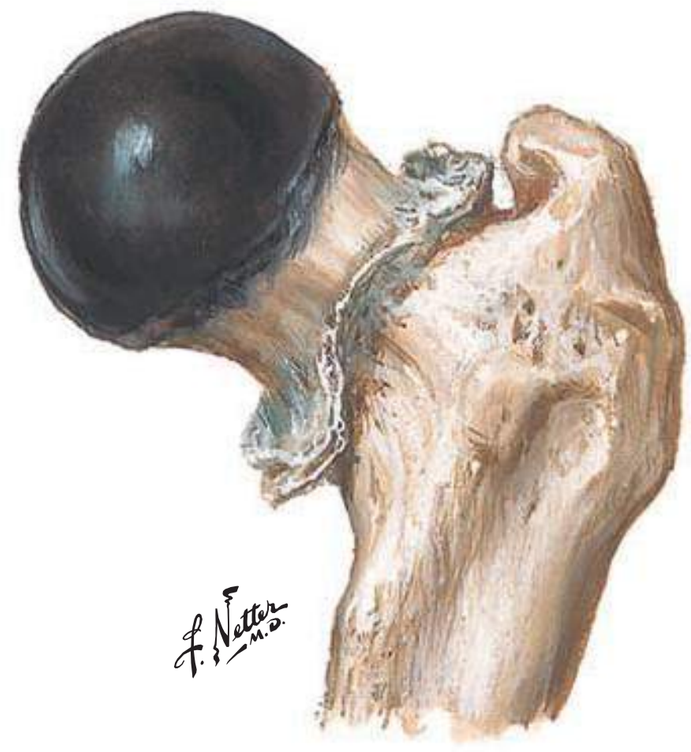


Fig. 35.20 **Ochronosis due to alkaptonuria.**

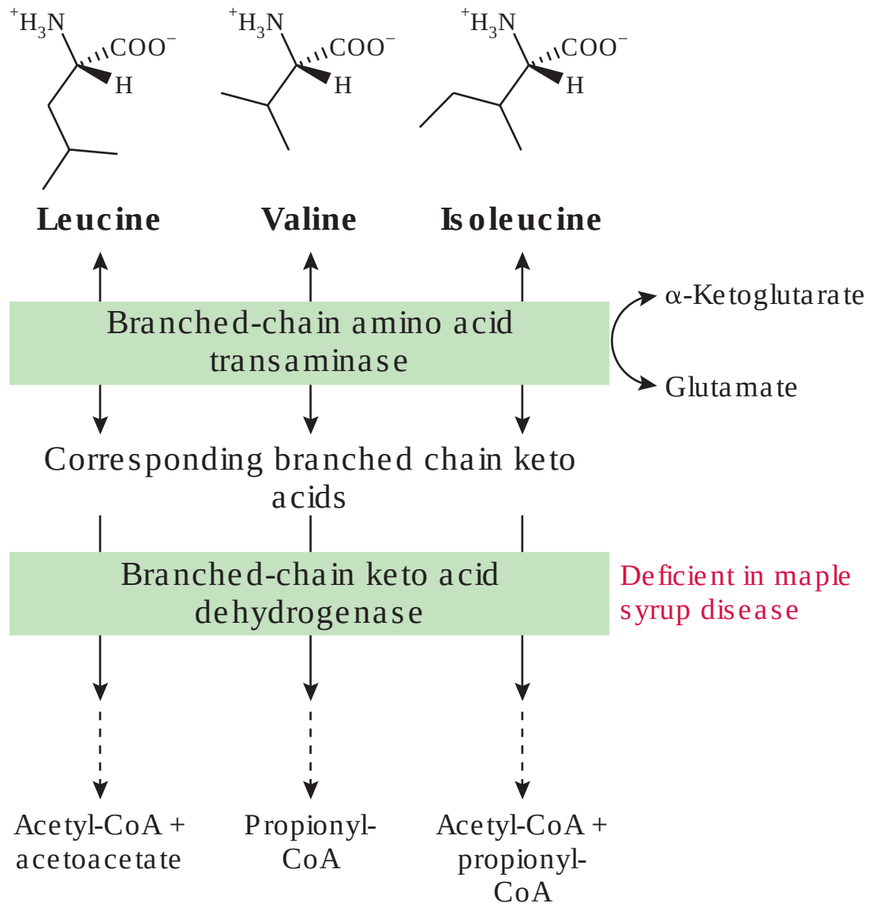


Fig. 35.21 **Degradation of the branched-chain amino acids.**

Table 35.3 Nitrogen Balance in Adults

	In	Out
Actual	N in dietary protein (~100% of all gains)	Urea, NH_3, creatinine, and uric acid in urine (~85% of all losses) Feces and miscellaneous (~15% of all losses)
Estimate	N in dietary protein (weight of amino acids in g/6.25)	Method I: N as part of urea in urine in g; add 4 g for baseline amount of other N losses Method II: (N as part of urea in urine in g) × 1.25

For more information about creatinine, see Section 1.5 in Chapter 23. For more information about uric acid, see Section 2 in Chapter 38. Fig. 35.11 shows the normal ranges of the contributions of urea, NH_3, creatinine, and uric acid to nitrogen losses via the urine.

branched-chain amino acids. Some patients respond favorably to extra **thiamine**, which is a cofactor of branched-chain keto acid dehydrogenase. Illnesses are challenging to manage because of increased degradation of body protein. Patients who are effectively treated show normal physical and mental development. Liver transplantation can normalize the concentrations of branched-chain amino acids in the blood and enable patients to eat a normal amount of protein in their diet.

Newborns can be screened for maple syrup disease by measuring the concentration of leucine (or the ratio of the concentrations of leucine and alanine) or the concentration of alloisoleucine (a diastereomer of leucine) in blood. This screening is commonly performed using chromatography or tandem mass spectrometry.

Isovaleric acidemia is caused by a deficiency of **isovaleryl-CoA dehydrogenase** in the pathway for leucine degradation. The disease is inherited in an autosomal recessive fashion and occurs in ~1 in 60,000 births in Germany and ~1 in 250,000 births in the United States. Some affected patients develop encephalopathy and severe acidosis in the newborn period, whereas others show episodes of vomiting, lethargy, coma, and acidosis only later on. These acute attacks are accompanied by hyperammonemia and ketosis. During episodes of major protein catabolism, the concentration of isovaleric acid in blood plasma may rise to as much as 5,000 µM (normal: <10 µM). During these episodes, patients exude an odor of sweaty feet. Isovaleric acidemia is primarily treated with carnitine and glycine supplementation to enhance the conversion of isovaleryl-CoA to isovalerylcarnitine and isovalerylglycine, which are excreted in the urine.

5. NITROGEN BALANCE

Nitrogen balance is the difference between nitrogen intake (in the form of protein) and nitrogen losses (mostly via the urine). If losses exceed intake, the balance is negative. A negative nitrogen balance is seen with starvation and to a much greater extent after delivery of a fetus, during sepsis, after trauma, or days after extensive burns.

5.1. Concept of Nitrogen Balance

Nitrogen balance is defined as nitrogen intake minus nitrogen losses. Table 35.3 provides an overview of individual contributors to the nitrogen balance sheet. In the clinic, estimates of nitrogen balance are usually based only on protein intake and loss of urea in urine (see Table 35.3). The nitrogen balance is most commonly measured in patients who receive enteral or parenteral nutrition because they cannot consume regular food. Parenterally fed patients cannot be infused with protein; rather, they are infused with a mixture of purified synthetic amino acids.

The nitrogen balance is normally **positive** in patients who increase the amount of protein in their body as, for example, in growing children and pregnant women. The nitrogen balance is **negative** during acute illness, trauma, sepsis, burns, and malnutrition as well as after delivery of a fetus. As these latter patients improve, they eventually develop a positive nitrogen balance and finally a zero balance when they are fully recovered. Fig. 35.22 illustrates the effects of excess and insufficient food intake on nitrogen balance.

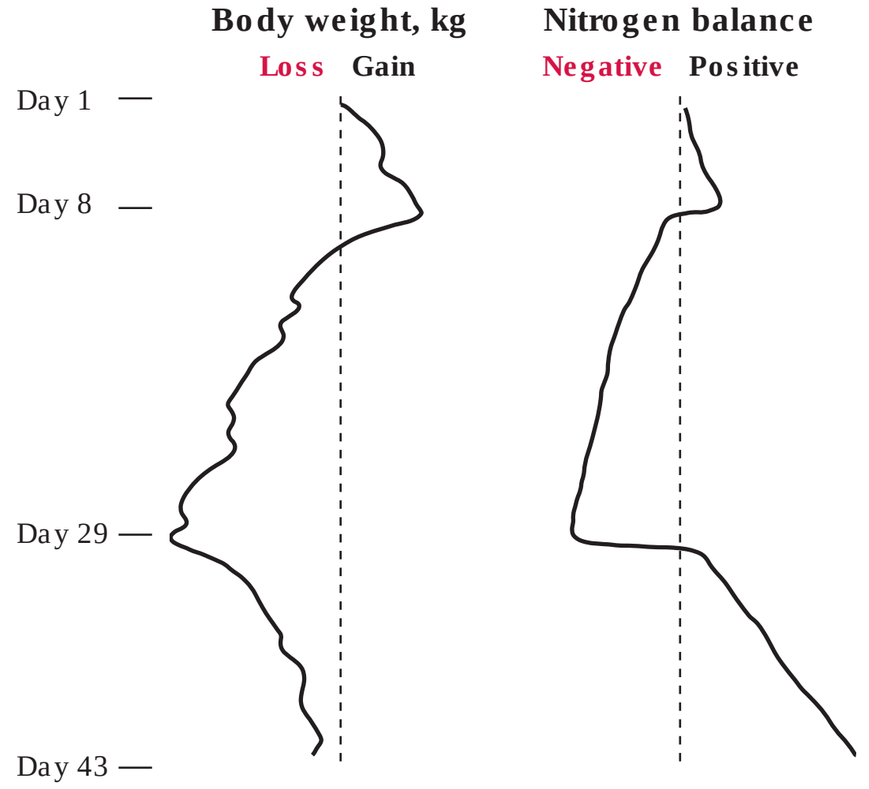


Fig. 35.22 Nitrogen balance with weight gain and weight loss. A total of 32 nonobese men consumed too many or too few calories in a prescribed pattern to change their body weight. Maximum weight loss was ~4 kg, and maximum positive nitrogen balance was ~0.14 g/day. Changes in weight are largely due to changes in the amount of body fat in the form of triglycerides. (Data from Müller MJ, Enderle J, Pourhassan M, et al. Metabolic adaptation to caloric restriction and subsequent refeeding: the Minnesota Starvation Experiment revisited. *Am J Clin Nutr.* 2015;102:807-19.)

5.2. Nitrogen Balance in Health and Illness

Consumption of a diet that is adequate in calories but deficient in protein for a protracted time leads to a major loss of protein mostly from visceral organs, such as the liver. Muscle protein is somewhat spared due to insulin secretion in response to meals. The condition is sometimes called **kwashiorkor** (a term from Ghana that refers to the disease a child gets who is displaced from breastfeeding). An affected person has a markedly protuberant abdomen and some loss of muscle mass. Children who have kwashiorkor often weigh only 60% to 80% of their recommended weight. The protein and amino acid deficit leads to decreased synthesis of proteins in the liver that are secreted into the blood, such as clotting factors, transferrin, and albumin. Hypoalbuminemia leads to edema, particularly of the abdomen. In addition, patients with kwashiorkor develop an enlarged, fatty liver due to inadequate synthesis of apoprotein B-100 for VLDL particles, which leads to retention of triglycerides. Low concentrations of albumin and transferrin (measured as total iron-binding capacity) in the blood can be used as indicators of protein depletion in visceral organs. Finally, persons who have kwashiorkor also develop atrophy of the villi and microvilli of the small intestine, leading to disaccharidase deficiency, impaired carbohydrate uptake, and diarrhea (see Chapter 18).

In persons who have a combined deficiency of calories and protein, there is a marked loss of muscle and fat mass, usually without a protuberant abdomen. This condition is often called **protein-energy malnutrition**. Protein loss is marked in both skeletal and cardiac muscle, whereas the visceral organs are somewhat spared. Muscle protein is degraded to support gluconeogenesis. The concentration of albumin in the blood is normal or nearly normal. However, affected persons are often anemic, and they are readily infected because they are immune deficient.

In Western countries, about 0.5% of all young women have **anorexia nervosa** (Fig. 35.23), a disease that is characterized by very low body weight. In the United States, a body mass index (BMI) of 16 to 17 kg/m^2 is indicative of moderate anorexia and one of 15 to 16 kg/m^2 is indicative of severe anorexia. The low BMI is the result of pathologically restricted food intake and often also excessive exercise. The disease is rare among men. Anorexia nervosa is an example of protein-energy malnutrition. Some patients have anemia and leukopenia. About 10% of affected patients die of the disease, often due to complications of a decreased mass of the heart.

Cachexia is an illness-induced loss of body weight in an individual who has access to food and is not trying to lose weight. Diagnostic signs include unexplained recent weight loss in excess of 5%, or a BMI less than 20 to 22 kg/m^2 (depending on age). There is wasting of both skeletal muscle and adipose tissue. The wasting is a result of a combination of decreased food intake and increased degradation of body protein due to an elevated concentration of **cytokines** in the blood. The cytokines (e.g., interleukins-1 and -2, interferon-γ, tumor necrosis factor-α) stimulate proteasome-mediated **proteolysis** of muscle protein (see Section 1).

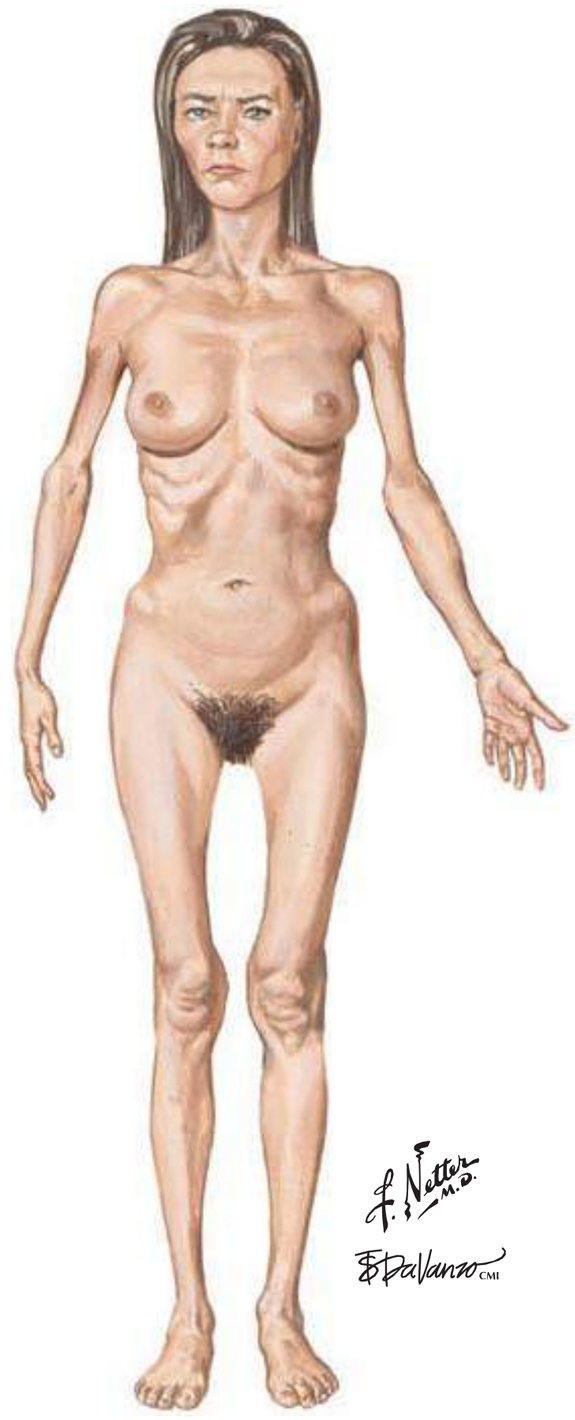


Fig. 35.23 Anorexia nervosa, an example of protein-energy malnutrition.

Cachexia is most commonly seen in patients who have a malignancy, a chronic inflammatory condition, or a degenerative disease; examples are chronic obstructive pulmonary disease; heart failure; acquired immunodeficiency syndrome; or cancer of the pancreas, stomach, colon, or lungs (Fig. 35.24). Cachexia is marked by general weakness, anemia, and a low concentration of albumin in blood plasma. Cachectic patients are immune deficient (a general feature of malnutrition) and commonly die of pneumonia.

Patients who have extensive **burns** (Fig. 35.25) exhibit some of the highest rates of proteolysis seen in hospitalized patients. For example, burns affecting about two-thirds of the body surface area double the basic energy expenditure and almost double the rate of degradation of muscle protein. Some of the resulting amino acids are used within muscle for resynthesis of protein and some are transported to other tissues for wound healing. In wounds, protein synthesis far exceeds protein degradation. While a healthy individual needs about 0.8 to 1.0 g of protein per kilogram of body weight per day to achieve zero nitrogen balance, patients with appreciable burns need about 2.0 to 2.5 g of protein per kilogram body weight per day. Provision of a relatively large amount of protein and calories to patients with burns greatly improves their rate of recovery. Estimates of nitrogen balance are routinely used to

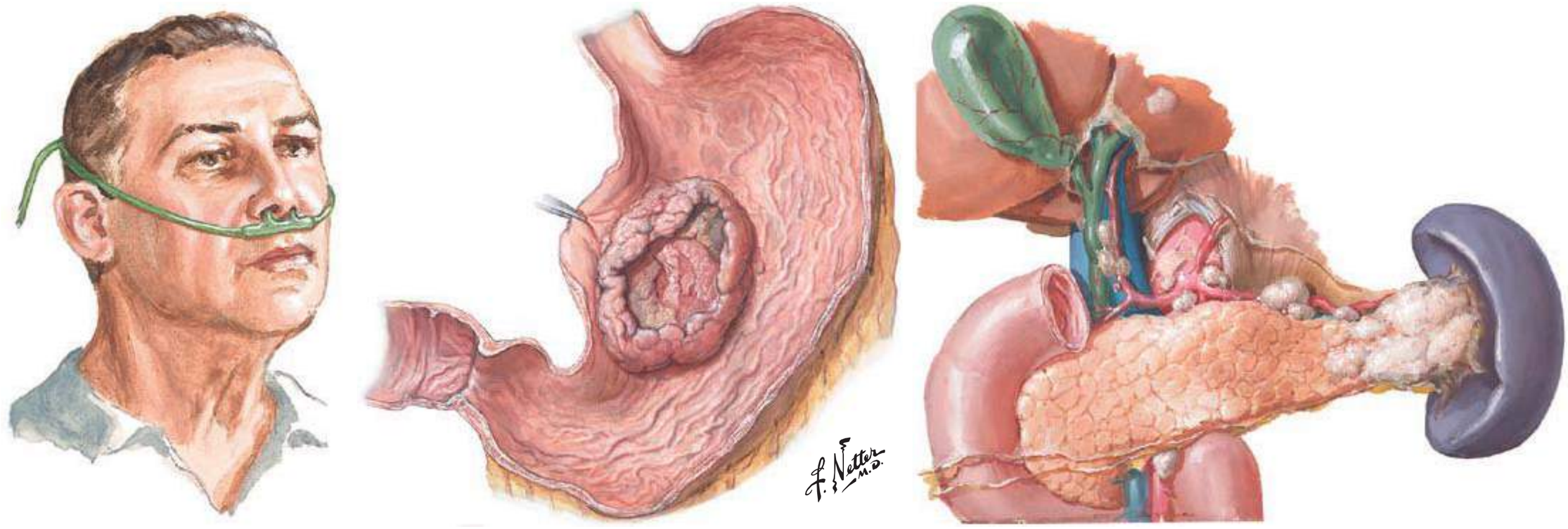


Fig. 35.24 **Some conditions often associated with cachexia: chronic obstructive pulmonary disease, cancer of the stomach, and cancer of the pancreas.**

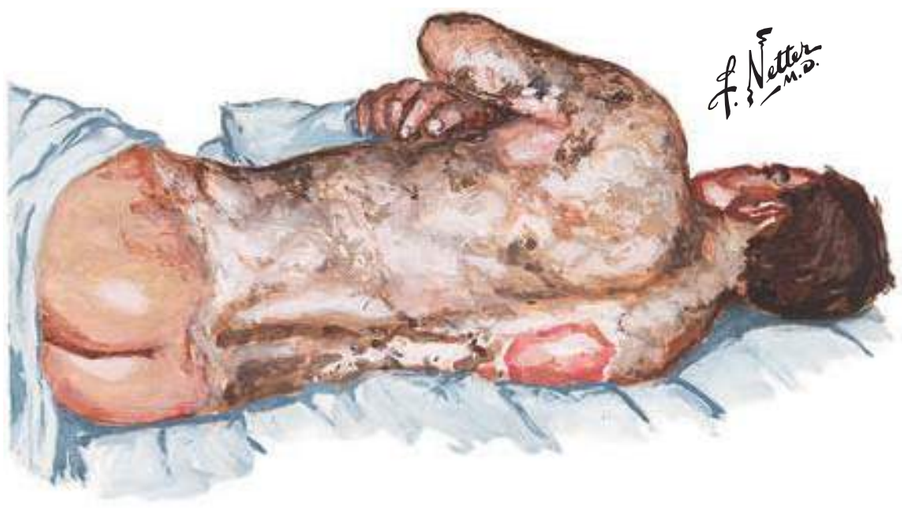


Fig. 35.25 **Extensive burn injuries are associated with a very high rate of nitrogen loss.**

guide nutrition during their hospital stay. Increased degradation of muscle protein in patients with burns is in part due to elevated concentrations of cortisol and cytokines.

After major burns or moderate to severe traumatic brain injury, it takes months for the basal rate of metabolism to return to normal.

SUMMARY

- Proteasomes recognize proteins that are conjugated with 4 or more ubiquitin polypeptides (linked via Lys-48) and degrade them into short peptides, thereby liberating ubiquitin for reuse. Peptidases in the cell then degrade the peptides into amino acids. T e concentrations of leucine, insulin, cortisol, and various cytokines (Il-1, IL-1, IFγ, and TNF-α) determine the overall rate of protein degradation in proteasomes. Within cells, the lifetime of proteins depends in part on the N-terminal amino acid sequence, the presence of a PEST motif, protein conformation, and posttranslational modifications.
- Aberrant ubiquitination is caused by altered E6AP ubiquitin-protein ligase activity following papilloma virus infection and also in patients who have Angelman syndrome, by mutant von Hippel Lindau (VHL)-factor in VHL disease and in many clear-cell renal carcinomas, and by altered ubiquitin E3 ligase activity of parkin in a form of hereditary Parkinson disease.
- Lysosomes have an acidic pH; cathepsins inside lysosomes degrade proteins in acquired extracellular material and in intracellular material obtained via autophagy.
- Degradation of amino acids requires disposal of nitrogen. T is disposal occurs primarily via excretion of urea and secondarily via excretion of NH_4^+ into the urine. T e full urea cycle is present in the liver, and portions of it are present in the intestine and in the kidneys. T e urea cycle converts NH_4^+ and the nitrogen from aspartate into urea. T e NH_4^+ stems mostly from the deamination of Gln and Glu. Almost all amino acids can participate in transamination, thereby giving rise to glutamate, glutamine, and aspartate. Transaminases (aminotransferases) that catalyze these reactions use pyridoxal phosphate as a cofactor and temporary acceptor of an amino group.
- Nitrogen is transported between tissues primarily in the form of glutamine and alanine. Muscle mainly releases alanine and glutamine. T e intestine converts glutamine to alanine and citrulline. T e kidneys convert citrulline to arginine. T e liver uses alanine and some of the arginine. Ammonia is toxic and normally is present in blood at concentrations well below 0.1 mM.
- Periportal hepatocytes convert ammonia (principally NH_4^+) to urea, and perivenous hepatocytes incorporate any remaining ammonia into glutamate, thereby forming nontoxic glutamine, which they release. For this reason, hyperglutaminemia precedes and accompanies hyperammonemia. T e concentrations in the blood of glutamine and ammonia are not measured routinely. However, respiratory alkalosis is an early sign of hyperammonemia, and abnormal cognition (or even coma) is seen at higher concentrations of ammonia.
- Patients who have severe liver disease may develop hepatic encephalopathy, which is accompanied by

hyperammonemia due to inadequate flux in the urea cycle. Besides ammonia, other neurotoxic substances likely play a role in the pathogenesis of the encephalopathy. Af ected patients can be treated with the disaccharides lactulose or lactitol or with the antibiotic rifaximin to decrease the concentration of ammonia in the blood via a change in gut pH and an ef ect on metabolism of microbes in the intestine.

- Some patients have hyperammonemia due to an inherited deficiency of an enzyme of the urea cycle. T e deficiency may cause symptoms a few days after birth or later, such as after a high-protein meal, during an extended fast, during illness or sepsis, or following major trauma or burns. Many women who are heterozygous for a deficient allele of the X-linked ornithine carbamoyltransferase develop their first episode of severe hyperammonemia following delivery of a fetus. T e more severely af ected patients with a urea cycle deficiency have to chronically limit protein intake and take oral phenylbutyrate daily to remove nitrogen as phenylacetyl glutamine.
- Treatment options for patients who are hospitalized due to hyperammonemia include dialysis and treatment with IV phenylacetate and benzoate to remove nitrogen by conjugation with glutamine and glycine.
- Classic phenylketonuria is due to homozygosity for deficient phenylalanine hydroxylase, which can be detected by newborn screening. Treatment with a low-phenylalanine diet prevents intellectual disability caused by altered amino acid transport into the brain. All phenylketonuria patients should be tested for a deficiency of tetrahydrobiopterin production.
- Maple syrup disease is due to homozygosity for deficient branched-chain keto acid dehydrogenase, which can be detected by newborn screening. Treatment with a low leucine diet reduces intellectual disability from altered amino acid transport into the brain.
- Oculocutaneous albinism can be caused by mutant tyrosinase, OCA2 protein, or tyrosinase-related protein 1, all of which impair melanin production and lead to impaired clarity of vision.
- Vitiligo is an inflammatory disease that leads to patchy loss of melanocytes in the skin.
- Patients with tyrosinemia type 1 accumulate the mutagen fumarylacetate and have a very high risk of developing hepatocellular carcinoma. Treatment with nitisinone inhibits an upstream enzyme and thereby reduces the formation of fumarylacetate.
- Alkaptonuria is due to a deficiency of homogentisic acid oxidase that causes ochronosis and debilitating joint pain in adults.
- T e nitrogen balance is positive when there is a net gain of body protein.
- A prolonged, severe deficiency of protein in the diet can lead to impaired protein synthesis, edema, and immune deficiency.
- T e rate of protein degradation is particularly high after delivery of a baby and after major burns.

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Review Questions

1. A 3-day-old boy was in the intensive care unit and received mechanical ventilation. Analysis of his blood revealed the following:

Ammonia	3,500 µM (normal: 0–59 µM)
Glutamine	6,400 µM (normal: 420–705 µM)
Citrulline	0 µM (normal: 17–43 µM)
Ornithine	1,100 µM (normal: 8–26 µM)

Analysis of his urine revealed the following:

Orotate	1,700 µmol/g creatinine (normal: 8–26 µmol/g creatinine)

T is boy most likely has a deficiency of which one of the following enzymes?

A. Argininosuccinate lyase
B. Argininosuccinate synthase
C. Carbamoyl-phosphate synthase I
D. N-Acetylglutamate synthase
E. Ornithine carbamoyltransferase

2. A 34-year-old morbidly obese woman underwent a gastric bypass procedure. During the ensuing 8 months, she was almost continuously hospitalized for various medical problems. Her presurgery weight was 400 lb; 8 months after surgery she weighed 160 lb. At the current admission, she displayed uncontrolled status epilepticus. T e concentration of ammonia in her serum was 440 µM (normal: 20–50 µM). Appropriate acute treatment of this patient is which of the following?

A. Hemodialysis, IV citrulline
B. Hemodialysis, IV phenylacetate and benzoate
C. IV essential amino acids, IV phenylbutyrate
D. IV glutamate, IV ornithine
E. Total parenteral nutrition

Chapter

36 One-Carbon Metabolism, Folate Deficiency, and Cobalamin Deficiency

SYNOPSIS

- One-carbon metabolism refers to reactions that involve one-carbon groups such as methyl-, methylene, methenyl, and formyl groups. These groups can be carried by tetrahydrofolic acid, which is derived from folic acid, a vitamin.
- Tetrahydrofolic acid is required for the detoxification of methanol.
- Folate-linked one-carbon metabolism feeds one-carbon groups into the synthesis of purine and pyrimidine nucleotides as well as into the activated methyl group cycle.
- The activated methyl group cycle uses S-adenosylmethionine as a methyl group donor in the synthesis of creatine and catecholamines, as well as in the methylation of DNA, RNA, lysine side chains, and arginine side chains. The activated methyl group cycle can drain into the transsulfuration pathway, yielding cysteine.
- Patients with a folate deficiency have impaired thymine nucleotide synthesis and hence impaired DNA replication; this causes diarrhea and megaloblastic anemia.
- Cobalamin (derived from vitamin B_{12}) is needed for the transfer of a methyl group from tetrahydrofolates to the activated methyl group cycle. Patients with a cobalamin deficiency may show the same symptoms as those with a primary folate deficiency. In addition, they have impaired peripheral nerve conduction and suffer damage to the central nervous system.
- Cobalamin is also needed for converting propionyl-CoA to succinyl-CoA (an intermediate of the citric acid cycle). Patients with a cobalamin deficiency cannot properly convert propionyl-CoA to succinyl-CoA and thus have an increased concentration of methylmalonic acid in their blood and urine. This is used clinically to distinguish between a primary folate deficiency and a secondary folate deficiency caused by cobalamin deficiency.

LEARNING OBJECTIVES

For mastery *of* this topic, you should be able to do the *f*ollowing:

- Define folates and list folate-rich foods.
- Describe the absorption of folates in the intestine and the transport to peripheral tissues.
- Describe the body's use of folates.
- Describe the pathobiochemistry and treatment of methanol poisoning.
- List cobalamin-rich foods.
- Describe the absorption of cobalamin.
- Describe the enzyme-catalyzed reactions that require cobalamin.
- Describe the synthesis of S-adenosylmethionine and its role in methylation reactions.
- Explain how a cobalamin deficiency can lead to a secondary folate deficiency.
- Interpret laboratory data (e.g., serum folic acid, cobalamin, and methylmalonic acid) to distinguish between a primary and a secondary folate deficiency.
- Select laboratory tests that contribute to a diagnosis of pernicious anemia.
- Explain the treatment of folate deficiency and cobalamin deficiency.

1. SOURCES AND ABSORPTION OF DIETARY FOLATES

T e human body cannot synthesize *f*olic acid. Folic acid is a vitamin that gives rise to tetrahydro*f*olic acid, which can carry a one-carbon group, such as a methyl group. Folic acid and its derivatives are *f*ound primarily in legumes, green lea*f*y vegetables, and grains. T e intestine releases mostly N^5-methyl-tetrahydro*f*olic acid into the blood.

1.1. Structure of Folates

Folic acid, sometimes called **vitamin B_9**, is absorbed and then reduced to **dihydrofolic acid** (**DHF**) and finally **tetrahydrofolic acid** (**THF**; Fig. 36.1 and Section 1.2).

Tetrahydrofolates can carry a methyl ($–CH_3$), methylene ($–CH_2–$), methenyl (also called methylidene; =CH–), formyl (–CH=O), or formimino group (–CH=NH; see Fig. 36.1). Inside peripheral cells, tetrahydrofolate **monoglutamates** can be extended with glutamate to produce tetrahydrofolate **polyglutamates**.

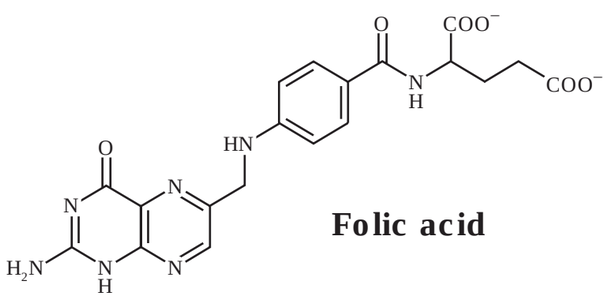
T e term **folates** customarily includes all folate compounds regardless of one-carbon group or number of glutamate residues. Folates are present in the body only in sub-µM concentrations.

1.2. Absorption of Folates in the Intestine and Transport in the Blood

Folates are contained in grains, citrus fruit, legumes, and green leafy vegetables (Table 36.1).

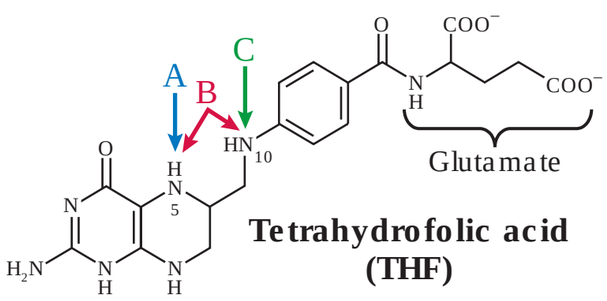
Folic acid is commonly used in supplements and food fortification. Natural foods mostly contain folates other than folic acid, principally N^5-methyl-tetrahydrofolate. T rough oxidation and enzymatic deconjugation (i.e., removal of the polyglutamate tail) by glutamate carboxypeptidase II in the intestine, these folates give rise to folic acid monoglutamate.

T e intestine takes up supplementary folic acid about twice as efficiently as natural folates. T e main reasons for this difference appear to be the enclosure of natural folates inside cells and the longer polyglutamate tail of natural folates that has to be removed before uptake. T e measure **"dietary folate equivalents"** (**DFE**) was introduced to account for dif erences in the



Folic acid

A: formimino, –CH=NH
formyl, –CH=O
methyl, –CH_3
B: methylene, –CH_2–
methenyl, =CH
C: formyl, –CH=O



Tetrahydrofolic acid (THF)

Fig. 36.1 Folic acid and tetrahydrofolates. THF carries one-carbon groups at N^5, N^{10}, or both N^5 and N^{10}. Inside cells, THF mostly has a polyglutamate tail; here, only one glutamate residue is shown.

Table 36.1 Folate Content of Various Foods

Food	Folates (µg/100 g Edible Portion)
Lentils	181
Asparagus	147
Spinach	146
Bread, white, fortified*	140
Pasta, cooked, fortified*	120
Broccoli	50
Peas	32
Orange juice	30
Ground beef	11
Potato, boiled	9
Milk	5
Ice cream	5
Apple	3

*In the United States and Canada, enriched cereal grain products must be fortified with folic acid.

efficiency of uptake: 1 µg of natural *f*olates in food is worth 1 µg of DFE, and 1 µg of supplemental *f*olic acid is worth 1.7 µg of DFE if taken with food but 2 µg of DFE if taken on an empty stomach.

The recommended daily folate allowance for healthy children 1 to 3 years old is 150 µg of DFE, for people 14 years of age and older it is 400 µg of DFE, and for pregnant women it is 600 µg of DFE. Patients who have increased tissue turnover (e.g., due to chronic **hemolytic disease**) need more folates than the recommended dietary amount.

The Food and Nutrition Board of the Institute of Medicine in the United States recommends that adults not take more than 1,000 µg of supplemental folic acid per day. The intake of folates from the diet is not limited. The major concern over a high intake of folic acid is that it may allow a cobalamin-deficient patient to not develop megaloblastic anemia, to not seek medical attention, and then unknowingly experience slowly progressive nerve damage (see Section 7.2 below).

Most **folate absorption** occurs in the duodenum and jejunum via the **proton-coupled folate transporter** (**PCFT**, **SLC46A1**). Folates produced by bacteria in the colon are also absorbed but correspond to only about 10% of the folate absorption in the small intestine if the diet contains adequate folates.

Patients who have **hereditary folate deficiency** are homozygous or compound heterozygous for a nonfunctional PCFT. The PCFT transports folates both from the lumen of the intestine into the epithelium and from the blood into the central nervous system. Accordingly, the serum and spinal fluid of affected patients have an abnormally low folate concentration. Without supplementary folate treatment, the defect causes severe anemia, as well as abnormal development of the brain, which may be associated with seizures. The disease is very rare.

Inside enterocytes, dihydrofolate reductase reduces folic acid first to **dihydrofolic acid** (**DHF**) and then to **THF**. THF is methylated to form **N^5,N^{10}-methylene-THF**, which is reduced to N^5-methyl-THF (Fig. 36.2). The intestinal epithelial cells then release N^5-methyl-THF into the bloodstream.

Efflux of folate from intestinal epithelial cells into the extracellular space and blood is at least partially mediated by **multidrug resistance-associated proteins** (**MRP**s).

In the bloodstream, about half of the N^5-methyl-THF is free; the rest is bound to albumin or a **soluble folate receptor** (see Fig. 36.2). Protein binding of folates reduces losses of folates by filtration in the kidneys. The proximal tubules in the kidneys express transport systems and receptors for the reuptake of free folates, albumin, and folate-binding protein.

The uptake of folates into peripheral cells occurs via the **reduced folate carrier** (**RFC**), also called **SLC19A1**. The RFC has a higher affinity for reduced folates, such as N^5-methyl-THF, than for folic acid. RFC transports folates into cells, probably in exchange for transporting organic phosphates into the extracellular space. Both thiamine phosphate and thiamine pyrophosphate can exit cells via the RFC (thiamine enters cells through other transporters, SLC19A2, and SLC19A3). The PCFT facilitates the transfer of folates from the blood to the central nervous system. Membrane-bound **folate receptors** (FRα and FRβ), which contain a glycosyl phosphatidyl inositol anchor and are endocytosed, are used for some of the folate uptake by hematopoietic cells, macrophages, and epithelial cells in the kidneys.

Endocytosis of a folate receptor is followed by the release of N^5-methyl-THF in endosomes. Cells liberate THF from

N^5-methyl-THF in a single step of the activated methyl group pathway.

Most **antifolate drugs**, which resemble folates but oppose their effects, are transported by the PCFT and/or the RFC. Antifolates are used against tumors and certain autoimmune diseases. Examples are **methotrexate** and **pemetrexed** (see Chapter 37).

A **tetrahydrofolate synthase** (also called **folylpolyglutamate synthase**) adds glutamate residues to THF monoglutamate. Compared with folylmonoglutamates, folylpolyglutamates are less likely lost from cells by diffusion (owing to increased charge), less likely transported out of the cell by membrane transporters, and have a greater affinity to many of the enzymes that are involved in folate metabolism.

Folates are **stored** in the liver and the kidneys.

Several drugs have an adverse effect on folate uptake or cellular folate content. The antifolate **methotrexate**, which is used in the treatment of rheumatoid arthritis and moderate to severe psoriasis, traps cellular folates as dihydrofolates (see Chapter 37). The antibiotic **trimethoprim** and the antimalarial **pyrimethamine** inhibit the dihydrofolate reductase of humans less than that of bacteria, plasmodia, or *Toxoplasma gondii*. These drugs are contraindicated in patients who are anemic due to a folate deficiency, and they are used cautiously in patients who are at risk of folate deficiency. Many antiepileptic drugs (e.g., **phenytoin**, **phenobarbital**) lead to low folate status, and patients treated with these drugs often need supplemental folate. Supplementation is especially important in women of child-bearing age (see neural tube defects in Section 8.1).

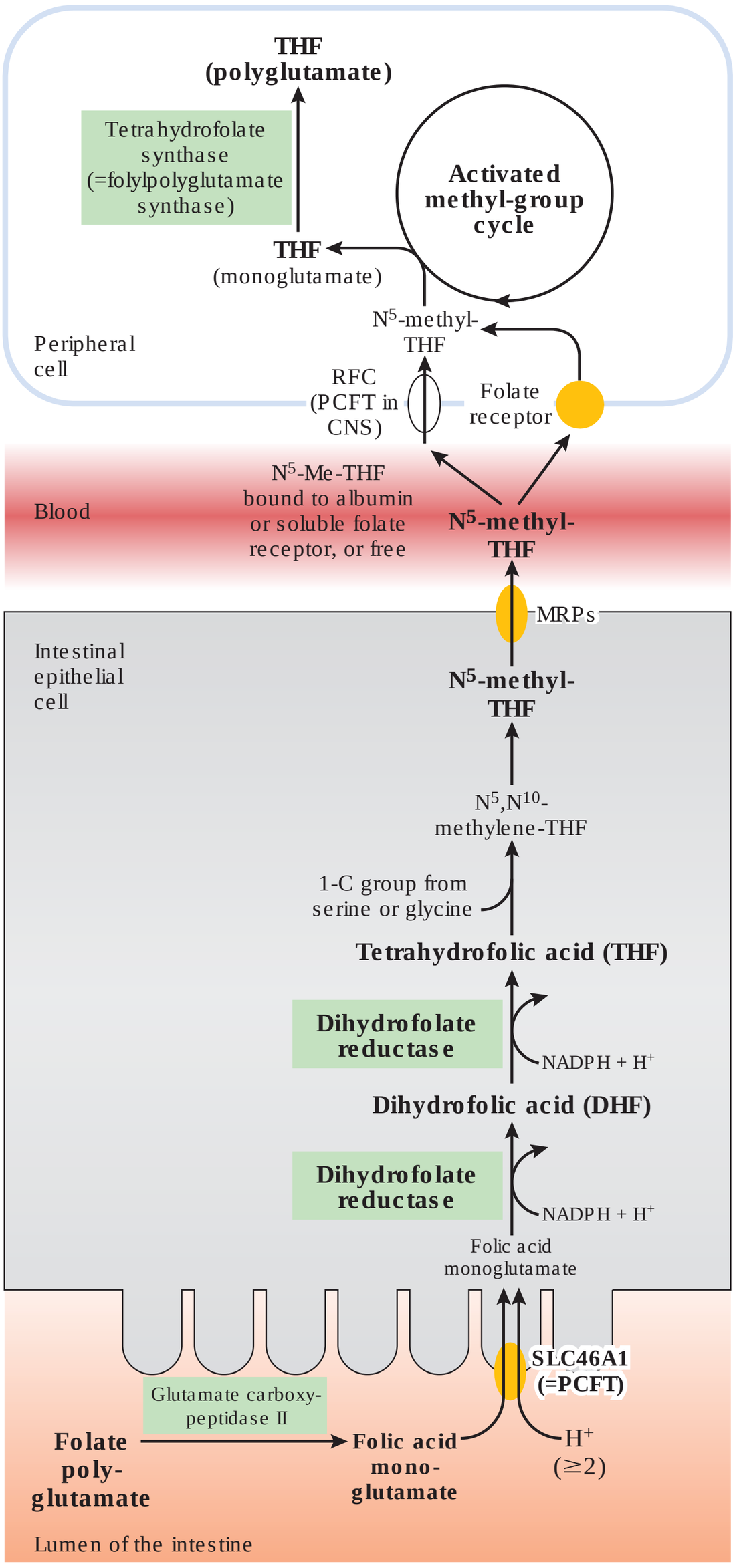


Fig. 36.2 **Uptake of folates into the intestine and transport to peripheral tissues (bottom to top).**

2. LOADING TETRAHYDROFOLATES WITH ONE-CARBON GROUPS

Serine and glycine are the main sources of one-carbon groups that are added to THF, giving rise to N^5,N^{10}-methylene-THF. N^5,N^{10}-methylene-THF in turn can give rise to other one-carbon THFs. The detoxification of methanol yields formate ($HCOO^-$), which is toxic and must form N^{10}-formyl-THF before it can be disposed of as CO_2. N^5-formyl-THF, also called leucovorin, is an injectable folate that rapidly becomes part of the pool of one-carbon tetrahydrofolates.

2.1. Glycine and Serine as Sources of One-Carbon Groups

Fig. 36.3 provides an overview of the reactions that add one-carbon groups to THFs.

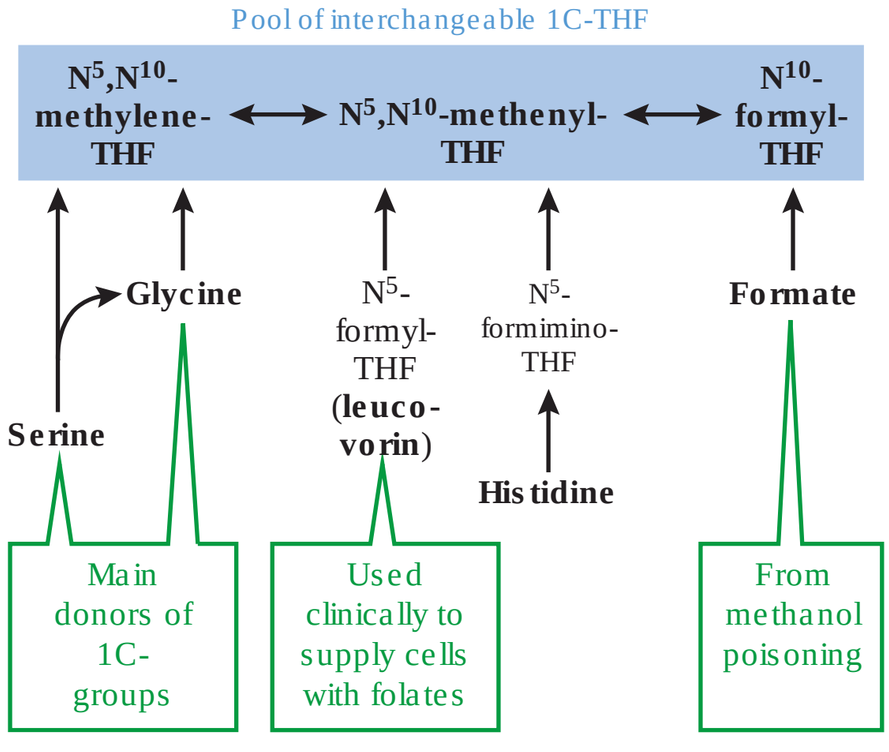


Fig. 36.3 **Overview of reactions that donate one-carbon groups to tetrahydrofolic acid.**

The majority of one-carbon groups loaded onto THF stems from **serine**, and a lesser amount from **glycine** (see Fig. 36.3). There is a limitless supply of serine because serine can readily be made de novo from an intermediate of glycolysis/gluconeogenesis (see Figs 19.11 and. 34.11). Removal of a one-carbon group from serine yields glycine. Glycine can also donate a one-carbon group to THF. For example, when intestinal enterocytes take up folic acid and export N^5-methyl-THF as discussed above (see Section 1.2 and Fig. 36.2), the one-carbon groups stem chiefly from serine and glycine.

2.2. Other Sources of One-Carbon Groups and Folates

Minor amounts of one-carbon groups on THF normally arise from the degradation of **formic acid** and **histidine**. The degradation of **formic acid** plays a crucial role in methanol detoxification (see Section 3.4). The degradation of **histidine** yields formiminoglutamate, which forms N^5-formimino-THF and glutamate (see Fig. 36.3).

Several one-carbon group–containing folates can readily be converted to other one-carbon group–containing folates (Fig. 36.4). The three folates N^5,N^{10}-methylene-THF, N^5,N^{10}-methenyl-THF, and N^{10}-formyl-THF form a pool of reversibly convertible folates. In an irreversible reaction, N^5,N^{10}-methylene-THF from this pool gives rise to N^5-methyl-THF; this reaction is important in understanding the induction of a secondary folate deficiency by a cobalamin deficiency (see Section 7.2).

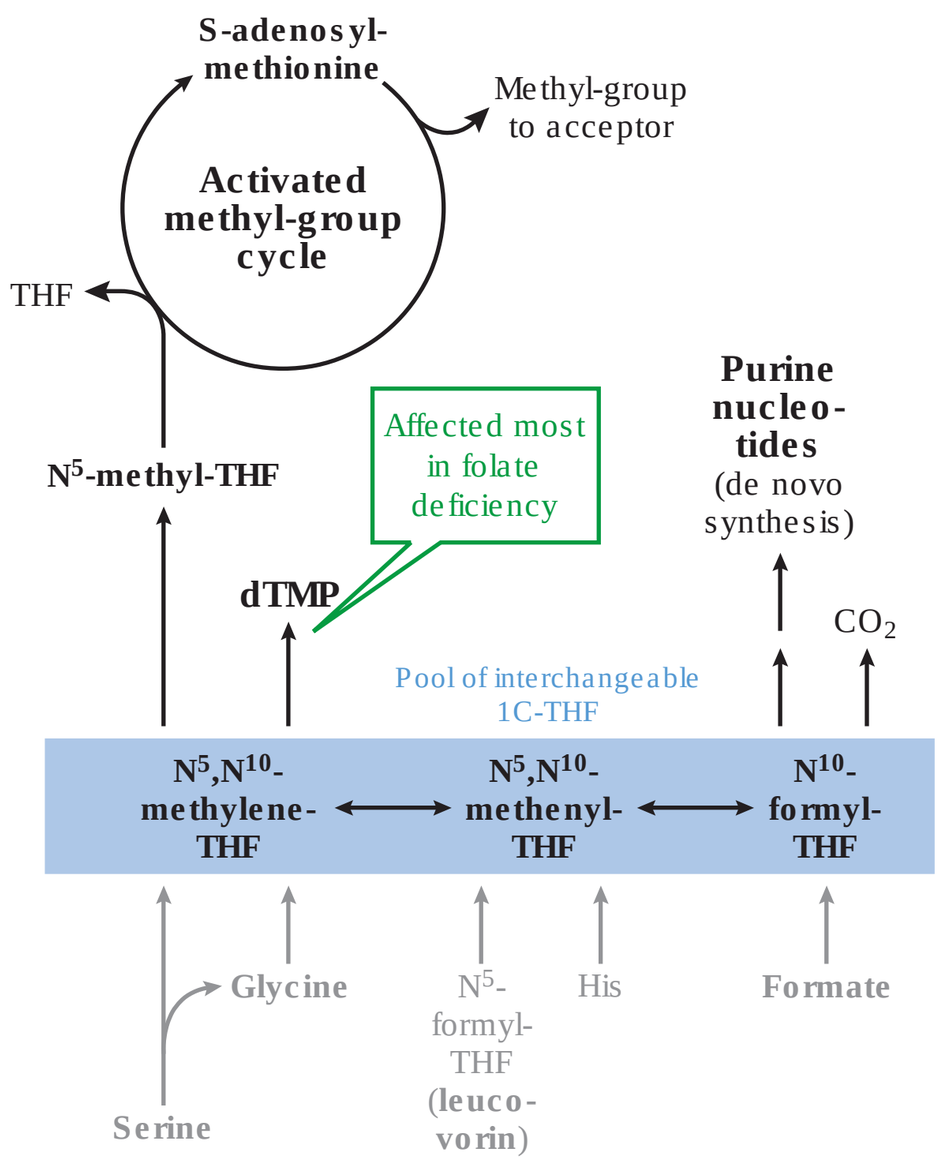


Fig. 36.4 Overview of the use of one-carbon groups from folates.

3. USE OF ONE-CARBON GROUPS ON TETRAHYDROFOLATES

Physiologically, most one-carbon groups are used *for* the synthesis *of* purine nucleotides (chie*fly* adenosine triphosphate) and the pyrimidine nucleotide deoxythymidine triphosphate. A lesser amount *of* one-carbon groups is *fed* into the activated methyl group cycle. N^{10}-*f*ormyl-*T*HF can release its one-carbon group as CO_2; this plays a role in the detoxif cation *of* methanol.

3.1. Overview

Fig. 36.4 provides an overview of the reactions that use one-carbon groups from folates.

N^{10}-formyl-THF can give of its one-carbon group as CO_2 (see Fig. 36.4). This reaction can be viewed as a way to bleed excess one-carbon groups from a pool of one-carbon-THF compounds. This reaction becomes important in the detoxification of methanol (see 3.4).

3.2. Synthesis of Inosine Monophosphate and Deoxythymidine Monophosphate

The major use of one-carbon groups is in the synthesis of inosine monophosphate **(IMP)**, a precursor for the **purine nucleotides** adenosine monophosphate (AMP) and guanosine monophosphate (GMP; see Fig. 36.4 and Chapter 38). However, a dietary folate deficiency (see Section 7.1) does not impair this pathway to a medically relevant extent.

One-carbon groups are also used for the synthesis of **thymidine** monophosphate (deoxythymidine monophosphate, dTMP; see Chapter 37). The synthesis of dTMP is impaired in folate-deficient and in cobalamin-deficient patients (see Section 7), as well as in patients who receive chemotherapy with certain antifolates. The synthesis of dTMP is unique in that it produces dihydrofolate, which has to be reduced to tetrahydrofolate before it can carry a one-carbon group. Chemotherapy of tumors exploits this situation as well as the dTMP need of fast-growing cells.

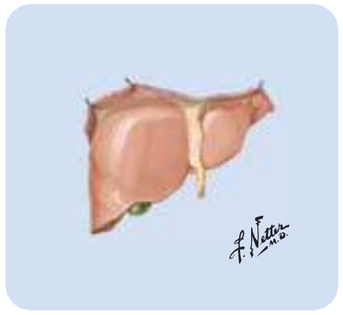
3.3. Transfer of Methyl Groups to the Activated Methyl Group Cycle

For most methylation reactions, the methyl group on N^5-methyl-THF is not sufficiently reactive. The methyl group of N^5-methyl-THF is transferred into the activated methyl group cycle. There, S-adenosyl-methionine carries a much more reactive methyl group (see Section 4).

3.4. Detoxification of Methanol

The basic reactions of oxidizing **methanol** to the corresponding aldehyde and acid are similar to those of the degradation of ethanol (Fig. 36.5; see also Chapter 30).

Methanol poisoning leads to a transient high concentration of **formate** in cells and blood (see Fig. 36.5). Catalyzing



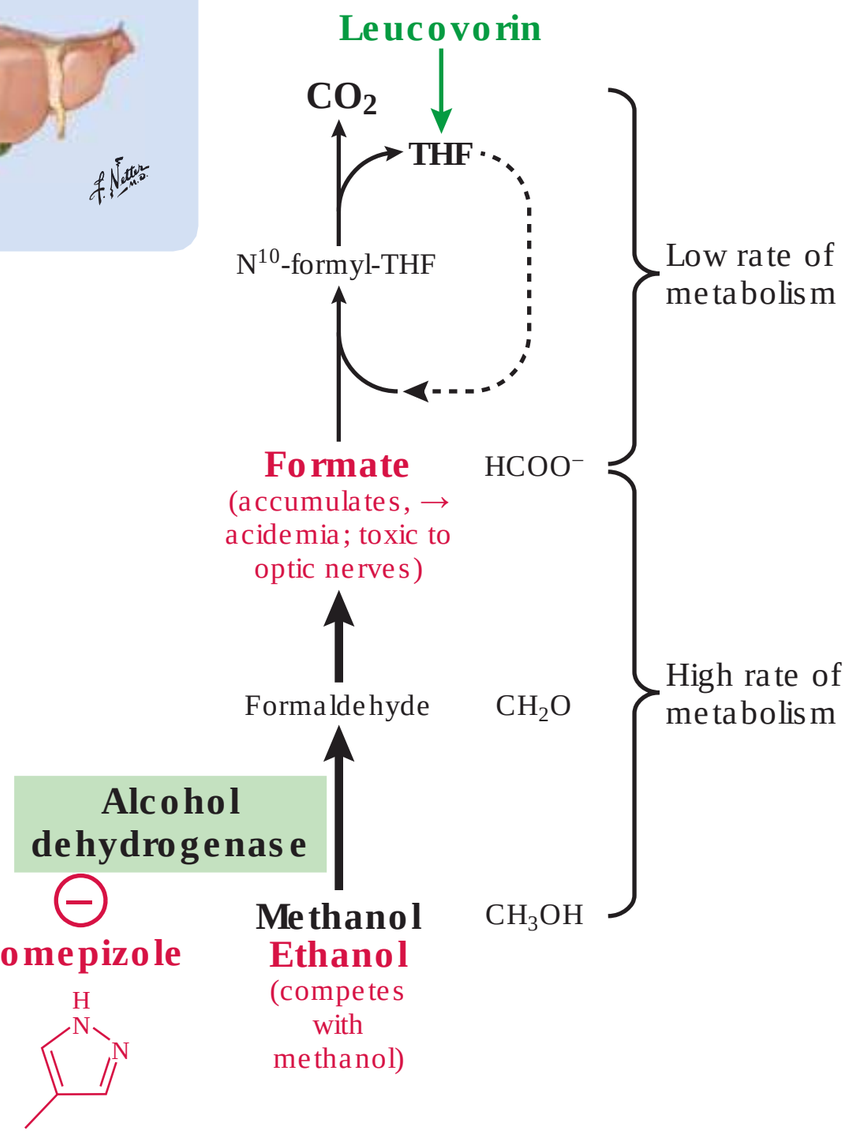


Fig. 36.5 Detoxification of methanol. Fomepizole or ethanol, as well as leucovorin, may be a part of treatment.

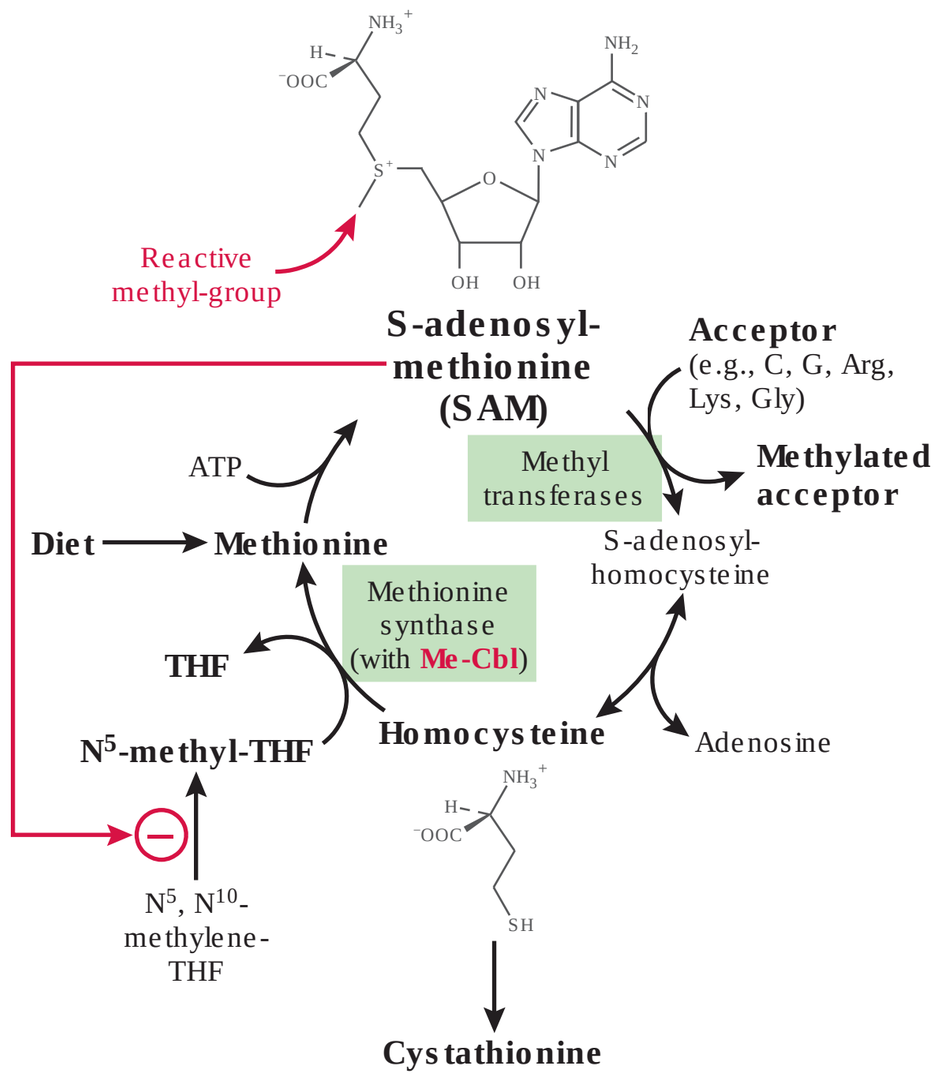


Fig. 36.6 The activated methyl group cycle.

sequential reactions, alcohol dehydrogenase, and formaldehyde dehydrogenase oxidize methanol to formate at a high rate. However, formate reacts with folates only at a comparatively low rate. Thus, formate can accumulate to millimolar concentrations; this leads to acidosis and damage to the optic nerve (mechanism unknown).

In the treatment of methanol poisoning, it is important to prevent the concentration of formate in the blood from rising into the toxic range. Soon after ingestion, gastrointestinal lavage can diminish the absorption of methanol. Conversion of methanol to formate can be slowed either with **ethanol** (which competes with methanol as a substrate of alcohol dehydrogenase) or with **fomepizole** (which inhibits alcohol dehydrogenase and is also used in the treatment of **ethylene glycol poisoning**; ethylene glycol is used in cars to prevent freezing or cooling of water). Patients can be injected with **leucovorin** (also called folinic acid, N^5-formyl-THF) to ensure an optimal concentration of folates for removing formate.

4. THE ACTIVATED METHYL GROUP CYCLE

The activated methyl group cycle produces S-adenosylmethionine, which is used *for* a large number *of* methylation reactions that involve DNA, RNA, or proteins. In addition, S-adenosylmethionine is used *for* the synthesis *of* creatine, epinephrine, and phosphatidylcholine.

4.1. Reactions of the Activated Methyl Group Cycle

The activated methyl group cycle is found in most cells, but it is most active in the liver, kidneys, intestine, and pancreas.

The activated methyl group cycle produces **S-adenosylmethionine** (**SAM**), which can donate a methyl group for methylation reactions (Fig. 36.6). Demethylation of SAM yields S-adenosylhomocysteine, which is nearly in equilibrium with **homocysteine**. On average, **methionine synthase** remethylates about half of the homocysteine to methionine, whereas the other half is converted to cystathionine and thus enters the transsulfuration pathway (see Section 9). After a high-protein meal, more homocysteine enters the transsulfuration pathway, whereas in the fasting state, more homocysteine stays in the activated methyl group cycle. Methionine synthase activity depends on two vitamins: **N^5-methyl-THF** and **cobalamin**.

In the liver and the brain, homocysteine can also be methylated to methionine by a second reaction catalyzed by **betaine-homocysteine S-methyltransferase**. This reaction uses **glycine betaine** (=trimethylglycine, $(CH_3)_3N^+–CH_2–COO^–$) as a one-carbon group donor. The reaction does not depend on either folates or cobalamin.

The concentration of SAM is regulated. If the concentration of SAM is low, there is increased conversion of N^5-N^{10}-methylene-THF to N^5-methyl-THF (see Fig. 36.6), which can then give rise to more SAM. If the concentration of SAM in the liver is high, SAM stimulates glycine N-methyltransferase, which methylates **glycine** to **sarcosine**. Sarcosine dehydrogenase then demethylates sarcosine to yield glycine and

N^5,N^{10}-methylene-THF. T is net transfer of a one-carbon group from the activated methyl group cycle to the THF pool also becomes active when there is an **excess of methionine in the diet**.

A buildup of S-adenosylhomocysteine product inhibits the activity of many SAM-dependent methyltransferases.

4.2. Use of Methyl Groups From the Activated Methyl Group Cycle

SAM is used in the biosynthesis of creatine, phosphatidylcholine, plasmenylcholine, and epinephrine; it is also used to methylate DNA, RNA, and proteins.

In muscle, the **phosphocreatine**/creatine pair participates in moving high-energy phosphate compounds from the site of ATP production in the mitochondria to the site of ATP consumption by the contractile machinery (see Fig. 23.5). In the brain, creatine phosphate presumably serves mostly as a reservoir of high-energy phosphate groups during periods of impaired ATP production.

Phosphocreatine spontaneously and irreversibly cyclizes to **creatinine** (Fig. 23.6), which is lost in the urine. On average, an adult excretes about 500 mg of creatinine per day (see Fig. 35.11). T is requires de novo synthesis of creatine.

T e synthesis of **creatine** requires the transfer of a methyl group from SAM to guanidino acetate. T is reaction uses about half of all SAM. T e liver releases creatine into the bloodstream; brain and muscles pick it up from there.

Creatine in the diet also reaches the bloodstream and is taken up into muscle. Some people consume a daily **supplement** of creatine in an ef ort to improve their physical performance. Creatine supplements may benefit patients with neurologic disorders that are caused by impaired energy production.

After the synthesis of creatine, the synthesis of **phosphatidylcholine** and **plasmenylcholine** requires the second-highest daily amount of SAM. SAM is used to methylate phosphatidylethanolamine threefold to produce **phosphatidylcholine** and, similarly, plasmenylethanolamine to produce **plasmenylcholine** (see Figs. 11.1 and 11.2).

T e synthesis of **epinephrine** from norepinephrine also requires a methyl group from SAM (see Fig. 35.14).

DNA contains methyl cytosine (Fig. 36.7; see also Fig. 1.1). T e methylation of cytosine occurs to inactivate retrotransposons (a class of movable genetic elements), the second X chromosome in females, imprinted regions of the genome (to silence select maternal or paternal genes; see Chapter 5), and gene expression at various stages of development (see Chapter 6).

T e 5′-ends of **mRNAs** contain a **7-methyl-guanosine** cap (see Fig. 36.7; also see Fig. 6.8), while the 5′-ends of some tRNAs and snRNAs in the spliceosomes contain a **trimethyl-guanosine** cap.

Proteins (e.g., histones; see Chapter 6) can be methylated on **lysine** or **arginine** side chains (see Fig. 36.7). T e amino group of the side chain of lysine residues can be methylated up to threefold, and the guanidino group of the side chain of arginine residues up to twofold (yielding up to three dif erent isomers).

5. ABSORPTION OF COBALAMIN

We take in generous amounts o*f* cobalamin when we eat meats, *f*owl, f sh, or dairy. T e stomach secretes intrinsic

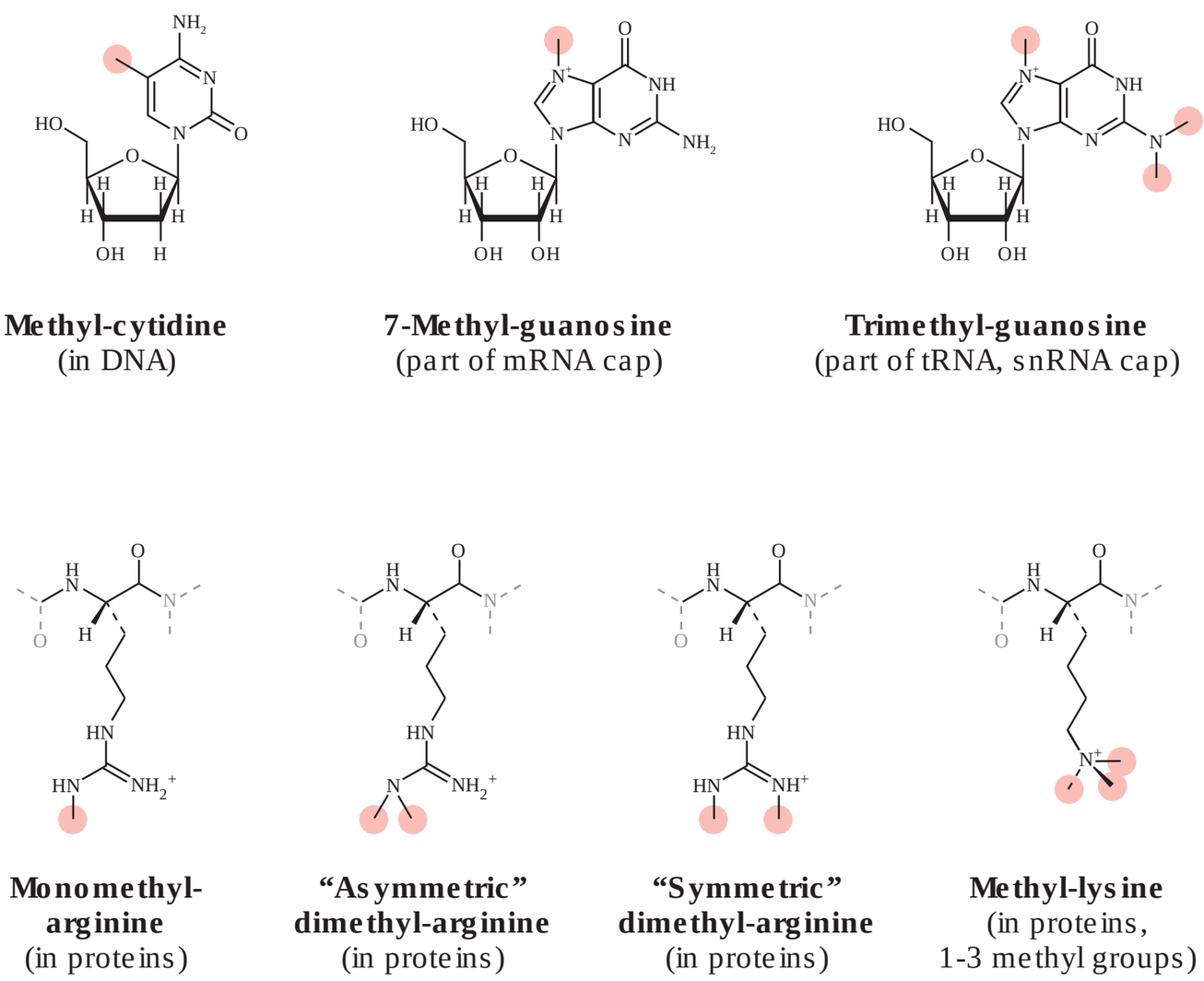


Fig. 36.7 Methylated bases in nucleic acids and methylated amino acid side chains in proteins. Pink circles highlight methyl groups transferred from S-adenosylmethionine.

*f*actor, and an intrinsic *f*actor-cobalamin complex is taken up a*ft*er binding to the cubam receptor in the distal portion o*f* the ileum. T e ileum then secretes a complex o*f* cobalamin and transcobalamin II into the blood.

Cobalamin is a vitamin principally acquired from eating foods that are derived from animals. Only bacteria can make cobalamin; animals acquire their cobalamin from bacteria. Table 36.2 states the cobalamin content of various foods.

T e U.S. Institute of Medicine **recommended daily allowance** of cobalamin for people aged 14 years or older is 2.4 µg; for pregnant and breastfeeding women it is 2.6 and 2.8 µg, respectively. No tolerable upper intake level for cobalamin is set because no adverse ef ects have been reported in healthy persons. Since there is a high prevalence of maldigestion among older adults, the Institute further recommends that **adults older than 50 years** receive the recommended amount of cobalamin mainly from fortified foods or cobalamin supplements.

Because of their diet, strict **vegans** need to take a supplement of cobalamin; this is especially important for pregnant women and nursing mothers to avoid cobalamin deficiency in their infants (see also Section 7.2).

T e structure of cobalamin is shown in Fig. 36.8. Cobalamin contains a substituted corrin ring with a cobalt (Co^{3+}) ion in its midst. T is Co^{3+} can bind to a methyl, 5′-deoxyadenosyl, hydroxyl, or cyano group. Cyanocobalamin is also called **vitamin B_{12}**. Cyanocobalamin is the most commonly used form of cobalamin in fortified foods and vitamin supplements. T e human body converts cyanocobalamin to the cofactors methylcobalamin and 5′-deoxyadenosylcobalamin (see below).

Efficient uptake of cobalamin requires a stomach that secretes acid, pepsin, and intrinsic factor; a pancreas that secretes proteases; and an ileum that expresses cubam. As a low pH and proteases in the stomach free dietary cobalamin from the food matrix, **haptocorrin**, secreted by salivary glands and the gastric mucosa, binds to cobalamin. Parietal cells in the stomach secrete **intrinsic factor**. In the small intestine, as chyme reaches a near-neutral pH, proteases from the pancreas degrade haptocorrin, and cobalamin leaves haptocorrin and instead binds to intrinsic factor (shown in abbreviated form in Fig. 36.9). In the terminal portion of the ileum, the intrinsic factor-cobalamin complex binds to **cubam** (a heterodimer of **cubilin** and **amnionless**); this binding requires Ca^{2+}, which is normally supplied by the pancreas. T e cubam-intrinsic factor-cobalamin complex is taken up via endocytosis. In the lysosomes, proteolysis of the complex liberates cobalamin, which then binds to **transcobalamin II**, a protein. Cubam is also liberated and recycled to the plasma membrane. Intrinsic factor is degraded.

Cubam is also expressed in the proximal tubules of the **kidneys** for the reabsorption of multiple proteins. Cubilin in cubam has multiple dif erent binding sites. T ese enable cubilin to bind not only the intrinsic factor-cobalamin complex but also **albumin**, **vitamin D–binding protein**, **transferrin**, and **apolipoprotein A1**.

T e cobalamin-transcobalamin II complex, which circulates in the blood, is commonly called **holotranscobalamin II**.

Cells take up holotranscobalamin II via receptor-mediated endocytosis. Lysosomes degrade transcobalamin II and thereby liberate cobalamin. Cobalamin is then either methylated or 5′-deoxyadenosylated to become methylcobalamin or 5′-deoxyadenosylcobalamin, respectively.

Of all the cobalamin in the blood, only about one quarter is bound to transcobalamin II, while about three-fourths are bound to **transcobalamin I** (also called **haptocorrin** or **R binder**, the same protein that the stomach secretes into its lumen). About 1% of patients who have a low serum cobalamin have a **deficiency of transcobalamin I**.

Table 36.2 Cobalamin Content of Various Foods

Food	Cobalamin (µg/100 g Edible Portion)
Salmon	5.8
Ground beef	2.7
Lamb, leg	2.6
Cheese (mozzarella)	2.3
Eggs (boiled)	1.1
Milk	0.4
Ice cream	0.4
Chicken breast	0.3
Potato, boiled	0.0
Broccoli	0.0
Bread, fortified*	0.0

*Fortification with folic acid and other vitamins but not cobalamin.

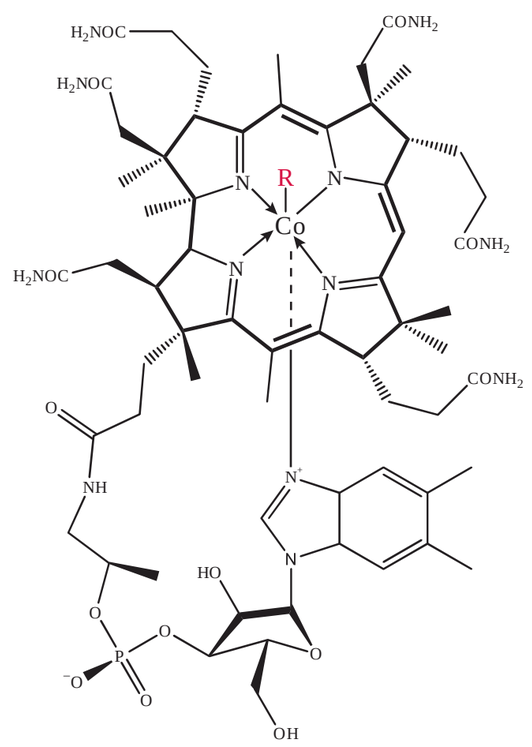


Fig. 36.8 Cobalamin. R can be a cyano group (–CN, as in vitamin B_{12}), a methyl group ($–CH_3$, used in methionine synthase), a 5′-deoxyadenosyl group (used in methylmalonyl-CoA mutase), or a hydroxyl group (–OH; in hydroxocobalamin, used in cyanide poisoning).

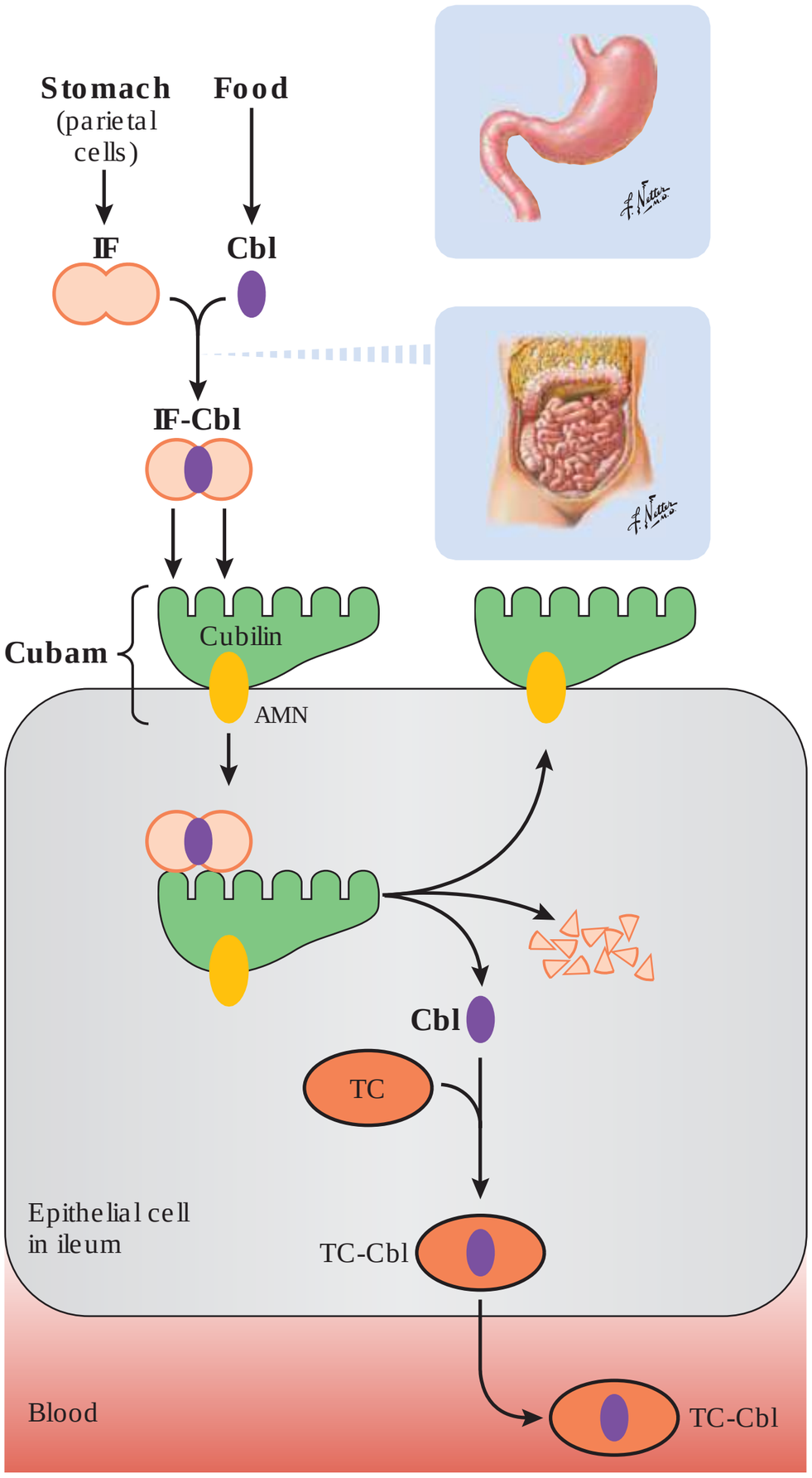


Fig. 36.9 Absorption of cobalamin. AMN, amnionless; Cbl, cobalamin; IF, intrinsic factor; TC, transcobalamin II.

6. ENZYMES THAT USE COBALAMIN AS A COFACTOR

Cobalamin is a cofactor for two enzymes: methionine synthase in the activated methyl group cycle and methylmalonyl-CoA mutase in the pathway that feeds propionyl-coenzyme A (proprionyl-CoA) into the citric acid cycle.

6.1. Methionine Synthase

Methionine synthase, the enzyme that transfers a methyl group from N^5-methyl-THF to homocysteine in the activated methyl group cycle, requires the cofactor **methylcobalamin** (see Figs. 36.6 and 36.8). As shown in Section 7.2, the rate of the cobalamin-dependent methyl transfer to homocysteine is abnormally low in patients who have a cobalamin deficiency.

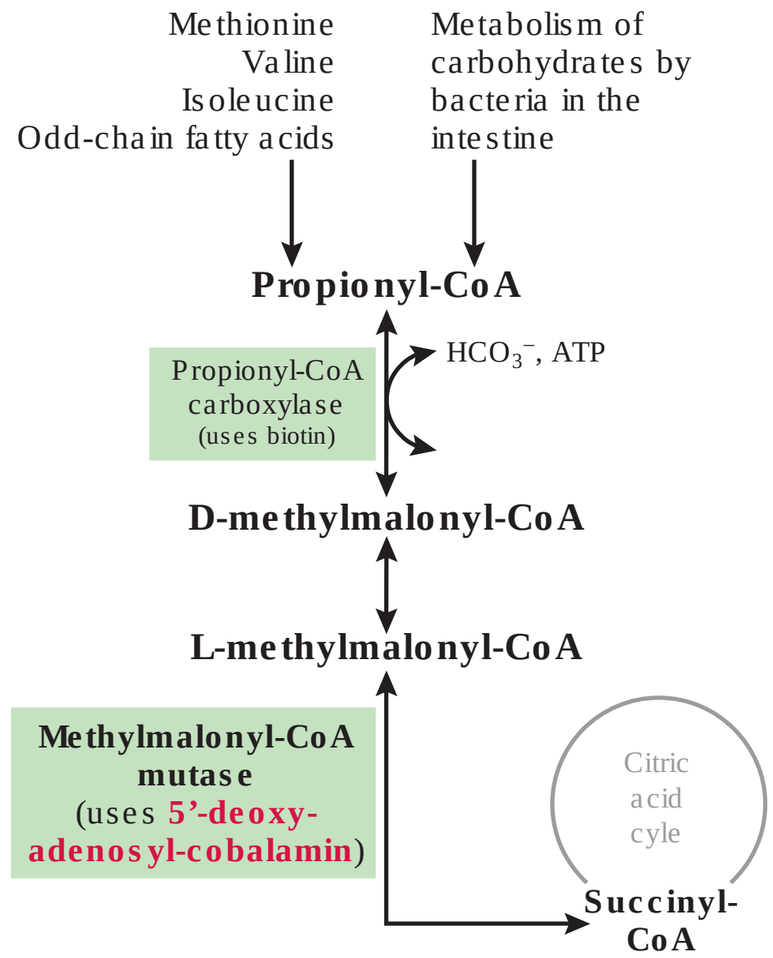


Fig. 36.10 Role of cobalamin in converting propionyl-CoA to succinyl-CoA.

6.2. Methylmalonyl-CoA Mutase

Methylmalonyl-CoA mutase requires 5′-deoxyadenosylcobalamin and catalyzes a reaction in the conversion of propionyl-CoA to succinyl-CoA. Propionyl-CoA arises from the metabolism of methionine (Fig. 36.10), the branched-chain amino acids valine and leucine (see Fig. 35-21), and odd-chain fatty acids (see Section 4 in Chapter 27). Succinyl-CoA is an intermediate of the citric acid cycle (see Fig. 22.6). A cobalamin deficiency leads to an increase in the concentration of methylmalonic acid in the blood, which is useful for a diagnosis (see Section 7.2).

7. MEGALOBLASTIC ANEMIA DUE TO FOLATE DEFICIENCY OR COBALAMIN DEFICIENCY

A folate deficiency impairs dTMP production. This impairs replication of rapidly dividing cells and thus leads to megaloblastic anemia and diarrhea. Cobalamin deficiency leads to a largely irreversible destruction of the myelin sheaths of the nervous system; in addition, by inactivating methionine synthase, a cobalamin deficiency may lead to a secondary folate deficiency.

7.1. Folate Deficiency

Folate deficiency is most common in countries without folic acid food fortification, among people who eat few foods of high folate content, and among persons who have a high rate of tissue turnover. We generally consume about as many folates as we need. Furthermore, normal folate stores (which are mainly in the liver) equal only a few weeks of folate needs. Persons who are at the highest risk of a folate deficiency are

those who consume a folate-deficient diet, regularly abuse alcohol, take certain drugs (e.g., methotrexate, trimethoprim, pyrimethamine; see Section 1.2), or produce new cells at a high rate (e.g., children, pregnant women, patients with rapid tumor growth, and patients who have persistent hemolysis or moderate to severe psoriasis).

A shortage of cellular folates af ects primarily the synthesis of **dTMP** (Fig. 36.11; see also Fig. 37.6) and hence DNA replication. T is means that a folate deficiency af ects rapidly dividing tissues; other tissues do not need to make a substantial amount of the thymidine nucleotides. In turn, this explains the principal ef ects of folate deficiency on the body: **megaloblastic anemia** (also called **macrocytic anemia**; see Fig. 36.13) and diarrhea. Any inhibition of DNA replication yields unusually large (megaloblastic, macrocytic), red blood cells (see Chapter 16). An inadequate supply of deoxythymidine triphosphate (dTTP) also limits the rate of red blood cell production and leads to cells of highly variable size, many of which are destroyed in the bone marrow instead of being released into the bloodstream (hence the anemia). Anemia makes patients fatigue early and experience shortness of breath with minor physical activity. A decrease in the number of absorptive epithelial cells in the intestines leads to malabsorption, increased metabolism of remaining nutrients by bacteria, and **diarrhea** when a significant amount of osmotically active compounds reaches the colon (see Chapter 18). T ere is no solid evidence that a folate deficiency impairs the synthesis of **purine nucleotides** in vivo (see Chapter 38).

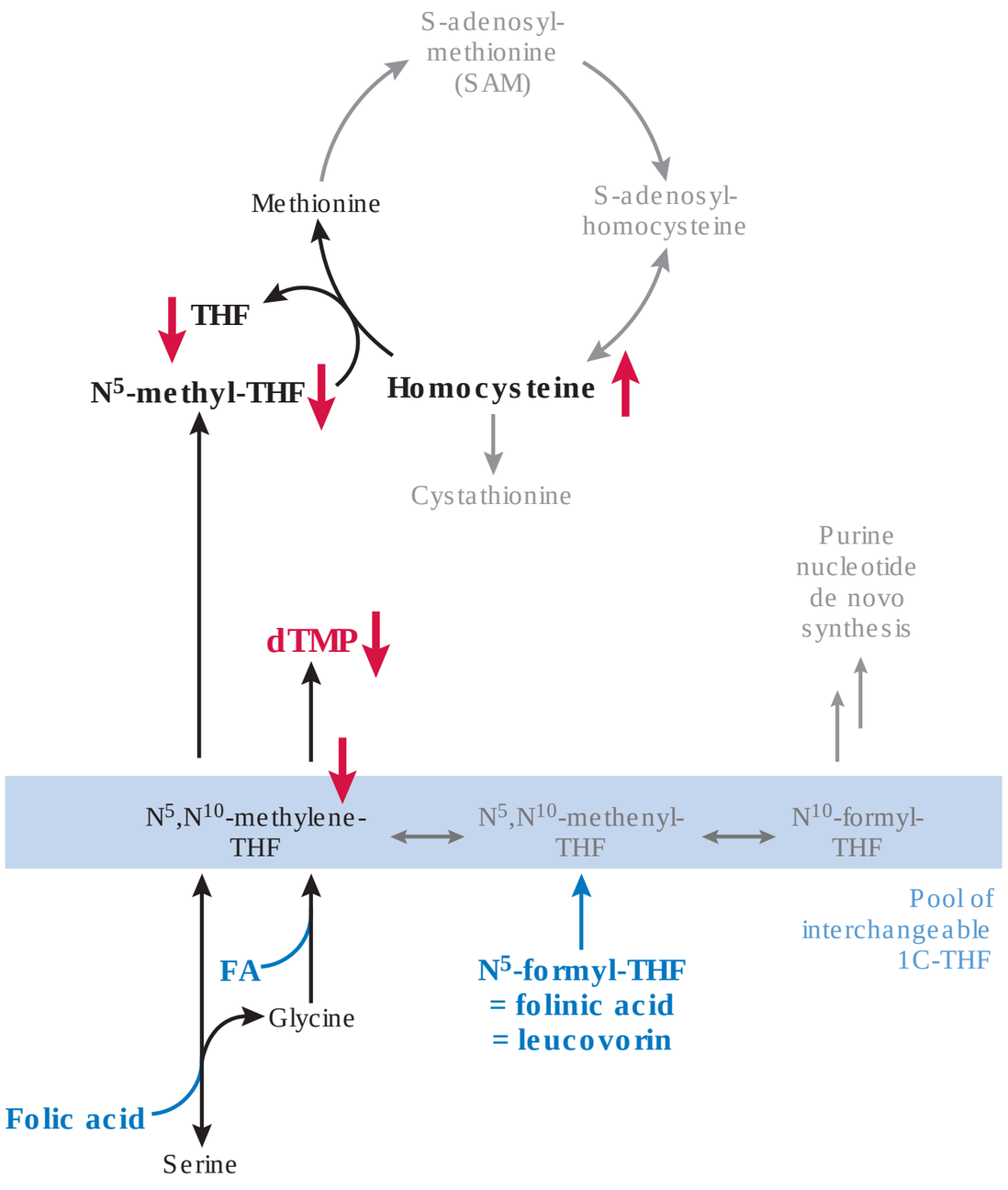


Fig. 36.11 Effects of folate deficiency on one-carbon metabolism. Treatment options are shown in blue. FA, folic acid.

A folate deficiency also leads to a decreased rate of methylation of **homocysteine** (see Fig. 36.11) and hence an increase in the concentration of homocysteine in the blood.

In patients with megaloblastic anemia, a **diagnosis** of folate deficiency is usually made based on finding a decreased concentration of folates in the serum. When necessary, the plasma concentration of homocysteine is also measured, but this test requires special sample handling. Whenever a folate deficiency is found, a cobalamin deficiency must be ruled out by findings of normal serum concentrations of cobalamin and/or methylmalonic acid (see Section 7.2).

A folate deficiency can readily be treated with a supplement of **folic acid**. Instead of folic acid, **N^5-formyl-THF** (**folinic acid**, **leucovorin**) can also be used, either orally or by injection, but this is typically done only in conjunction with antifolate therapy (see Chapter 37). When N^5-formyl-THF enters peripheral cells, it becomes part of the folate pool for thymidine synthesis without having to go through cobalamin-dependent methyl transfer to the activated methyl group cycle.

7.2. Cobalamin Deficiency

A cobalamin deficiency is commonly due to a low dietary intake or an impaired absorption of cobalamin. Dietary sources of cobalamin are listed in Table 36.2, and the normal absorption of cobalamin is described in Section 5. Patients who consume a strict **vegan** diet are at an increased risk of cobalamin deficiency. **Age** is another risk factor, as malabsorption of food-bound cobalamin, sometimes due to insufficient secretion of intrinsic factor, becomes more prevalent among older persons. In developed countries, about 5% of those aged 60 years or older are cobalamin deficient.

Normal **cobalamin stores** amount to about 2 to 3 years of the recommended daily intake of cobalamin. Hence, if a patient develops a deficiency in the uptake of cobalamin, symptoms generally appear only on a scale of months to years later.

T e release of cobalamin from the diet is decreased in patients who secrete an abnormally low amount of **hydrochloric acid** (HCl) in their stomach. For example, patients who take a **proton pump inhibitor** (e.g., omeprazole) for the long-term treatment of gastroesophageal reflux disease (GERD) release an abnormally low fraction of cobalamin from their diet. After several years of drug use, these patients may develop cobalamin deficiency. Long-term use of **H_2-receptor antagonists** (e.g., cimetidine, famotidine, and ranitidine) does not cause a cobalamin deficiency, probably because these drugs are less ef ective at blocking acid secretion. A deficiency in acid secretion does not impair absorption of supplemental cobalamin.

T e absorption of cobalamin into the intestinal epithelium is impaired in patients who do not produce a sufficient amount of **intrinsic factor**. T is occurs in patients whose parietal cells are under attack, either because of **atrophic gastritis** (a chronic inflammation of the stomach mucosa) or **pernicious anemia** (an autoimmune disease of the stomach);

both diseases are most prevalent among the elderly. Tests for pernicious anemia typically involve measurement of antibodies to intrinsic factor. Patients who have undergone **bariatric surgery** to reduce the size of their stomach also secrete less intrinsic factor.

The uptake of the cobalamin-intrinsic factor complex in the **ileum** is impaired in patients who have **chronic ileitis**, **Crohn disease**, **sprue** (often also called celiac disease, a disease of marked intolerance to gluten) or **tropical sprue** (probably due to infection), or whose ileum has been resected. **Imerslund-Gräsbeck syndrome** (also called megaloblastic anemia type I) is recessively inherited, rare, and caused by mutant cubam (mutation either in the CUBN/cubilin or AMN/amnionless gene). Affected patients often show signs of cobalamin deficiency at 2 to 4 years of age.

Patients who for several years take **metformin** to treat type 2 diabetes (see Chapter 39) have a decreased concentration of cobalamin in serum.

The symptoms of cobalamin deficiency include damage to the nervous system and symptoms of a primary folate deficiency. A cobalamin deficiency leads to demyelination of the nervous system; the mechanism for this is unclear. Impaired peripheral nerve conduction can be tested with a **tuning fork** held to the sole of a patient's foot. Demyelination of the central nervous system manifests itself as a gradual onset of **dementia**.

By slowing the rate of methyl group transfer from N^5-methyl-THF to homocysteine, a cobalamin deficiency induces a **secondary folate deficiency**. Because this leads to a low concentration of SAM, methylene-THF reductase becomes more active, thereby funneling folates into the formation of an excessive amount of N^5-methyl-THF (Fig. 36.12). This process is often referred to as the **folate trap** or folate trap hypothesis (the hypothesis is now thought to be correct). Hence, a patient with a cobalamin deficiency not only has a damaged nervous system but may also have a **megaloblastic anemia** (Fig. 36.13) and **diarrhea**.

The laboratory-based diagnosis of cobalamin deficiency relies on a decreased concentration of **total cobalamin** and/or an elevated concentration of **methylmalonic acid** in the serum. The serum cobalamin assay is a screening test that may be diagnostic. The more expensive methylmalonic acid test is definitive. Decreased activity of methylmalonyl-CoA mutase leads to an accumulation of methylmalonyl-CoA (see Fig. 36.12B) and subsequently, an increased concentration of methylmalonic acid in the blood and urine. As in a primary folate deficiency, the concentration of homocysteine in the plasma is elevated due to decreased activity of methionine synthase.

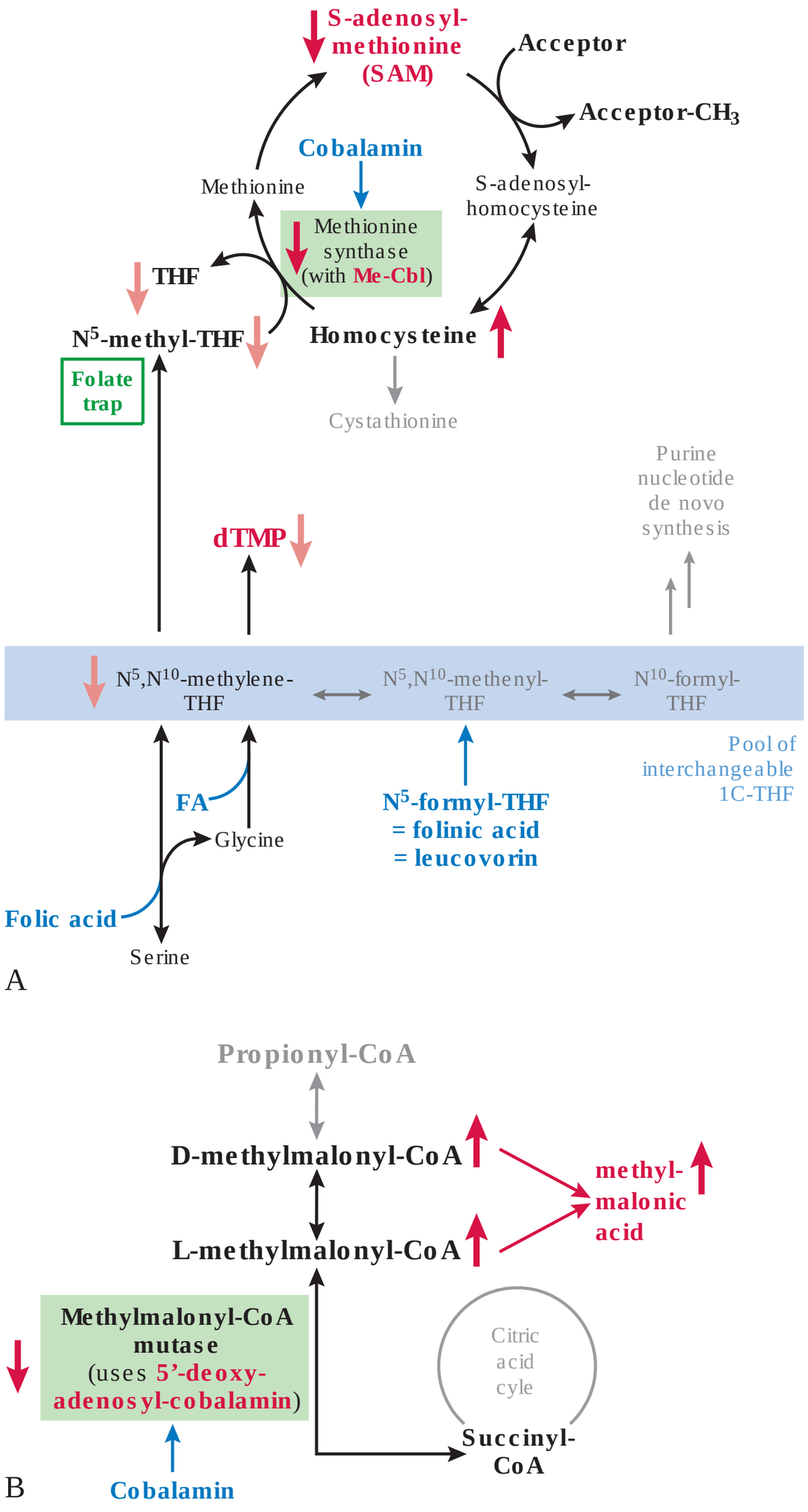


Fig. 36.12 Effects of cobalamin deficiency on one-carbon metabolism and propionyl-CoA metabolism. (A) Cobalamin deficiency often induces a secondary folate deficiency and changes in metabolite concentrations (brown arrows). (B) Cobalamin deficiency leads to an elevated concentration of methylmalonic acid in the blood and urine.

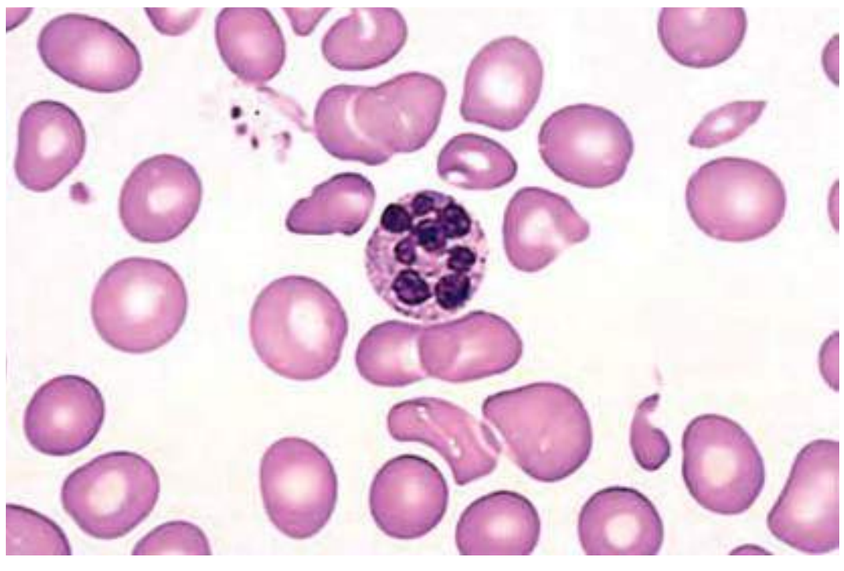


Fig. 36.13 Macrocytosis and hypersegmentation of neutrophils in secondary folate deficiency due to a cobalamin deficiency. Blood smears of primary and secondary folate deficiency are indistinguishable.

A cobalamin deficiency can be treated with supplementary cobalamin. T is is obvious in the case of patients whose diet is cobalamin deficient. Patients who have a deficiency in cobalamin uptake can either be injected intramuscularly with cobalamin approximately monthly, or they can be treated with very large daily oral doses of cobalamin because ~1% of oral cobalamin is absorbed via a process that does not depend on intrinsic factor and cubam.

T ere are several rare inborn errors of cobalamin metabolism. T ese disorders are named cblA through cblG. **cblA** and **cblB** both diminish synthesis of 5′-deoxyadenosyl-cobalamin, the cofactor of methylmalonyl-CoA mutase, causing methylmalonic aciduria. **cblC**, **cblD,** and **cblF** all diminish the synthesis of both methyl-cobalamin and 5′-deoxyadenosyl-cobalamin, the cofactors of methionine synthase and methylmalonyl-CoA mutase, causing both homocystinuria and methylmalonic aciduria. **cblE** and **cblG** are associated with impaired activity of methionine synthase, causing homocystinuria.

8. OTHER DISEASES LINKED TO FOLATES

A low *f*olate status around the time o*f* conception and early pregnancy is associated with an increased risk o*f* neural tube de*f*ects, conotruncal heart disease, cle*ft* lip, and cle*ft* palate. In all adults, low *f*olate status increases the risk o*f* tumorigenesis, yet many tumors grow *f*aster at a high *f*olate status.

8.1. Neural Tube Defects and Other Folate-Dependent Congenital Anomalies

Failure of the neural tube to close during embryogenesis gives rise mainly to **spina bifida** or **anencephaly** (Fig. 36.14). To date, it is unclear how an absolute or relative folate deficiency leads to neural tube defects. In most countries, neural tube defects occur in about 1 in 300 to 1 in 1,700 newborns. Any part of the neural cord may fail to close in the first 3 to 4 weeks of pregnancy, a time when women often do not know that they are pregnant.

In many developed countries, at about 16 to 18 weeks of gestation, the serum concentration of **α-fetoprotein** (**AFP**) of most women is determined to screen for developmental disorders of the fetus. α-Fetoprotein is produced by the liver of the fetus, and some of it crosses into the maternal bloodstream. T e concentration of AFP depends on the gestational age of the fetus. An abnormal concentration of AFP is commonly followed by ultrasound examination of the fetus, repeat determination of AFP, and, if needed, determination of the concentration of AFP in the fluid of the amnion. For neural tube defects, the AFP screening test has approximately 80% sensitivity.

Some newborns with neural tube defects die shortly after birth, and others may have lifelong paralysis. Surgery in utero, shortly after birth, or later can reduce the degree of disability.

Congenital neural tube defects are partially preventable with **supplemental folic acid**. Epidemiological studies revealed that 30% to 70% of the neural tube defects can be prevented with supplements of folic acid given to women who contemplate pregnancy or who have just become pregnant. Accordingly, some countries require that grain products be **enriched with folic acid**. In the United States and Canada, certain grain products (e.g., flour, bread, and pasta) have been fortified with folic acid since about 1998. T is change reduced the birth prevalences of spina bifida and anencephaly by about 30% in the United States and by about 45% in Canada. T e U.S. Food and Nutrition Board recommends that all women of childbearing age, particularly those who contemplate pregnancy, take a daily supplement of 400 µg of folic acid. Women who are pregnant are given a daily supplement of 600 µg folic

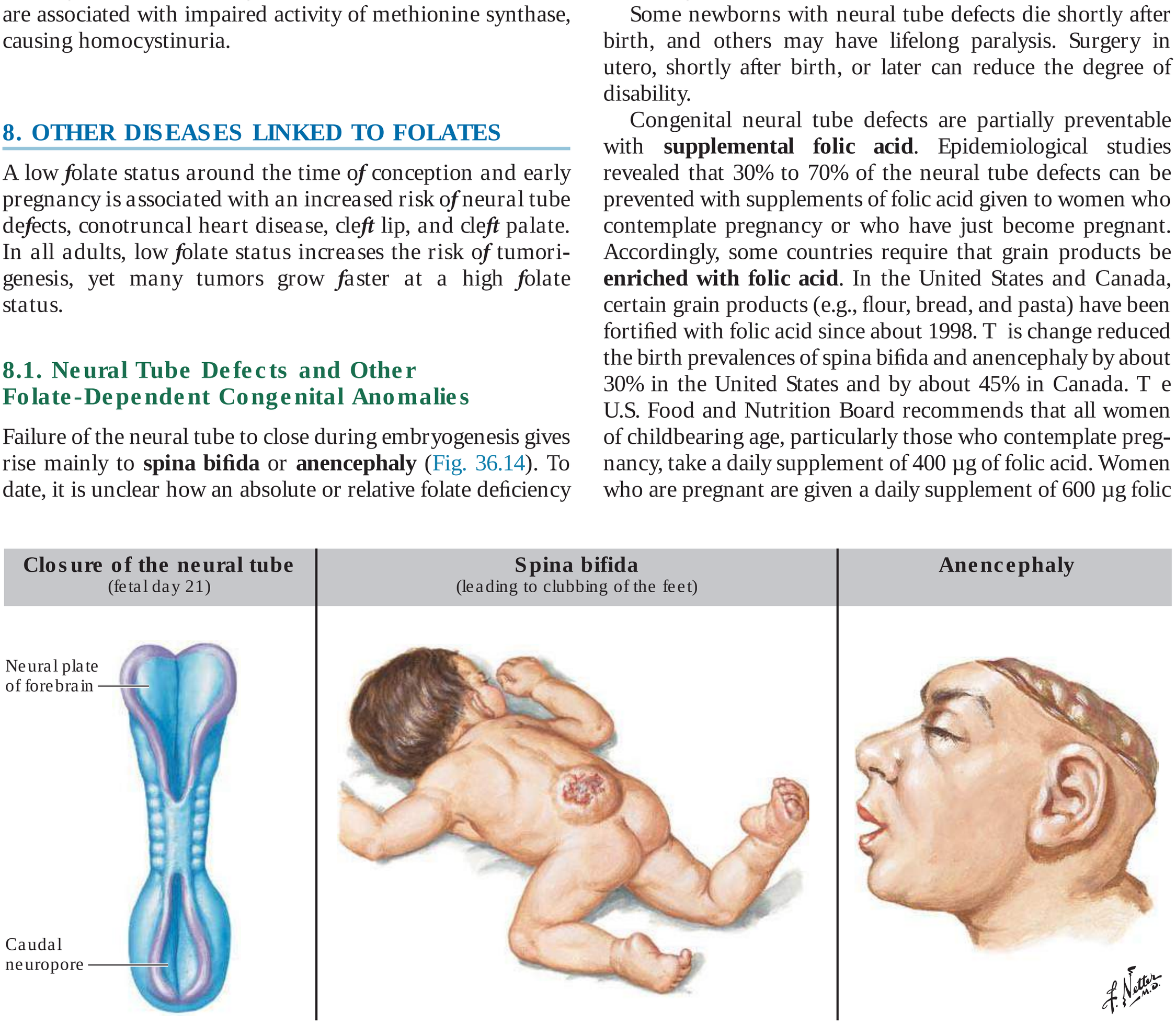


Fig. 36.14 **Normal and abnormal closure of the neural tube.**

acid to accommodate the needs for tissue production in the mother and fetus.

Mothers who are homozygous for the C677T mutation of **N^5,N^{10}-methylenetetrahydrofolate reductase** (the enzyme that reduces N^5,N^{10}-methylene-THF to N^5-methyl-THF) are somewhat more likely to have a child with a neural tube defect. Fetuses who are homozygous for 677T likewise are at an increased risk of a neural tube defect. Among studied populations, 10% to 25% of individuals are homozygous for 677T. T e C677T mutation results in an Ala → Val substitution, which renders the enzyme less stable. When folate intake is low, this mutant enzyme is associated with an increased concentration of homocysteine in the blood.

A low intake of folic acid early in pregnancy is also associated with an increased incidence of **conotruncal heart defects**, **cleft lip**, and **cleft palate**. Cells for the lips and palate arise from the same cells as the neural tube.

Although in the United States a complete **cobalamin deficiency** is rare among pregnant women, women who have a low concentration of cobalamin are more likely to have a child with a neural tube defect, perhaps because of trapping of N^5-methyl-THF (see Section 7.2).

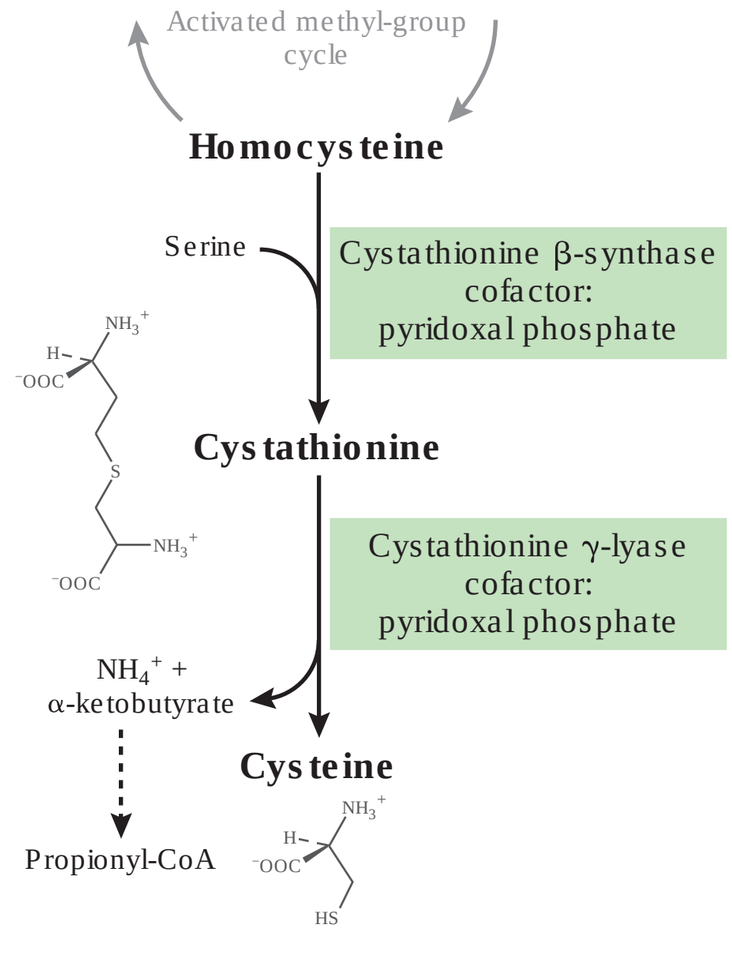


Fig. 36.15 Transsulfuration pathway. Cystathionine γ-lyase is also called cystathioninase or cystathionase. The metabolism of propionyl-CoA to succinyl-CoA is described in Section 6.2 and Fig. 36.10.

8.2. Folates and Cancer

In normal tissues, a low concentration of folates presents an increased risk of neoplastic transformation. In contrast, once a tumor has developed, folate deficiency has the beneficial ef ect of slowing the replication of cells (this is the basis of the ef ect of methotrexate and other antifolate chemotherapeutic agents; see Chapter 37). T e beneficial ef ect of normal folate status before tumorigenesis may relate to normal concentrations of deoxyribonucleotides and SAM, which allow normal DNA replication and repair, as well as normal methylation of DNA and associated proteins.

9. TRANSSULFURATION PATHWAY AND METABOLISM OF CYSTEINE

T e transsul*f*uration pathway is most active in the liver and converts homocysteine to cysteine. Cysteine, in turn, is used *f*or the synthesis o*f* glutathione, taurine, and conjugated bile salts. Catabolism o*f* cysteine yields sul*f*ate, some o*f* which is conjugated with xenobiotics and drugs.

T e transsulfuration pathway commonly refers to the conversion of homocysteine to cysteine (Fig. 36.15). T e pathway is most active in the liver and, to a lesser extent, the kidneys, intestines, and pancreas.

T e enzymes that convert homocysteine to cystathionine and then cysteine require pyridoxal phosphate (a derivative of vitamin B_6). For yet unknown reasons, **cystathionine** is present in the brain at millimolar concentrations and is needed for the proper function of the brain.

A **cystathionine β-synthase deficiency** is the cause of classical **homocystinuria**; patients with this disease have severe hyperhomocysteinemia, mental retardation, osteoporosis in childhood, thromboembolisms in their teens and twenties, subluxation of their lenses before age 30 years, and a reduced life span. T e disease is inherited in autosomal recessive fashion and occurs in 1 of about 50,000 newborns. In addition to homocysteine, the concentrations in the blood of methionine, SAM, S-adenosylhomocysteine, and sarcosine are also elevated. Most patients are treated with a low-methionine diet and given a supplement of cystine. Some patients respond to large doses of vitamin B_6, which gives rise to pyridoxal phosphate, the cofactor of cystathionine β-synthase. Many patients who do not respond to extra vitamin B_6 do respond to supplemental betaine; betaine helps methylate homocysteine to methionine in a reaction that occurs parallel to the one that is catalyzed by methionine synthase.

Cysteine plays a central role in the metabolism of sulfur-containing compounds (Fig. 36.16). Free cysteine is used for the synthesis of **glutathione**, an antioxidant and radical scavenger (see Chapter 21). Many cells contain millimolar concentrations of glutathione, and glutathione also serves as a reservoir for cysteine. T e concentration of cysteine is the rate-limiting factor in the synthesis of glutathione.

Free cysteine itself, together with iron, forms toxic free radicals, and the intracellular concentration of cysteine is kept relatively low (~<0.2 mM). To this end, cysteine is degraded into cysteine sulfinate, which is either degraded further or used for the synthesis of **taurine** (see Fig. 36.16). Taurine is an amino acid with which bile acids are conjugated to increase their hydrophilicity (see Chapter 29). Some cells produce taurine to compensate for extracellular hyperosmolality. Otherwise, the degradation of cysteine yields sulfate, which can react with ATP to form **3′-phosphoadenosine 5′-phosphosulfate** (**PAPS**). PAPS can be used to sulfate proteins (see Chapters 7 and 13), steroids, or xenobiotics, thereby making them more water soluble.

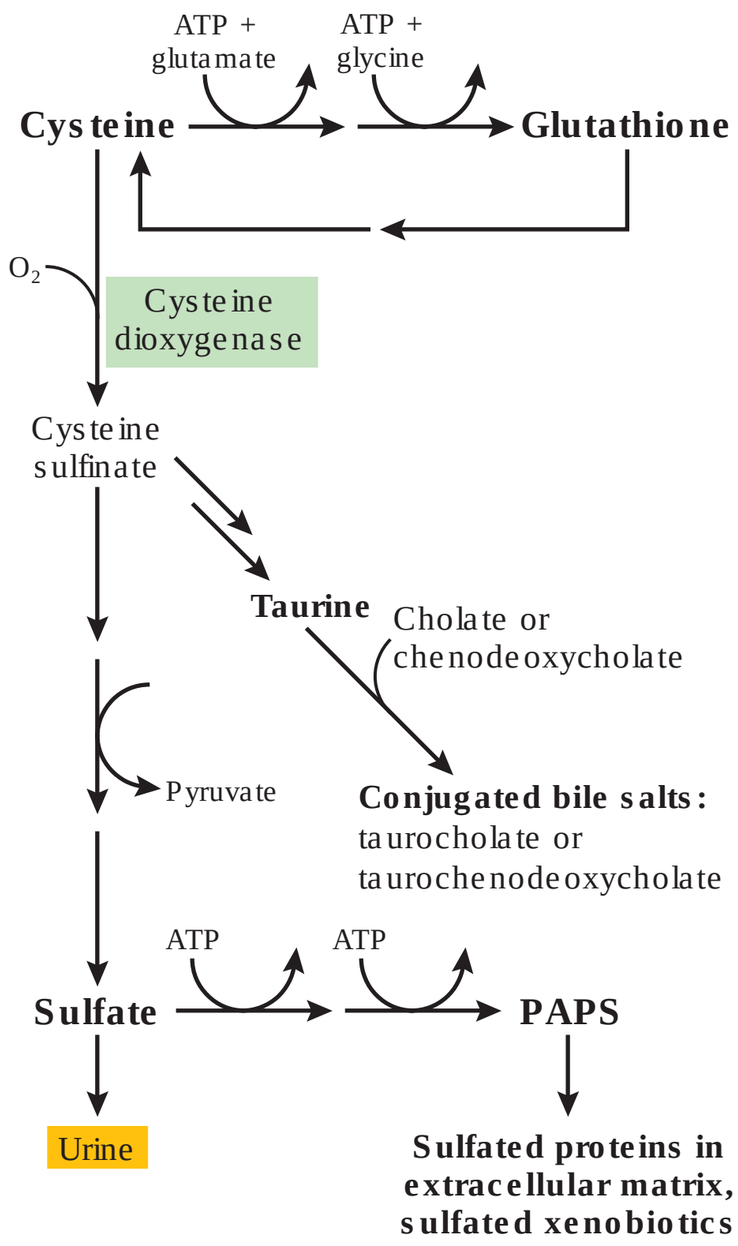


Fig. 36.16 **Role of cysteine in metabolism.**

SUMMARY

- The term *f*olates includes folic acid, dihydrofolic acid (DHF), tetrahydrofolic acid (THF), N^5-methyl-THF, N^5,N^{10}-methylene-THF, N^5,N^{10}-methenyl-THF, N^5-formyl-THF, N^{10}-formyl-THF, and N^5-formimino-THF, regardless of the length of the polyglutamate tail.
- Folates are found in legumes, green leafy vegetables, citrus fruit, and grains. In some countries, grain products are fortified with folic acid, a vitamin. Folic acid enters the intestine via the proton-coupled folate transporter (PCFT). DHF reductase then reduces folic acid to THF. THF is loaded with a one-carbon group from serine or glycine and exported as N^5-methyl-THF.
- Peripheral cells take up N^5-methyl-THF via the RFC or endocytosis of folate receptors. The PCFT is involved in transporting folates from the blood into the brain. Once inside a cell, N^5-methyl-THF must donate its methyl group to homocysteine in the activated methyl group cycle; the resulting THF receives a polyglutamate tail and can then accept a one-carbon group and become part of the general one-carbon-THF pool.
- The methyl transfer from N^5-methyl-THF to homocysteine is catalyzed by methionine synthase, which uses methylcobalamin as a cofactor. In cobalamin-deficient patients, this reaction is impaired such that cells have a reduced capacity to gain THF from N^5-methyl-THF in the bloodstream. Hence, cobalamin deficiency often leads to a secondary folate deficiency.
- One-carbon loaded THFs are primarily required for the de novo synthesis of inosine monophosphate (IMP, from which AMP and GMP are made) and the conversion of deoxyuridine monophosphate (dUMP) to dTMP. In folate-deficient patients, only the synthesis of dTMP is decreased in a pathogenic manner, thereby slowing the replication of cells in the bone marrow and intestinal epithelium; this causes megaloblastic anemia and diarrhea.
- Patients who have megaloblastic anemia most often have a primary or secondary folate deficiency. Laboratory data that support a primary folate deficiency include a low concentration of folate and a normal concentration of cobalamin and methylmalonic acid in the serum. A secondary folate deficiency can be caused by a cobalamin deficiency via decreased activity of methionine synthase and the trapping of most folates as N^5-methyl-THF. Laboratory data that support a cobalamin deficiency are a low concentration of cobalamin and an elevated concentration of methylmalonic acid in the serum. Because a cobalamin deficiency damages the nervous system and high doses of folates can cure the megaloblastic anemia, it is important that folate-deficient patients be tested for cobalamin deficiency and given cobalamin if needed. A folate deficiency is treated with oral folic acid or with oral or injected leucovorin.
- Several conditions can lead to cobalamin deficiency: decreased acid secretion by the stomach (which leads to decreased liberation of dietary cobalamin) due to the chronic use of a proton pump inhibitor for gastroesophageal reflux disease; reduced numbers of intrinsic factor–secreting parietal cells owing to atrophic gastritis, pernicious anemia, or bariatric surgery; and impaired absorption of the cobalamin-intrinsic factor complex via cubam in the ileum due to chronic inflammation or surgical resection of the ileum.
- The activated methyl group cycle generates S-adenosylmethionine (SAM), which is used as a methyl donor for almost all methylation reactions in the body. Most of the SAM is needed for the synthesis of creatine (to make up for the loss of creatinine), phosphatidylcholine, and plasmenylcholine. The remaining SAM is used for the synthesis of epinephrine from norepinephrine, and by numerous enzymes that methylate lysine or arginine residues in proteins, cytosine bases in DNA, or guanine bases in RNA.
- The degradation of methanol by alcohol dehydrogenase and formaldehyde dehydrogenase yields formate, which must be removed via N^{10}-formyl-THF. Methanol poisoning causes the transient accumulation of millimolar concentrations of formate in the blood, which can cause severe metabolic acidosis as well as severe damage to the optic nerve. The accumulation of formate can be prevented with fomepizole (an inhibitor of alcohol dehydrogenase) or with ethanol (another substrate of alcohol dehydrogenase). In patients poisoned with ethylene glycol, fomepizole or ethanol are used in a similar manner.
- The transsulfuration pathway converts homocysteine to cysteine. This pathway is most active in the liver.

- Free cysteine is toxic. Cysteine is needed for the synthesis of glutathione, which is used for diverse redox reactions. Degradation of glutathione yields cysteine, and glutathione thus also serves as a reservoir for cysteine. When cysteine is metabolized, it can give rise to taurine, which serves as an intracellular osmolyte and which the liver conjugates with bile acids. The degradation of cysteine yields sulfate, which can be activated to 3′-phosphoadenosine 5′-phosphosulfate (PAPS) for the sulfation of xenobiotics or extracellular matrix proteins.

FURTHER READING

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- Quadros EV. Advances in the understanding of cobalamin assimilation and metabolism. Br J Haematol. 2010;148: 195-204.
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Review Questions

1. A 2½-year-old child is treated for methanol poisoning. She is given an intravenous infusion of ethanol. In addition, intravenous administration of which one of the following would be most appropriate to prevent damage to her optic nerve?

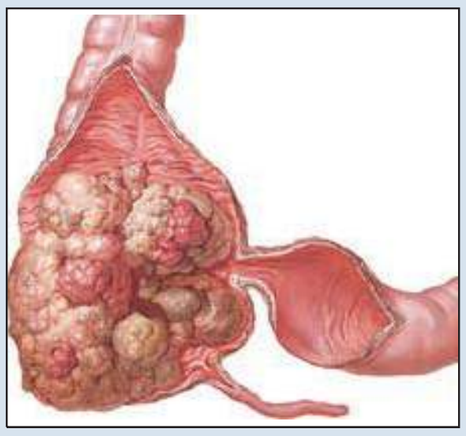
   A. Biotin
   B. Cobalamin
   C. Leucovorin
   D. Niacin
   E. Thiamine

2. An anemic patient is found to have a low serum folate level and a serum cobalamin level that is inconclusive. A common test to distinguish primary folate deficiency from cobalamin-induced secondary folate deficiency involves measuring which of the following?

   A. Red blood cell folate
   B. Serum antibodies to haptocorrin (R-binder)
   C. Serum homocysteine
   D. Serum methylmalonic acid

3. Many patients who have type 2 diabetes are treated with the drug metformin. Based on preliminary data, investigators hypothesized that cobalamin deficiency is a side effect of metformin treatment. Serum of metformin-treated and untreated control diabetic patients was analyzed. A significantly elevated concentration in metformin-treated patients of which one of the following metabolites would be the strongest indication that the hypothesis is correct?

   A. Homocysteine
   B. Methionine
   C. Methylmalonate
   D. S-adeonosylmethionine



Chapter

37 Pyrimidine Nucleotides and Chemotherapy

SYNOPSIS

- A pyrimidine nucleotide contains a uracil (U), cytosine (C), or thymine (T) base and a ribose or deoxyribose.
- A person needs to synthesize about half a gram of pyrimidine nucleotides per day. The main clinical interest in pyrimidine nucleotides is derived from the fact that DNA replication and repair are the only processes that depend on the production of deoxythymidine triphosphate (dTTP).
- Persons who are folate deficient synthesize an inadequate amount of deoxythymidine monophosphate (dTMP) and dTTP (see Chapter 36).
- One way of inhibiting the growth of a tumor is to deprive its cells of dTTP (see Fig. 37.1). This can be achieved by inhibiting the enzyme that makes dTMP with 5F-deoxyuridine monophosphate (5F-dUMP) or with pemetrexed. It can also be achieved by inhibiting the reduction of dihydrofolate (DHF) to tetrahydrofolate (THF) with the antifolate methotrexate.
- The deoxyribonucleotides deoxyuridine diphosphate (dUDP), deoxycytidine diphosphate (dCDP), deoxyadenosine diphosphate (dADP), and deoxyguanosine diphosphate (dGDP) are made from the respective ribonucleotides by a single enzyme (see Fig. 37.1). This enzyme can be inhibited by hydroxyurea or the active metabolite of gemcitabine. Gemcitabine and hydroxyurea are used for the treatment of a variety of cancers. Hydroxyurea is also used to reduce the frequency of sickle cell crises in patients who have a sickle cell disease.
- Pyrimidine nucleotides are also used in the synthesis of RNAs, phospholipids, and glycogen, as well as for glucuronidation (a detoxification reaction). For the most part, nucleotides used in these processes are recycled.

LEARNING OBJECTIVES

For mastery *of* this topic, you should be able to do the *f*ollowing:

- Describe the biosynthesis of pyrimidine nucleotides with emphasis on the key regulated steps.
- Explain the relationship between folates and thymidine synthesis.
- Describe the ribonucleotide reductase reaction and its regulation, and explain its role in cancer chemotherapy with hydroxyurea or gemcitabine.
- Compare and contrast the effects of 5-fluoruracil, pemetrexed, and methotrexate on the synthesis of thymidine, paying special attention to the mechanisms of action.

1. DE NOVO SYNTHESIS OF URIDINE MONOPHOSPHATE, A PRECURSOR FOR ALL PYRIMIDINE NUCLEOTIDES

De novo synthesis o*f* a precursor *f*or all pyrimidine nucleotides, uridine monophosphate (UMP), takes place mainly in the liver and in dividing cells. Use o*f* a ribose *f*rom the pentose phosphate pathway yields phosphoribosylpyrophosphate (PRPP). Synthesis o*f* a pyrimidine base starts with bicarbonate and yields orotate. PRPP and orotate then give rise to UMP. T e liver converts UMP to uridine and releases uridine into the blood. From there, other cells can pick up uridine and phosphorylate it to UMP.

T e synthesis of pyrimidine nucleotides involves the following steps: (1) synthesis of PRPP; (2) synthesis of the base orotate; (3) the combination of PRPP and orotate to form the nucleotide UMP; (4) conversion of UMP to cytidine triphosphate (CTP); (5) the reduction of ribonucleotides to deoxyribonucleotides; and (6) the conversion of dUMP to dTMP. T is section deals only with the synthesis of UMP.

Pyrimidine nucleotides are needed for the synthesis of **DNA**, **RNA**, **phospholipids**, and **glycogen**, as well as for **glycosylation** and **glucuronidation** reactions. DNA replication and repair require dCTP and dTTP (see Chapters 2 and 3). RNA synthesis requires UTP and CTP (see Chapter 6). Phospholipid de novo synthesis needs CTP. Glycogen synthesis, glycosylation reactions, and glucuronidation reactions require UTP (see Chapters 7, 14, and 24). DNA is degraded only at a low rate and yields dCMP and dTMP, which can be reused (see Section 6). Similarly, RNA degradation yields CMP and UMP, which can be reused (see Section 6).

Most pyrimidine nucleotide synthesis takes place in the **liver** and in **dividing cells,** such as intestinal epithelial cells, erythropoietic cells in the bone marrow, and tumor cells. T e liver provides a service to other cells by synthesizing uridine and releasing it into the blood. Other cells then take up uridine and phosphorylate it to UMP. Total daily pyrimidine nucleotide synthesis amounts to about 0.5 g per day, comparable to the de novo synthesis of purine nucleotides (see Chapter 38).

PRPP is a phosphorylated ribose that plays a role in the production of all nucleotides and that cells synthesize only when they have normal energy production (see Fig. 37.3). **PRPP synthetase** makes PRPP from ribose 5-phosphate, an intermediate of the pentose phosphate pathway (see Chapter 21). PRPP synthetase is active when the concentration of **phosphate** is near normal and that of **ADP** is low. PRPP is also used for the salvage of purine bases and the de novo synthesis of purine nucleotides (see Chapter 38).

Orotate is synthesized mostly in response to a need for **DNA replication** or **repair** (Fig. 37.2). T e rate-limiting enzyme for orotate synthesis is the carbamoyl-phosphate synthase II moiety of CAD, a trifunctional enzyme. PRPP feed forward activates carbamoyl-phosphate synthase activity. At

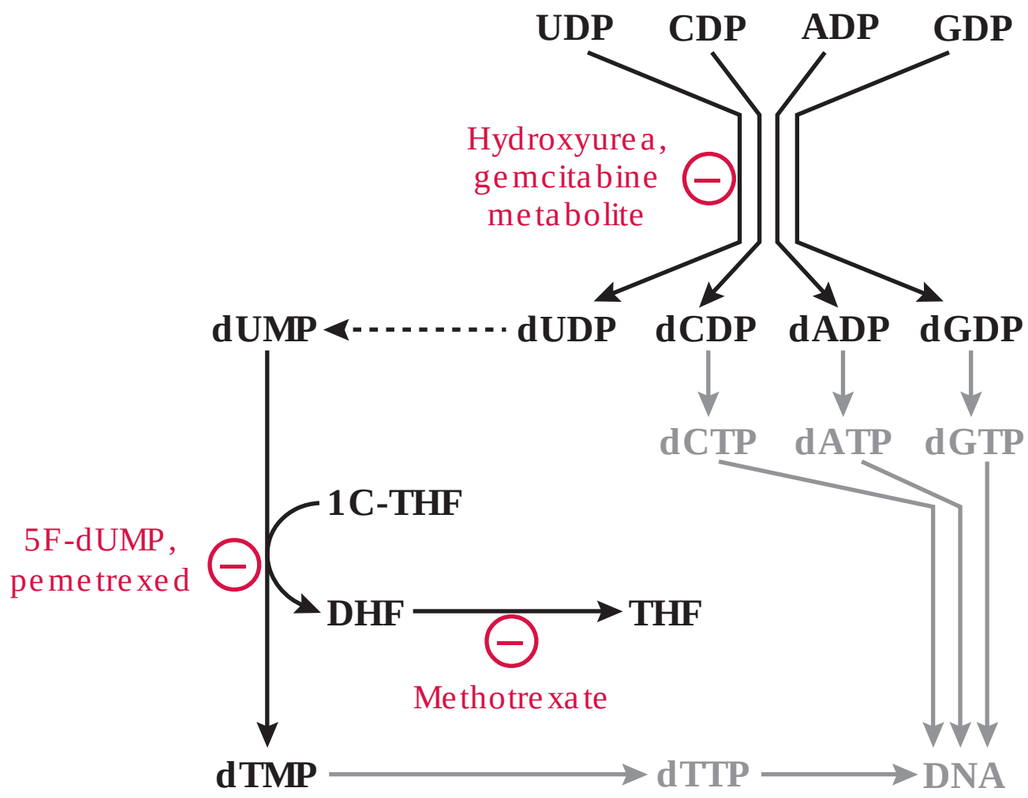


Fig. 37.1 Overview of clinical interference with pyrimidine nucleotide metabolism.

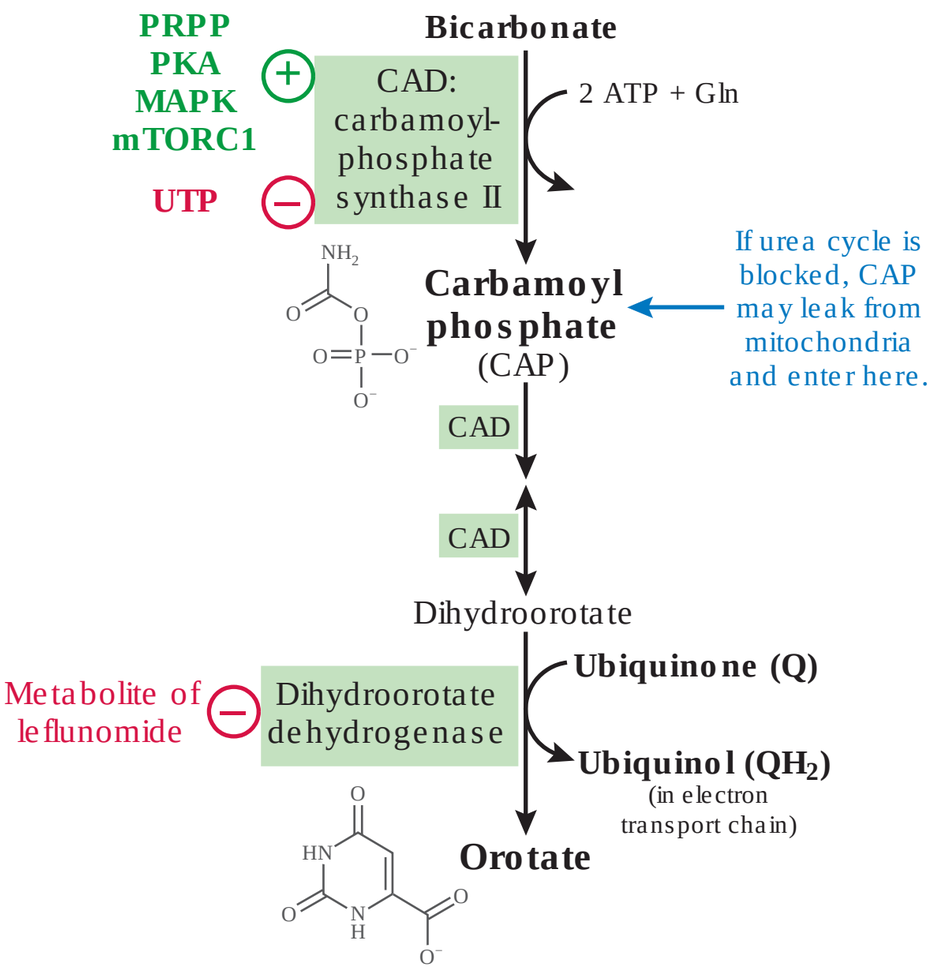


Fig. 37.2 Synthesis of orotate. CAD, carbamoyl-phosphate synthase II/aspartate carbamoyl-transferase/dihydroorotase (a trifunctional enzyme); Gln, glutamine; MAPK, mitogen-activated protein kinase; PKA, protein kinase A; PRPP, phosphoribosylpyrophosphate. Ubiquinone is also called coenzyme Q. Leflunomide is used in the treatment of rheumatoid arthritis.

rest (in the G_0 phase of the cell cycle), orotate synthesis is mostly limited by feedback inhibition from UTP. When cells synthesize DNA, active protein kinase A (PKA), MAP kinase (MAPK), and mTORC1 (see Chapters 8 and 34) lead to the phosphorylation of CAD. Phosphorylated CAD is less sensitive to UTP feedback, allowing for increased production of orotate and hence pyrimidine nucleotides.

T e carbamoyl-phosphate synthase II moiety of CAD carries the number II because a dif erent carbamoyl-phosphate synthase (I) was first discovered in mitochondria. Carbamoyl-phosphate synthase I produces carbamoylphosphate (CAP) for the urea cycle (see Chapter 35).

In patients who have a disease of the **urea cycle** (see Fig. 35.12 and Section 3.3 in Chapter 35) that leads to the accumulation of CAP, CAP leaks from the mitochondria into the cytosol. T is leaked CAP bypasses the regulation of the activity of carbamoyl phosphate synthase II. Leaked CAP therefore gives rise to excess **orotate** via the reactions shown in Fig. 37.2. T e excess orotate spills into the blood and the urine. T e concentration of orotate in the urine is used to determine the cause of urea cycle defects.

T e early steps in orotate synthesis take place in the **cytosol** and **nucleus**, but the final step takes place in the intermembrane space of **mitochondria**. **Dihydroorotate dehydrogenase** is an integral protein of the inner mitochondrial membrane that oxidizes dihydroorotate in the intermembrane space. T ereby, the enzyme reduces ubiquinone in the membrane to ubiquinol, which is part of the electron transport chain (see Fig. 23.3). T is reaction is not an important contributor to oxidative phosphorylation.

Leflunomide is used as a disease-modifying antirheumatic drug (DMARD) for the treatment of the autoimmune disease **rheumatoid arthritis** (see Fig. 37.10 and Section 5.3). In the body, leflunomide is metabolized to teriflunomide, which inhibits dihydroorotate dehydrogenase and thus leads to a deficiency of pyrimidine nucleotides, particularly in lymphocytes.

UMP synthase joins **PRPP** with **orotate** to generate **UMP** (Fig. 37.3). UMP synthase is found both in the cytosol and in the nucleus. A hereditary **deficiency** of UMP synthase, which is rare, causes an orotic aciduria (also compare to the above discussion of urea cycle defects). T e deficiency can be treated with uridine supplementation.

T e liver dephosphorylates UMP to **uridine** and releases uridine into the bloodstream; from there, peripheral cells take up uridine (see Fig. 37.3). Uridine and other unphosphorylated nucleosides cross cell membranes through **nucleoside transporters**, some of which of er facilitated passive dif usion, whereas others actively pump nucleosides. Peripheral cells then phosphorylate uridine to UMP, a process that is also called **salvage**. Apart from biosynthesis in the liver, uridine can also stem from the degradation of RNA in other cells (see Section 6 and Fig. 37.12).

A **deficiency of pyrimidine 5′-nucleotidase** is accompanied by hemolytic anemia. T e deficiency is usually a consequence of the direct inhibition of the enzyme by Pb^{2+} in **lead poisoning**; an inherited deficiency is rare. It is unclear how the deficiency is detrimental to erythrocytes.

Chemotherapeutic and antiviral drugs that are analogs of normal pyrimidine nucleosides enter cells through transporters for uridine or thymidine. Inside cells, kinases triphosphorylate these drugs. Examples of such chemotherapeutic drugs are **5-fluorouracil** and **gemcitabine** (2′,2′-difluoro-2′-deoxycytidine). 5-Fluoruracil is discussed in Section 5 and gemcitabine in Section 3. Examples of antiviral drugs taken up via nucleoside transporters are **zidovudine** (**AZT**; 3′-azido-2′,3′-dideoxythymidine) and **stavudine** (2′,3′-didehydro-2′,3′-dideoxythymidine).

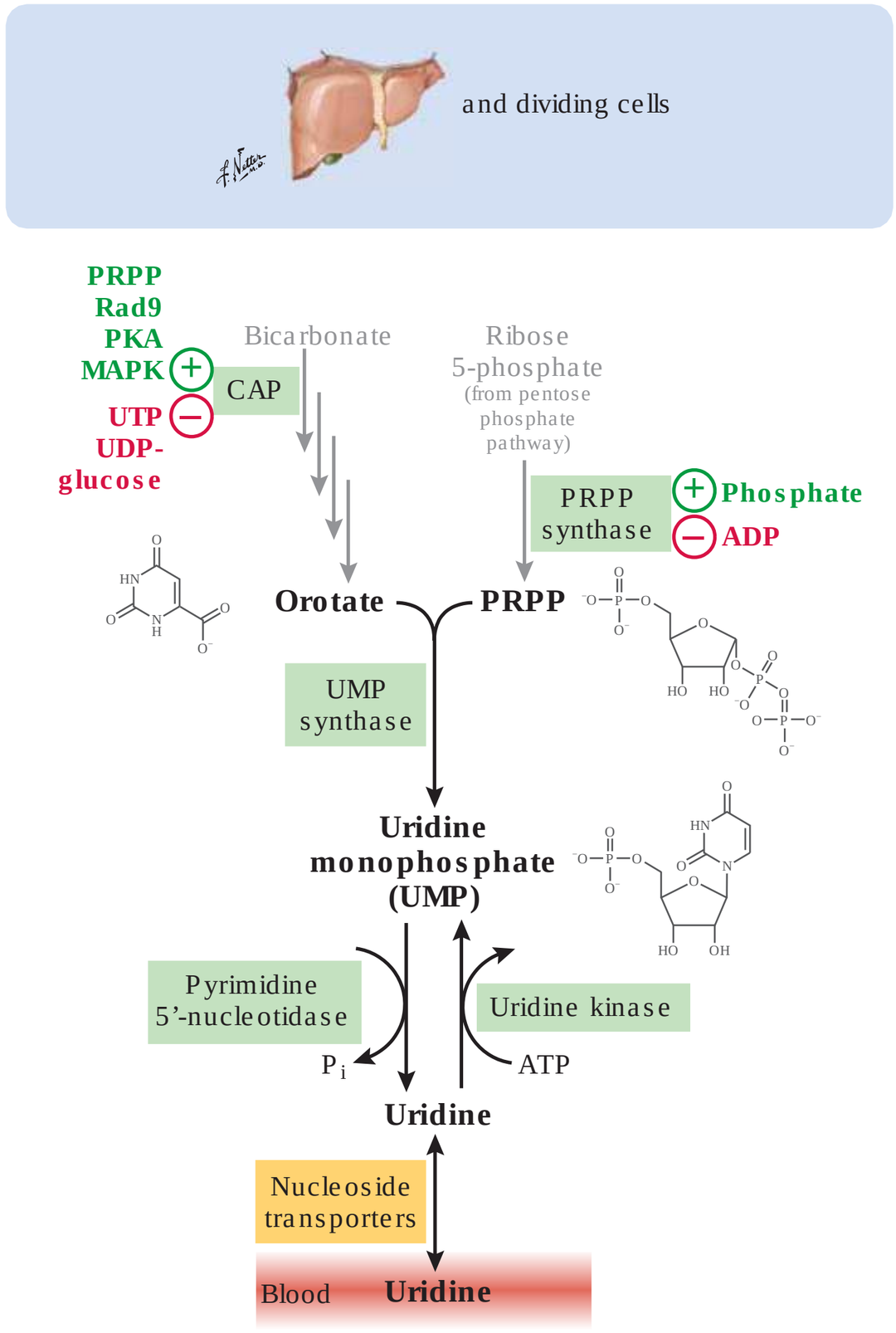


Fig. 37.3 **Synthesis of UMP from orotate**. The liver exports uridine and other cells import it from the blood. ADP, adenosine diphosphate; CAP, carbamoyl phosphate; MAPK, mitogen-activated protein kinase; PKA, protein kinase A; PRPP, phosphoribosylpyrophosphate; UDP, uridine diphosphate; UTP, uridine triphosphate; Rad9 is a protein that plays a role in DNA repair and cell cycle control.

2. SYNTHESIS AND USES OF UTP AND CTP

Cells phosphorylate uridine and UMP to U*T*P. U*T*P gives rise to C*T*P. U*T*P and C*T*P are used *f*or the synthesis o*f* RNA, phospholipids, and *f*or UDP-glucose, which is used in a variety o*f* processes.

Uridine is phosphorylated to UTP, which can be converted to CTP (Fig 37.4; see also Fig. 37.3). Averaged over an entire cell (i.e., disregarding compartmentation), the concentration of UTP is on the order of 0.3 mM (i.e., about one-tenth the concentration of ATP). T e concentration of CTP is about 0.1 mM.

T e chemotherapeutic drugs gemcitabine (see Section 3) and 5-fluorouracil (see Section 5.1) are phosphorylated by the same enzymes as the uridine and cytidine nucleotides.

UTP gives rise to **UDP-glucose**, which is used for several dif erent processes. UDP-glucose is used in **glycogen** synthesis (see Figs. 24.1 and 24.5); in the synthesis of **lactose** in the

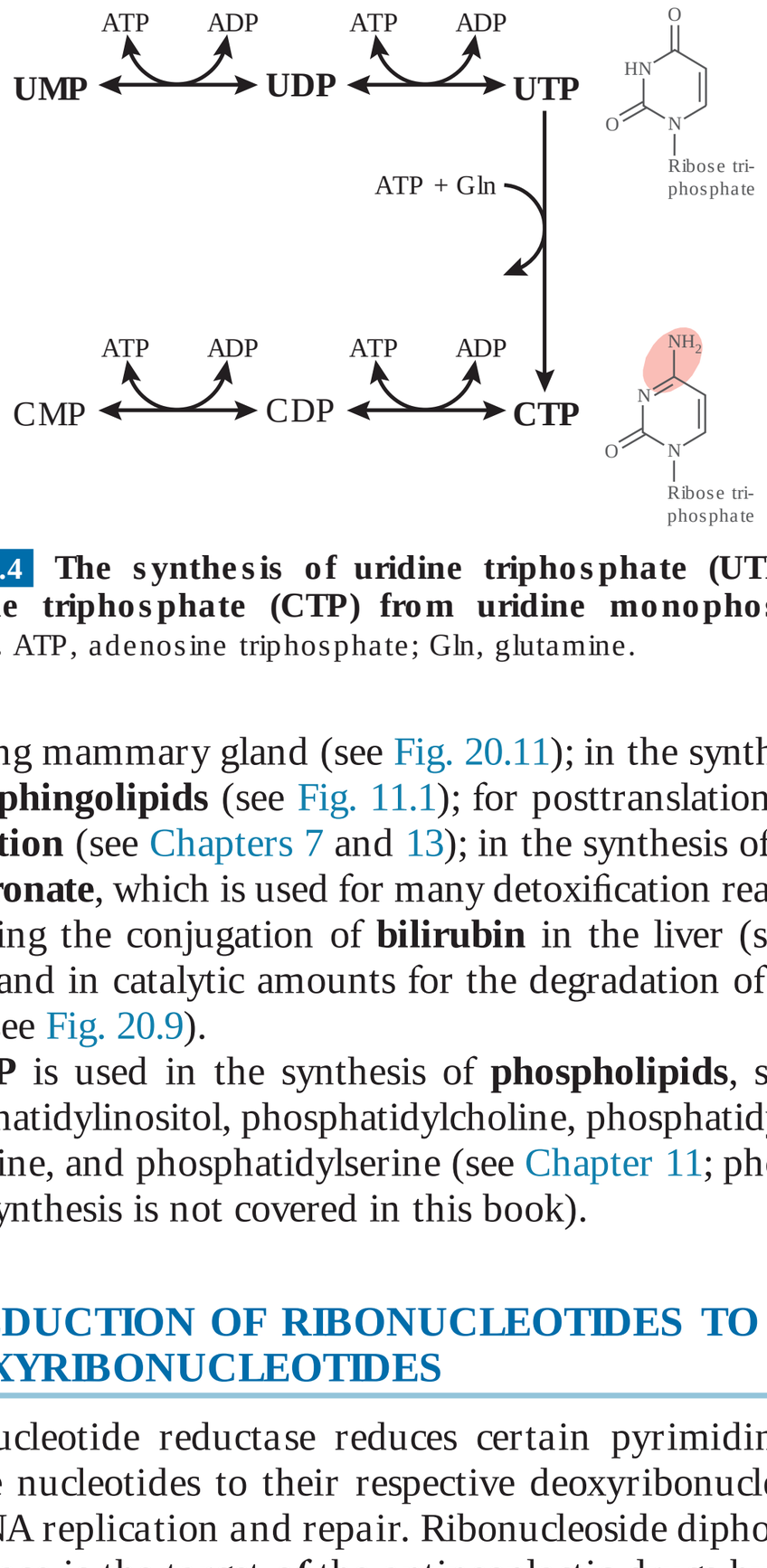


Fig. 37.4 **The synthesis of uridine triphosphate (UTP) and cytidine triphosphate (CTP) from uridine monophosphate (UMP).** ATP, adenosine triphosphate; Gln, glutamine.

lactating mammary gland (see Fig. 20.11); in the synthesis of **glycosphingolipids** (see Fig. 11.1); for posttranslational **glycosylation** (see Chapters 7 and 13); in the synthesis of **UDP-glucuronate**, which is used for many detoxification reactions, including the conjugation of **bilirubin** in the liver (see Fig. 14.7); and in catalytic amounts for the degradation of **galactose** (see Fig. 20.9).

CTP is used in the synthesis of **phospholipids**, such as phosphatidylinositol, phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine (see Chapter 11; phospholipid synthesis is not covered in this book).

3. REDUCTION OF RIBONUCLEOTIDES TO DEOXYRIBONUCLEOTIDES

Ribonucleotide reductase reduces certain pyrimidine and purine nucleotides to their respective deoxyribonucleotides *f*or DNA replication and repair. Ribonucleoside diphosphate reductase is the target o*f* the antineoplastic drugs hydroxyurea and gemcitabine.

Ribonucleoside-diphosphate reductase (also called **ribonucleotide reductase**) catalyzes the reduction of ribonucleotides to deoxyribonucleotides in the **cytosol** (Fig. 37.5). T is reduction physiologically occurs only with the nucleoside diphosphates UDP, CDP, ADP, and GDP. Ribonucleotide reductase activity is regulated allosterically by nucleotide binding to two distinct sites (activity sites and specificity sites), by transcription, and by regulatory proteins.

In **quiescent** cells, the concentration of deoxyribonucleotides is extremely low. During **DNA replication** or **repair**, the concentration of deoxyribonucleotides is relatively high but still lower than the concentration of ribonucleotides. T anks to two dif erent subunit compositions, the ribonucleotide reductase holoenzyme is active only during DNA replication or only during DNA repair.

Ribonucleotide reductase uses **thioredoxin** to reduce ribonucleotides to deoxyribonucleotides. **T ioredoxins** are proteins of about 100 amino acids that contain a Cys-X-X-Cys active-site sequence (X can be any amino acid). In reduced thioredoxin, the Cys side chains are free (i.e., –SH). In oxidized thioredoxin (i.e., thioredoxin disulfide), the two active-site

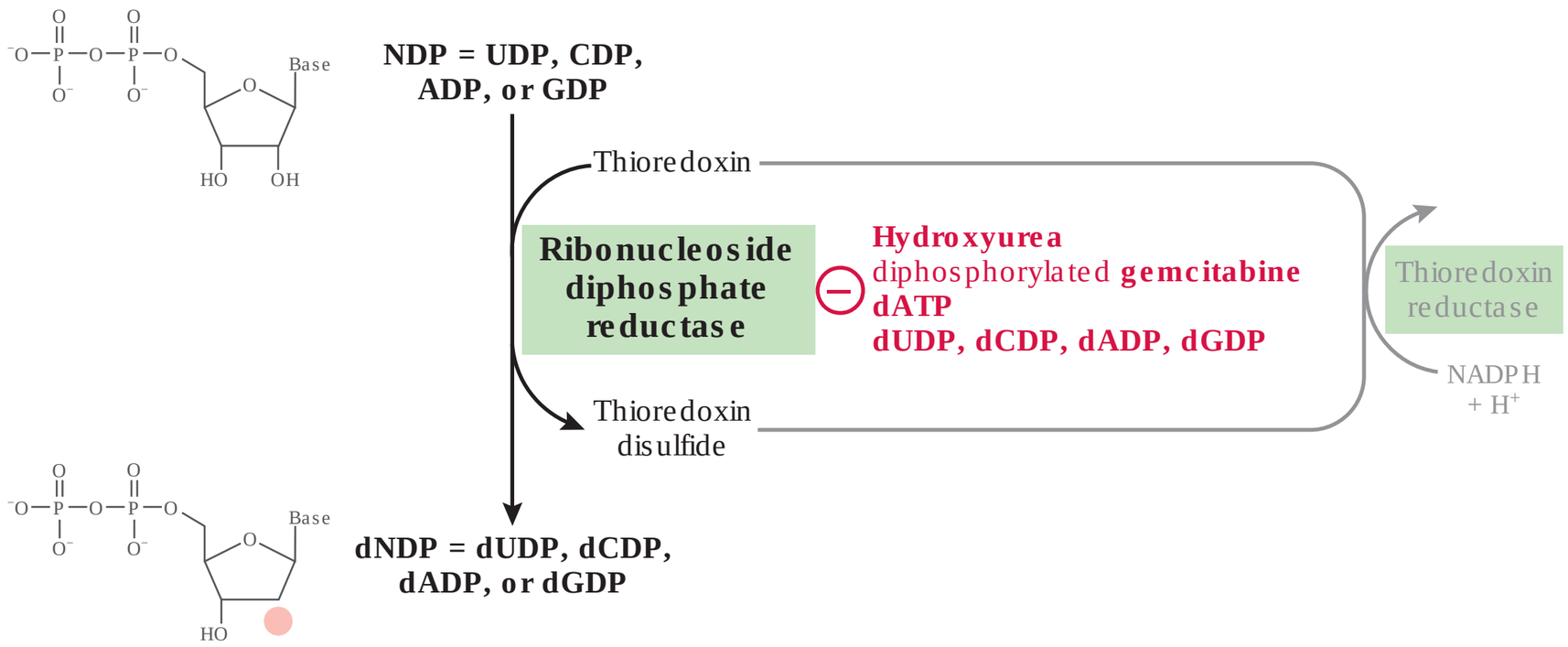


Fig. 37.5 The reduction of ribonucleotides to deoxyribonucleotides. ATP, dTTP, dATP, and dGTP regulate the *substrate* specificity of ribonucleotide reductase (not shown). NDP, nucleoside diphosphate.

cysteine side chains form a disulfide bridge. Besides the reduction of ribonucleotides, thioredoxins also play a role in the reestablishment of –SH groups in proteins, antioxidant defense, immune defense, and apoptosis.

T ioredoxin reductases use NADPH to reduce thioredoxin disulfides to thioredoxins (see Fig. 37.5). T ioredoxin reductases contain **selenocysteine** in their active site (see Section 1.1 in Chapter 9). T ioredoxin reductase activity is increased in many cancer cells.

Hydroxyurea and **gemcitabine** are chemotherapeutic drugs that lead to a decreased rate of deoxyribonucleotide production (see Fig. 37.5). Hydroxyurea ($H_2N–CO–NHOH$) inhibits ribonucleotide reductase by reducing a protein tyrosyl radical that is crucial for enzyme activity. Hydroxyurea is used for patients who have **polycythemia vera** and are at high risk of thrombosis. Polycythemia vera is due to an abnormally increased red blood cell production. T e disease is commonly treated with phlebotomy. Hydroxyurea also plays a role in the treatment of several other forms of cancer. Finally, hydroxyurea is used in patients with **sickle cell anemia** to decrease the incidence of vasoocclusive episodes. Hydroxyurea decreases sickling by leading to enhanced synthesis of hemoglobin F (attributed in part to nitric oxide that is released from hydroxyurea). Diphosphorylated gemcitabine (2′,2′-difluorodeoxycytidine diphosphate) inhibits ribonucleotide reductase irreversibly. In addition, triphosphorylated gemcitabine is incorporated into DNA and thereby inhibits chain elongation. Gemcitabine is used in the treatment of metastatic **breast cancer**, certain stages of **non–small cell lung cancer**, advanced **ovarian cancer**, advanced **pancreatic cancer**, and a number of other tumors.

4. SYNTHESIS OF dTMP

dUMP can *f*orm by various pathways. T ymidine synthase uses a one-carbon tetrahydro*f*olate to methylate dUMP to d*T*MP.

Sometimes, dTMP, dTDP, and dTTP are abbreviated simply as TMP, TDP, and TTP, since only the thymidine deoxyribonucleotides play a known physiological role. Similarly, deoxythymidine is usually abbreviated to thymidine. For clarity, use of the prefixes "d" or "deoxy" is preferable.

dUDP, dUTP, or dCMP can give rise to **dUMP** (see Fig. 37.6), although little is known about the physiological relevance of these reactions.

Thymidylate synthase uses **N^5,N^{10}-methylene-THF** to reduce and methylate dUMP to **dTMP** (see Fig. 37.6). T e reaction produces **DHF**. **DHF reductase** reduces DHF to THF (see Fig. 36.2), which is subsequently converted to N^5,N^{10}-methylene-THF. Control of dTTP synthesis most likely occurs via control of the production of dUMP.

5. CHEMOTHERAPEUTIC AGENTS THAT INTERFERE WITH dTMP SYNTHESIS

T e drug *fl*uorouracil gives rise to *fl*uoro-dUMP, which permanently inactivates thymidylate synthase and thus impairs the production o*f* d*TT*P *f*or DNA replication. Pemetrexed is also an inhibitor o*f* thymidylate synthase. In contrast, methotrexate is a competitive inhibitor o*f* dihydro*f*olate reductase. Methotrexate-induced accumulation o*f* dihydro*f*olate and the depletion o*f* methylene-tetrahydro*f*olate leads to decreased activity o*f* thymidylate synthase, thus also impairing the production o*f* d*TT*P *f*or DNA replication.

5.1. 5-Fluorouracil and Related Drugs

Capecitabine, **tegafur**, and **5-fluorouracil** all give rise to the same active metabolite, **5-fluoro-dUMP**, that impairs thymidylate synthase and hence DNA synthesis (Fig. 37.7). Capecitabine and tegafur are converted to 5-fluorouracil. 5-Fluorouracil gives rise to two toxic metabolites: 5-fluoro-UTP and 5-fluoro-dUMP. 5-Fluoro-UTP is incorporated into RNA. T ere is no proofreading mechanism for RNA synthesis,

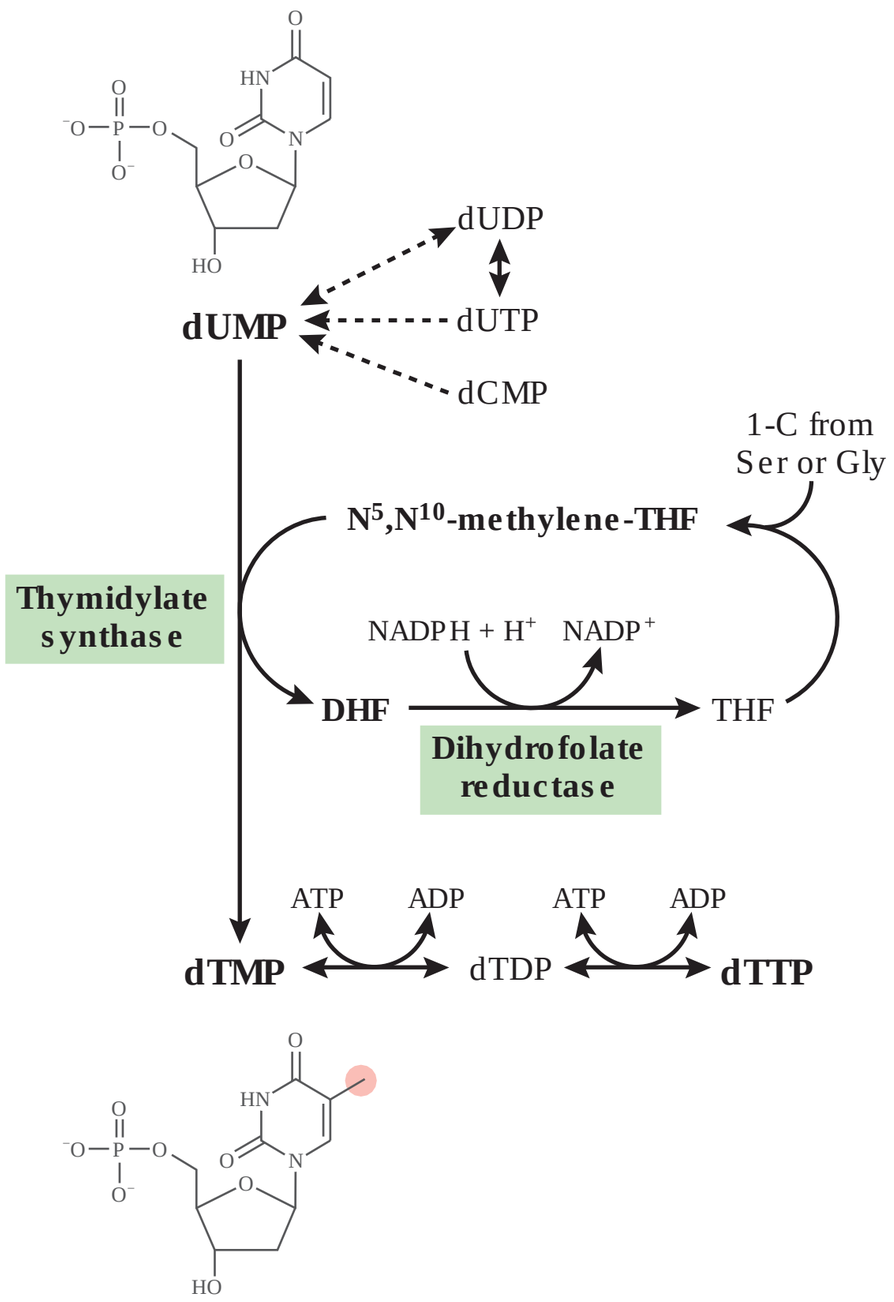


Fig. 37.6 Synthesis of deoxythymidine monophosphate (dTMP) from deoxyuridine monophosphate (dUMP). The transfer of a one-carbon group to tetrahydrofolate (THF) to form N^5,N^{10}-methylene-THF is shown in Figs. 36.2 and 36.3.

and RNA that contains 5-fluorouracil is not fully functional. 5-Fluoro-dUMP is a **suicide substrate** for **thymidylate synthase** (i.e., it inactivates the enzyme irreversibly); it slows the synthesis of dTTP and thus inhibits DNA synthesis.

N^5-formyl-THF (also called **leucovorin**; see Chapter 36) is often administered together with 5-fluorouracil. N^5-formyl-THF can be converted into N^5,N^{10}-methylene-THF (see Fig. 36.3), an elevated concentration of which increases the substrate saturation of thymidylate synthase. Thus, N^5-formyl-THF increases the rate at which 5-fluorouracil incapacitates thymidylate synthase.

The effect of 5-fluorouracil on tumor cells also depends on the relative activities of **thymidylate synthase** and **dihydropyrimidine dehydrogenase**. The lower the thymidylate synthase activity is, the more tumor cells die from 5-fluorouracil treatment. Dihydropyrimidine dehydrogenase normally degrades 80% to 90% of administered 5-fluorouracil to dihydrofluorouracil (see Fig. 37.7). If a patient has decreased activity of this enzyme, 5-fluorouracil is unexpectedly toxic to the patient. Patients can be screened for dihydropyrimidine dehydrogenase deficiency. In patients who do not have this deficiency, the drug **gimeracil** is sometimes used to inhibit dihydropyrimidine dehydrogenase and thus boost the toxicity of 5-fluorouracil. Gimeracil is currently given together with tegafur.

5-Fluorouracil, capecitabine, and tegafur are used in the treatment of a wide variety of solid tumors, sometimes as part of a multidrug regimen. 5-Fluorouracil is also used topically for warts.

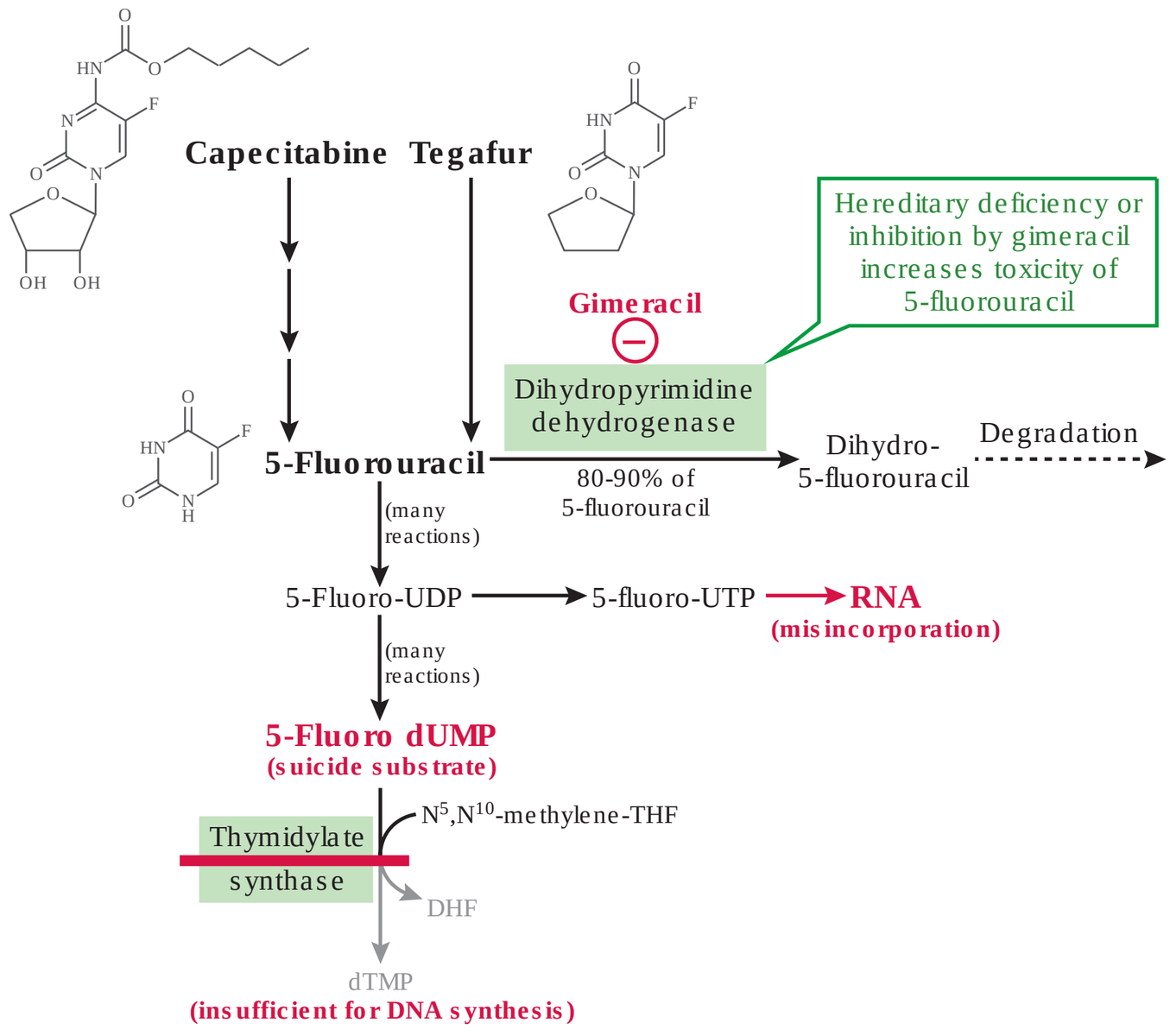


Fig. 37.7 Mechanism of action of 5-fluorouracil, capecitabine, and tegafur. DHF, dihydrofolate; dTMP, deoxythymidine monophosphate; dUMP, deoxyuridine monophosphate; UDP, uridine diphosphate; UTP, uridine triphosphate.

5.2. Pemetrexed

Pemetrexed, like 5-fluouracil, inhibits **thymidylate synthase**, but it is not a suicide inhibitor. Pemetrexed also inhibits de novo synthesis of purine nucleotides, although this is of uncertain clinical relevance. Pemetrexed is used in the treatment of mesothelioma and nonsquamous non–small cell lung cancer.

5.3. Methotrexate

T e antifolate drug methotrexate is a competitive inhibitor of **dihydrofolate reductase** and thus indirectly reduces thymidylate synthase activity (Fig. 37.8). Methotrexate is an analog of dihydrofolate. Methotrexate crosses the brush-border membrane via the reduced folate carrier (RFC; see Chapter 36). Inside cells, it is polyglutamylated and then stays in cells for many days. Methotrexate competes for the substrate binding site of dihydrofolate reductase and thereby inhibits binding of DHF. In the presence of methotrexate, the concentrations of THF and one-carbon-loaded THFs (e.g., N^5,N^{10}-methylene-THF) decrease. With a lower concentration of N^5,N^{10}-methylene-THF as a substrate, thymidylate kinase activity is decreased. Methotrexate also leads to an accumulation of DHF. DHF product inhibits thymidylate synthase. Hence, substrate deficiency and product inhibition both decrease thymidylate synthase activity.

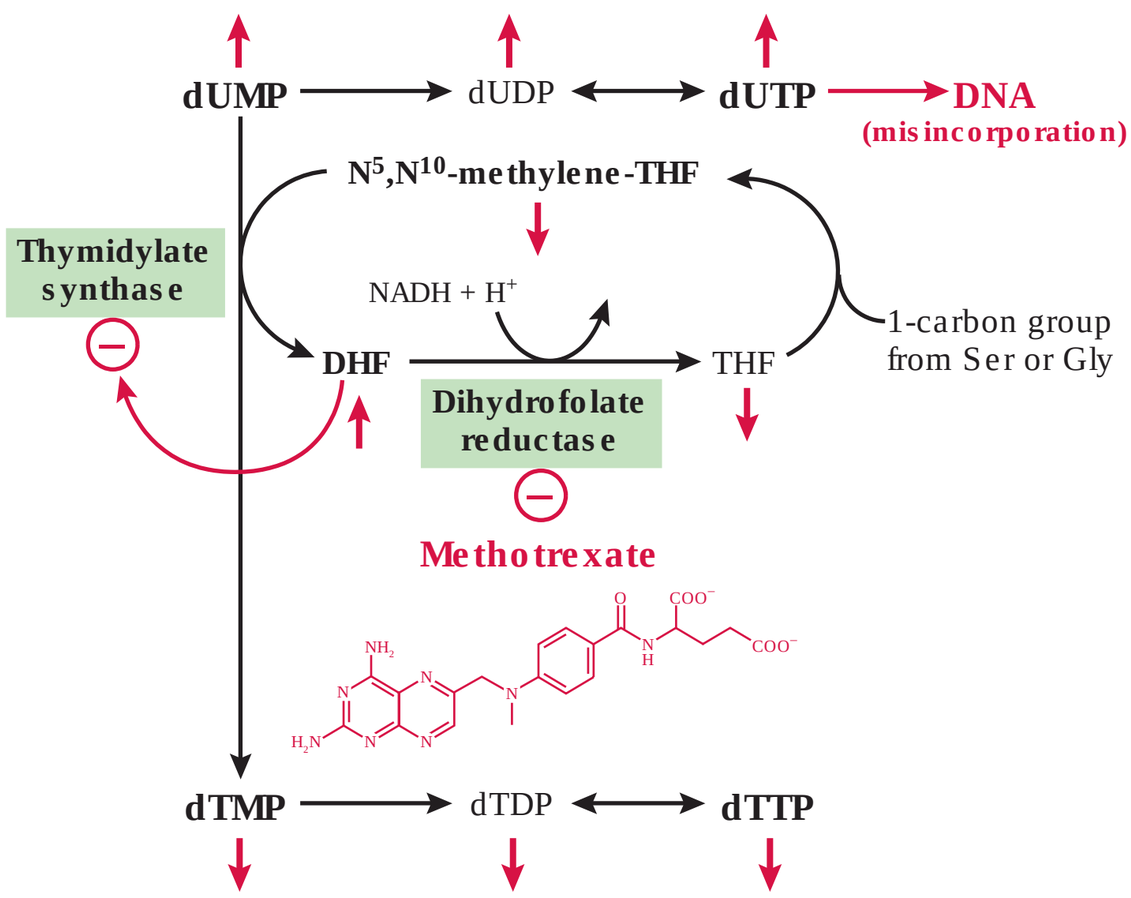
Methotrexate has essentially two medical uses: one is related to the inhibition of DHF reductase, which is of use in treating malignancies, and the other is related to an immunosuppressive ef ect that is of use in the treatment of autoimmune diseases.

Methotrexate is used in the treatment of various leukemias (see Fig. 37.8) and solid tumors. T e dose may be more than 0.5 g/m^2 of body surface. Neoplastic cells, normal cells in the bone marrow, normal fetal cells, and normal cells of the mucosa of the mouth, intestine, and bladder are especially sensitive to methotrexate.

Several **multidrug resistance proteins** (**MRPs**) pump methotrexate out of cells. T ese MRPs represent a subfamily of the **ATP-binding cassette** (**ABC**) **transporters**. ABC refers to a protein motif in these ATP-driven transporters; the genes for the MRPs contain the letters ABCC. Some of these transporters remove mono-, di-, and tri-glutamyl methotrexate from the cytosol. Methotrexate molecules with longer polyglutamate tails are less likely to be pumped out of cells, and they are more ef ective at inhibiting dTMP synthesis. T e rare **Dubin-Johnson syndrome**, due to a heterozygous loss-of-function mutation in the ABCC2 gene, is associated not only with an impaired excretion of conjugated bilirubin (see Section 5.2.2 in Chapter 14) but also with an impaired elimination of methotrexate. Hence, such patients need an unusually low dose of methotrexate.

Resistance to methotrexate can develop in several ways: reduced activity of folylpolyglutamate synthase leads to reduced retention of methotrexate inside cells and thus enhanced excretion of the drug. Active pumping of methotrexate by ABC transporters out of cells likewise diminishes the concentration of methotrexate inside cells. Gene amplification (insertion of many extra copies of the normal gene) of thymidylate synthase permits adequate dTMP synthesis despite the presence of methotrexate. Gene amplification of dihydrofolate reductase and mutations in the dihydrofolate reductase gene that decrease the methotrexate affinity of the enzyme facilitate reduction of DHF in the presence of methotrexate.

In some treatment protocols, methotrexate is infused at concentrations that would prove toxic were they not followed,



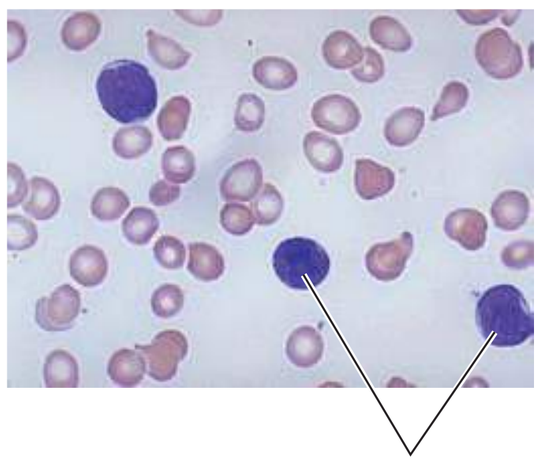


Fig. 37.8 Mechanism of action of methotrexate, and blood smear of a patient who has acute lymphoblastic leukemia (ALL). Red arrows indicate changes in concentration. Methotrexate is often part of the treatment regimen for ALL.

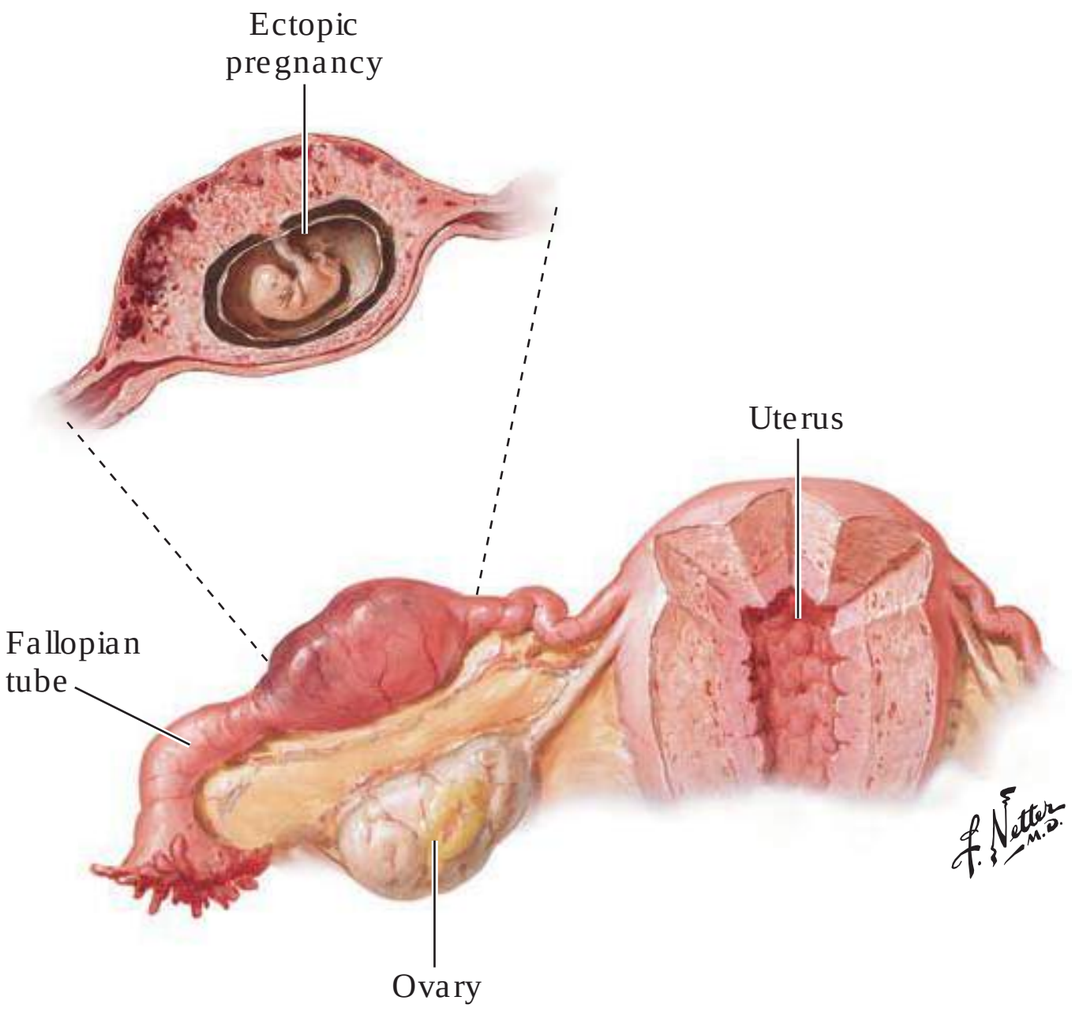


Fig. 37.9 **Ectopic pregnancy in the fallopian tube.**

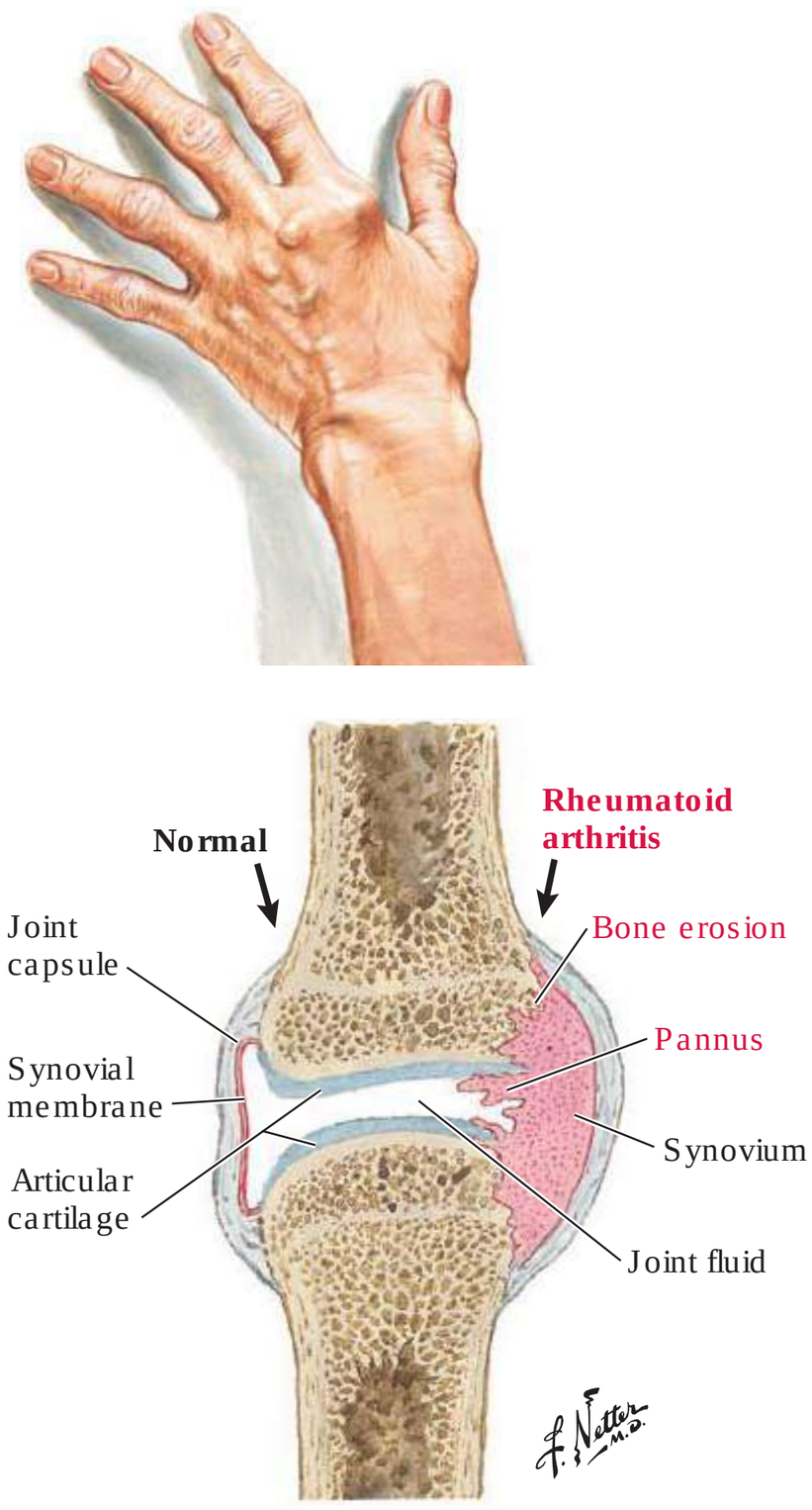


Fig. 37.10 **Rheumatoid arthritis.**

about a day later, by leucovorin (N^5-formyl-THF). T is maneuver is sometimes called **leucovorin rescue**. With this protocol, more cancer cells are exposed to a therapeutic concentration of methotrexate and fewer cancer cells escape because of resistance to methotrexate.

Methotrexate is used in place of surgery to treat **ectopic pregnancies** (Fig. 37.9). An ectopic pregnancy is a pregnancy outside the uterus, usually in the fallopian tube. Methotrexate therapy preserves the af ected fallopian tube, which would otherwise often be severed or removed during surgery. Methotrexate treatment is often reserved for patients who have amniotic sacs that are smaller than about 4 cm. Patients are commonly given a single dose of methotrexate (in the 0.15 g range) and a second or third dose if indicated. Resolution of the pregnancy is followed by measuring the concentration of chorionic gonadotropin in the serum. It usually takes about a month for the fetal tissue to resolve.

Sometimes, methotrexate is also used together with other drugs for **abortions** within the first 2 months of gestation.

Methotrexate is used weekly in relatively small doses (often at about 0.015 g/wk) as an **immunosuppressant** in the treatment of autoimmune diseases. T e mechanisms by which methotrexate suppresses the immune system are poorly understood and likely multifaceted. Inhibition of purine nucleotide metabolism may play a role (see Section 2.1 and Chapter 38). Inhibition of dihydrofolate reductase likely plays only a minor role, and folic acid intake does not significantly diminish the ef ectiveness of methotrexate.

In the treatment of autoimmune diseases, methotrexate is most often prescribed to patients who have **rheumatoid arthritis** (Fig. 37.10), a disease that af ects about 0.5% to 1% of the world's population. Af ected patients often have joint pain, joint swelling, and severe morning stif ness. T e joints of the hands and wrists are most often involved early in the disease. T e synovium is inflamed, and nearby cartilage and bone are destroyed. T erapy is aimed at preventing inflammation and destruction of the joint. Methotrexate, like leflunomide (see Section 1), is a **DMARD**.

Another drug used in the treatment of rheumatoid arthritis is **leflunomide**, which gives rise to a metabolite that inhibits dihydroorotate dehydrogenase in the orotate synthesis pathway (see Section 1 and Fig. 37.2).

Other modes of treatment of rheumatoid arthritis involve **biologicals** that bind **tumor necrosis factor-α** (**TNF-α**) or act as antagonists at the **interleukin-1** (**IL-1**) or **interleukin-6** (**IL-6**) receptor.

Methotrexate is sometimes used to treat **psoriasis** (Fig. 37.11) that covers more than 10% of the body. Psoriasis results in chronic hyperproliferation of keratinocytes that gives rise to scaly, sometimes itchy plaques. About 2% of the world population has psoriasis. T ere are several forms of the condition, with plaque psoriasis the most common. In place of methotrexate, patients can also be treated with a biological that binds TNF-α.

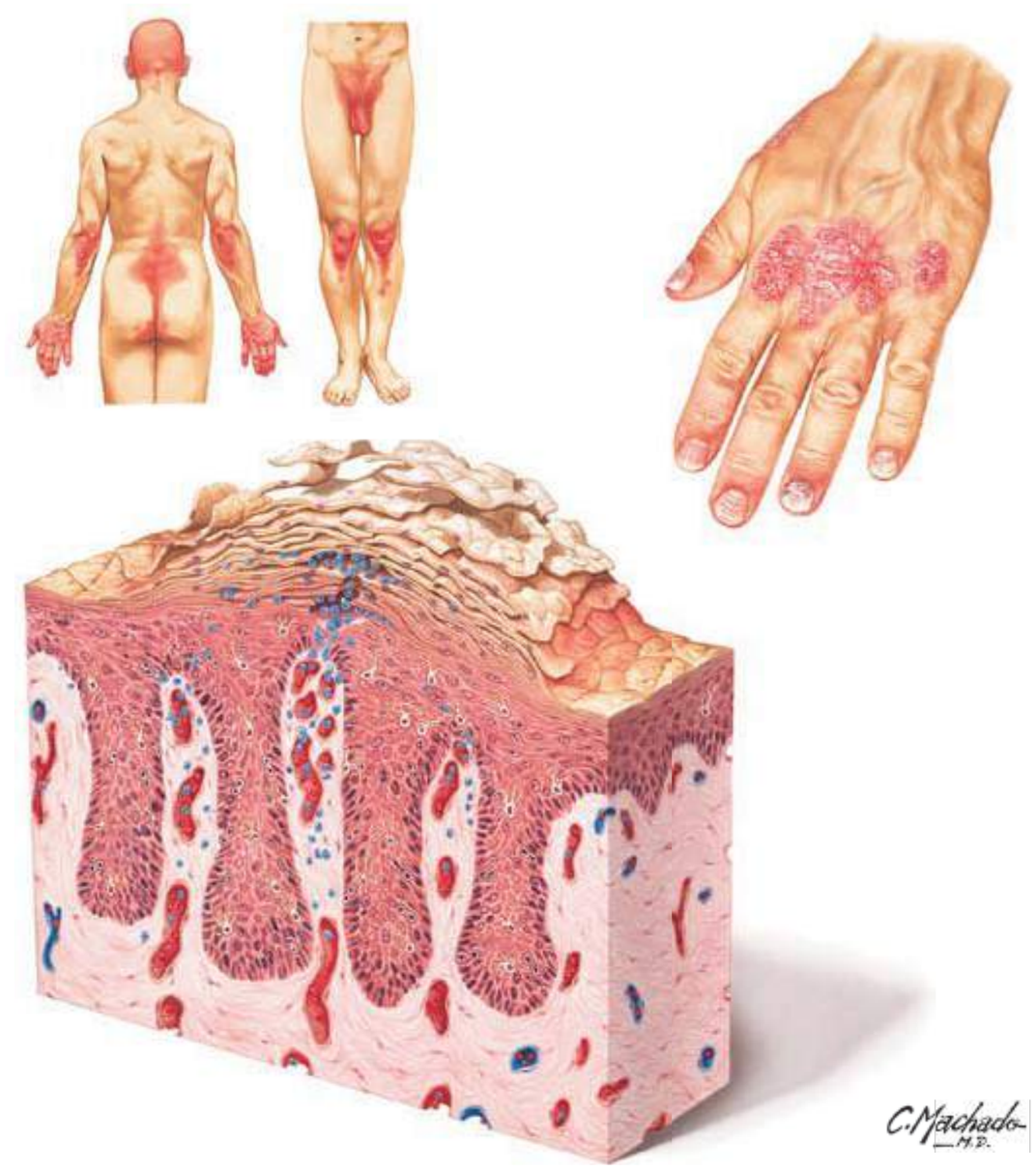


Fig. 37.11 **Psoriasis.**

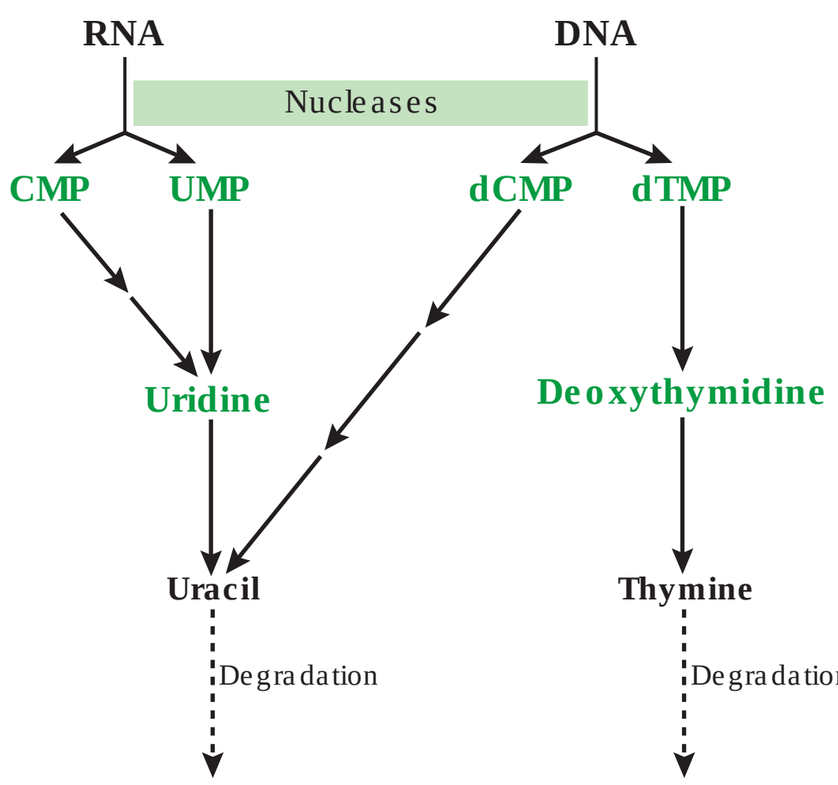


Fig. 37.12 **Degradation of RNA, DNA, and pyrimidine nucleotides.** Compounds in green can be rephosphorylated and reused.

6. DEGRADATION OF PYRIMIDINE NUCLEOTIDES

T e degradation o*f* RNA and DNA yields nucleosides, which can be rephosphorylated and reused.

Degradation of RNA and DNA by nucleases yields pyrimidine mononucleotides (CMP, UMP, dCMP, and dTMP; Fig. 37.12) that can be rephosphorylated to pyrimidine trinucleotides and thus reused for the synthesis of RNA or the synthesis and repair of DNA. CMP and UMP can be degraded to uridine, which can also be rephosphorylated (see Fig. 37.3). Similarly, dTMP can be degraded to deoxythymidine, which can be rephosphorylated.

SUMMARY

- Pyrimidine nucleotides are synthesized de novo mainly in the liver and in dividing cells. Synthesis involves the production of phosphoribosylpyrophosphate (PRPP) using ribose 5-phosphate from the pentose phosphate pathway. PRPP is produced only when the concentration of phosphate is adequate and the concentration of ADP is low (i.e., when a cell has normal ATP production). Pyrimidine nucleotide synthesis also involves the production of orotate, a pyrimidine base. Orotate is mostly synthesized when a cell performs DNA replication or repair. PRPP and orotate are joined to produce UMP.
- T e liver dephosphorylates UMP to uridine and releases uridine into the bloodstream. Peripheral cells salvage uridine and phosphorylate it to UMP. UMP is the starting point for the synthesis of all other pyrimidine nucleotides.
- When carbamoylphosphate (CAP) accumulates in mitochondria due to a problem with the urea cycle, it leaks into the cytosol and enters the pathway for orotate synthesis. Measurement of orotate excretion in urine is useful in pinpointing the location of a urea cycle defect.
- Leflunomide, used in the treatment of rheumatoid arthritis, inhibits the synthesis of orotate, which in turn impairs lymphocyte production.
- UTP is aminated to CTP. Besides the synthesis of nucleic acids, UTP is used for the synthesis of UDP-glucose and UDP-galactose, whereas CTP is used for phospholipid synthesis.
- Ribonucleotide reductase reduces UDP, CDP, ADP, and GDP to dUDP, dCDP, dADP, and dGDP, respectively. T e protein thioredoxin is the reducing agent, and NADPH, in turn, reduces thioredoxins. T e drugs hydroxyurea and gemcitabine inhibit ribonucleotide reductase. Hydroxyurea is used for instance in the treatment of polycythemia vera and sickle cell disease; gemcitabine is used against a diverse set of solid tumors.
- T ymidylate synthase uses N^5,N^{10}-methylene-THF to methylate dUMP to dTMP; this reaction is unique in that it produces DHF. DHF reductase reduces DHF to THF. THF can then be loaded with another one-carbon group for further dTMP synthesis. dTMP synthesis is impaired in patients with a primary or secondary folate deficiency.
- T e antifolate methotrexate inhibits DHF reductase. As a result, DHF accumulates and feedback inhibits thymidylate synthase. In addition, N^5,N^{10}-methylene-THF becomes depleted, thus decreasing thymidylate synthase activity. T is ef ect is useful in the treatment of certain leukemias, solid tumors, and ectopic pregnancies.
- Patients with Dubin-Johnson syndrome are unusually sensitive to methotrexate because they have an inadequate activity of a transporter that removes methotrexate.

Cells can become resistant to methotrexate by reduced polyglutamylation of methotrexate, by amplification of the dihydrofolate reductase or thymidylate synthase gene, or by point mutation of the dihydrofolate reductase gene.

- Low-dose methotrexate has an immunosuppressive effect that is useful in the treatment of rheumatoid arthritis and psoriasis. The immunosuppressive effect seems to be independent of folates.
- Metabolism of the chemotherapeutic drugs capecitabine and tegafur yields 5-fluorouracil. 5-Fluorouracil can also be administered directly. The drug gimeracil inhibits an enzyme that degrades 5-fluorouracil. 5-Fluorouracil gives rise to 5-fluoro-dUTP, which is incorporated into RNA, making the RNA nonfunctional; it also gives rise to 5-fluoro-dUMP, which is a suicide substrate for thymidylate synthase. Without adequate thymidylate synthase activity, DNA replication and repair are impaired. Capecitabine, tegafur, and 5-fluorouracil are mainly used in the treatment of solid tumors.
- The drug pemetrexed also inhibits thymidylate synthase and is used in the treatment of mesothelioma and nonsquamous non–small-cell lung cancer.
- Degradation of RNA gives rise to CMP, UMP, and uridine, all of which can be reused.
- Degradation of DNA yields dTMP and deoxythymidine, which can be reused; dCMP is also formed but cannot be reused.

FURTHER READING

- Capmas P, Bouyer J, Fernandez H. Treatment of ectopic pregnancies in 2014: new answers to some old questions. Fertil Steril. 2014;101:615-620.
- How Renoir coped with rheumatoid arthritis. BMJ. 1997;315:1704-1708. This paper is written by experts in rheumatoid arthritis. It nicely illustrates the natural history of untreated rheumatoid arthritis and dramatically describes the effect of the disease on the daily life of the famous painter.

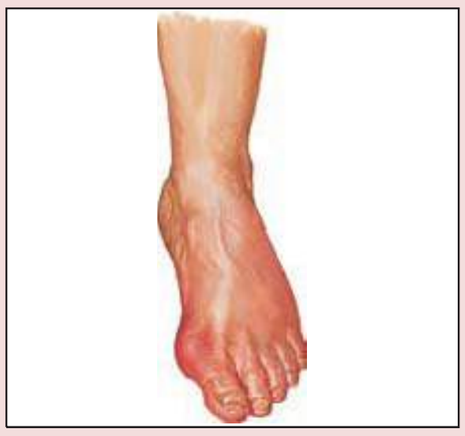
Review Questions

1. De novo pyrimidine biosynthesis is stimulated by which of the following?

   A. 5-Fluorouracil
   B. N-acetyl-glutamate
   C. Ornithine
   D. Phosphoribosylpyrophosphate
   E. UTP

2. An 85-year-old patient was taking methotrexate for his rheumatoid arthritis. When he moved into an assisted living community, the dose of methotrexate was accidentally changed from 15 mg once a week to 15 mg daily. After 9 days, the patient became very ill. He had labored breathing, nausea, fever, and chills. The patient was hospitalized. A blood sample revealed a low number of red and white blood cells as well as platelets. Which one of the following can be administered to the patient to counteract the toxic effects of methotrexate?

   A. Cobalamin
   B. Gemcitabine
   C. Gimeracil
   D. N^5-formyltetrahydrofolic acid
   E. S-adenosyl methionine
   F. Uridine



Chapter

38 Gout and Other Diseases Related to the Metabolism of Purine Nucleotides

SYNOPSIS

- The purine nucleotides contain the bases adenine or guanine. Adenosine triphosphate (ATP) is the predominant cellular purine nucleotide.
- Purine nucleotide metabolism is of clinical interest chiefly because of the high incidence of gout.
- Fig. 38.1 provides an overview of the relevant steps of adenine nucleotide metabolism. Guanine nucleotide metabolism is comparable but quantitatively less important.
- When needed, most cells can synthesize adenine and guanine nucleotides de novo.
- In cells with impaired energy production, the concentration of adenosine monophosphate (AMP) is increased. Such cells rapidly degrade AMP to hypoxanthine, which they release into the blood.
- Other cells salvage most of the hypoxanthine in the blood and reuse it to produce purine nucleotides. The liver and the intestine degrade the remaining hypoxanthine to urate, which they release into the blood.
- Urate serves as an antioxidant. In men and postmenopausal women, urate is present in blood plasma near the limit of its solubility.
- Urate is actively excreted by the kidneys. Certain diuretics and organic acids impair the excretion of uric acid (the protonated form of urate). Excretion is also impaired in patients with kidney disease.
- If uric acid is present in urine beyond its solubility, it crystallizes and forms kidney stones.
- The concentration of urate in the blood depends on the rates of urate production and excretion. If the concentration of urate in blood is abnormally high, needle-like crystals of sodium urate tend to form in joints and soft tissues. Occasionally, the crystals in joints give rise to an inflammatory reaction that is extremely painful.
- Gout is characterized by one or both of the following during a patient's life: kidney stones made up of uric acid, and highly painful inflammatory reactions to crystals of sodium urate in peripheral joints. Joint pain can be lessened by treatment with an antiinflammatory drug.
- Drugs available to treat the cause of gout inhibit the formation of urate, promote the excretion of urate, or degrade urate.
- Tumor lysis syndrome can develop in patients who receive cytolytic therapy. Damage to kidney tubules by uric acid crystals can be prevented with an enzyme that degrades urate.

LEARNING OBJECTIVES

For mastery *of* this topic, you should be able to do the *f*ollowing:

- Explain how the pathways of de novo synthesis, degradation, and salvage of purine nucleotides fit together and how they are regulated.
- List the factors that influence the concentration of urate in the blood, considering urate overproduction and underexcretion.
- For the population at large, describe the effect of gender and age on the concentration of urate in the blood.
- Explain why patients with preeclampsia or eclampsia have hyperuricemia.
- Describe the cause of tumor lysis syndrome, the major risk for patients who have this syndrome, and the role of blood urate–lowering drugs in preventing kidney damage.
- Explain the pathogenesis of an acute attack of gout, starting with hyperuricemia.
- Describe the gold standard for the diagnosis of gout and describe how gout can be differentiated from pseudogout.
- Describe the natural history of an untreated acute attack of gout, as well as the long-term consequences of untreated or treatment-resistant gout.
- Explain the options for acute and long-term treatment of gout with lifestyle intervention and drugs, paying attention to the mechanisms of action.
- Describe the mechanisms by which the following contribute to hyperuricemia and gout: ethanol consumption, fructose consumption, hypoxia, psoriasis, hemolytic anemias, lead poisoning, lactic acidemia, and Lesch-Nyhan disease and its variants.
- Explain the pathogenesis of uric acid nephrolithiasis and list appropriate treatment options.

1. DE NOVO SYNTHESIS OF PURINE NUCLEOTIDES

Adenine and guanine are purines. A*T*P and adenosine diphosphate (ADP) in mitochondria and the cytoplasm constitute the majority o*f* all purine nucleotides. Adenine and guanine nucleotides can be synthesized de novo, starting with a 5-carbon sugar *f*rom the pentose phosphate shunt pathway.

In biochemistry, the term **purines** entails a large collection of compounds that can be derived chemically from purine (Fig. 38.2) by substitution, such as adenine, adenosine, guanine, guanosine, inosine, hypoxanthine, xanthine, uric acid, the purine nucleotides inosine monophosphate (IMP), AMP, ADP, ATP, guanosine monophosphate (GMP), guanosine diphosphate (GDP), guanosine triphosphate (GTP), and the deoxyribose analogs of these nucleotides.

T e concentration of some purine nucleotides in cells is as follows (tissues dif er appreciably): ~4 mM ATP, ~1 mM ADP (only ~30 µM free ADP), ~0.1 mM AMP (<1 µM free AMP), and ~0.5 mM GTP. Fig. 38.2 provides a more detailed visual summary. ATP, ADP, and AMP in the mitochondria and the cytoplasm are involved in energy transfer. Almost all of the ADP and AMP is bound to proteins. T e GTP/GDP/GMP ratio resembles the ATP/ADP/AMP ratio. T e purine nucleotides contained in DNA and RNA make up only about one-tenth of a cell's purine nucleotide content.

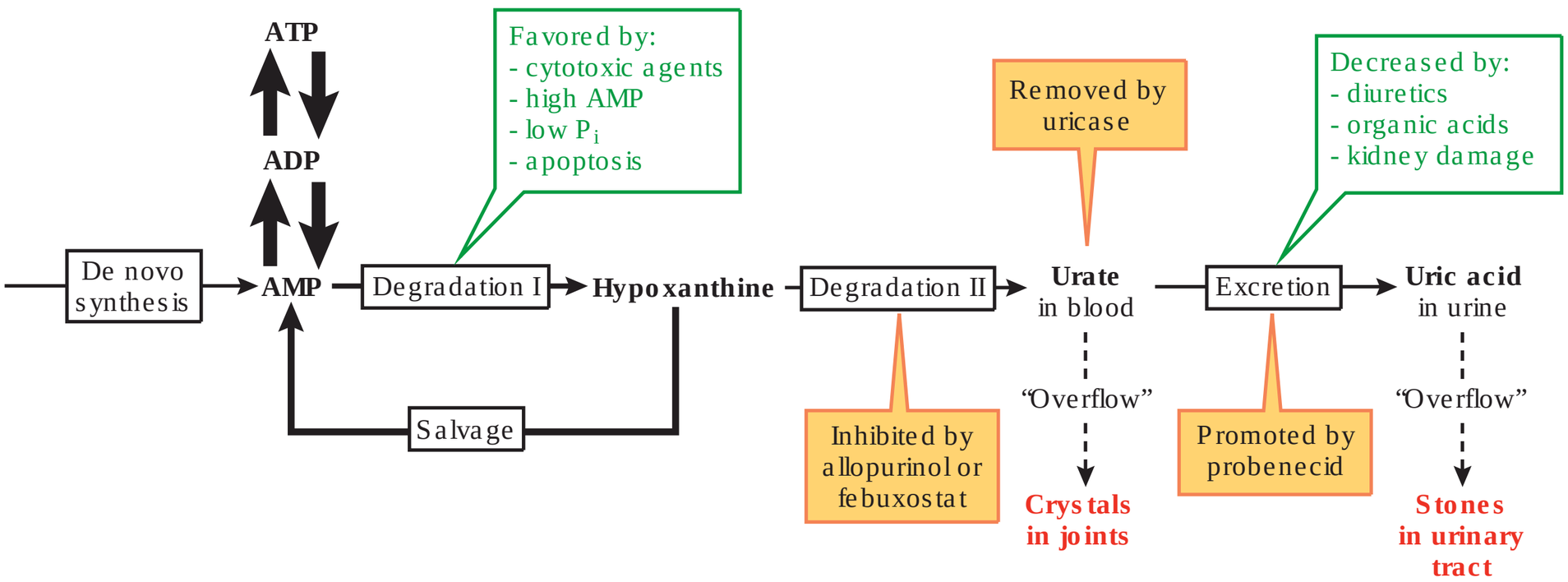


Fig. 38.1 **Overview of adenine nucleotide metabolism as it pertains to gout.**

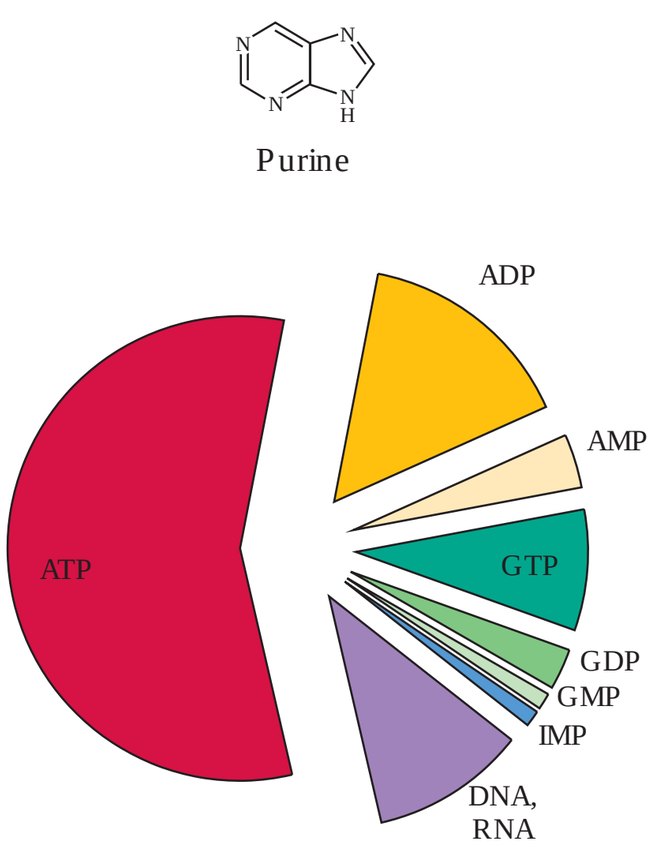


Fig. 38.2 **Structure of purine and the approximate purine nucleotide content of cells.** The concentration of ATP is usually several mmol/L cell water. Structures of AMP and GMP are shown in Fig. 38.3. (Data from Traut TW. Physiological concentrations of purines and pyrimidines. *Mol Cell Biochem*. 1994;140:1-22.)

Almost all cells can synthesize AMP and GMP both by de novo synthesis and by salvage (see Section 2.4). In the entire human body, de novo synthesis constitutes only about 3% of all AMP synthesis; the rest occurs via salvage. In the de novo synthesis of AMP or GMP, IMP—a precursor purine nucleotide—is built from the ribose phosphoribosylpyrophosphate (PRPP) in many steps. IMP is then converted to AMP or GMP. Fig. 38.3 summarizes these reactions.

Details of purine nucleotide synthesis are as follows. **Well-energized cells** with an adequate concentration of phosphate synthesize **PRPP** from ribose 5-phosphate, an intermediate of the pentose phosphate shunt pathway (see Fig. 38.3; see also Chapter 21). PRPP synthetase is chiefly activated by **phosphate** and inhibited by **ADP**. As will be shown in Section 2.4, PRPP is also used for the salvage of hypoxanthine and guanine. As detailed in Chapter 37, PRPP is also used for the de novo synthesis of pyrimidine nucleotides.

The purine base is built up stepwise on the ribose of PRPP to form **IMP** (which contains the hypoxanthine ring structure; compare to Fig. 38.5). The first of these reactions is irreversible and catalyzed by **glutamine PRPP amidotransferase** (also termed **amido phosphoribosyl transferase**). The activity of this enzyme limits the rate of de novo synthesis. Glutamine PRPP amidotransferase shows cooperativity toward PRPP. As shown in Section 2.5, when IMP cannot be produced from salvage of hypoxanthine, the concentration of PRPP rises and activates the glutamine PRPP amidotransferase. AMP and GMP feedback inhibit this enzyme.

The immunosuppressant drug **mycophenolic acid** inhibits the conversion of IMP to GMP. This drug selectively decreases the concentration of GMP in lymphocytes because these cells have insufficient salvage to produce GMP from guanine and PRPP (see Section 2.4).

Controls on the conversions of IMP to **AMP** and **GMP** are such that appropriate amounts of AMP and GMP are produced.

Even though two of the reactions in purine nucleotide de novo synthesis require **N^{10}-formyltetrahydrofolate**, a **folate deficiency** in a patient does not diminish purine synthesis to a clinically significant extent (see Chapter 36).

Under normal circumstances, most ADP is phosphorylated to ATP by **oxidative phosphorylation**, whereby the creatine kinase–catalyzed reaction ADP + **phosphocreatine** + $H^+ \leftrightarrow$ ATP + creatine plays a major accessory role in muscle and the central nervous system (see Chapter 23). About one-tenth as much ATP is derived from substrate-level phosphorylation in glycolysis (see Chapter 19). **Adenylate kinase** catalyzes the reversible reaction AMP + ATP $\leftrightarrow$ 2 ADP. Because of this reaction, the physiological concentration of AMP is tied to that of ADP (i.e., when the concentration of ADP is high, that of AMP is also high). Furthermore, the concentration of AMP changes with the square of the concentration of ADP, making AMP particularly suitable as an indicator of a cell's phosphorylation state.

The concentrations of free GMP, GDP, and GTP are related to those of AMP, ADP, and ATP. **Guanylate kinase** uses ATP to phosphorylate GMP reversibly to GDP. **Nucleoside**

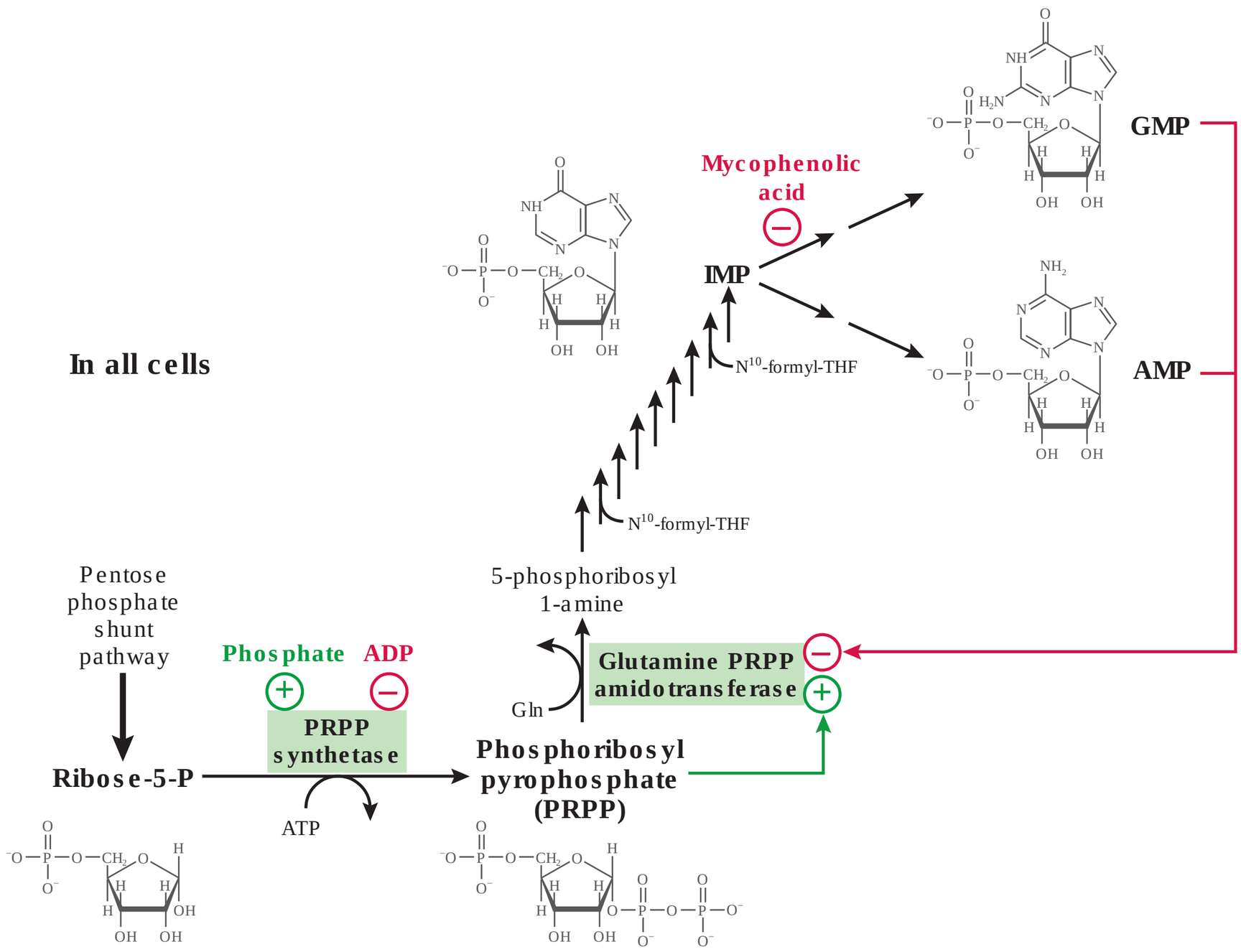


Fig. 38.3 De novo synthesis of adenine and guanine nucleotides. Only well-energized cells (i.e., cells with an adequate concentration of phosphate and a low concentration of free ADP) synthesize PRPP. The de novo synthesis pathway is primarily active when the concentration of PRPP is elevated and the concentration of nucleoside mono- and diphosphates is low.

diphosphokinase uses ATP to phosphorylate reversibly GDP to GTP. Succinyl-coenzyme A (succinyl-CoA) synthetase in the citric acid cycle also phosphorylates GDP to GTP. As a consequence of the prevalence of these enzymes, the degree of phosphorylation of guanine nucleotides generally resembles the degree of phosphorylation of adenine nucleotides.

A large number of reactions dephosphorylate either ATP to ADP or AMP, or GTP to GDP or GMP.

2. DEGRADATION AND SALVAGE OF PURINE NUCLEOTIDES

Whenever the concentrations of AMP and ADP in a cell rise substantially, or whenever the concentration of phosphate in a cell drops substantially, AMP is degraded. During intense exercise, skeletal muscle degrades AMP mostly to IMP and then resynthesizes AMP as part of a purine nucleotide cycle. Nonmuscle cells degrade AMP to hypoxanthine, which they release into the blood. The liver and the intestine oxidize excess hypoxanthine into urate. Most hypoxanthine is salvaged by cells that need more adenine nucleotides. Salvage is always preferred over de novo synthesis.

GMP is degraded to guanine under the same conditions as AMP. Cells can salvage guanine and produce GMP. Guanine deaminase in the blood degrades guanine to xanthine, which is taken up mostly by the liver and oxidized to urate.

The kidneys excrete about 7% to 10% of the urate that is present in the glomerular filtrate. Organic acids such as ketone bodies, lactic acid, and thiazide diuretics lower this percentage, whereas uricosuric drugs (e.g., probenecid) increase it.

2.1. Degradation of AMP and GMP to Hypoxanthine and Guanine

Almost all of a cell's AMP is bound to proteins, and **free AMP** is present only in **nanomolar** concentrations.

If the concentration of free **AMP** in a cell is abnormally high, or if the cytosol contains an abnormally low concentration of **phosphate**, AMP is rapidly degraded. The concentration of AMP may be high because certain reactions produce AMP at an increased rate (e.g., muscle contraction, metabolism of ethanol, phagocytic degradation of dying cells, pathologic futile cycling in glycolysis and gluconeogenesis), or because mitochondrial ATP production is impaired (e.g., due to hypoxia, poisoning of the electron transport chain, low intracellular free phosphate, or substrate limitation).

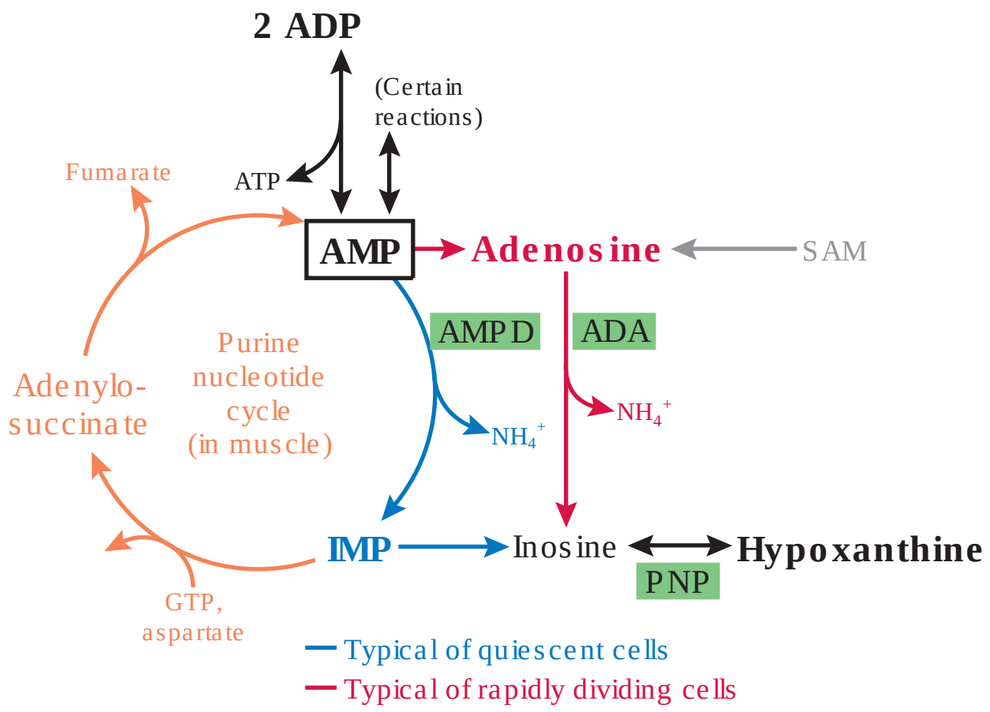


Fig. 38.4 Degradation of ADP and AMP to hypoxanthine. Adenosine deaminase (ADA) and purine nucleoside phosphorylase (PNP) also handle deoxyadenosine and deoxyinosine, respectively. AMPD, AMP deaminase; SAM, S-adenosyl homocysteine, an intermediate in the activated methyl group cycle (see Chapter 36).

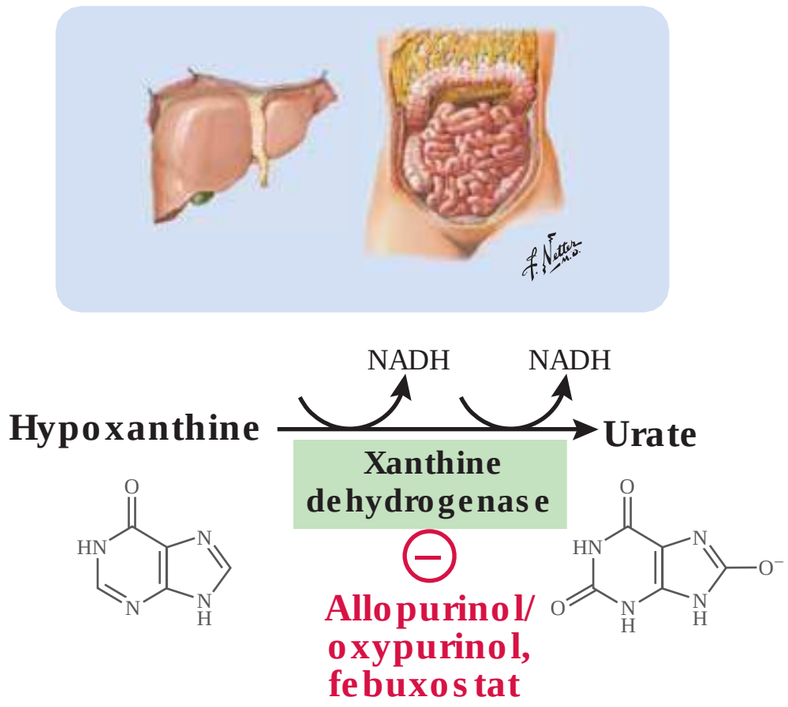


Fig. 38.5 Degradation of hypoxanthine to urate and inhibition by allopurinol and febuxostat. Xanthine dehydrogenase oxidizes allopurinol to oxypurinol, a virtually irreversible inhibitor of xanthine dehydrogenase.

In skeletal muscle, **AMP deaminase** degrades AMP to IMP, which can be reaminated in two reactions to reform AMP (Fig. 38.4). T is cycle is called the **purine nucleotide cycle**. No agreement exists yet on the perceived role of this cycle. AMP deaminase is most active when the concentration of ATP is low and that of ADP is high.

In the United States, about 2% of the population has **no active type 1 AMP deaminase**, the predominant AMP deaminase in muscle (other tissues express type 2 or type 3 AMP deaminase). T e majority of type 1 AMP deaminase–deficient patients remain asymptomatic, but a minority experience early **exercise fatigue** and **muscle cramps**. In these af ected patients, exercise may also lead to an abnormal increase in serum urate (see Section 2.2). For yet unclear reasons, partial and complete AMP deaminase deficiency is associated with improved **recovery from ischemic heart disease**.

While **nondividing cells** resemble skeletal muscle cells in that they degrade AMP via IMP, **rapidly dividing cells** usually degrade AMP via adenosine (see Fig. 38.4). Most tissues must release their hypoxanthine into the blood while the liver and the intestine can convert hypoxanthine all the way to urate (see Section 2.2)

Near-complete **adenosine deaminase** (**ADA**) **deficiency** leads to **severe combined immunodeficiency** (**SCID**) that develops over the first few months of life. ADA deficiency (see Fig. 38.4) leads to increased concentrations of the ADA substrates adenosine and deoxyadenosine, which in turn are phosphorylated to ATP and dATP. Deoxyadenosine and dATP are toxic to lymphocytes. T e loss of B-, T-, and NK-lymphocytes can be followed as the disease develops. Patients who have on the order of 5% of the normal ADA activity experience later disease onset, sometimes only in adulthood. In North America, SCID develops in about 1 in 50,000 newborns, and ADA deficiency is responsible for about 20% of all SCID.

T e immunosuppressive ef ect of the antifolate drug **methotrexate** is thought to involve inhibition of both adenosine deaminase and AMP deaminase, thereby leading to the accumulation of adenosine, which binds to adenosine receptors that inhibit neutrophils, monocytes, and macrophages.

Purine nucleoside phosphorylase (**PNP**) **deficiency** (see Fig. 38.4) also leads to SCID via increased, lymphotoxic concentrations of deoxyguanosine and dGTP. PNP deficiency is a very rare disease.

Most cells degrade **GMP** to **guanine**, which they release into the blood. Other cells that are in need of guanine nucleotides take up guanine and salvage it (see Section 2.4). Since the phosphorylation states of adenine nucleotides and guanine nucleotides are coupled, GMP and AMP are degraded in parallel.

Blood contains the enzyme **guanine deaminase** (also called **guanase**), which degrades guanine to xanthine. T e liver takes up xanthine and oxidizes it to **urate** (see Section 2.2 below).

2.2. Degradation of Hypoxanthine to Xanthine and Urate

Hypoxanthine and xanthine are degraded to urate chiefly in the **liver**, and to some degree in the **intestine**. **Xanthine dehydrogenase** catalyzes the sequential oxidation of hypoxanthine to xanthine and xanthine to urate (Fig. 38.5). T e intestine converts about one-third of the **adenine nucleotides in the diet** to urate. Both the liver and the intestine release urate into the blood.

In the bloodstream, urate functions as an **antioxidant** that reacts preferentially with **peroxynitrite** ($O{=}N{-}O{-}O^-$). Peroxynitrite results from the reaction of superoxide anion ($\bullet O_2^-$) and nitric oxide ($\bullet NO$). If not removed by urate, peroxynitrite directly reacts with or gives rise to compounds that damage DNA and proteins. In contrast to its antioxidant ef ect, uric acid is also a **prooxidant** that favors lipid peroxidation.

T e drugs **allopurinol** and **febuxostat** inhibit xanthine dehydrogenase (see Fig. 38.5). Xanthine dehydrogenase oxidizes allopurinol (an analog of hypoxanthine) to **oxypurinol**

(alloxanthine). Oxypurinol then inhibits xanthine dehydrogenase virtually irreversibly. Febuxostat inhibits the molybdenum pterin catalytic center of xanthine dehydrogenase. As shown below, allopurinol and febuxostat are used to decrease urate production in certain patients who have gout (see Section 4; hypoxanthine is then excreted as such, or as its metabolite xanthine). Since xanthine dehydrogenase also metabolizes certain drugs, allopurinol and febuxostat also inhibit the degradation of drugs such as **azathioprine** and **6-mercaptopurine** (see Section 5 and Fig. 38.19).

Oxidation or proteolysis, for instance during **ischemia-reperfusion injury,** turns xanthine dehydrogenase into a **xanthine oxidase** that uses O_2 in place of NAD^+ and then produces **H_2O_2** (hydrogen peroxide) in place of $NADH + H^+$. Ischemia-reperfusion injury occurs when tissues experience inadequate perfusion or hypoxia, followed by adequate perfusion and oxygenation. T is happens, for instance, in the course of a **myocardial infarction** or a **stroke**. Injury leads to the activation of proteases, which convert xanthine dehydrogenase to xanthine oxidase. T e H_2O_2 from hypoxanthine oxidation by xanthine oxidase gives rise to reactive oxygen species that damage proteins, lipids, and nucleic acids (see Chapter 21).

Pharmacological information generally refers to allopurinol and febuxostat as inhibitors of **xanthine oxidase** even though the normal pharmacological target is xanthine dehydrogenase. In fact, these drugs inhibit both enzymes.

Recombinant urate oxidase (also called **uricase**), such as **rasburicase** or **pegloticase**, can be infused into patients to reduce the concentration of urate in the blood by converting uric acid into **allantoin**. Allantoin is excreted in the kidneys. Virtually all animals express urate oxidase, but humans do not. T e recombinant uricases are somewhat immunogenic. Rasburicase has a short half-life in the blood and is used principally for the treatment of tumor lysis syndrome. Pegloticase has a longer half-life in blood and is used principally for the treatment of gout when every other treatment fails.

Recombinant uricases are contraindicated in patients who have **glucose 6-phosphate dehydrogenase** (**G6PD**) **deficiency**. As the uricases oxidize uric acid to allantoin, they produce H_2O_2. T e H_2O_2 gives rise to reactive oxygen species (ROS) and oxidative damage (see Chapter 21). G6PD-deficient patients may have an insufficient capacity to generate NADPH in red blood cells to counter the ROS and the oxidative damage. As a result, G6PD-deficient patients injected with a uricase may develop acute hemolysis and sometimes even methemoglobinemia.

2.3. Excretion of Urate by the Kidneys

T e kidneys function as follows (Fig. 38.6): Blood flow through the kidneys is quite independent of blood flow in the rest of the body. T e **glomeruli** of the kidneys filter some of the blood that flows through them; cells and proteins are retained, whereas about one-fifth of water and small molecules in blood plasma end up in the filtrate. T e filtrate enters the **proximal tubule**, which actively reabsorbs small molecules such as glucose, amino acids, and ions; about 70% of the filtered water

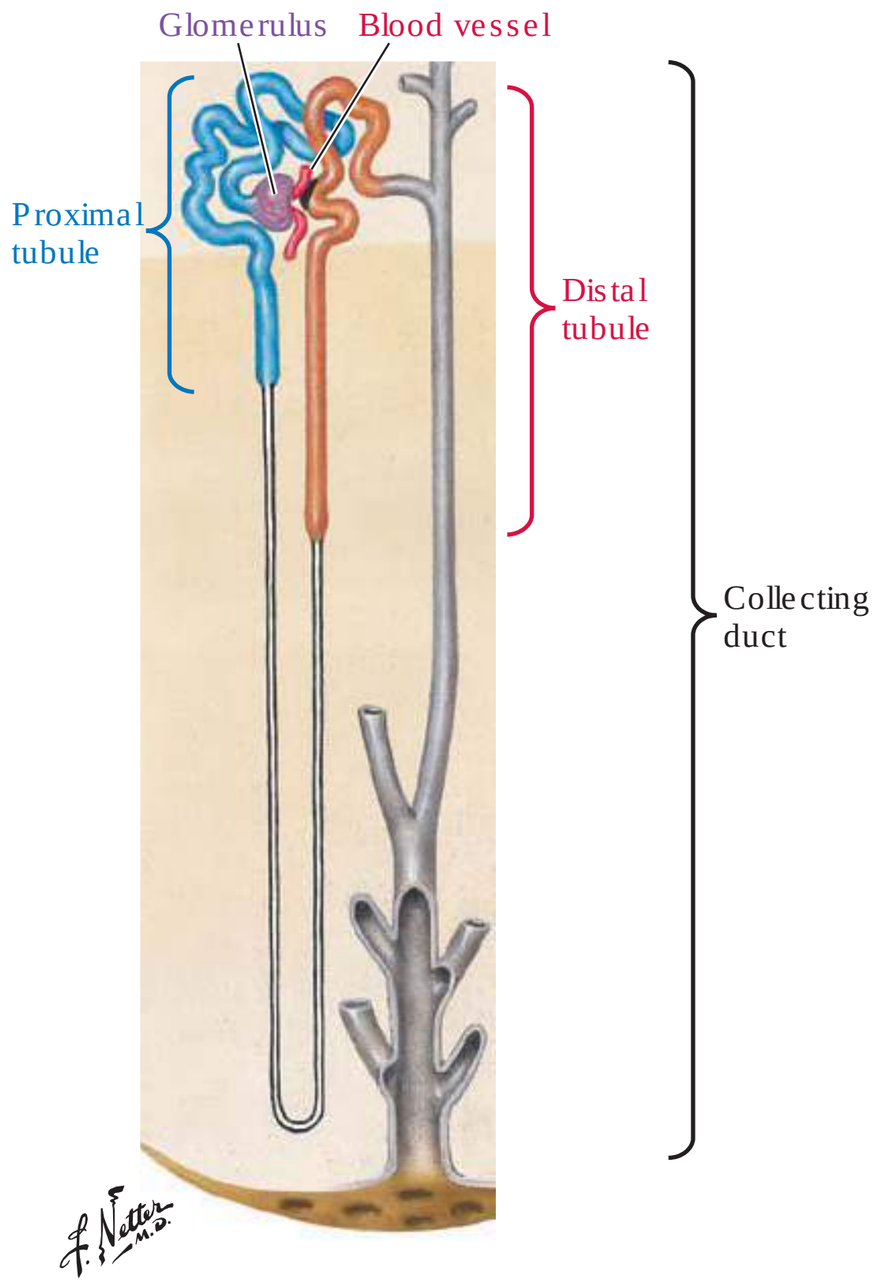


Fig. 38.6 **Architecture of the kidneys.**

is passively reabsorbed through osmosis. T e proximal tubules also secrete organic ions that are destined for excretion. T e remaining liquid in the proximal tubule enters the **loop of Henle**, which also removes inorganic ions and water. In the **distal tubule** and the **collecting duct**, the further reabsorption of salt and water is regulated by hormones depending on the needs of the body to secrete either small amounts of hyperosmolar urine or large amounts of hypoosmolar urine. T e distal tubules can also acidify the urine.

About **7% to 10%** of the urate in the glomerular filtrate ends up in the **urine**, and organic acids lower this fraction (Fig. 38.7). T e excretion of urate by the kidneys is an area of active research. Our understanding of the physiological function and relevance of transporters is therefore preliminary. As a small molecule, urate passes through the glomerular filter. T e proximal convoluted tubules take up about 99% of the urate in the filtrate. A subsequent portion of the tubules secretes about half of this urate via the transporters ABCG2 and NPT4. T ese transporters also secrete lactic acid, ketone bodies, and aspirin. T rough competition for the transporter, an elevated concentration of these organic acids decreases the rate of urate excretion. About 40% of the secreted urate is then reabsorbed from the proximal tubules by several **urate transporters**. **URAT1** among these transporters is inhibited by the uricosuric drug **probenecid**, and, as a side ef ect, by other drugs (see Section 4.2).

T e urine is often supersaturated with uric acid. In some patients, **stones of uric acid** may form in the kidneys (see Section 4.3).

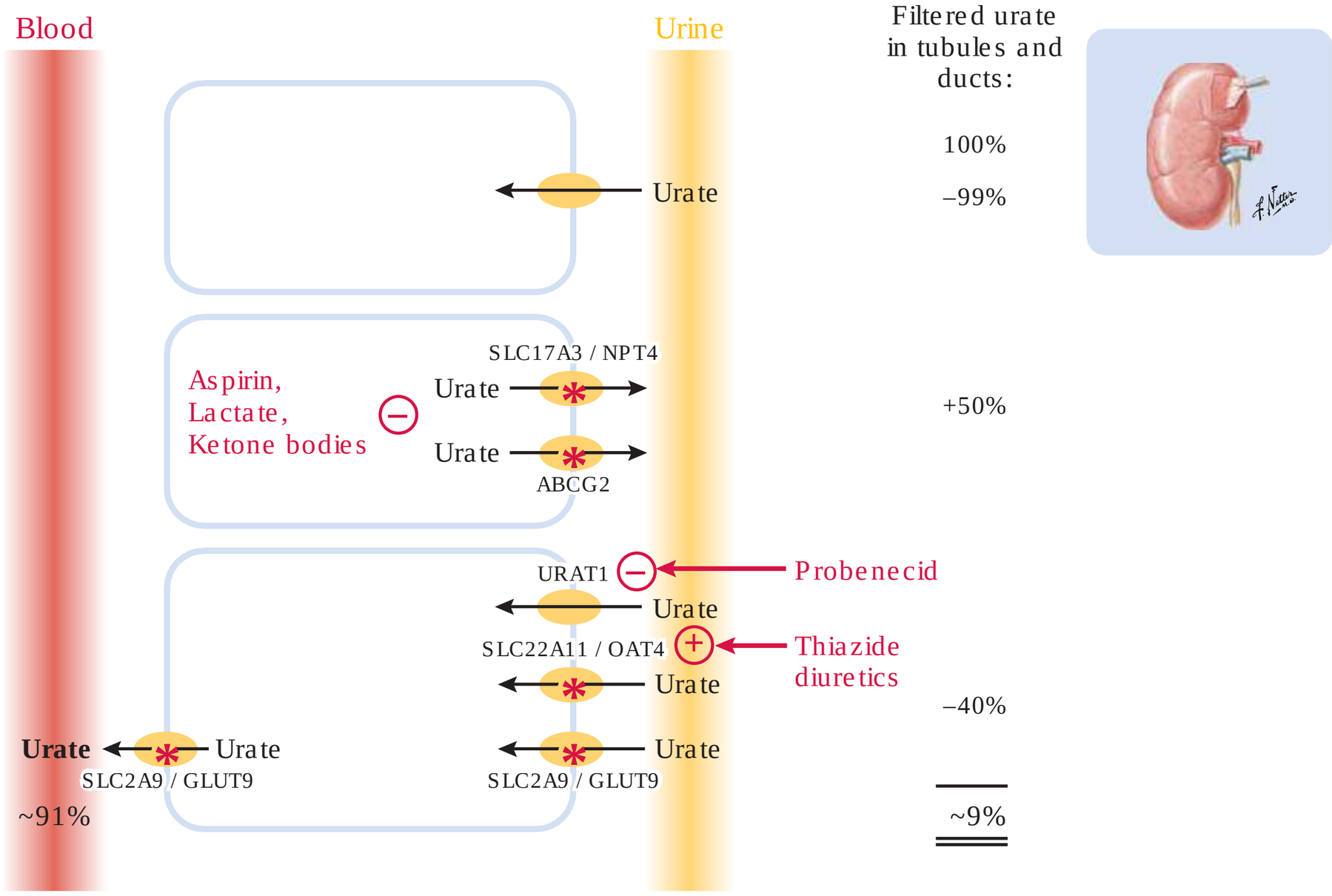


Fig. 38.7 **Excretion of urate in the proximal tubules of the kidneys.** Only about 9% of the filtered urate ends up in the urine. Asterisks denote a genetic locus in or near the transporter gene associated with a person's genetic risk for gout.

About 25% of all urate is excreted via the **feces**. ABCG2, which also transports urate into the urine, is thought to play a significant role in urate excretion into the intestine. T ere are no established means of influencing fecal urate excretion.

2.4. Salvage of Hypoxanthine and Guanine

Cells that need to increase their adenine nucleotide content may take up the purine base **hypoxanthine** from the blood and react it with the ribose **PRPP** to make the nucleotide **IMP**, a precursor of **AMP** and **GMP** (Fig. 38.8). Cells synthesize PRPP only when they have an adequate concentration of phosphate and when the concentration of free ADP is low. T e salvage reaction is catalyzed by **hypoxanthine guanine phosphoribosyl transferase** (commonly abbreviated **HGPRT** or **HPRT**). As indicated by the name of this enzyme, HGPRT can also salvage **guanine**, thereby producing GMP.

Patients who have **classic Lesch-Nyhan disease** have less than 2% of the normal **HGPRT** activity. T e deficiency leads to mental retardation, profound motor disability, and a strong inclination toward self-mutilation. HGPRT is encoded on the X chromosome; hence boys are af ected more often than girls. Female heterozygote carriers are usually normal. Patients with classic Lesch-Nyhan disease recycle almost no hypoxanthine. As a result, de novo synthesis and urate production are about 30-fold increased. T e neurologic problems are likely due to an inadequate synthesis of purine nucleotides, especially in

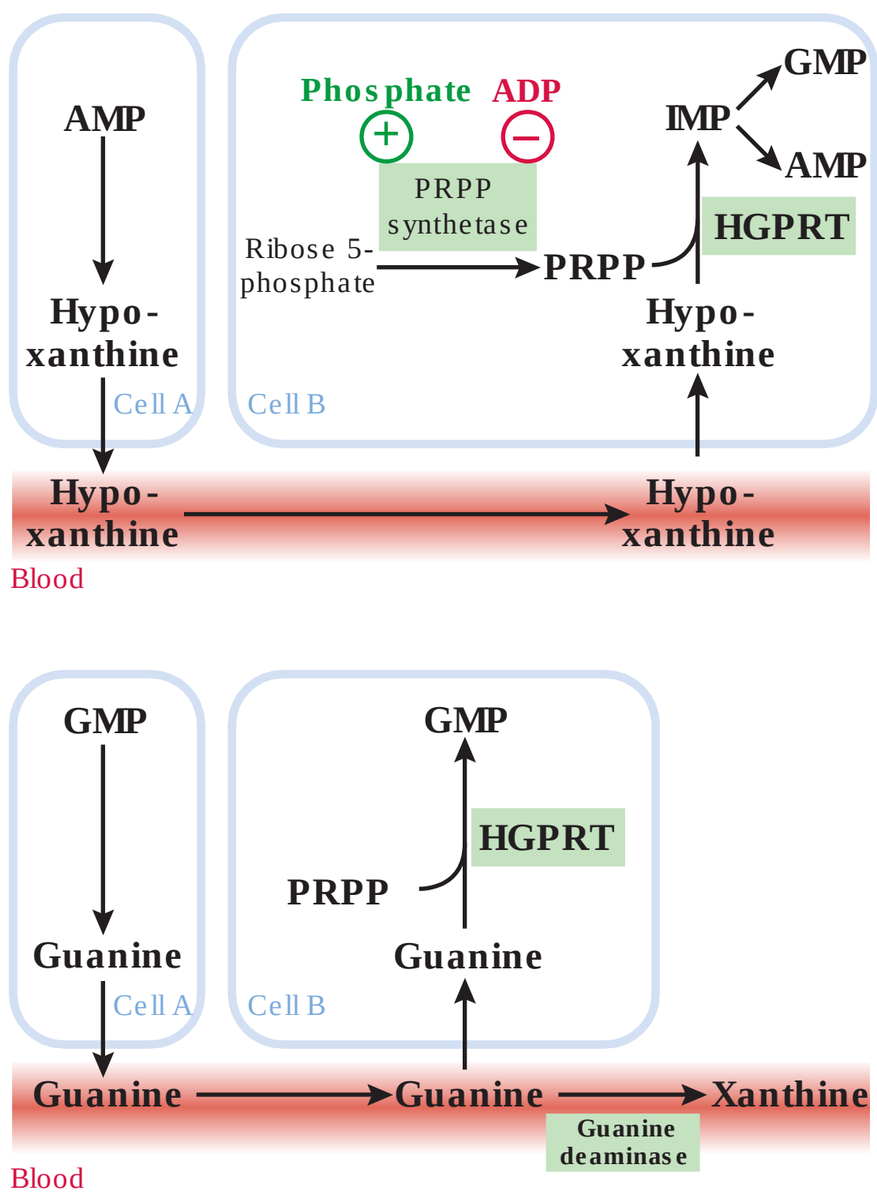


Fig. 38.8 **Salvage of hypoxanthine and guanine.** Control over the salvage pathway rests with the production of PRPP. The liver degrades hypoxanthine and xanthine that have not been salvaged.

the basal ganglia. Urate overproduction leads to gout (see Section 4.1). Classic Lesch-Nyhan disease is very rare, and it represents one extreme of a spectrum of HGPRT deficiencies.

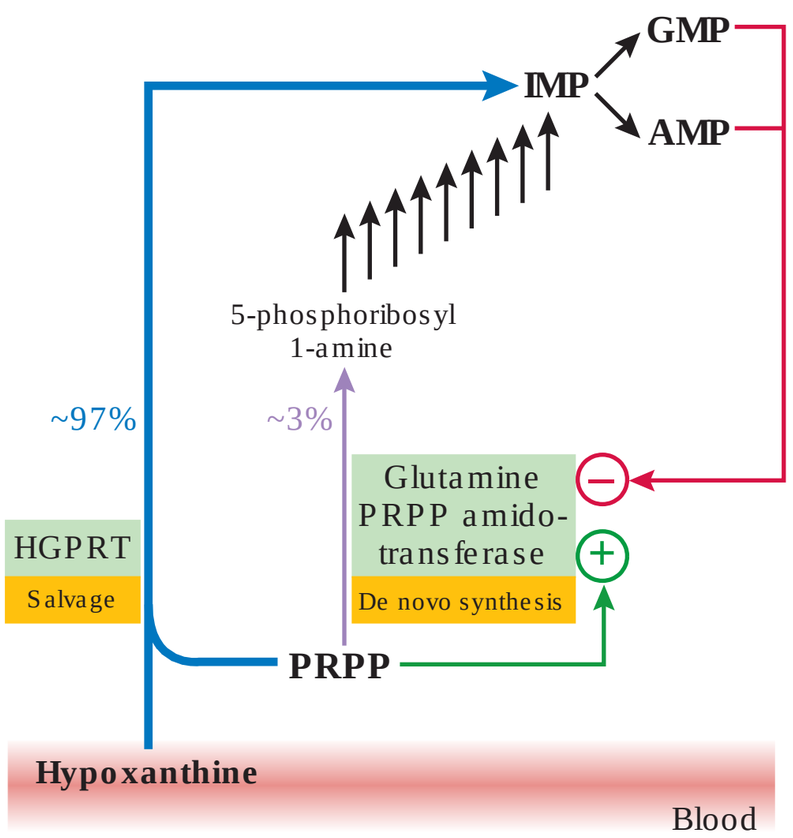
Variant Lesch-Nyhan disease is caused by milder deficiencies of HGPRT activity than classic Lesch-Nyhan disease. The mildest phenotype is associated only with hyperuricemia, but this seems to be uncommon. A more severe deficiency of HGPRT also causes motor handicaps and cognitive problems.

2.5. Balancing the Production of IMP From Salvage and De Novo Synthesis

For the production of IMP, **salvage** of hypoxanthine is **preferred over de novo synthesis**. In the human body overall, about **97%** of the production of purine nucleotides stems from the **salvage** of hypoxanthine, and about **3%** stems from **de novo** synthesis. The salvage enzyme, **HGPRT**, follows Michaelis-Menten type kinetics (Fig. 38.9). HGPRT has a greater affinity for **PRPP** than does glutamine PRPP amidotransferase in the de novo synthesis pathway. In addition, in many tissues, maximal enzyme activity for HGPRT is larger than for glutamine PRPP amidotransferase. Hence, IMP is made mainly from hypoxanthine as long as sufficient hypoxanthine is available. **Glutamine PRPP amidotransferase**, the enzyme that limits the rate of de novo synthesis, shows cooperativity toward PRPP. Only when the concentration of PRPP is relatively high, usually because insufficient hypoxanthine is available for salvage, does glutamine PRPP amidotransferase become active.

Cells build up their adenine nucleotide content only relatively slowly. After losing adenine nucleotides, cells generally require many **hours** to several **days** to regain more than 95% of their normal adenine nucleotide content.

For the production of **GMP**, the same principles apply to balancing de novo synthesis and salvage from either guanine or hypoxanthine as for the production of AMP.



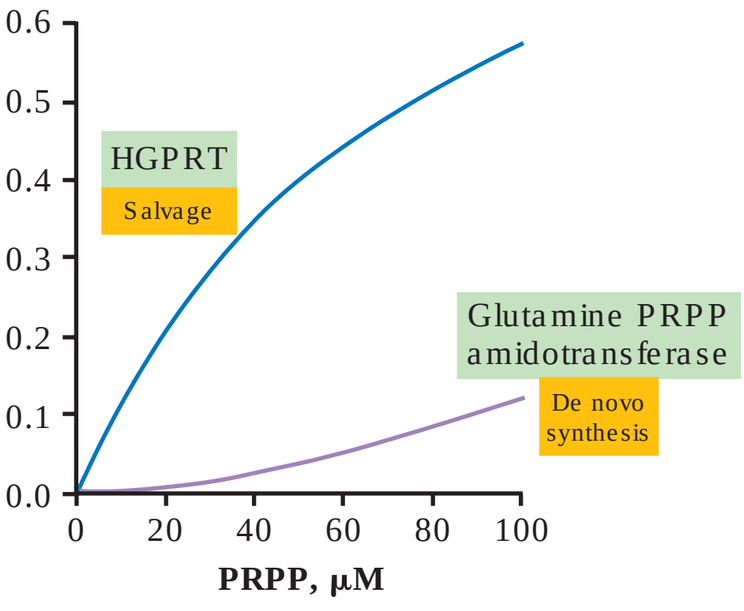


Fig. 38.9 Balancing salvage of hypoxanthine and de novo synthesis of IMP.

2.6. Daily Purine Turnover and Urate Excretion

There are two contributors to the daily purine load: the degradation of hypoxanthine and guanine to urate, and the conversion of dietary purines to urate (Fig. 38.10). About three-fourths of the daily purine load are excreted via the urine and one-fourth via the feces.

3. HYPERURICEMIA

Men and postmenopausal women have the highest concentrations of urate in plasma. Whenever the body produces too much urate, the concentration of urate in the blood also rises, thereby giving rise to hyperuricemia. Likewise, whenever the kidneys inadequately filter blood or excrete a smaller fraction than the normal 7% to 10% of the filtered urate, hyperuricemia ensues. Pregnant women who have preeclampsia overproduce and underexcrete urate; serum urate therefore serves as one of several indicators of the severity of the disease. In persons who have chronic hyperuricemia, crystals of sodium urate tend to form in joints.

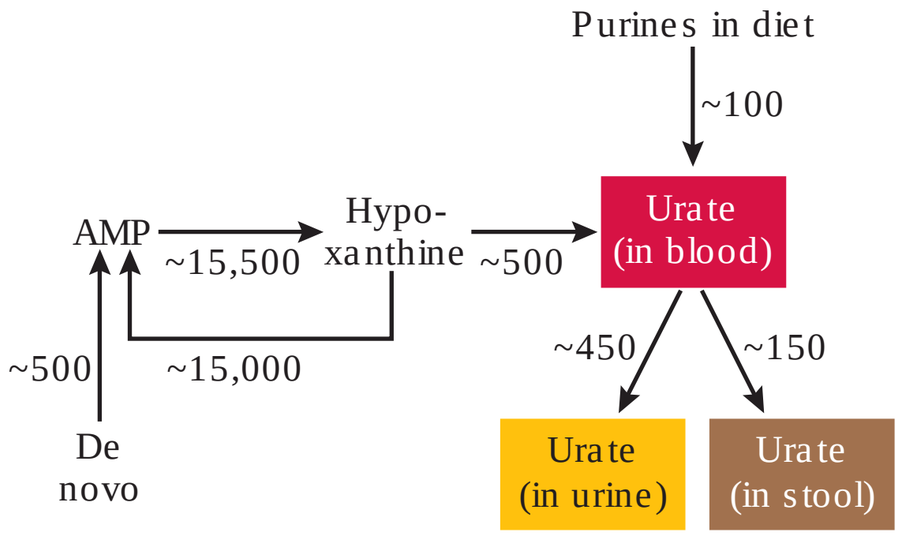


Fig. 38.10 Normal purine flux. Numbers are approximate and given in milligrams per day.

3.1. Plasma Urate as a Function of Gender and Age

The concentration of urate in blood plasma is **age and gender dependent** (Fig. 38.11). In adults, estrogen increases uric acid clearance by the kidneys. Hence, premenopausal women excrete a greater percentage of filtered urate than men. Pregnancy normally further lowers the concentration of urate in women (this is also partly due to increased glomerular filtration). In transsexual persons, treatment with estrogens lowers serum urate, whereas treatment with testosterone increases it.

In population studies, the concentration of urate in blood is strongly **hereditary**. More than 25 different genetic loci are known to correlate with the serum urate concentration, although the individual contributions to the population variation are small. To date, about half of these loci could also be associated with protection from, or susceptibility to, gout.

3.2. Overproduction of Urate

The relationship between plasma urate concentration and the amount of urate the kidneys excrete per day is shown in Fig. 38.12. The kidneys of a patient who has a high urate production can excrete the larger amount of urate only under the condition of hyperuricemia. In other words, persons who overproduce urate have hyperuricemia. There is no physiological body store for urate. Any daily pathological deposition of urate as crystals of sodium urate in the joints is small compared with the daily urate production.

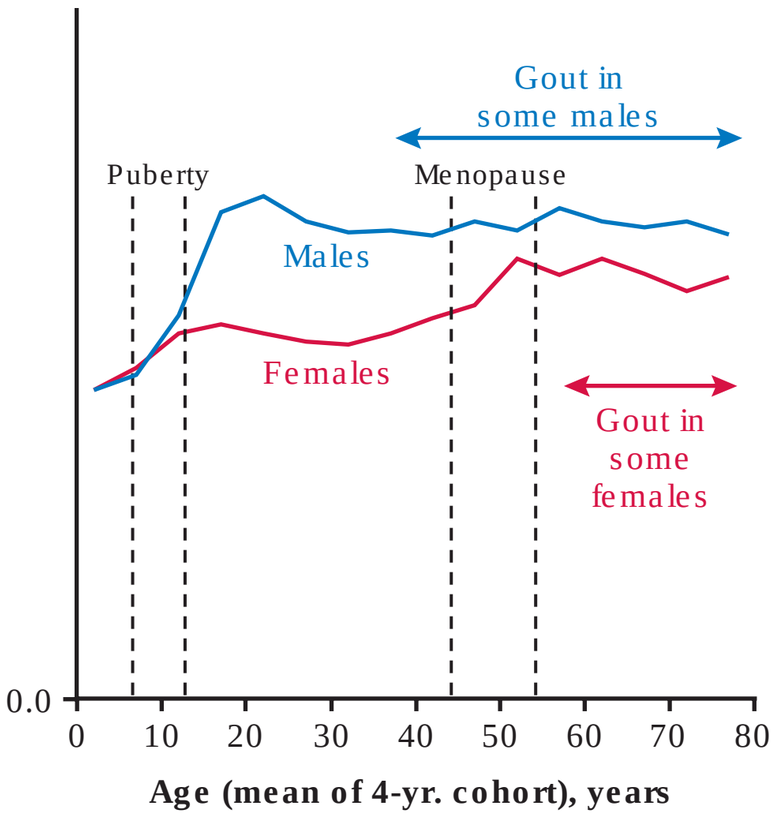


Fig. 38.11 Serum urate concentration in the population of Tecumseh, MI, in 1959–1960. The data include both normal and abnormal values. The exact concentration of urate is omitted because current assays produce different numbers. The trends remain the same, however. (Modified from Mikkelsen WM, Dodge HJ, Valkenburg H. The distribution of serum uric acid values in a population unselected as to gout or hyperuricemia: Tecumsah, Michigan 1959-1960. *Am J Med*. 1965;39:242-251.)

3.3. Underexcretion of Urate

If a patient has impaired perfusion of the kidneys, impaired glomerular filtration, decreased resecretion of uric acid into the proximal tubules of the kidneys, or increased reabsorption from the distal portion of the proximal tubules, the excretion of uric acid amounts to less than the normal 7% to 10%. The concentration of urate in the blood rises until daily uric acid excretion matches urate production (Fig. 38.13). This problem is commonly called urate **underexcretion**. However, this can be a misleading term because persons who produce a normal amount of urate and who "underexcrete" still excrete a normal total amount of urate per day. Underexcretion only refers to a low rate of urate excretion at a normal plasma urate concentration.

Hyperuricemia is thus present in both urate overproducers and urate underexcretors.

In patients who have hyperuricemia but are free of symptoms, possible causes of the hyperuricemia are usually explored, and diet, alcohol intake, and pharmacotherapy are adjusted, if possible. Drug therapy is usually not instituted.

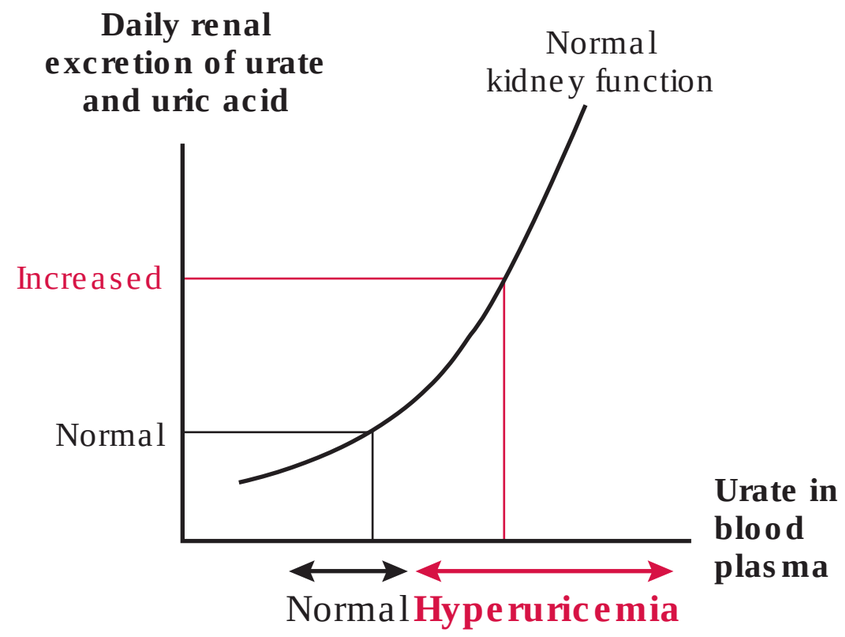


Fig. 38.12 Relationship between daily renal urate excretion and the concentration of urate in blood plasma.

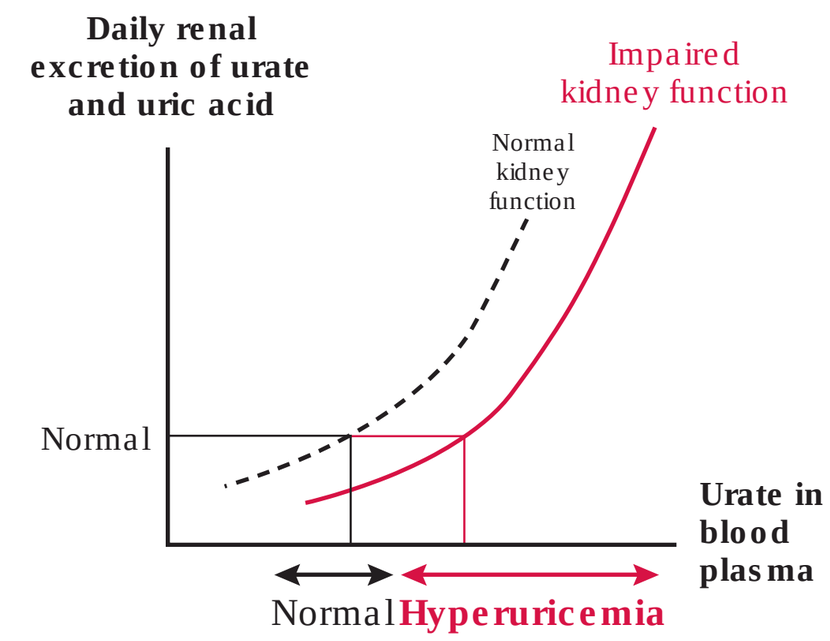


Fig. 38.13 Relationship between daily renal urate excretion and concentration of urate in blood plasma in patients who are urate underexcretors.

3.4. Plasma Urate and Preeclampsia

Preeclampsia and eclampsia are a collection of life-threatening diseases that af ect about **5%of pregnant women**, most often in their third trimester (Fig. 38.14). Vasospasms, dif use intravascular coagulation, and the loss of fluid into the intravascular space (causing edema) decrease perfusion to almost all organs. Decreased renal perfusion and function lead to **underexcretion of urate**. Tissue hypoxia and necrosis, as well as degradation of red blood cells in clots, lead to **overproduction of urate**. Underexcretion and overproduction each increases the concentration of urate in serum. T e concentration of total uric acid in blood plasma is a valuable predictor of preeclampsia and eclampsia, as well as an **indicator of the severity** and course of this disease. T e principal treatment of preeclampsia and eclampsia is the delivery of the fetus. Seizures can be prevented with an infusion of magnesium sulfate.

3.5. Crystallization of Urate

Uric acid has a pK of 5.75; thus, above pH 5.75 (such as in blood), **urate** predominates, whereas below pH 5.75 (e.g., in acidic urine), **uric acid** predominates.

Sodium urate can form needle-like crystals. Sodium urate is present in blood near the limit of its solubility. T is can be considered advantageous because of the antioxidant activity of urate. On the other hand, when plasma is **supersaturated** with sodium urate, sodium urate tends to crystallize on existing nuclei, preferentially in **joint synovia** and **soft cartilaginous tissues** (Fig. 38.15). T is tendency is more pronounced at a lower temperature, which may explain why sodium urate crystals preferentially form in the **cooler** parts of the human body (e.g., limbs and auricles).

Depending on the pH of urine and the total concentrations of uric acid and sodium in it, crystals of either sodium urate or uric acid can form in the urinary tract (see Fig. 38.15). A **low daily urine volume** and a **high daily total uric acid production** both increase the chance of crystal formation. While a **low urine pH** favors the formation of **uric acid** crystals, a **high daily sodium excretion** and a **high urine pH** favor the formation of **sodium urate** crystals. T e crystals can aggregate to form kidney stones.

3.6. Tumor Lysis Syndrome

Patients who undergo chemotherapy for certain neoplasms are at a particularly high risk of developing uric acid nephrolithiasis as part of tumor lysis syndrome. When tumor cells die, they liberate large amounts of hypoxanthine, potassium, and phosphate. T e liver and intestine convert hypoxanthine to urate. T is can lead to severe hyperuricemia and damage to kidney tubules by precipitation of calcium phosphate and/or the crystallization of uric acid. Tumor lysis syndrome can be fatal and is a feared complication of chemotherapy or radiotherapy, particularly in patients who have certain lymphomas or leukemias. Onset is usually 2 to 3 days after the start of therapy. In a current classification system, patients who have

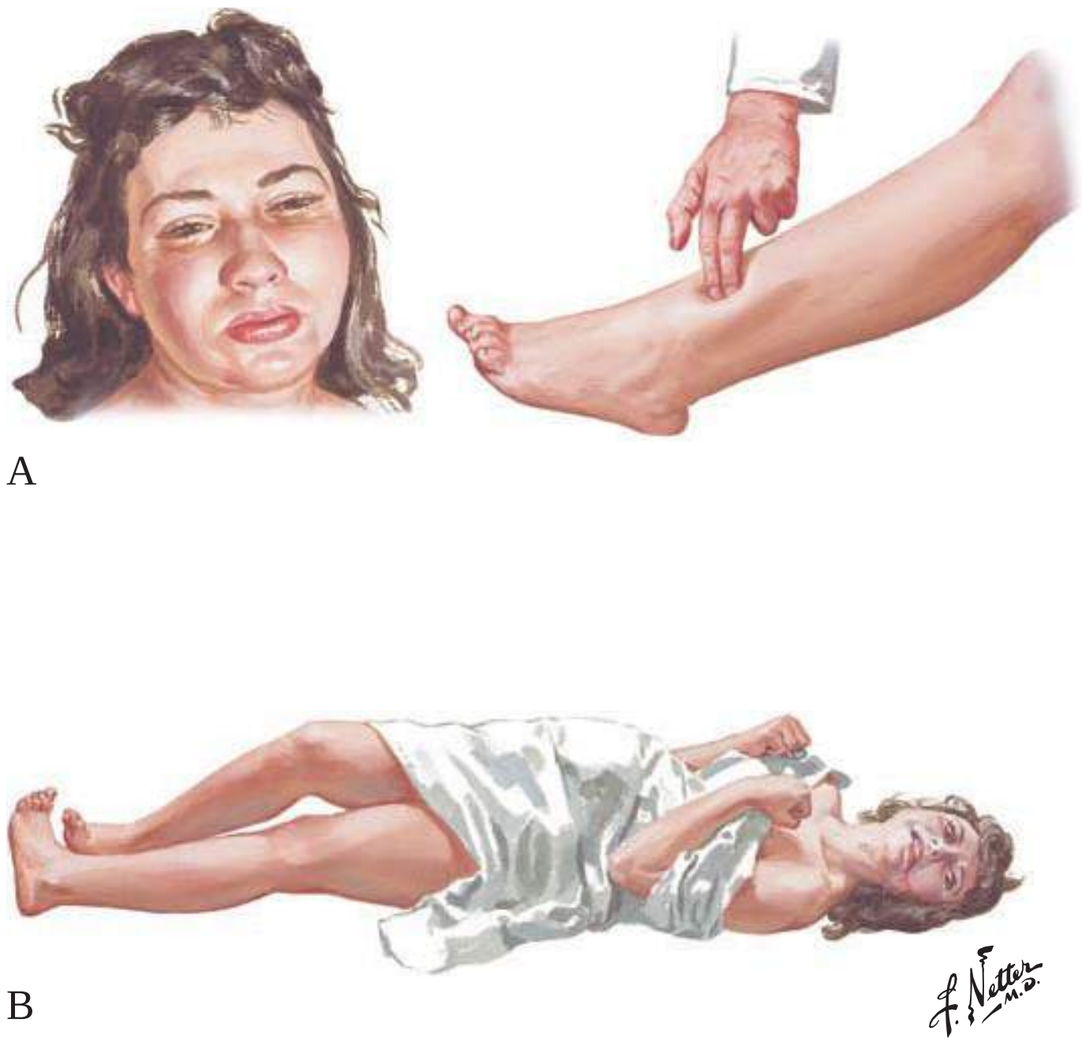


Fig. 38.14 Preeclampsia and eclampsia are accompanied by hyperuricemia. (A) Preeclampsia is characterized by edema, high blood pressure, and proteinuria. (B) Eclampsia is accompanied by life-threatening seizures and internal hemorrhage.

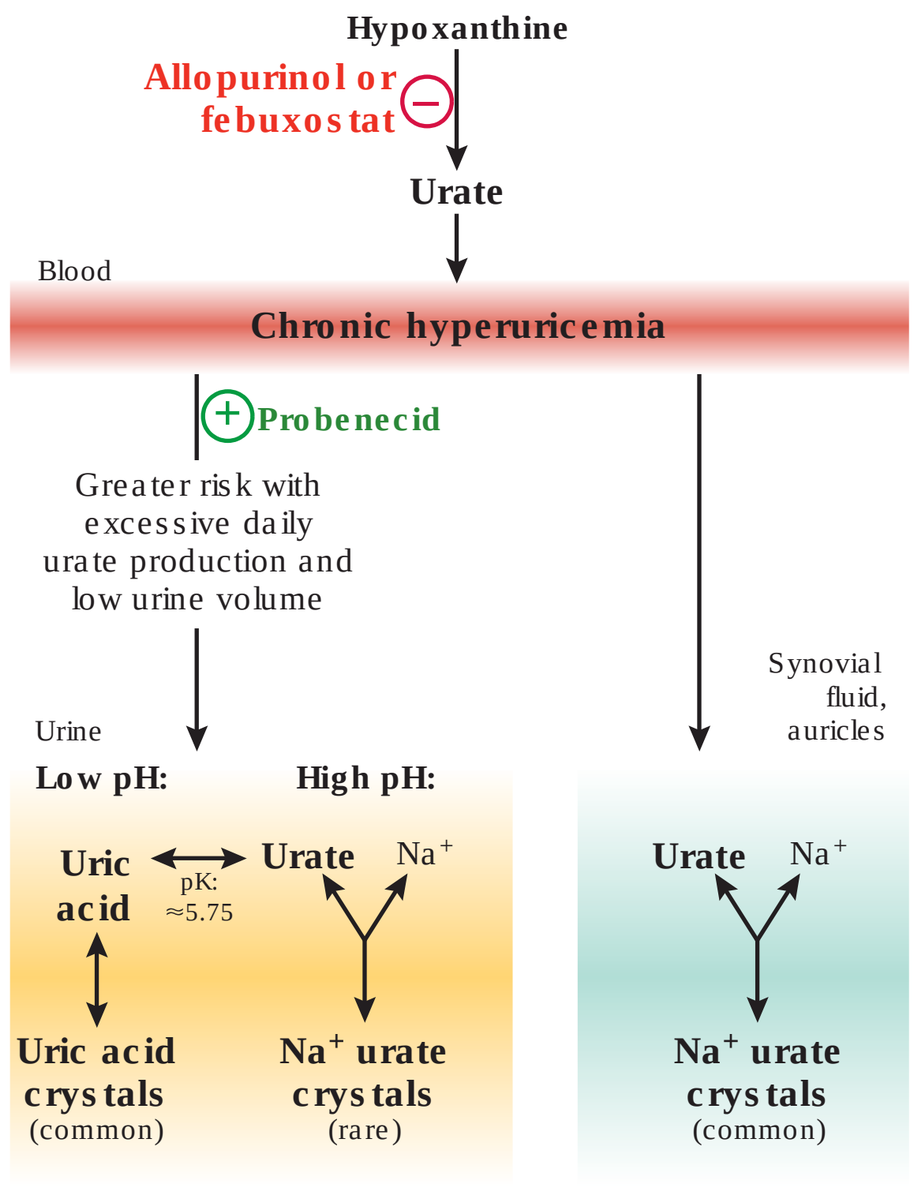


Fig. 38.15 Pathologic formation of crystals of sodium urate and uric acid.

tumor lysis syndrome have two of the following abnormalities: increased K^+, increased phosphate, decreased Ca^{2+} (Ca^{2+} is low due to precipitation of calcium phosphate in the kidneys), and increased serum urate, or more than a 25% change in these values 7 days after starting chemotherapy.

Uric acid stone formation with tumor lysis can be minimized with a large fluid intake, oral bicarbonate or citrate, oral allopurinol, or intravenous recombinant urate oxidase (e.g., **rasburicase**). Compared with rasburicase, allopurinol has a slower onset, is not as effective in lowering blood urate, and may lead to the precipitation of xanthine in renal tubules. Hence, allopurinol is used in patients who are expected to produce only a moderate amount of extra urate. Hyperkalemia and hyperphosphatemia are addressed by the induction of diuresis and/or dialysis.

4. GOUT

Patients who have chronic hyperuricemia may develop gout, which can manifest itself either as acute arthritis or kidney stones. Gout is a very common disease, particularly among elderly men. Acute gouty arthritis is due to joint inflammation in response to the presence of sodium urate. Kidney stones are usually due to aggregates of uric acid crystals. Urate-lowering therapy may involve inhibitors of xanthine dehydrogenase, drugs that increase the excretion of urate in the kidneys, and recombinant uric acid oxidase to destroy urate in the blood.

4.1. General Comments About Gout

The term **gout** is sometimes used in reference to urate-dependent joint inflammation only, and sometimes in reference to both joint inflammation and formation of kidney stones. Both of these pathologies stem from aberrations in urate production and excretion. Joint inflammation stems from sodium urate crystals in joints that occasionally give rise to a highly painful inflammatory reaction, an **acute gouty arthritis**. **Nephrolithiasis** (presence of kidney stones) is due to the aggregation of crystals of uric acid or, rarely, sodium urate in the urine. Another form of **nephropathy**, although uncommon, results from crystallization of sodium urate in the hyperosmolar interstitial fluid of the renal medulla. Both nephropathy (related to uric acid or sodium urate) and arthritis are usually preceded by months to years of symptom-free **hyperuricemia**. During their lives, patients who have symptomatic hyperuricemia may present with acute gouty arthritis, nephrolithiasis, or both. However, during a single, painful episode, patients usually present only with arthritis or with nephrolithiasis.

Adult men have the highest concentration of urate in their blood for the longest time during their lives (see Fig. 38.11) and are most likely to develop gout. As a rule of thumb, **children and premenopausal women do not get gout**. If gout is present in a child or premenopausal woman, it may be due to a metabolic disease.

It is estimated that more than 95% of patients who have gout are urate **underexcretors** (see Section 3.3), often for unknown reasons. It is also not known why **hypertriglyceridemia** (see Chapter 28), **hypertension**, and the **metabolic syndrome** (see Chapter 39) are associated with impaired uric acid excretion. Chronic urate underexcretion is seen in the following circumstances:

- Decreased glomerular filtration in patients with advanced chronic **renal failure** and in patients who have **lead** poisoning.
- Inhibition of the excretion of uric acid into the kidney tubules by organic acids (see Fig. 38.7) in patients with chronic **lactic acidemia**, **ketoacidosis**, or **propionic acidosis**, as well as in patients who frequently metabolize large amounts of **ethanol**, and in patients who take certain diuretic drugs (especially **thiazides**, which are organic acids) or **aspirin** (acetylsalicylic acid) at a dose of 1 to 2 g/day.
- In chronically **dehydrated** persons, uric acid reabsorption by URAT1 is increased and excretion is decreased.

Perhaps as little as **1%** of all patients who have gout are urate **overproducers** (see Section 3.2), mostly for unknown reasons. Known causes of increased urate production are diseases and physiological states that lead to an **increased concentration of AMP** or **PRPP** or to decreased **salvage**. Urate production can be increased by the following:

- Metabolism of **ethanol** (see Chapter 30).
- Metabolism of **fructose** (see Chapter 20).
- Increased tissue turnover due to one of the following: **obesity**, **psoriasis** (see Chapter 37), **leukemia**, **lymphoma**, **hemolytic anemias**, **sickle cell** disease and **thalassemia** (see Chapter 17), **polycythemia vera**, **hemorrhage**, **infection**, **trauma**, or **cytolytic therapy**.
- **Increased AMP production** due to **hypoxia** or **ethanol metabolism**.
- Exercise in patients with a deficiency in muscle **glycogen** metabolism (e.g., muscle glycogen phosphorylase deficiency and glycogen debranching enzyme deficiency) (see Chapter 24).
- **ATP-consuming futile cycles** in patients who have a deficiency in the gluconeogenic pathway, such as glucose 6-phosphatase deficiency or fructose bisphosphatase deficiency (see Chapter 25).
- Exercise in patients with a **medium-chain acyl-CoA dehydrogenase** (**MCAD**) deficiency (see Chapter 27).
- **Lesch-Nyhan** disease (due to lack of salvage; see Section 2.4).
- Excessive activity of **PRPP synthetase** (a very rare mutation; increased de novo purine nucleotide synthesis must be balanced by an increased production of urate).

4.2. Acute Gouty Arthritis

Acute gouty arthritis is the most common presentation of symptomatic, long-term hyperuricemia. The higher

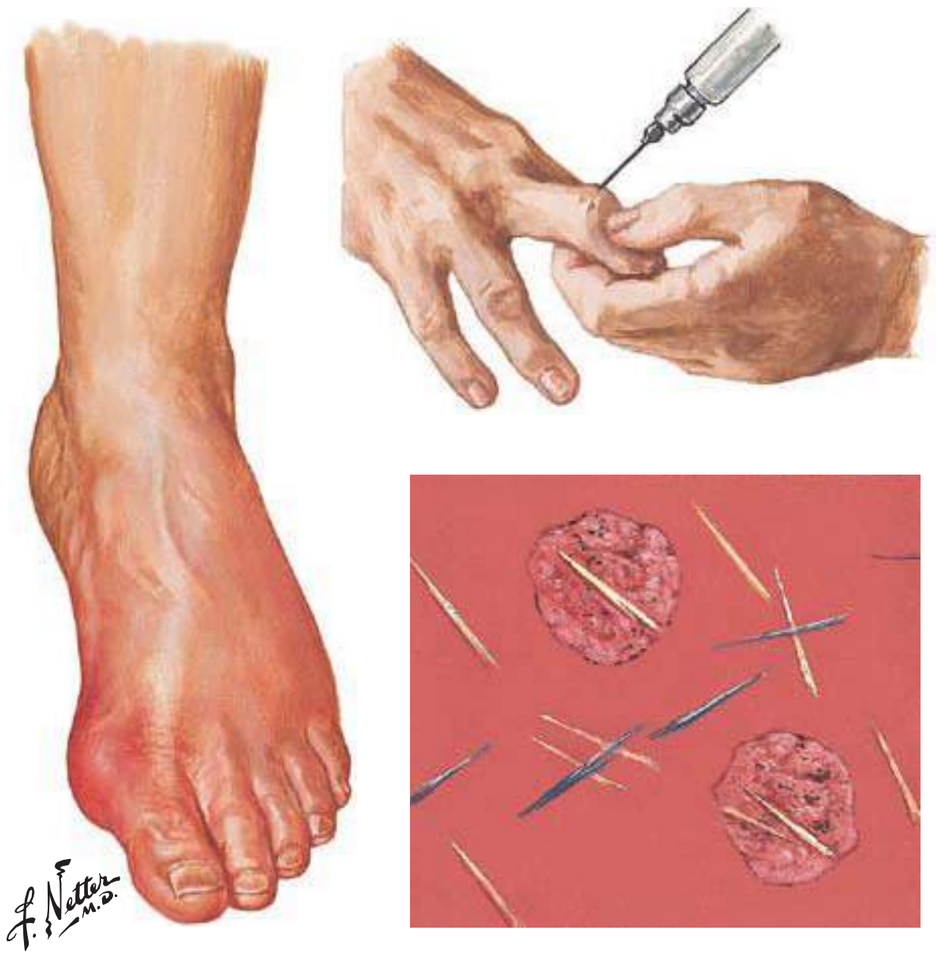


Fig. 38.16 Acute gouty arthritis and its diagnosis.

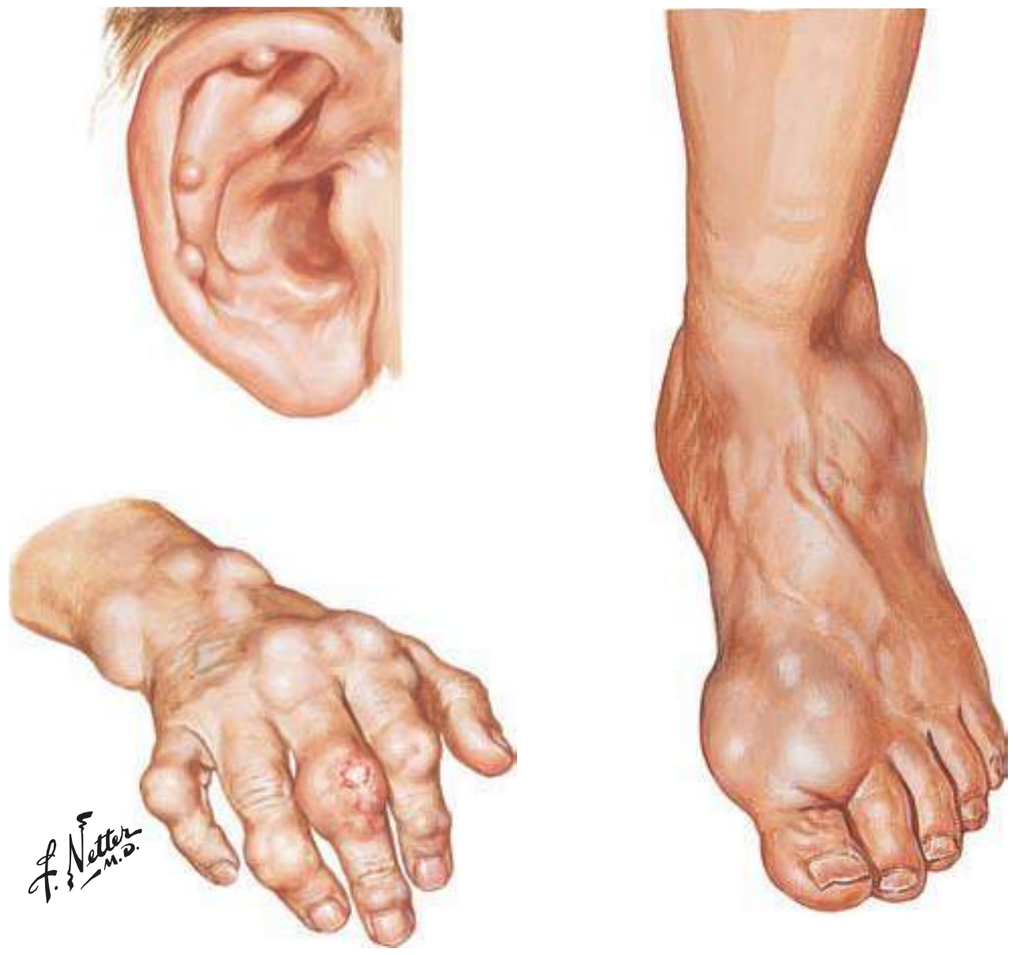


Fig. 38.17 Tophaceous gout. The sodium urate deposits are likely accompanied by repeated episodes of acute gouty arthritis and also by the erosion of nearby bone that can destroy a joint.

the concentration of urate in the blood, the greater is the chance that crystals of sodium urate form in the **synovial fluid**. Such crystals occasionally give rise to a very painful, acute inflammation called acute gouty arthritis. The inflammation typically affects a single joint, usually of the lower extremity, and most commonly the metatarsophalangeal joint (Fig. 38.16).

The **diagnosis** of an acute gouty attack often relies on aspirating fluid from the affected joint and finding negatively birefringent crystals under a polarizing microscope (see Fig. 38.16). If the compensator is oriented parallel to the crystals, the crystals appear yellow; if it is perpendicular to the crystals, they appear blue. The method allows crystals of sodium urate to be distinguished from crystals of calcium pyrophosphate, which are the cause of **pseudogout**.

The joint inflammation is self-limiting and, in the absence of treatment, resolves by itself within hours to weeks. The duration of the inflammation can be shortened with nonsteroidal antiinflammatory drugs (**NSAIDs;** e.g., indomethacin, naproxen, ibuprofen), **corticosteroids**, or **colchicine** (an inhibitor of leukocyte chemotaxis). None of these drugs appreciably affects the concentration of urate in the blood.

All patients with acute gouty arthritis should be informed about **lifestyle modification**. Obese patients should lose weight by using a combination of diet and exercise. Patients can reduce the dietary purine load by not eating **liver**, **kidney**, or **sweetbreads** (thymus) as well as limiting their consumption of **red meat** and **shellfish**. Patients can also limit their intake of **fructose** from sucrose, high-fructose corn syrup, and fruit juices. Finally, patients can limit their consumption of **ethanol**, particularly **beer (**because of its high purine content).

After one or more episodes of acute gouty arthritis, patients decide whether to start long-term **urate-lowering drug therapy**. In principle, the concentration of urate in the blood can be lowered by decreasing urate production, increasing the efficiency of renal uric acid excretion, or a combination of these two processes. The major complication to be avoided is **nephrolithiasis**. Kidney stones with uric acid form most readily in patients with a low urine pH, a low urine volume, and an excessive production of urate.

The major urate-lowering drugs for long-term use are allopurinol, febuxostat, probenecid, and pegloticase. **Allopurinol**, an inhibitor of xanthine dehydrogenase (see Section 2.2), is currently preferred as long as patients do not have an HLA haplotype that is associated with allopurinol hypersensitivity syndrome, which in turn has about a 20% mortality rate. If allopurinol is contraindicated, **febuxostat**, another inhibitor of xanthine dehydrogenase, is used. **Probenecid** enhances renal urate excretion by inhibiting urate reuptake via the URAT1 transporter (see Section 2.3); it is thus a **uricosuric drug**. The use of probenecid is generally restricted to patients who are unlikely to form kidney stones. Patients can reduce their risk of uric acid stones by increasing their fluid intake and taking oral bicarbonate or citrate to alkalinize their urine. At times, patients are given both allopurinol and probenecid. If none of these drugs work, **pegloticase**, a uricase (see Section 2.2), can be used. The American College of Rheumatology recommends that the goal of urate-lowering therapy be a serum urate of 6 mg/dL or less.

Fenofibrate, **losartan**, and **amlodipine** have a uricosuric **side effect** that may be helpful in the treatment of gout. Like probenecid, these drugs inhibit the URAT1 transporter (see Section 2.3). Fenofibrate is used to treat hyperlipidemia. Losartan and amlodipine are both used to lower blood pressure.

If consecutive episodes of acute gouty arthritis are not, or cannot be, adequately treated, **tophi** (visible accumulations of

sodium urate crystals; Fig. 38.17) form as a result of a **chronic** excess of urate production over urate excretion. The longer a patient is hyperuricemic, and the greater the hyperuricemia, the more likely a patient is to develop such tophi. Tophi are rarely present at the time of the first gouty attack. With current therapies, the formation of tophi can be prevented in almost all patients.

Patients who have gout due to **lead poisoning**, often referred to as **saturnine gout**, most commonly present with acute gouty arthritis in a **knee**. By contrast, patients who have the usual primary gout usually present with acute arthritis in the first metatarsophalangeal joint. Most patients in the United States with saturnine gout have absorbed too much lead from solder in equipment used for illegal alcohol distillation. Almost all of these patients have lead-induced kidney damage. Lead is stored in bone and stays in the body for decades (it has a half-life of ~20 years).

Treatment of saturnine gout is the same as for primary gout, but steps are also taken to minimize further lead poisoning and reduce lead in the body through **chelation** with Ca^{2+}-EDTA (Pb^{2+} binds to EDTA in place of Ca^{2+}; Ca^{2+} is present to prevent the hypocalcemia that EDTA would otherwise cause), dimercaprol, or succimer. Some of the lead-induced kidney damage is reversible.

4.3. Uric Acid and Sodium Urate in Nephrolithiasis

About 25% of patients who have symptomatic hyperuricemia first present with **uric acid nephrolithiasis** (uric acid **kidney stone**; Fig. 38.18). Most of these patients produce urine of a low pH and a high concentration of uric acid. **Sodium urate** is rarely found in stones.

The long-term prevention of uric acid nephrolithiasis is aimed at reducing the concentration of uric acid in urine. The patient can increase **urine volume** by increasing fluid intake, increase **urine pH** with oral **bicarbonate** or **citrate**, and decrease **urate production** with lifestyle changes (e.g., weight loss, decreased consumption of alcohol, fructose, and foods with high purine contents, such as organs or seafood). The physician should also treat any underlying disease that contributes to urate production (e.g., psoriasis or myeloproliferative disease). The patient can be prescribed **allopurinol** or **febuxostat**. If indicated by special circumstances, patients can be infused with recombinant **urate oxidase**.

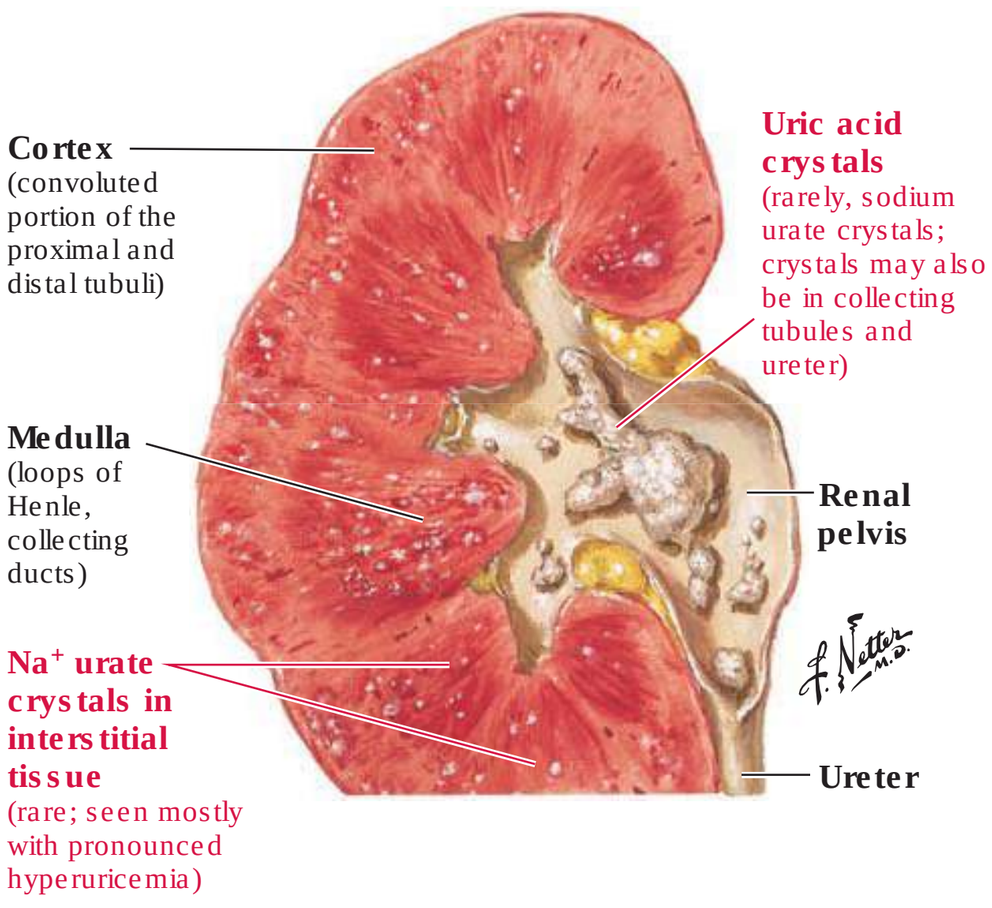


Fig. 38.18 **Uric acid nephrolithiasis.**

5. THIOPURINES

Thiopurines are purine analogs that have a mutagenic and immunosuppressive effect. They are used in the treatment of neoplasms, autoimmune diseases, and inflammatory diseases.

The thiopurines have short-term anticancer and immunosuppression effects, yet their long-term use by transplant recipients is associated with an increased incidence of neoplasms, particularly skin cancer. Thiopurines are no longer used much in organ transplantation, but they are used for the treatment of acute lymphoblastic leukemia or inflammatory bowel disease (e.g., Crohn disease or ulcerative colitis).

While most cells can synthesize AMP either by the de novo synthesis pathway (see Fig. 38.3) or by salvage of hypoxanthine (see Fig. 38.8), activated lymphocytes synthesize most of their AMP by de novo synthesis. Therefore they are particularly susceptible to inhibitors of the purine de novo synthesis pathway.

The main clinically used thiopurines are **azathioprine**, **6-mercaptopurine**, and **6-thioguanine**. With the help of glutathione inside red blood cells, azathioprine is converted to 6-mercaptopurine (Fig. 38.19). In lymphocytes, HGPRT (see Section 2.4) acts on 6-mercaptopurine to form thio-IMP, some of which is methylated to methylthio-IMP, an inhibitor of glutamine PRPP amidotransferase, the first enzyme of de novo purine nucleotide synthesis. Both 6-mercaptopurine and 6-thioguanine are enzymatically converted to thio-dGTP, which is incorporated into DNA and may then be methylated. It seems likely that methylated thio-G presents a problem for DNA mismatch repair in a subsequent round of DNA replication, leading to cell cycle arrest and apoptosis.

About 0.3% of all patients are deficient in **thiopurine S-methyltransferase** (**TPMT**), degrade thiopurines abnormally slowly, and are therefore at substantial risk of a fatal adverse reaction. Before treatment, patients can be tested for erythrocyte TPMT activity if they have not recently received a blood transfusion.

Patients who take **allopurinol** or **febuxostat** need to be given a smaller dose of azathioprine or 6-mercaptopurine to account for decreased 6-mercaptopurine inactivation by xanthine dehydrogenase.

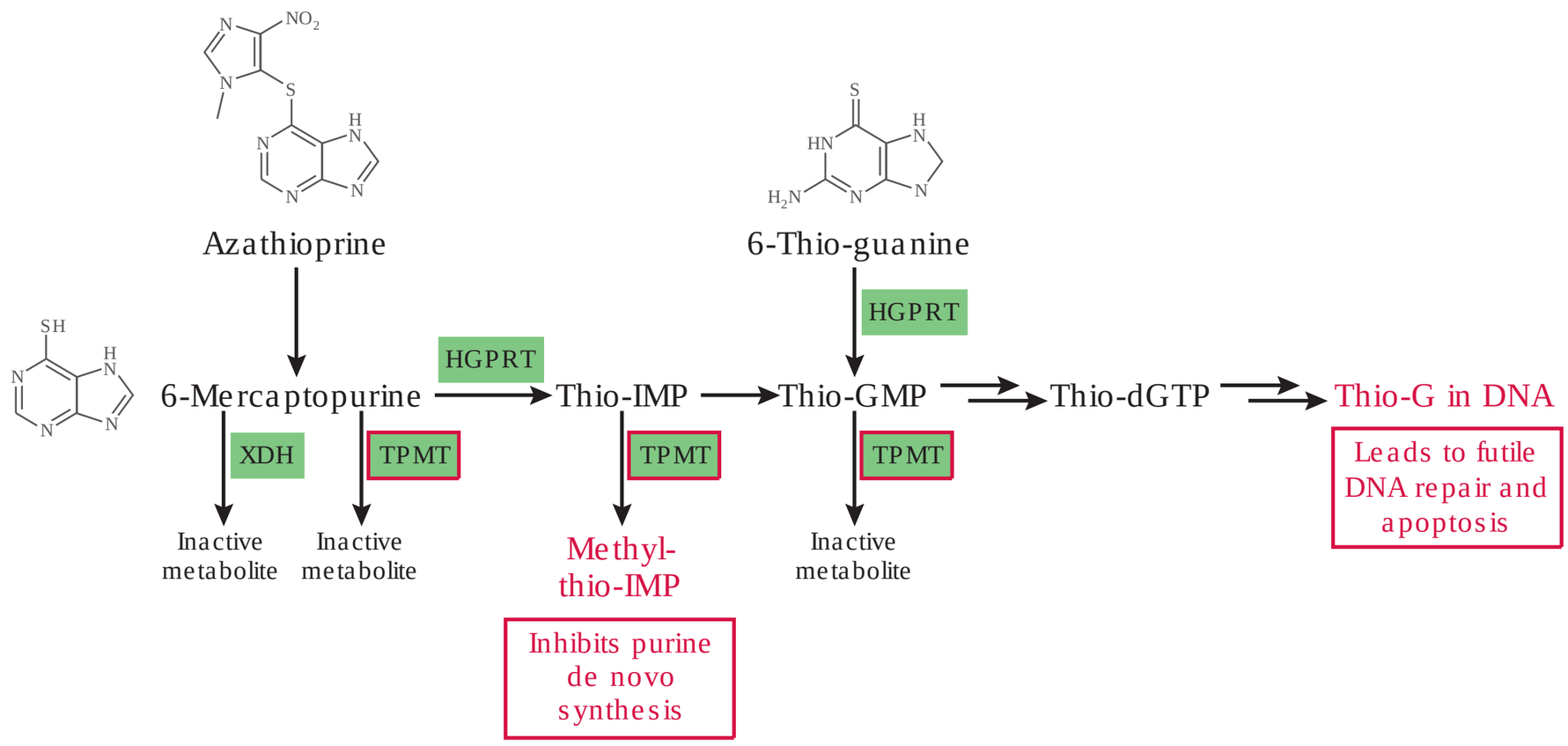


Fig. 38.19 Metabolism of the thiopurines azathioprine, 6-mercaptopurine, and 6-thioguanine.
TPMT-deficient persons are at risk for thiopurine toxicity. HGPRT, hypoxanthine guanine phosphoribosyl transferase; TPMT, thiopurine S-methyltransferase; XDH, xanthine dehydrogenase.

SUMMARY

- When the concentration of phosphate is adequate, and that of ADP low, cells can synthesize phosphoribosylpyrophosphate (PRPP) from ribose 5-phosphate, an intermediate of the pentose phosphate pathway.
- De novo synthesis of AMP and GMP starts with PRPP and proceeds in many steps, two of which involve N^{10}-formyl-THF. However, a dietary folate deficiency does not impair purine de novo synthesis (it impairs only synthesis of dTMP).
- When cells accumulate purine nucleoside monophosphates, they rapidly degrade AMP to hypoxanthine and GMP to guanine, both of which they release into the blood. Other cells salvage ~97% of this hypoxanthine and guanine, thereby generating AMP and GMP.
- Cells that have a depleted nucleotide pool slowly reestablish the pool through salvage or de novo synthesis. Enzyme controls ensure that salvage is preferred over de novo synthesis.
- Only about 3% of the hypoxanthine is degraded to urate; this happens mostly in the liver and to a lesser extent in the intestine. The intestine also degrades some of the dietary purines to urate, which it releases into the blood.
- The intestine excretes about one-fourth of the daily amount of urate produced, and the kidneys excrete about three-fourths, mostly as uric acid.
- Men and postmenopausal women have the highest concentration of urate in the blood. In addition, the concentration of urate in the blood is strongly hereditary.
- Hyperuricemia can be the result of urate overproduction, urate underexcretion, or both overproduction and underexcretion. Chronic hyperuricemia leads to the formation of sodium urate crystals in the joints and can also lead to the formation of uric acid stones in the kidneys. In turn, these deposits can lead to acute gouty arthritis or nephrolithiasis, respectively.
- Further episodes of acute gouty arthritis can be prevented with urate-lowering therapy with the xanthine dehydrogenase inhibitors allopurinol or febuxostat, the uricosuric drug probenecid, or the recombinant uricase pegloticase. Further episodes of uric acid nephrolithiasis can be prevented with an increased volume and pH of the urine, allopurinol or febuxostat, or pegloticase. Patients with gout should also be counseled about weight loss as well as the dietary intake of purines, alcohol, and fructose.
- The thiopurines azathioprine, 6-mercaptopurine, and 6-thioguanine are converted to thio-GMP with the help of the salvage enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT). Thio-dGTP is incorporated into DNA and eventually leads to apoptosis. Thiopurines are used for their immunosuppressive and acutely antineoplastic effects, but they increase patients' long-term cancer risk.

FURTHER READING

- Dalbeth N, Doyle AJ. Imaging of gout: an overview. Best Pract Res Clin Rheumatol. 2012;26:823-838.
- Merriman TR, Choi HK, Dalbeth N. The genetic basis of gout. Rheum Dis Clin North Am. 2014;40:279-290.
- Preston R. An error in the code. New Yorker. 2007;Aug 13:30-36. This is an account of Lesch and Nyhan's pioneering work on elucidating the cause of a syndrome that came to be called Lesch-Nyhan syndrome. Available at: <http://archives.newyorker.com/?i=2007-08-13#folio=004>.

Review Questions

1. Patients with certain malignancies who undergo chemotherapy are at a particularly high risk of tumor lysis syndrome. When the concentration of urate in plasma is already high, uric acid nephrolithiasis can best be minimized by which of the following?

   A. Intravenous leucovorin
   B. Intravenous rasburicase
   C. Oral 6-mercaptopurine
   D. Oral probenecid
   E. Reduced fluid intake

2. T e hyperuricemia of a 21-year-old male patient who has chronic gout and variant Lesch-Nyhan syndrome is best treated with which of the following?

   A. Intravenous lactic acid
   B. Intravenous rasburicase
   C. Oral allopurinol
   D. Oral probenecid

3. Most patients who have gout show which of the following?

   A. Excessive de novo synthesis of IMP
   B. Excessive degradation of AMP
   C. Insufficient salvage of hypoxanthine
   D. Overly active PRPP synthetase
   E. Overly active xanthine dehydrogenase
   F. Urate underexcretion in the intestine
   G. Urate underexcretion in the kidneys

Chapter

39

Diabetes

SYNOPSIS

- Type 1 diabetes is characterized by autoimmune destruction of pancreatic β-cells. When the remaining β-cells no longer secrete enough insulin, the concentration of glucose and fatty acids in the blood rises; eventually, diabetic ketoacidosis develops.
- The development of type 1 diabetes depends on both genetic predisposition and the environment.
- Patients with type 1 diabetes treat themselves with insulin, the amount of which they estimate based on carbohydrate intake, prevailing blood glucose concentration, and insulin sensitivity. Almost all currently used insulins are recombinant insulins.
- In type 2 diabetic patients, insulin secretion from pancreatic β-cells is inadequate to maintain the concentration of blood glucose within a normal range. The majority of patients with type 2 diabetes are overweight and have low sensitivity to insulin.
- Treatments for patients with type 2 diabetes include exercise, weight loss, and insulin as well as drugs that stimulate insulin secretion, increase insulin sensitivity, inhibit gluconeogenesis, slow glucose absorption in the intestine, or promote loss of glucose into the urine.
- Gestational diabetes is diabetes that becomes evident during pregnancy, a time of low insulin sensitivity caused by hormones from the placenta. During the postpartum period, most patients with this form of diabetes regain control of the concentration of glucose in the blood. However, they often develop type 2 diabetes years later.

LEARNING OBJECTIVES

For mastery *of* this topic, you should be able to do the *f*ollowing:

- Describe the pathogenesis of type 1 diabetes and explain the major predisposing factor for this form of diabetes.
- Explain the procedure for diagnosing type 1 diabetes.
- Compare and contrast type 1 diabetes and neonatal diabetes.
- Explain the biochemistry of diabetic ketoacidosis, paying special attention to the role of pancreatic hormones.
- Describe the effects of an overdose of injected insulin on the metabolism of glucose, fatty acids, and ketone bodies.
- Describe the defense against insulin-induced hypoglycemia in healthy and in type 1 diabetic patients.
- Create a procedure for the screening and diagnosis of type 2 diabetes.
- Explain the changes in insulin sensitivity and insulin secretion that typically concur with the development of type 2 diabetes in obese patients.
- Describe the abnormalities that occur in glycogen metabolism and gluconeogenesis in patients who have type 2 diabetes.
- Describe the mechanisms by which weight loss and exercise can improve blood glucose control in type 2 diabetic patients.
- Compare and contrast the mechanisms of action of drugs that are used to treat type 2 diabetes, such as metformin, insulin sensitizers, α-glucosidase inhibitors, glucagon-like peptide 1 receptor agonists, dipeptidyl peptidase-4 inhibitors, sodium-glucose cotransporter-2 inhibitors, inhibitors of adenosine triphosphate–sensitive K^+ channels, and insulin.
- Compare and contrast the pathogenesis, blood chemistry, and treatment of hyperosmolar hyperglycemic state and diabetic ketoacidosis.
- Describe testing and treatment for gestational diabetes and estimate the likelihood that a patient with gestational diabetes will develop type 2 diabetes later on.
- Explain the acronym MODY, describe the inheritance pattern and prevalence of MODY, and discuss how a mutation in glucokinase can lead to diabetes.

1. OVERVIEW OF THE CLASSIFICATION OF DIABETES

Diabetes is characterized by a blood glucose concentration that is *f*requently above a predetermined range. Patients with diabetes have a relative or absolute def ciency in insulin secretion, and they may not properly respond to insulin. Patients who lose most o*f* their pancreatic β-cells to autoimmune attack have type 1 diabetes. Patients whose tissues have an abnormally low response to insulin and who no longer secrete an adequate amount o*f* insulin have type 2 diabetes. Patients who develop diabetes during pregnancy have gestational diabetes. Patients who develop severe diabetes during the f rst 6 months o*f* li*f*e and some patients who develop a milder *f*orm o*f* diabetes as children or young adults have mutations in genes, the products o*f* which a*ff*ect pancreatic β-cell development or *f*unction. Patients with maturity-onset diabetes o*f* the young (MODY) carry mutations that mostly lead to impaired insulin secretion.

Diabetes is characterized by an abnormally high concentration of glucose in blood plasma. At a very early, preclinical stage of the disease, hyperglycemia occurs mostly after a meal; later, hyperglycemia is also present in the fasting state. When diabetes is recognized, most patients have hyperglycemia in both the fed and the fasting state.

Many dif erent pathological processes can give rise to diabetes, and our understanding of these processes influences the way dif erent forms of diabetes are categorized. Table 39.1 provides an overview of major, currently accepted types of diabetes.

In the Western world, most patients with diabetes have type 2 diabetes. Only about 5% to 10% of all diabetic patients have type 1 diabetes, about 1% to 5% have MODY, and about 1% to 2% have mitochondrial diabetes. Gestational diabetes af ects about 5% of all pregnant women. Neonatal diabetes is

Table 39.1 Common Classification of Types of Diabetes

Type	Description
Type 1 diabetes	Autoimmune destruction of pancreatic β-cells. Without treatment with insulin, patients develop life-threatening diabetic ketoacidosis.
Type 2 diabetes	Some reduction in volume of β-cells. Insufficient and somewhat altered insulin secretion, often coupled with insulin resistance. About 85% of patients are obese.
Gestational diabetes	Diabetes that is recognized for the first time during pregnancy. Years later, most patients develop type 2 diabetes.
Neonatal diabetes	Diabetes onset during the first 6 months of life.
Transient form	Intrauterine growth retardation. Hyperglycemia often around the fourth day of life. Remission often around 3 months of age. Most patients have excessive transcription of the imprinted genes for the protein PLAGL1 and the noncoding RNA HYMAI.
Permanent form	Caused by mutations in the genes encoding the inward rectifying K-channel 6.2, the sulfonylurea receptor type 1 (these two proteins give rise to K_{ATP}-channels), insulin, insulin promoter factor-1, or glucokinase, which lead to impaired insulin secretion. Some patients instead show altered imprinting of chromosome 6q.
Maturity-onset diabetes of the young (MODY)	Onset of diabetes is typically before 25 years of age. Many causes, but most patients have a mutation in the gene for HNF-4α, glucokinase, HNF-1α, or HNF-1β. Some forms can present as neonatal diabetes or increase the risk for gestational diabetes.
Latent autoimmune diabetes in adults (LADA)	Clinical picture is intermediate between type 1 and type 2 diabetes. Patients are adult and have autoantibodies to islet proteins, but they do not have ketoacidosis and initially do not require insulin.
Mitochondrial diabetes	In some patients who have a mitochondrial disease that impairs ATP production, often due to mutant mitochondrial DNA.
Diabetes due to a disease of the exocrine pancreas	In patients who do not have functional β-cells due to pancreatectomy, chronic pancreatitis, severe cystic fibrosis, or severe hemochromatosis.

rare. The prevalence of latent autoimmune diabetes in adults (LADA) is difficult to judge because the disease is poorly defined and patients often receive a diagnosis of type 1 or type 2 diabetes. By some estimates, ~5% of type 2 diabetic patients have LADA, and LADA is up to twice as prevalent as type 1 diabetes.

2. METABOLISM DURING SEVERE INSULIN DEFICIENCY

A severe deficiency of insulin gives rise to diabetic ketoacidosis, which is characterized by high concentrations of glucose and ketone bodies in the blood. Patients who have a somewhat less severe deficiency of insulin may instead develop the hyperosmolar hyperglycemic state, which is characterized by severe hyperglycemia and severe dehydration. Diabetic ketoacidosis is principally seen in type 1 diabetic and hyperosmolar hyperglycemic state in type 2 diabetic patients.

2.1. Diabetic Ketoacidosis

As insulin deficiency develops due to autoimmune destruction of pancreatic β-cells, there is first diminished use of glucose, then excessive endogenous glucose production, and finally, excessive lipolysis and ketone body production. This sequence of events is explained by the insulin sensitivity of metabolism, as shown in Fig. 39.1. Glucose uptake requires the highest concentration of insulin; inhibition of glucose production by the liver and kidneys requires an intermediate concentration of insulin, and inhibition of lipolysis requires the lowest concentration of insulin.

Diabetic ketoacidosis is commonly defined as a condition during which the concentration of glucose is exceedingly high (>250 to 300 mg/dL, or >14 to 17 mM), the concentration of bicarbonate is low (≤18 mEq/L), the pH is low (≤7.30), and the serum osmolality is only modestly elevated (~290 to 320 mOsm/kg; Table 39.2). In addition, calculation of serum "anion gap" (Na^+ minus Cl^- minus bicarbonate) yields a value greater than 10 mEq/L. Diabetic ketoacidosis develops only when the insulin deficiency is very severe. Hence, diabetic ketoacidosis is commonly seen in patients who have type 1 diabetes, and it is uncommon in patients who have type 2 diabetes.

During diabetic ketoacidosis, glucose production is near maximal, glucose consumption is minimal, lipolysis is excessive, and ketone body production far exceeds consumption (Fig. 39.2).

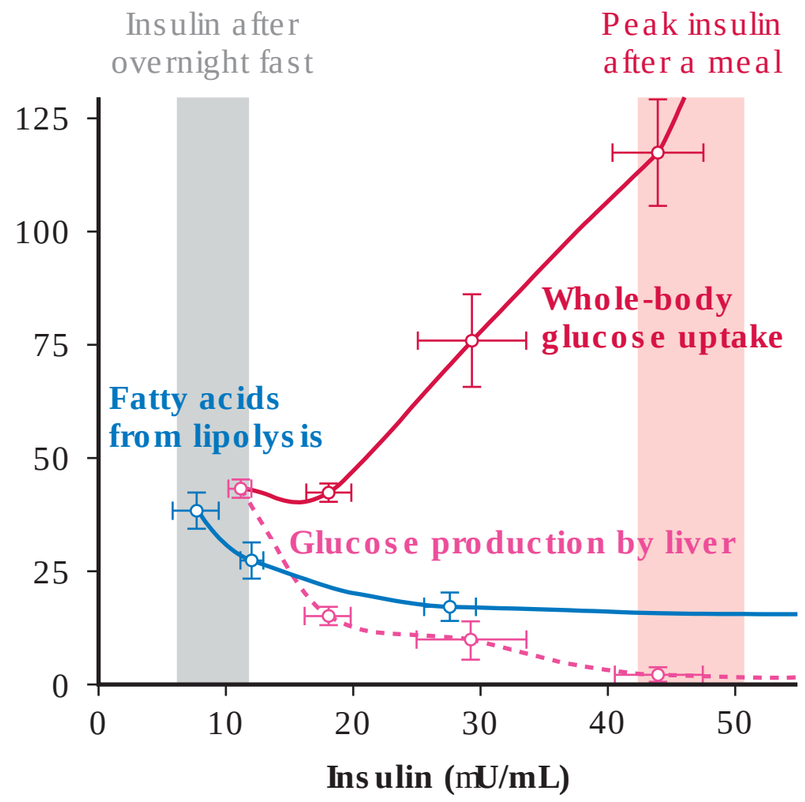


Fig. 39.1 Relative insulin sensitivity of adipose tissue lipolysis, glucose production by the liver, and whole-body glucose uptake. Fifty millimoles of glucose are equal to 9 g of glucose (~36 kcal), and 50 mmol of fatty acids is equal to ~14 g of fatty acids (~120 kcal). 100 mM insulin equals ~14 μU/mL. (Data from Bonadonna RC, Groop L, Kraemer N, Ferrannini E, Del Prato S, DeFronzo RA. Obesity and insulin resistance in humans: a dose-response study. *Metabolism*. 1990;39:452–459; and Campbell PJ, Carlson MG, Hill JO, Nurjhan N. Regulation of free fatty acid metabolism by insulin in humans: role of lipolysis and reesterification. *Am J Physiol*. 1992;263:E1063–E1069.)

Table 39.2 Typical Laboratory Values for Patients With Hyperosmolar Hyperglycemic State and Patients With Diabetic Ketoacidosis

Analyte in Serum or Blood	Pure Diabetic Ketoacidosis	Pure Hyperosmolar Hyperglycemic State
Glucose		
mg/dL	≥250	≥600
mM	≥14	≥33
Ketone bodies	Moderate to high	None or low
Bicarbonate (mEq/L)	≤18	≥18
pH	≤7.30	≥7.30
Osmolality (mOsm/kg)	variable	≥320

Modified from Kitabchi AE, Umpierrez GE, Miles JM, Fisher JN. Hyperglycemic crises in adult patients with diabetes. Diab Care. 2009;32:1335–1343.

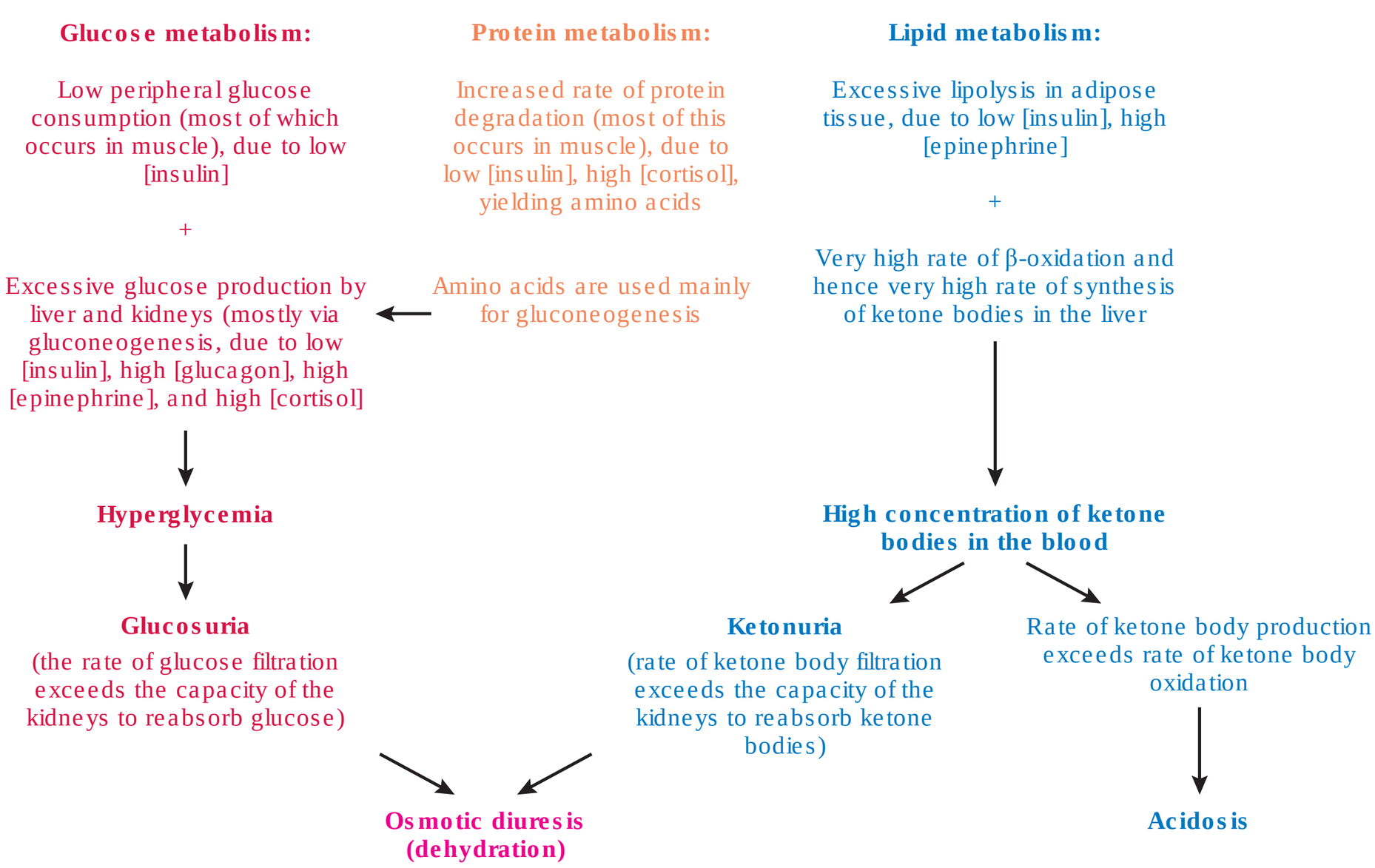


Fig. 39.2 Metabolism during diabetic ketoacidosis. Brackets indicate concentration.

Treatment of patients who have diabetic ketoacidosis involves infusing saline solution, insulin, potassium, and glucose. The saline solution helps rehydrate the patient. Insulin stops the lipolysis and hence the production of ketone bodies. Acidosis, dehydration, diuresis, and insulin deficiency all alter potassium homeostasis. During acidosis, cells absorb H^+ (and buffer it) in exchange for losing K^+ thus causing hyperkalemia. When insulin is administered, K^+ flows back into cells, potentially causing hypokalemia. Hence, potassium (as KCl) is given as needed. Hypokalemia can cause cardiac arrhythmia

and respiratory arrest, whereas hyperkalemia can cause cardiac arrest. Glucose can also be infused so as to lower the concentration of glucose in the blood only gradually, which may lower the incidence of brain swelling that accompanies some episodes of severe diabetic ketoacidosis.

2.2. Hyperosmolar Hyperglycemic State

The hyperosmolar hyperglycemic state develops in diabetic patients who secrete too little insulin to inhibit gluconeogenesis, yet enough insulin to inhibit lipolysis. The syndrome is chiefly seen in a minority of type 2 diabetic patients, and it takes days to develop. During this time, the concentration of glucose in the blood climbs steadily. The concentration of insulin in the blood is too low to stimulate insulin-induced glucose uptake (this is the process that requires the highest concentration of insulin; see Fig. 39.1). Hyperglycemia induces somnolence, but per se it is not acutely toxic. When the concentration of glucose in the blood exceeds about 10 mM (180 mg/dL), the kidneys no longer reclaim all glucose from the glomerular filtrate, and glucose appears in the urine. The osmotic activity of glucose induces loss of extra water, which favors dehydration. With drinking, affected patients do not replace all of the water they lose. The osmolarity of serum increases. After several days, the dehydration and consequent decrease in blood volume impair oxygen delivery to the brain, causing mental impairment and then coma.

Hyperosmolar hyperglycemic state is often defined by laboratory values of the concentration of glucose in excess of about 600 mg/dL (33 mM), an osmolarity equal to or greater than 320 mOsm/kg, a pH equal to or greater than 7.3, and bicarbonate (HCO_3^-) in plasma equal to or greater than 15 mEq/L (see also Table 39.2). Comparison with the lab values of patients who have pure diabetic ketoacidosis shows differences in the concentrations of glucose, ketone bodies, and bicarbonate as well as in the osmolality.

In practice, the metabolic derangement of diabetic patients is frequently intermediate between diabetic ketoacidosis and hyperosmolar hyperglycemic state, reflecting a spectrum of insulin secretion and insulin sensitivity.

The **treatment** of patients who have hyperosmolar hyperglycemic state involves infusions of saline solution, insulin, potassium, and glucose. Saline solution is infused for rehydration. Insulin is infused to curb endogenous glucose production. Potassium is infused as needed to maintain a normal concentration of potassium in the blood (see also the treatment of diabetic ketoacidosis above). Glucose is infused to lower the concentration of glucose in blood plasma only gradually. In response to the prevailing high osmolarity, the brain of an affected patient has produced sizable concentrations of osmolytes (such as myo-inositol, N-acetyl-aspartate, taurine, creatine, and phosphocreatine). These osmolytes are degraded only slowly. Lethal swelling of the brain may occur during treatment of hyperosmolar hyperglycemic state, and there is concern that this may be due to water flowing into the brain due to the high concentration of intracellular osmolytes. For this reason, many algorithms call for a gradual reduction in blood glucose. However, while the above theory is plausible, a thorough understanding of the cause of the brain edema is still lacking. About 20% of patients who present with the hyperosmolar hyperglycemic state do not survive the episode.

3. DIAGNOSIS OF DIABETES

The diagnosis of diabetes is based on the finding of an abnormally high concentration of glucose or an abnormally high fraction of hemoglobin A_{1c} in the blood. The testing procedures yield groups of patients who have either frank diabetes or abnormalities that are associated with an increased risk of developing diabetes in the future.

Diabetes is defined in the following ways: (1) as an abnormally high concentration of **glucose** in blood plasma after an overnight fast, at any time during the day, or 2 hours into an oral glucose tolerance test (described below); or (2) as an elevated fraction of **hemoglobin A_{1c}** (**HbA_{1c}**). Criteria for glucose testing of nonpregnant adults are shown in Fig. 39.3. HbA_{1c} is described in Table 16.1. Blood for an HbA_{1c} test can be collected at any time. This test should not be used in pregnant patients, those with hemolysis, or patients who receive a

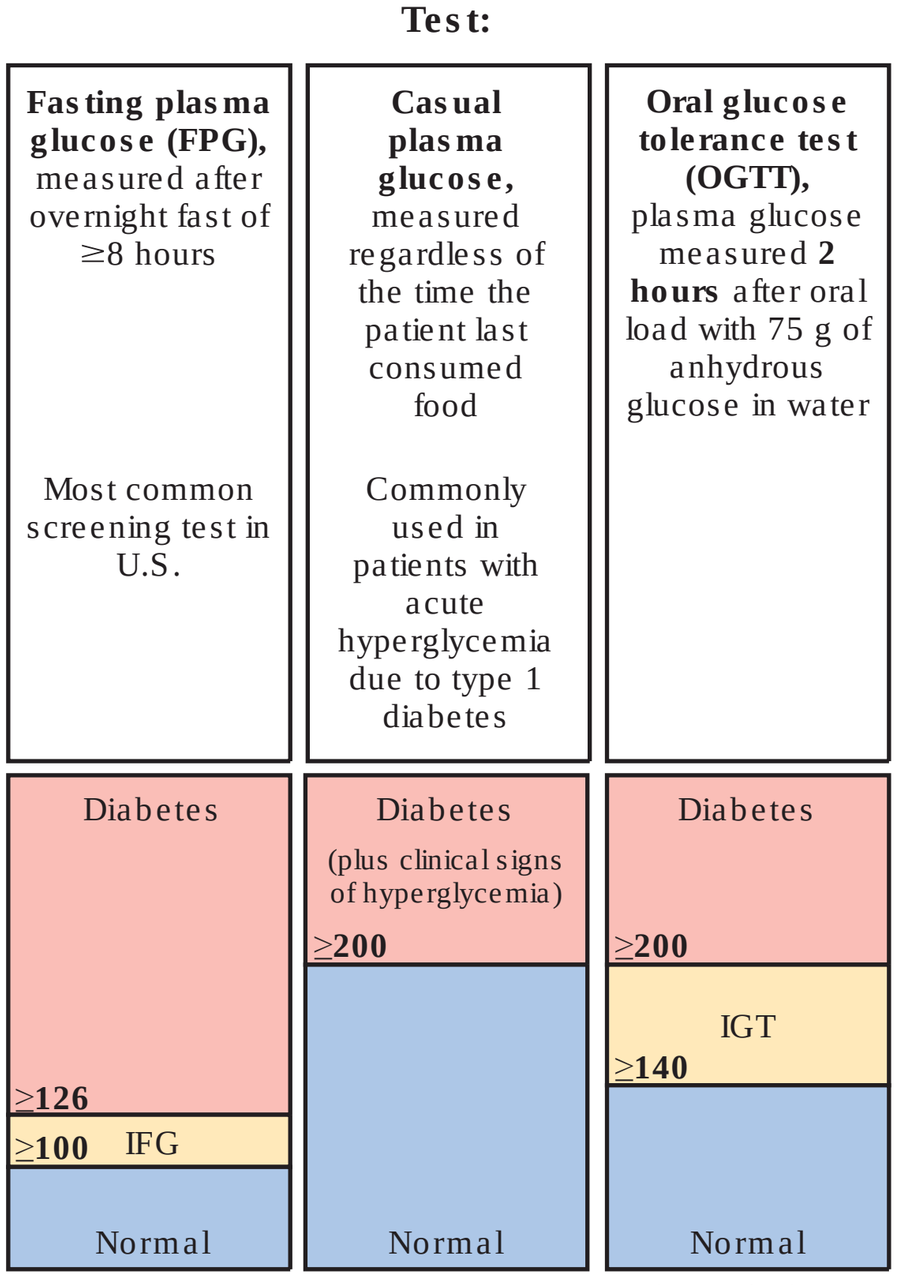


Fig. 39.3 Tests for screening and diagnosis of diabetes. Cutoff numbers are those of the American Diabetes Association. The World Health Organization and the International Diabetes Federation mostly use the same values. Conversion of glucose concentrations: 100 mg/dL = 5.56 mM, 110 mg/dL = 6.11 mM, 126 mg/dL = 7.00 mM, 140 mg/dL = 7.78 mM, 200 mg/dL = 11.11 mM. IFG, impaired fasting glucose; IGT, impaired fasting glucose tolerance.

blood transfusion or erythropoietin therapy. It is also inappropriate to perform this test in patients with certain hemoglobinopathies. An HbA_{1c} value (standardized to the Diabetes Control and Complications Trial in the United States) of 6.5% or more is positive for diabetes.

T ere is incomplete concordance between tests for diabetes. T e oral glucose tolerance test with determination of plasma glucose after 2 hours is the most sensitive test. T is is largely attributable to the fact that most patients lose control over blood glucose after a meal before they lose control in the fasting state.

For a **definitive diagnosis** of diabetes, there must be clear clinical evidence of diabetes, a dif erent test that also shows diabetes, or an immediate repeat test with a new blood sample that also shows diabetes. Similar principles apply to the testing of children and pregnant women.

Prediabetes is a popular term for impaired fasting plasma glucose, impaired glucose tolerance (Fig. 39.3), or an HbA_{1c} of 5.7% to 6.4%, all of which are risk factors for diabetes. T ese patients can lower their risk of developing diabetes by exercising regularly and maintaining or achieving a normal body weight.

A **glucose tolerance test** measures the body's ability to clear glucose from the blood without excessive hyperglycemia. A patient is given a standardized amount of glucose, usually by mouth, and the concentration of glucose in the patient's blood is determined at two or more time points (Fig. 39.4). Pancreatic insulin secretion and the body's response to insulin both af ect glucose clearance. In the absence of diabetes, the β-cells secrete sufficient insulin to compensate for any deficit in the body's response to insulin. Currently, tests with oral glucose are clinically limited mostly to the detection of gestational diabetes in pregnant women and to their follow-up. T e oral glucose test is also frequently used in diabetes research.

Infants who develop diabetes before the age of 6 months have **neonatal diabetes** or MODY and should undergo DNA testing for mutations in genes that have been linked to these forms of diabetes. In the 6- to 12-month age cohort, type 1 diabetes is more likely than neonatal diabetes, and genetic testing can be limited to those who do not have antibodies against islets. T e incidence of neonatal diabetes is at least ~1 in 400,000. T e major causes of neonatal diabetes are listed in Table 39.1.

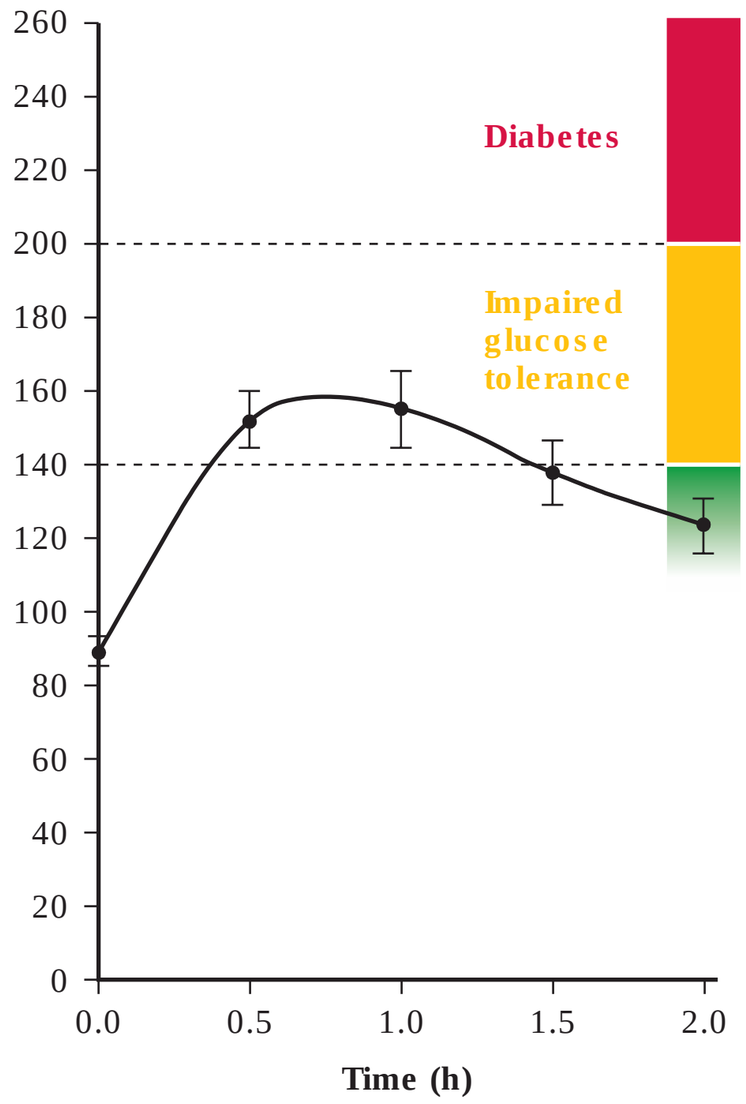


Fig. 39.4 The 75-g oral glucose tolerance test and its interpretation according to the American Diabetes Association. The time course of the concentration of glucose represents the mean of ~150 nondiabetic individuals. (Data from Man C, Campioni M, Polonsky KS, et al. Two-hour seven-sample oral glucose tolerance test and meal protocol: minimal model assessment of beta-cell responsivity and insulin sensitivity in nondiabetic individuals. *Diabetes*. 2005;54:3265-3273; and Gerich J, Van Haeften T. Insulin resistance versus impaired insulin secretion as the genetic basic for type 2 diabetes. *Curr Opin Endocrinol Diabetes*. 1998;5:144-148.)

4. PATHOGENESIS, DIAGNOSIS, AND TREATMENT OF TYPE 1 DIABETES

T e likelihood that a person develops type 1 diabetes depends on the person's makeup o*f* antigen-presenting proteins, other heritable *f*actors, and the environment. Over a period o*f* months or years, the pancreatic β-cells are destroyed by an autoimmune attack. Patients who develop type 1 diabetes present with pronounced hyperglycemia and *f*requently also with ketoacidosis. *T*ype 1 diabetic patients need to be treated with insulin. T e carbohydrate content o*f* meals as well as other *f*actors are used to estimate insulin needs. T ere are long- and short-acting insulins, which are best suited to cover the body's needs between and a*ft*er meals, respectively.

4.1. Definitions

Type 1 diabetes is sometimes subdivided into type 1A and type 1B diabetes. At the time of diagnosis, type 1A diabetes is accompanied by autoantibodies to islet proteins. In contrast, patients with type 1B diabetes do not have islet autoantibodies. Among patients with type 1 diabetes, most have type 1A diabetes. T e term type 1 diabetes in this text refers to type 1A diabetes.

4.2. Pathogenesis, Heredity, and Diagnosis

T e development of type 1 diabetes depends on a person's genotype and environment.

Type 1 diabetes shows a polygenic pattern of inheritance. Certain alleles for class II **human leukocyte antigens** (**HLA**s) are the most common predisposing factor, and alleles for the **insulin promoter** are second most important. Other genes have a comparatively small influence.

Pancreatic β-cells die due to a direct attack by cytotoxic T cells, a bystander ef ect, or a combination of these. HLA

proteins are located in the membrane of antigen-presenting cells, such as dendritic cells, activated macrophages, and activated B cells (i.e., lymphocytes, not pancreatic β-cells); on the surface of those cells, HLA proteins present short peptides to T cells. If T cells consider these peptides foreign, they might attack β-cells directly. Alternatively, β-cells might sustain damage due to the release of free radicals, NO, interleukin-1, and enzymes from nearby macrophages and neutrophils. Patients who have had clinically recognized type 1 diabetes for several years have only about 2% of the normal number of β-cells left. Most type 1 diabetic patients produce β-cells at a low rate throughout their lives; however, autoimmune killing keeps the total number of β-cells very low.

Antibodies to islet cell antigens are detectable years before onset of glucose intolerance due to type 1 diabetes and can be used to predict who will develop type 1 diabetes. T ese antibodies are typically directed against one or more of the following: insulin, glutamic acid decarboxylase (GAD), IA-2 (protein tyrosine phosphatase 2), and ZnT8 (also called SLC30A8, a zinc transporter in islet β-cells). T e number of nucleotide repeats in a region 5′ to the insulin gene determines the expression of (a tiny amount of) insulin in the thymus. Insufficient insulin synthesis in the thymus might give rise to inadequate central tolerance.

Years after islet cell antibodies are first apparent, impaired glucose tolerance and glucose intolerance usually set in over a period of many months. As β-cells die and insulin secretion diminishes, there is first diminished use of glucose, then excessive endogenous glucose production, and finally excessive lipolysis and ketone body production (see Fig. 39.1; further details are provided in Chapters 24, 25, 27, and 28). Accordingly, patients who develop type 1 diabetes first have postprandial hyperglycemia and then fasting hyperglycemia. Typically, the concentration of glucose 2 hours after an oral glucose tolerance test rises markedly (by ~2 mM or ~35 mg/dL per month) only during the few months before onset of diabetes. With marked hyperglycemia, glucosuria (glucose in the urine) and polyuria (excessive urination due to the osmotic ef ect of glucose in the urine) set in. T e af ected person commonly does not recognize the gradual loss of glucose tolerance. Sometimes, glucose intolerance is accompanied by candidiasis, a yeast infection. Excessive lipolysis develops at a later stage, which gives rise to diabetic ketoacidosis. Patients with yet undiagnosed type 1 diabetes typically present with a history of polydipsia, polyuria, weight loss, and malaise and, in ~25% of cases, diabetic ketoacidosis. Ketoacidotic patients breathe deeply and rapidly, and their breath smells of acetone (acetone is spontaneously formed from acetoacetate, a ketone body; see Sections 5.1 and 7.3 in Chapter 27).

Once a patient is diagnosed with type 1 diabetes and receives treatment, **diabetic ketoacidosis** sets in whenever the concentration of insulin is extremely low compared with needs (see Section 2.1). In the absence of insulin, a type 1 diabetic patient develops diabetic ketoacidosis within hours to a day (depending on the half-life of the insulin that was last administered). Sometimes, a patient has the misperception that no insulin is needed when no food is consumed during an acute illness. In truth, ill patients may have to increase their insulin dose beyond their normal baseline dose because illness causes the body to secrete relatively large amounts of glucagon, epinephrine, and cortisol.

In patients with type 1 diabetes, **diagnosis** is usually urgent and straightforward. T e diagnosis of diabetes typically rests on finding a plasma glucose concentration of 200 mg/dL or higher (≥11.1 mM; see Fig. 39.3) as well as polyuria, polydipsia, and unexplained weight loss. An elevated concentration of ketone bodies in serum, a reduced concentration of bicarbonate, and a low blood pH can provide further support for a diagnosis of type 1 diabetes. In special cases, a test for islet cell antibodies may help distinguish type 1 from type 2 diabetes.

4.3. Treatment of Type 1 Diabetes

Type 1 diabetic patients depend on exogenous **insulin** for immediate survival. Current chief regimens are either a minimum of three to four injections per day with short- and long-acting analogs of human insulin (see below) or continuous and bolus infusion of short-acting insulin by a pump. Patients measure their blood glucose concentration by finger prick multiple times per day and adjust their insulin dose accordingly. A continuous glucose monitor with a sensor under the skin can provide additional data on interstitial glucose. T e U.S. Food and Drug Administration has recently approved a wearable artificial pancreas, which consists of a glucose sensor, algorithms, and an insulin pump.

A few weeks after diagnosis, type 1 diabetic patients sometimes experience a **honeymoon** phase during which they require a reduced amount of exogenous insulin.

Type 1 diabetic patients need a **basal** concentration of insulin in the blood at all times and **boluses** of insulin to accommodate meals. Figs. 26.5, 26.8, 28.9, and 39.6 show that there is a basal concentration of insulin in nondiabetic volunteers. T e basal concentration of insulin suppresses lipolysis (see Section 5.1 in Chapter 28) and endogenous glucose production (glycogenolysis and gluconeogenesis; see Section 2.2 in Chapter 24 and Section 3 in Chapter 25). A bolus of insulin covers the diet-derived spike in blood glucose by activating glucose consumption (see Fig. 39.1).

Patients who receive multiple insulin **injections** per day use a **rapid-acting insulin** to cover meal-related needs for insulin and a **long-acting insulin** to cover the baseline need for insulin. A rapid-acting insulin peaks 0.5 to 2 hours after injection, whereas a long-acting insulin does not have a clearly identifiable peak action and is ef ective for a half-day to a full day. Typically, patients inject about 50% of their daily insulin as a long-acting insulin (toddlers need only about 30% of the daily total). Insulin cannot be given orally because the digestive tract would degrade most of it before it reaches the bloodstream.

Patients who wear an insulin **pump** use only rapid-acting insulin. T e pump delivers a bolus of programmable duration to cover meals and an ongoing infusion to provide for baseline needs.

Rapid-acting, intermediate-acting, and long-acting insulins are the result of applying knowledge of protein chemistry to a clinical problem and producing modified proteins with methods of molecular biology. The majority of insulin is now purified from transformed bacteria that synthesize a normal or mutated human preproinsulin, proinsulin, and insulin. Insulin is purified from an extract of such bacteria. This purified insulin does not contain C-peptide. The insulin is sometimes crystallized with $\mathbf{Zn^{2+}}$; however, it can instead also be complexed with **protamine**, a DNA-binding protein that contains many positive charges.

A challenge in producing rapid- and long-acting mutant insulins is to produce molecules that do not lead to excessive activation of insulin-like growth factor-1 (IGF-1) receptors. Excessive activation of IGF-1 receptors leads to an increased rate of cell division and may give rise to a **neoplasm**.

The commonly used **rapid-acting insulins** are insulin lispro, aspart, and glulisine. Insulin **lispro** differs from normal human insulin in that it has a Pro28Lys and a Lys29Pro mutation. Insulin **aspart** has a Pro28Asp mutation. **Glulisine** has Asn3Lys and Lys29Gln mutations. These sequence changes impede dimerization of insulin and formation of hexamers around a zinc atom. Hence, in the subcutaneous space, these insulin analogs are mainly present as monomers and they therefore diffuse into the bloodstream at a higher rate than do insulins that form hexamers with Zn and then crystallize. The appearance of short-acting insulins in the bloodstream resembles the food-induced appearance of insulin in nondiabetic individuals.

The main currently used **intermediate-acting insulin** is insulin **NPH**. NPH stands for neutral protamine Hagedorn (Dr. Hagedorn in Denmark was one of the inventors of this technology). When insulin is complexed to protamine, the subcutaneous depot releases insulin into the bloodstream more gradually.

The main currently used **long-acting insulins** are insulin detemir, insulin glargine, and insulin degludec. Insulin **detemir** has a β-chain without Thr30, but with the fatty acid myristic acid (14 carbons) covalently linked to Lys29. As supplied, insulin detemir forms hexamers around a zinc atom. After injection, solute (phenol and cresol) is lost from the subcutaneous depot, and the hexamers pair up. Because large molecules diffuse only slowly, this dihexamer complex diffuses into the bloodstream only slowly. Insulin **glargine** has decreased solubility at physiological pH but it is soluble at low pH. Accordingly, insulin glargine is injected in a solution that has a pH of about 4. In the tissue, where the pH is above 7, insulin glargine precipitates and then dissolves relatively slowly. Insulin **degludec** misses ThrB30 and has LysB29 linked to glutamate and then two fatty acids. It dissolves in phenol and cresol, precipitates after injection, and forms dihexamers. The half-life is longer than 24 hours.

The control a diabetic patient has over the concentration of glucose in the blood is limited by multiple factors, including the accuracy with which the patient predicts his or her body's need for insulin, the patient's counterregulatory responses to hypoglycemia, stress, and the level of exercise. Most patients estimate their insulin needs for a meal based on meal carbohydrate content, insulin sensitivity, and current blood glucose concentration (Fig. 39.5). Consistently accurate prediction of insulin needs is impossible because the body's daily responses differ too much. If a patient injects too much insulin, a so-called **insulin reaction** can occur, which is characterized by a period of transient **hypoglycemia**, during which glucose consumption is excessive and endogenous glucose production is minimal. Hypoglycemia is dangerous because—depending on severity—it can lead to poor judgment, loss of consciousness, permanent vegetative state, or death.

Patients typically treat **hypoglycemia** with rapidly absorbed carbohydrate or, if severe, with glucagon. The carbohydrate can be consumed as glucose in solid or liquid form or as a regular food from which carbohydrate is readily available (e.g., sugar-containing liquids, such as fruit juice or soft drinks). **Glucagon** liberates glucose from liver glycogen stores and must be injected. Although a typical pharmacological dose of glucagon cannot stimulate lipolysis, it can always stimulate breakdown of liver glycogen. Glucagon is commonly used in children who are unconscious or who cannot swallow; however, it frequently elicits nausea or vomiting.

At the time of diagnosis, type 1 diabetic patients usually secrete a normal amount of **glucagon** and **epinephrine**, but these responses are lost after many years of the disease. Glucagon and epinephrine are the chief hormones that help counteract insulin-induced hypoglycemia. Without either hormone, a patient's body is defenseless against an overdose of insulin. Hence, patients with long-term type 1 diabetes are at increased risk of insulin-induced hypoglycemia.

Recurrent hypoglycemia leads to **hypoglycemia unawareness** and reduced secretion of counterregulatory hormones during hypoglycemia, components of the hypoglycemia-associated autonomic failure (HAAF) syndrome. About one-fourth of adults who have type 1 diabetes also have hypoglycemia unawareness. The molecular pathogenesis of HAAF is poorly understood. After several weeks without

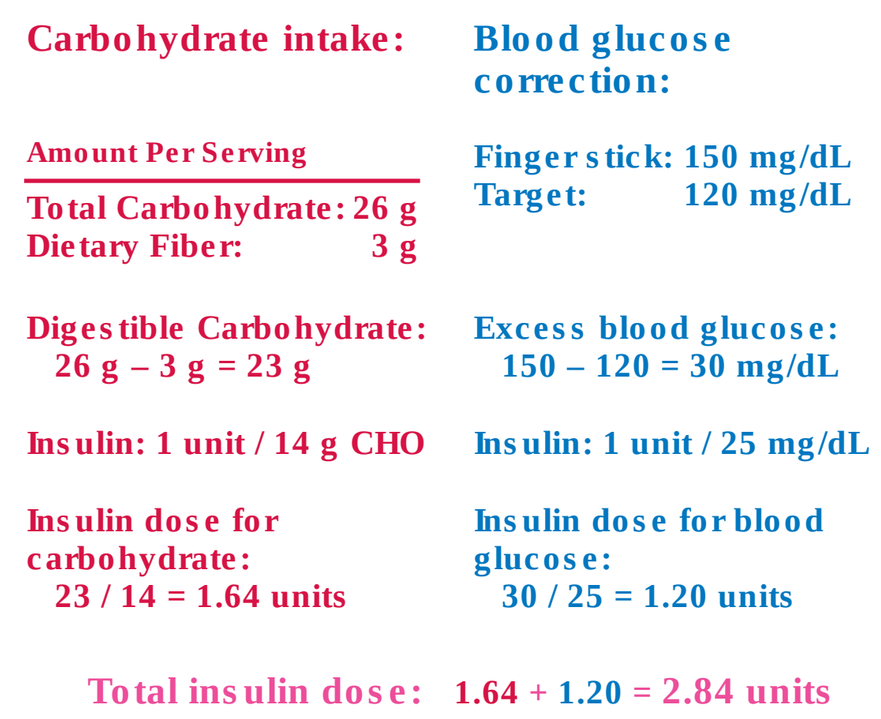


Fig. 39.5 Calculation of insulin dose for one serving of a packaged food. The person's insulin sensitivity determines how much carbohydrate 1 U insulin "covers" and by how much it lowers a high concentration of blood glucose. CHO, carbohydrate.

hypoglycemia, patients can become aware of hypoglycemia once again.

The **diet** of a type 1 diabetic patient is essentially that of a healthy person, except for an avoidance of rapidly absorbed sugars. Current insulins do not reach the bloodstream quickly enough to cover a large and rapid increase in blood glucose.

Treating physicians assess diabetic patients' control of their diabetes every 3 months by measuring the fraction of **HbA_{1c}**. This value reflects the plasma glucose concentration over a period of ~2 months (red blood cells are typically 0 to 4 months old). Two methods for the determination of HbA_{1c} are currently in use. The United States uses a traditional method that is traced to a standard that contains several glycated hemoglobins and was used during a large diabetes treatment trial. European countries use a newer method that refers to a single glycated hemoglobin species and yields lower values than those yielded by the traditional method. In an attempt to minimize confusion, results from the old method are reported as a percentage, and results from the new method as mmol/mol. A patient with type 1 diabetes, on average, always has some hyperglycemia, which typically leads to an elevated HbA_{1c}. An HbA_{1c} value less than 6.5% to 7.0% is considered acceptable (the HbA_{1c} of a nondiabetic person is ≤5.6%), whereas a larger value should be a call to improved control of plasma glucose.

The HbA_{1c} is misleadingly low in patients who have a shortened lifetime of red blood cells or certain other conditions (see Section 3).

Continuous glucose monitors measure the concentration of glucose in the interstitial fluid continuously and can often provide helpful information about daily excursions of the concentration of glucose. This information can be used to improve control of blood glucose.

5. PATHOGENESIS, DIAGNOSIS, AND TREATMENT OF TYPE 2 DIABETES

*T*ype 2 diabetes is usually diagnosed a*ft*er a screening procedure or when a patient has a typical complication o*f* type 2 diabetes, such as a stroke or myocardial in*f*arction. Almost all type 2 diabetic patients take up an abnormally small amount o*f* glucose in response to insulin, and all type 2 diabetic patients secrete an inadequate amount o*f* insulin to maintain a normal concentration o*f* glucose in the blood. A person's chance *f*or developing type 2 diabetes depends on genetic *f*actors, body weight, and age. Available treatments *f*or type 2 diabetes can be well rationalized based on knowledge o*f* glucose homeostasis. *T*reatments decrease the rate o*f* in*fl*ux o*f* glucose into the blood, increase insulin secretion, increase the e*ff*ect o*f* insulin on metabolism, attenuate glucose production by liver and kidneys, or induce a signif cant loss o*f* glucose into the urine.

5.1. Pathogenesis

Type 2 diabetes is largely a result of the interplay of a patient's **genotype**, **diet**, **exercise**, and **age**.

More than 60 genetic loci are known to af ect the risk of developing type 2 diabetes, and many more loci remain to be discovered to account for the contribution of genetics to a person's risk for type 2 diabetes. The genetic predisposition to type 2 diabetes is somewhat smaller than the genetic predisposition to type 1 diabetes. There is little overlap between genetic loci that predispose to type 1 and type 2 diabetes.

The locus that confers the highest risk for type 2 diabetes is in the *T*CF7L2 gene (which encodes a transcription factor), and it increases risk only by a factor of ~1.4. Therefore the genetic risk for diabetes is an aggregate of many small risks. Surprisingly, diabetic persons and matched nondiabetic persons have about the same number of risk factors. As a result, testing of patients for diabetes-related loci does not yet provide useful clinical information.

Age, **family history**, and **obesity** are the major risk factors for diabetes that are used clinically. For instance, compared with a 25-year-old person, the diabetes risk of a 50-year-old is ~13 times and that of a 70-year-old is ~26 times higher. Also, the ~8% of the U.S. population with the highest familial risk for diabetes has 5 times the risk of the ~70% of the population with only an average familial risk. **Obesity** in **adults** is commonly assessed using **body mass index** (**BMI**). The BMI is a useful measure of body fat in populations, not individuals, yet in clinical practice BMI is widely used with individual patients. The formula for calculating the BMI is as follows:

$$\text{BMI} = (\text{weight in kg})/(\text{height in m})^2$$

According to the widely used World Health Organization (WHO) definition, the term **overweight** is used for BMIs from 25 kg/m^2 to less than 30 kg/m^2 and the term **obese** for BMIs of 30 kg/m^2 or higher. **Morbid** or **extreme obesity** is sometimes defined as a BMI of 40 kg/m^2 or higher. Compared with a person with a BMI of 25 kg/m^2, a person with a BMI of 30 kg/m^2 is ~3 times more likely to develop diabetes and a person with a BMI of 40 kg/m^2 ~9 times more likely. About 80% to 90% of patients who have type 2 diabetes are obese. Obesity in **children** is assessed using special tables that take age into account, such as those published by the U.S. Centers for Disease Control and Prevention (see Kuczmarski et al. under the Further Reading section). **Insulin resistance** is a state in which the body, at a normal concentration of insulin, does not remove a normal amount of glucose from the blood. As a result, the pancreatic β-cells need to secrete more insulin to maintain a normal concentration of glucose in the blood. Hence, insulin resistance is accompanied by **hyperinsulinemia**. **Insulin sensitivity** is the opposite of insulin resistance.

In a research setting, insulin resistance can be assessed by infusing a patient with insulin to obtain a stable concentration of circulating insulin and by infusing glucose to maintain plasma glucose near a predetermined concentration. The less glucose that has to be infused, the more insulin resistant a patient is. An infusion of labeled glucose is commonly used to correct the data for endogenous glucose production by the liver and by the kidneys.

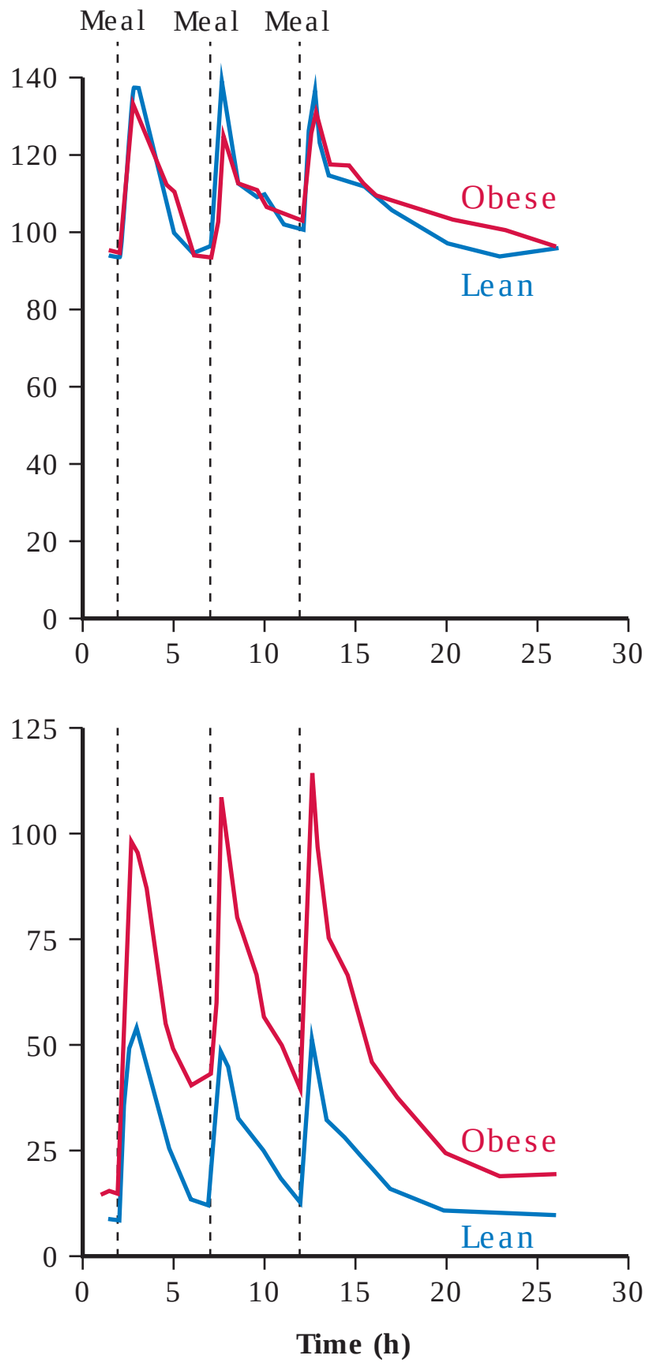


Fig. 39.6 Effect of obesity on the concentrations of glucose and insulin. Mean of two studies with a total of ~20 volunteers in each group. Meals contained about 50% carbohydrate and were approximately appropriate for maintaining weight. (Data from Polonsky KS, Given BD, Van Cauter E. Twenty-four-hour profiles and pulsatile patterns of insulin secretion in normal and obese subjects. *J Clin Invest*. 1988;81:442-448; and McQuaid SE, Hodson L, Neville MJ, et al. Down-regulation of adipose tissue fatty acid trafficking in obesity: a driver for ectopic fat deposition? *Diabetes*. 2011;60:47-55.)

Clinically, insulin resistance is apparent from a high plasma concentration of insulin in the fasting state or from a high daily amount of insulin needed to control blood glucose.

Obesity renders people **insulin resistant**. As a result, β-cells need to secrete more insulin to maintain normoglycemia (Fig. 39.6). Insulin resistance can be lessened by physical activity and weight loss.

Obese patients show insulin resistance of their skeletal muscles and adipose tissue. In response to insulin, the skeletal muscles thus take up an abnormally small amount of glucose. T e skeletal muscles normally account for most of the insulin-induced whole-body glucose uptake. T e adipose tissue of obese patients releases fatty acids at an abnormally high rate, and other tissues also oxidize fatty acids at an elevated rate so that the change in the concentration of circulating fatty acids is very small. Whether this contributes to insulin resistance in skeletal muscle is unclear. Furthermore, triglyceride-laden adipocytes alter their secretion of adipokines (hormones that stem from adipocytes). For instance, they secrete less **adiponectin**. Adiponectin makes tissues more sensitive to insulin. A decreased secretion of adiponectin, as in obesity, thus renders the body more insulin resistant. Obese patients with moderate insulin resistance typically have two to five times the concentration of insulin in the blood that is found in a lean, insulin-sensitive person (see Fig. 39.6).

Among patients with insulin resistance, the insulin desensitization is only partial, so that even markedly insulin-resistant patients always maintain a metabolic response to an increased concentration of insulin. Furthermore, the insulin resistance does not af ect all organs and metabolic pathways to the same extent. Indeed, the hyperinsulinemia that accompanies insulin resistance can lead to a seemingly paradoxical ef ect of increased activity in an insulin signaling pathway in a particular organ even though whole-body glucose uptake in response to insulin is decreased.

A longitudinal study of Pima Indians showed that the following changes occurred as volunteers progressed from normal glucose tolerance to diabetes: increase in body weight, decline in insulin-stimulated glucose removal from the blood (i.e., increasing insulin resistance), decline in insulin secretion in response to an intravenous bolus of glucose, and finally, an increase in the body's endogenous glucose production (this glucose comes mostly from the liver and the kidneys; see Chapter 25). T e concentration of glucose in the fasting state increases only late in the pathogenic process, and it appears to be due mostly to excessive endogenous glucose production. T e immediate causes of the decline in insulin secretion are not known; however, chronically elevated concentrations of glucose and fatty acids, changes in the concentrations of adipose tissue–derived hormones, and decreased insulin sensitivity of the β-cells themselves have been blamed.

Most type 2 diabetic patients who are **lean** are insulin resistant like their obese counterparts. T ese lean patients presumably develop diabetes in the same way as some obese patients. A minority of lean type 2 diabetic patients have normal insulin sensitivity but secrete insulin at a reduced rate. T e causes of impaired insulin secretion and signaling in these lean patients are unknown.

Type 2 diabetic patients still secrete insulin, but not enough to control the concentration of glucose in the blood. Autopsies of type 2 diabetic patients show that the pancreas contains ~50% of the normal volume of β-cells, which show signs of inflammation.

5.2. Diagnosis

Type 2 diabetes is usually discovered either on the basis of routine clinical vigilance and screening or after a patient has had a heart attack or stroke. Heart attack and stroke are often a consequence of protracted, unrecognized hyperglycemia. T e American Diabetes Association recommends that screens involve only a fasting plasma glucose concentration (see Section 3). T e WHO recommends that patients who have "impaired fasting glucose" also undergo an oral glucose tolerance test. For patients who have had a heart attack or stroke, the diagnostic test is performed following recovery from the acute illness; that is, at a time when the concentration of stress

hormones (also called counterregulatory hormones, including glucagon, epinephrine, and cortisol) is no longer markedly elevated. However, an immediate determination of an HbA_{1c} value allows the degree of the patient's hyperglycemia in the ~2 months before the acute illness to be assessed.

As a rough rule of thumb, type 2 diabetic patients are older than 40 years. However, as obesity increases in the developed world, this limit is shifting; a remarkable fraction of obese teenagers currently have type 2 diabetes.

About 7% of the U.S. population has type 2 diabetes. In other developed countries, the incidence of type 2 diabetes is moving toward a similar value.

Hyperosmolar hyperglycemic state is more often seen in patients previously diagnosed with diabetes than on first presentation. This state (see Section 2.2) develops only in a small number of patients who have type 2 diabetes. Patients who are most susceptible to developing the syndrome are those who have had diabetes for a long time and therefore have poor secretion of insulin from β-cells, patients who forget to take their antidiabetic medication (e.g., due to dementia), and patients who live alone and without a care provider.

Type 2 diabetic patients develop **ketoacidosis** only very rarely. Patients with type 2 diabetes usually secrete enough insulin to have normal inhibition of lipolysis and hence do not develop ketoacidosis.

The **distinction between type 1 and type 2 diabetes** is often made on clinical grounds. A young, lean child with ketoacidosis is exceedingly more likely to have type 1 rather than type 2 diabetes. Conversely, an overweight, 70-year-old person is much more likely to have type 2 rather than type 1 diabetes. Nonetheless, autoimmune destruction of pancreatic β-cells is thought to be possible at any age. Testing of a patient's serum for antibodies to islet cells is possible; however, this test is only rarely performed because its outcome would not change the management of the patient's diabetes.

Some patients with diabetes have characteristics that are intermediate between type 1 and type 2 diabetes; this type of diabetes is called **latent autoimmune diabetes in adults** (**LADA**; Table 39.1). In Europe and the United States, about 4% of patients who have a diagnosis of type 2 diabetes have antibodies to glutamic acid dehydrogenase (an enzyme that produces the neurotransmitter γ-aminobutyric acid), a finding that is typical (but not diagnostic) of type 1 diabetes. For the first half year after diagnosis, patients with LADA generally do not need insulin; however, they need exogenous insulin sooner than other patients who have type 2 diabetes. In clinical care, no distinction is made between patients who have type 2 diabetes and patients who have LADA.

5.3. Treatment

Available treatments for type 2 diabetes can be well rationalized based on our knowledge of glucose homeostasis. Treatments decrease the rate of influx of glucose from the intestine into the blood, increase insulin secretion, increase the effect of insulin on metabolism, attenuate glucose production by liver and kidneys, or lead to a loss of glucose into the urine.

The American Diabetes Association recommends that patients who receive a diagnosis of type 2 diabetes first try a regimen of **diet**, **exercise**, and **metformin**. Weight loss and exercise are accompanied by increased insulin sensitivity, though they do not mend the defect in insulin secretion. Still, the increase in insulin sensitivity may allow the pancreatic β-cells to regain control of the concentration of glucose in the blood. Metformin decreases glucose production by the liver (see below). Additional drugs are recommended only if these measures fail to lower the HbA_{1c} below 7% (according to the traditional method of measurement; see Section 4.3).

Type 2 diabetes is thought of as a slowly progressive disease. It may be controlled early on with diet, exercise, and perhaps one oral drug; however, it will eventually require further oral drugs or injected drugs. In the opinion of most physicians, type 2 diabetes does not vanish when a patient achieves normal glucose homeostasis with weight loss and exercise alone; it is then called controlled diabetes.

Patients who have type 2 diabetes do best when consuming a **diet** from which glucose enters the bloodstream only slowly. A steady, low flux of glucose from the intestine into the bloodstream requires secretion of less insulin than does a sudden, high influx of the same amount of glucose. In general, foods that contain **soluble fiber** are digested more slowly than foods that contain no fiber (see Section 1 in Chapter 18). Foods that release glucose only slowly are said to have a low glycemic index. **Fat** in a meal also slows gastric emptying and intestinal digestion of a meal. Although this effect is beneficial, most patients with type 2 diabetes also have lipid abnormalities, and they should therefore consume saturated fats and trans-fats only sparingly. A **low-carbohydrate, high-fat diet** (an Atkins type diet) requires considerably less insulin secretion than a high-carbohydrate diet. Multiple small meals also require less insulin to be secreted than do one or two large meals.

The drugs **acarbose** and **miglitol** competitively inhibit **α-glucosidases** and thus inhibit the production of glucose from dietary carbohydrates in the intestine. α-Glucosidases in the intestine are amylase, sucrase-isomaltase, and maltase-glucoamylase (see Section 2.2 in Chapter 18). Partial inhibition of α-glucosidases slows the degradation of starch and sucrose and thus allows for a more steady and lower flux of glucose into the blood; this glucose flux can often still be handled by insulin secretion from the failing β-cells. Side effects of these drugs are bloating and borborygmi, which are caused by bacteria that get to degrade an unusually large amount of carbohydrates (see Section 4 in Chapter 18).

Inhibitors of **ATP-sensitive K^+ channels (**K_{ATP}-channels)**, glucagon-like peptide 1** (**GLP-1**) **receptor agonists**, and inhibitors of the degradation of GLP-1 are used to boost insulin secretion from pancreatic β-cells as follows:

Sulfonylurea drugs and "**glinide" drugs** inhibit K_{ATP}-channels; this leads to increased insulin secretion (see Chapter 26). The different classes of drugs differ mainly in their chemical structure. Some of the inhibitors of K_{ATP}, such as repaglinide and nateglinide are short acting and can be taken before each meal; others, such as the sulfonylurea glyburide (also called glibenclamide), are long acting (several days) and are

taken only once a day. Since the secretagogue effect of all K_{ATP}-channel inhibitors shows only a mild dependence on the concentration of glucose, all of these inhibitors can cause **hypoglycemia** (which may be life threatening). K_{ATP}-channel inhibitors cause mild weight gain because the increased amount of insulin secretion leads to increased synthesis of triglycerides and their deposition in adipose tissue.

GLP-1 receptor agonists enhance glucose-induced insulin secretion via GLP-1 receptors, which signal via cyclic adenosine monophosphate (cAMP; see Chapter 26). GLP-1 itself has too short of a half-life in the blood to be of pharmacological use. The currently approved drugs in this class are **exenatide**, which is a synthetic recombinant form of the Gila monster toxin **exendin-4**, a 39–amino acid peptide that is ~50% homologous to human GLP-1; **liraglutide**, an analog of human GLP-1 that is acylated with palmitic acid; **albiglutide**, a recombinant peptide consisting of two copies of modified human GLP-1 and one copy of albumin; and **dulaglutide**, a recombinant fusion protein of modified human GLP-1 and a fragment of a modified human immunoglobulin. All of these peptides must be injected because, if taken orally, they would be degraded in the intestine. GLP-1 receptor agonists reduce postprandial hyperglycemia (Fig. 39.7) by slowing gastric emptying, inhibiting glucagon secretion, and stimulating insulin secretion. GLP-1 and its analogs enhance insulin secretion only at a concentration of glucose above ~90 mg/dL (~5 mM). Hence, if taken alone, analogs of GLP-1 should not cause hypoglycemia. (Nevertheless, these drugs do carry a warning of hypoglycemia because hypoglycemia is possible in conjunction with drugs that produce hypoglycemia, such as insulin and inhibitors of K_{ATP}-channels). GLP-1 normally has an appetite-suppressing effect; perhaps because of this, a side effect of GLP-1 receptor agonists is decreased appetite and nausea. A decrease in appetite obviously helps with weight loss.

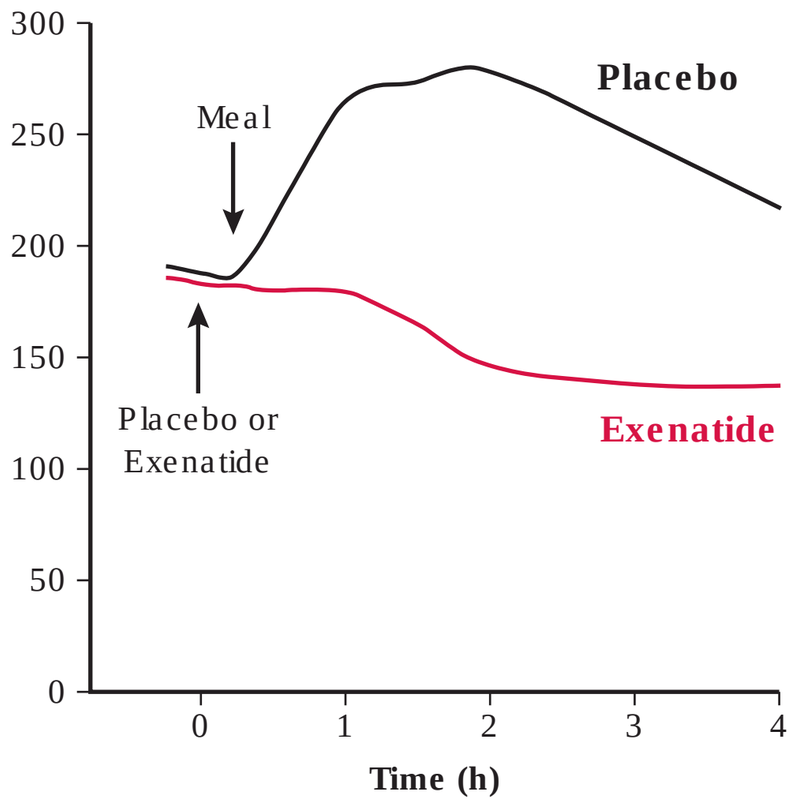


Fig. 39.7 Effect of exenatide on plasma glucose after a meal. Volunteers with type 2 diabetes received an injection of exenatide (~0.09 µg/kg body weight) or placebo 15 minutes before a standardized breakfast. (Data from Fineman MS, Bicsak TA, Shen LZ, et al. Effect on glycemic control of exenatide (synthetic exendin-4) additive to existing metformin and/or sulfonylurea treatment in patients with type 2 diabetes. *Diabetes Care*. 2003;26:2370-2377; and Kolterman OG, Buse JB, Fineman MS, et al. Synthetic exendin-4 (exenatide) significantly reduces postprandial and fasting plasma glucose in subjects with type 2 diabetes. *J Clin Endocrinol Metab*. 2003;88:3082-3089.)

The "**gliptins**" inhibit the degradation of the patients' own GLP-1 and thus lead to an elevated concentration of GLP-1. Gliptins currently approved in the United States are **sitagliptin**, **saxagliptin**, **linagliptin**, and **alogliptin**. The gliptins inhibit **dipeptidyl peptidase-4** (**DPP-4**), which degrades GLP-1 as well as other hormones, such as gastric inhibitory peptide (an incretin-like GLP-1; see Section 3.3.2 in Chapter 26). Gliptins are low-molecular-weight, nonpeptide compounds, and they can be taken by mouth. Like GLP-1 receptor agonists (see above), the gliptins by themselves do not cause hypoglycemia. An advantage of gliptins over GLP-1 receptor agonists is that they do not cause nausea; a disadvantage is that they are less effective in lowering HbA_{1c}.

Pramlintide, an analog of the peptide amylin (see Section 8.4 in Chapter 9 and Section 2.2 in Chapter 26), decreases food intake, slows gastric emptying, and inhibits glucagon secretion. These effects require that patients approximately halve their dose of short-acting insulin. Pramlintide is best suited for patients who cannot control their blood glucose concentration with insulin alone or with insulin plus oral antidiabetic drugs. Amylin is normally contained in secretory vesicles in pancreatic β-cells. Type 1 diabetic patients secrete virtually no amylin, whereas type 2 diabetic patients secrete progressively less amylin as they age. Amylin irreversibly aggregates when present at elevated concentrations (see Section 8.4 in Chapter 9). Pramlintide differs from amylin in three amino acid residues; this change renders pramlintide much more soluble than amylin.

The use of **sodium-glucose cotransporter-2** (**SGLT2**) **inhibitors** in the treatment of type 2 diabetes is discussed in Section 6 of Chapter 18. SGLT2 is a Na^+-dependent glucose transporter located in the kidney tubules that normally recovers most of the glucose from the glomerular filtrate. Treatment with an SGLT2 inhibitor leads to glucosuria (glucose in the urine). Urinary tract infection is one of the most common side effects of SGLT2 inhibition and can be dangerous.

Metformin chiefly attenuates endogenous glucose production. Most patients with type 2 diabetes produce too much glucose, mostly from gluconeogenesis and especially during the overnight fast. In addition, type 2 diabetic patients also have an elevated concentration of free fatty acids in their blood and therefore have an abnormally low need for endogenously produced glucose. The mechanism of action of metformin is poorly understood. In the liver, metformin decreases flux in gluconeogenesis, perhaps by activating AMP-activated protein kinase (AMPK; see Fig. 25.7 and Sections 3 and 4.2.2 in Chapter 25). In muscle, metformin leads to the insertion of glucose transporters into the plasma membrane and to activation of glycogen phosphorylase; this increases glucose uptake and the production of glucose 6-phosphate from glycogen. In muscle and in liver, metformin increases β-oxidation of fatty acids (see Section 4 in Chapter 27). Predominantly in patients

who have compromised function of the heart, lungs, liver, or kidneys, metformin may cause lactic acidosis. The lactic acidosis may be caused by inhibition of complex I of oxidative phosphorylation (see Sections 1.3 and 2 in Chapter 23). Metformin slightly decreases appetite.

The **thiazolidinediones** increase insulin sensitivity by increasing the activity of **peroxisome proliferator-activated receptor γ** (**PPAR-γ**). Currently, the thiazolidinediones **rosiglitazone** and **pioglitazone** are approved for use in the United States; however, there are major concerns that these drugs may damage the heart and its vasculature as well as have other adverse effects. Thiazolidinediones greatly increase insulin-stimulated glucose removal from the blood. The mechanism of action is only partially understood. PPAR-γ are nuclear receptors that heterodimerize with retinoid X receptors to form a functional transcription factor. This complex regulates transcription of genes that play a role in the metabolism of glucose and lipids. PPAR-γ is especially abundant in adipose tissue. There, activation by thiazolidinediones gives rise to more, younger, more insulin-sensitive adipocytes in the subcutaneous adipose tissue as well as increased activity of lipoprotein lipase and increased secretion of adiponectin from fat cells. Adiponectin, in turn, increases the insulin sensitivity of muscle and liver. In muscle, thiazolidinedione use is associated with increased AMPK activity (similar to metformin, see above). If used by themselves, thiazolidinediones do not cause hypoglycemia.

After about 3 to 5 years of use, monotherapy with one of the aforementioned oral agents generally fails to be sufficiently effective, most likely because of a further decline in function of pancreatic β-cells. At this point, agents with a different, noninterfering mechanism of action can be added, or **insulin** injections can be instituted. Indeed, insulin injections are used in all patients whose blood glucose cannot be controlled with oral medications.

Short-acting insulins are needed for patients whose β-cells secrete little or no insulin in response to meals, whereas **long-acting "baseline" insulins** can be used in patients who still have appreciable β-cell function. Because most patients with type 2 diabetes are insulin resistant, they need to inject much more insulin than do the typical type 1 diabetic patients.

Patients who have type 2 diabetes who start a new drug or take a drug that can cause hypoglycemia (particularly insulin) commonly measure their blood glucose concentration using a **glucose meter**.

6. MODY

Maturity-onset diabetes *of* the young (MODY) is a collection *of* diseases that are caused by mutations in individual proteins that are essential *for* the normal *function of* β-cells.

The term MODY dates from a time when type 2 diabetes was referred to as "maturity onset" and when the causes of MODY were still unknown. Patients with MODY are often misdiagnosed as having type 1 or type 2 diabetes.

All known types of MODY are due to mutations in proteins that are important for normal development and function of pancreatic β-cells; often, these proteins also affect the function of the liver. For the most common types of MODY, numerous mutations are known, and there is therefore an appreciable diversity of phenotypes.

The overall prevalence of MODY is likely ~1 in 1,000, though many persons are undiagnosed, especially if they have MODY-2 (see below).

A diagnosis of MODY is commonly entertained if a patient is lean, develops diabetes before 30 years of age, has a family member who also developed diabetes at such a young age, has no signs of insulin resistance (e.g., acanthosis nigricans), and either does not require insulin or has a significant plasma concentration of C-peptide (an indicator of endogenous insulin secretion; see Chapter 26). In the original working definition of MODY, patients also had to be free of diabetic ketoacidosis and islet cell autoantibodies. Among teenagers who do not have type 1 diabetes, one can expect the leaner patients to have MODY and the more obese to have type 2 diabetes. A calculator to estimate the probability that a patient has MODY is available at http://diabetesgenes.org/content/mody-probability-calculator.

The most common forms of MODY are MODY-2 and MODY-3. Both are inherited in an autosomal dominant fashion.

MODY-2 is caused by a heterozygous loss-of-function mutation in the GCK gene, which encodes **glucokinase**. In Europe, the prevalence of MODY-2 is ~1 in 1,000. Glucokinase is the glucose sensor of the pancreatic β-cell (see Fig. 26.7). In the liver, in the fed state, glucokinase helps direct glucose into glycolysis and glycogen synthesis (see Section 5.6 in Chapter 19 and Sections 1.3 and 3.1 in Chapter 24). Affected patients have a higher set point for glucose-induced insulin secretion and therefore have mild hyperglycemia even in the fasting state. Hyperglycemia is recognizable at birth and increases slightly with age. Patients with MODY-2 do not seem to have a noticeably increased risk for diabetic complications. Treatment is usually not necessary, except during pregnancy. Oral antidiabetic agents are ineffective.

MODY-3 is caused by a mutation in the HNF1A gene, which encodes HNF1 homeobox A (**HNF1A**). HNF1A is a transcription factor that stimulates the transcription of genes, the products of which are involved in the metabolism of glucose or fatty acids. At birth, patients have a normal concentration of blood glucose. However, they have a severe defect in insulin secretion that worsens with age. Typical onset of diabetes is at ~20 years of age. Some patients present with pronounced hyperglycemia and are misdiagnosed as having type 1 diabetes. Patients are typically treated with diet and a sulfonylurea. When endogenous insulin secretion has become insufficient, patients need to be switched to insulin therapy.

7. GESTATIONAL DIABETES

Pregnancy is a time *of* marked insulin resistance and thus increased need *for* insulin secretion. Diabetes first shows up during pregnancy in about 5% *of* women. Patients who have

gestational diabetes are treated with small modifications in diet and, if necessary, with insulin, glyburide, or metformin.

Pregnancy requires a large increase in insulin secretion from the mother's pancreatic β-cells. After ~20 weeks of gestation, insulin resistance normally develops to the point that the pancreas has to secrete several times the usual amount of insulin, yet the β-cell mass increases only by a factor of ~1.4. Insulin resistance of the mother likely helps shunt glucose to the placenta to satisfy the fetus's large demand for glucose. The insulin resistance has been attributed in part to relatively high concentrations of placental growth hormone, placental lactogen, and progesterone. Age- and obesity-induced insulin resistance both increase a pregnant woman's chance of developing gestational diabetes. Approximately 5% of pregnant women do not secrete enough insulin during late pregnancy and thus have gestational diabetes.

When the fetus is exposed to chronic hyperglycemia, it synthesizes an inordinately large amount of fat. The fetal pancreas secretes insulin according to the prevailing concentration of glucose. The concentration of glucose in fetal blood is heavily dependent on the concentration of glucose in the mother's blood. The hyperinsulinemia in the fetus of a hyperglycemic mother stimulates the synthesis of fatty acids and triglycerides in the fetus. Excessive fetal lipid stores are the cause of **macrosomia** (weight in excess of 9 lb or 4.0 kg) and its attendant risks during delivery (e.g., shoulder dystocia; Fig. 39.8). Fetuses of hyperglycemic mothers also have relatively immature lungs.

In **newborns** of markedly hyperglycemic mothers, β-cells fail to properly reduce insulin secretion during normoglycemia or hypoglycemia. Accordingly, newborns of mothers with poorly controlled diabetes hypersecrete insulin and develop **hypoglycemia**. This must be treated with glucose infusions. Over a period of days, provided the concentration of glucose is close to normal, the infant's β-cells adapt and eventually secrete the appropriate amount of insulin for normal glucose homeostasis.

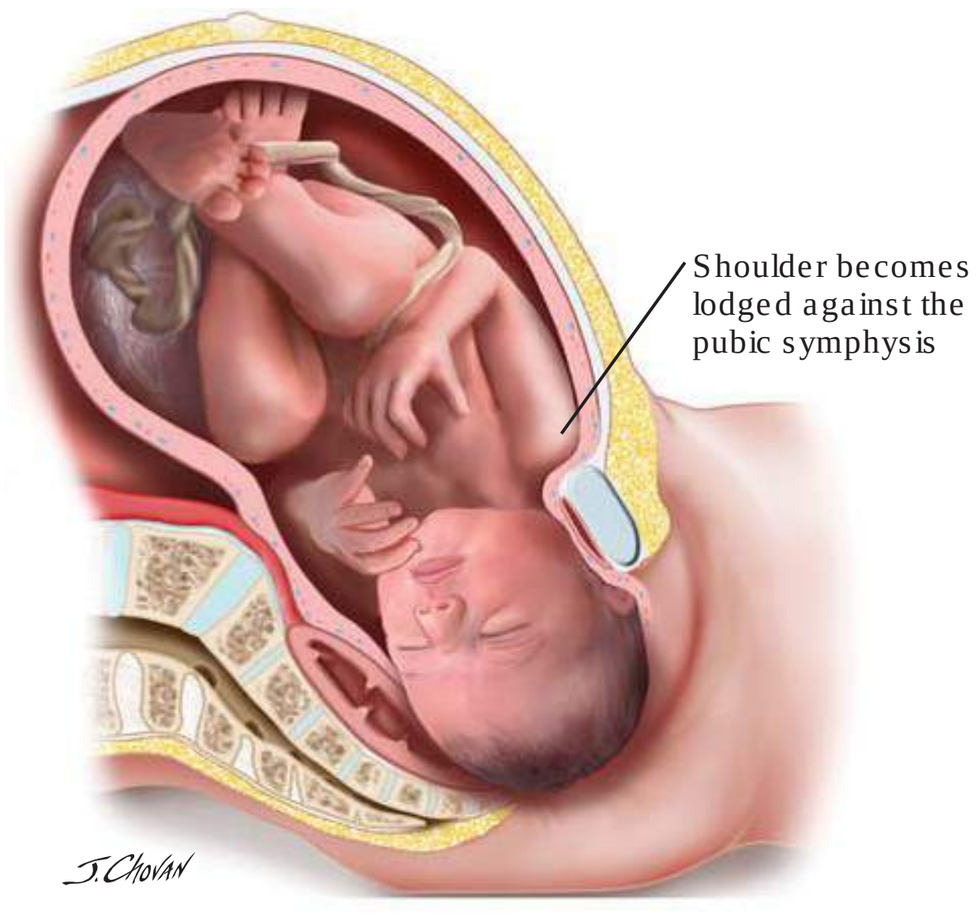


Fig. 39.8 Shoulder dystocia. Macrosomia from gestational diabetes can cause shoulder dystocia, a serious complication of a vaginal delivery. Shoulder dystocia may lead to permanent damage of the brachial plexus.

Because untreated gestational diabetes can lead to the abovementioned serious complications, pregnant patients generally undergo **screening** for diabetes with a modified oral glucose tolerance test (see Section 3). This test is given at 24 to 28 weeks of gestation; that is, at a time when a woman's inability to secrete insulin in the face of mounting insulin resistance becomes apparent and when the fetus has not yet deposited a damaging amount of adipose tissue. In the United States, **glucose tolerance** during pregnancy is commonly tested as follows. Patients are given 50 g of glucose by mouth (without prior fasting), and a blood sample is taken 1 hour later. If the plasma glucose is above 135 to 140 mg/dL, the patient is tested further. In the second test, the patient fasts overnight and then undergoes a 100-g oral glucose tolerance test with samples taken at time points 0, 1, 2, and 3 hours. If plasma glucose values for two or more time points are above certain limits, the patient has gestational diabetes.

Treatment of patients who have gestational diabetes involves modest changes in **diet** and is augmented with drugs as needed. Even markedly overweight pregnant patients should not fast aggressively because pregnancy itself requires increased food intake for the health of both the mother and fetus. Only a few antidiabetic drugs are approved for use in pregnant women. Injected **insulin** to reduce baseline insulin secretion is usually adequate because the patient's β-cells can still take care of increased demand after meals. **Glyburide** (**glibenclamide**) is a long-acting oral sulfonylurea antidiabetic drug (see Section 5.3) that is used in place of insulin. **Metformin** (oral) mostly reduces endogenous glucose production (see Section 5.3).

Most women who have gestational diabetes become nondiabetic in the postpartum period. Unfortunately, gestational diabetes is a sign of future trouble. Within 10 years of being diagnosed with gestational diabetes, a majority of patients develop **type 2 diabetes.**

8. COMPLICATIONS OF DIABETES

The main complications of diabetes lead to decreased vision, impaired peripheral nerve sensation, decreased kidney function, and dysfunction of the heart or large blood vessels. The main processes that cause these complications are thought to be spontaneous glycation of proteins and lipids; increased formation of damaging, oxygen-containing radicals; and insulin resistance.

8.1. General Comments

Epidemiological studies have shown that diabetic patients who receive instructions to achieve good control of the concentration of **glucose** in plasma have fewer microvascular

and macrovascular complications (see Section 8.2) of diabetes than patients who do not receive counseling and who have poor control. The difference in treatment results in a difference in the fraction of **HbA_{1c}**, which in turn correlates with the frequency of microvascular and macrovascular complications. Many of the complications of diabetes seem to result from chronic hyperglycemia, but there may be other causes as well. Complications of diabetes are also seen in patients who have forms of MODY that cause marked hyperglycemia. Patients who are obese or who have abnormalities of lipid metabolism are at risk of additional complications.

The current standard of care is a regimen of blood glucose testing and administration of drugs so that the concentration of glucose is fairly close to the normal concentration, yet not dangerously low.

8.2. Clinical Aspects of Complications of Diabetes

Diabetic complications are often divided into microvascular and macrovascular complications. **Microvascular complications** (Fig. 39.9) affect blood flow predominantly in the capillaries of the eyes, peripheral nerves, and kidneys. The capillaries have an abnormally small lumen, and the walls are leaky. This is associated with increased blood pressure, edema, and decreased blood flow to tissues (ischemia). For compensation, new blood vessels are produced. Major microvascular complications of diabetes are **retinopathy**, peripheral **neuropathy**, and **nephropathy**. **Macrovascular complications** include cardiovascular disease and peripheral vascular disease (arteries that supply the brain, heart, or lower extremities). Major macrovascular complications of diabetes are **heart disease**, **stroke**, **ulcers**, and **gangrene**.

Retinopathy may be accompanied by reduced blood flow to the retina due to vasoconstriction, local hypertension, leaky vessels, ischemia, proliferation of new blood vessels, and reduced visual acuity (see Fig. 20.4). Blindness can be the result of detachment of the retina, bleeding into the retina or vitreous, or ischemia of the macula (the region of the retina that provides vision of the highest acuity).

Diabetic kidney disease is accompanied by a decreased rate of filtration of blood (evident from serum creatinine), leakage of albumin into the urine (albuminuria), or both of these abnormalities. In patients with chronic hyperglycemia, capillaries to the glomeruli are lost and nearby cells fill the space with extracellular matrix (Fig. 39.10). In the glomerulus, the basement membrane and the foot processes of podocytes together form a size-exclusion filter. In patients with chronic hyperglycemia, a lack of adequate podocyte foot processes compromises this filter and allows albumin to pass into the filtrate.

Diabetic kidney disease is currently treated mainly with strict control of blood glucose and blood pressure. Blood pressure is reduced with angiotensin-converting enzyme inhibitors and angiotensin receptor blockers (see Fig. 31.17 and Section 4.1 in Chapter 31).

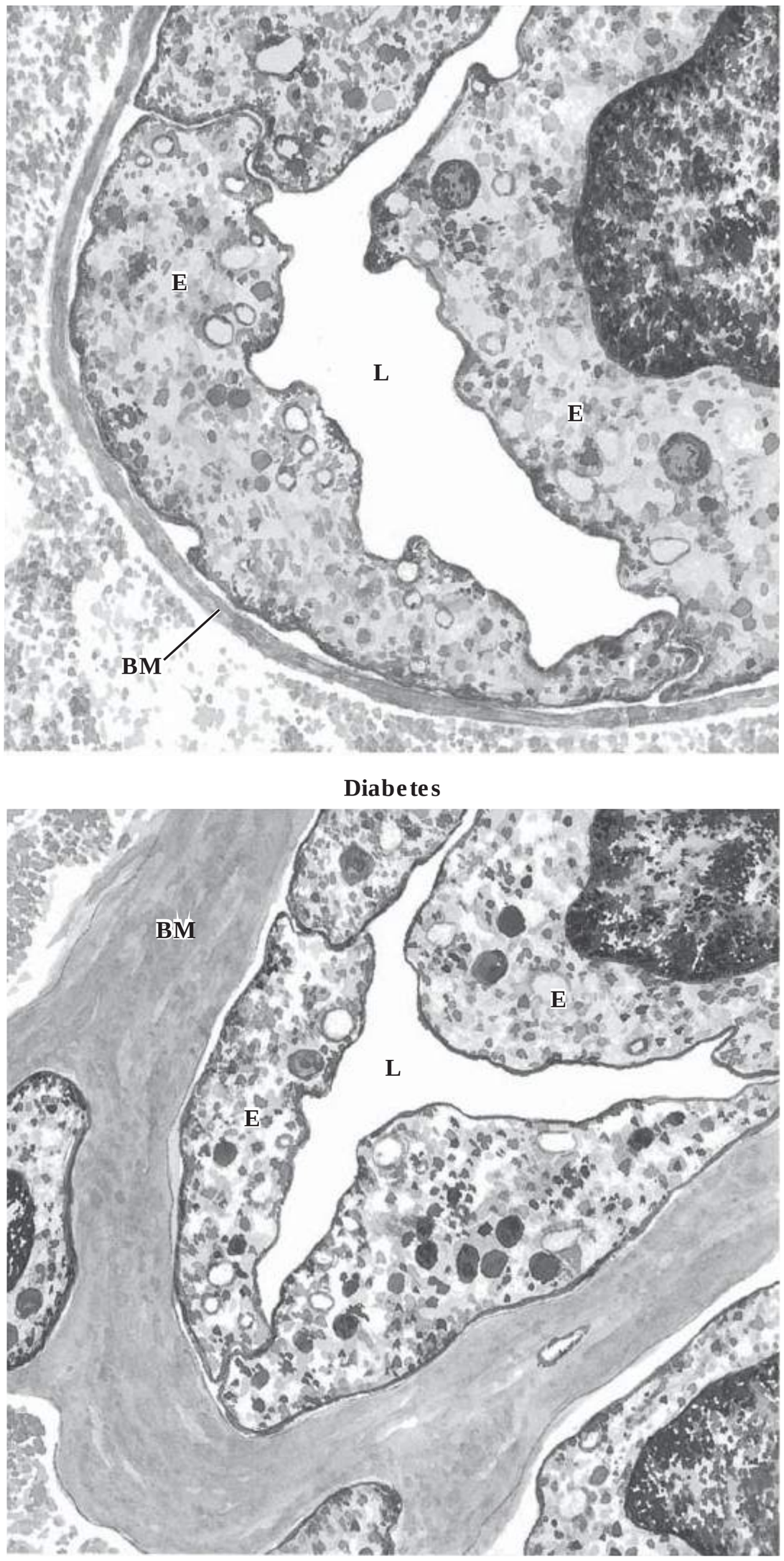
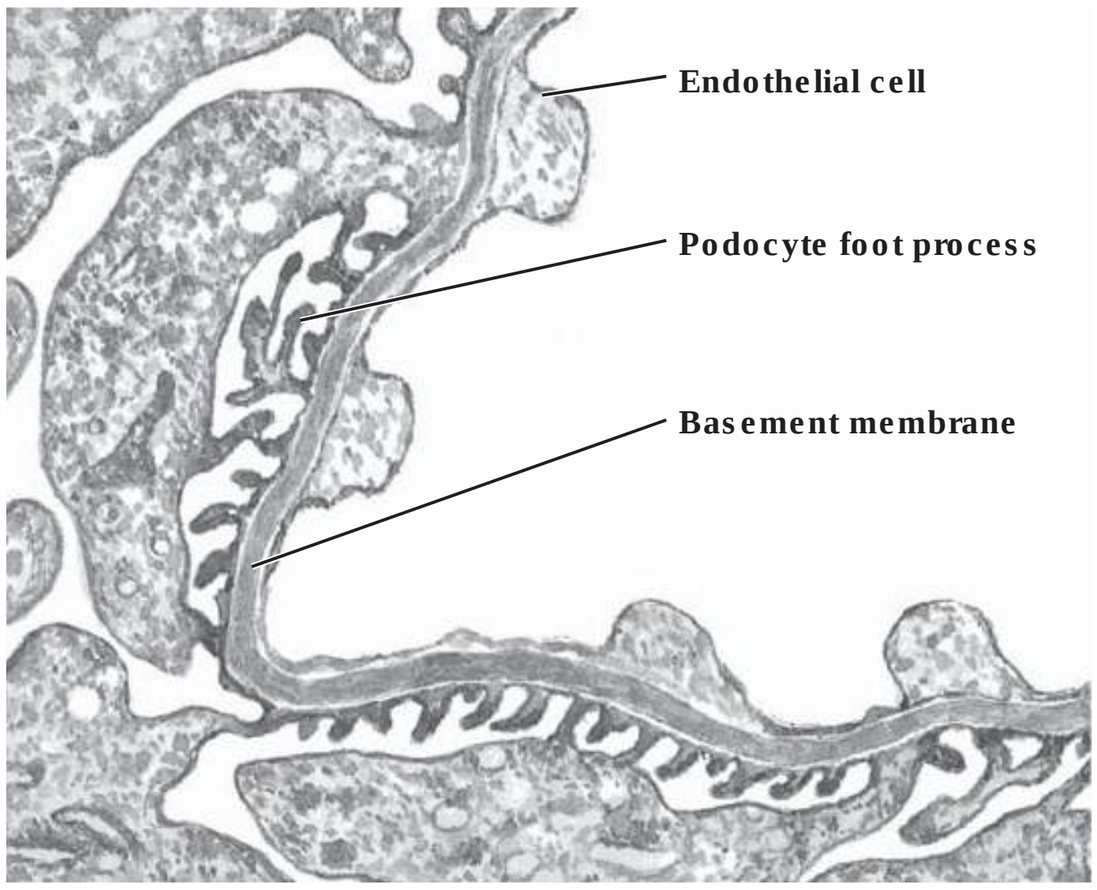


Fig. 39.9 Microvascular disease in diabetes. Electron microscopic image of cross section of a skin capillary. BM, basement membrane; E, epithelial cell; L, lumen. The basement membrane is thickened and the lumen reduced.

Peripheral neuropathy is seen in almost half of all patients with diabetes. The neuropathy usually manifests itself with numbness. Painful neuropathy occurs in about 5% of all



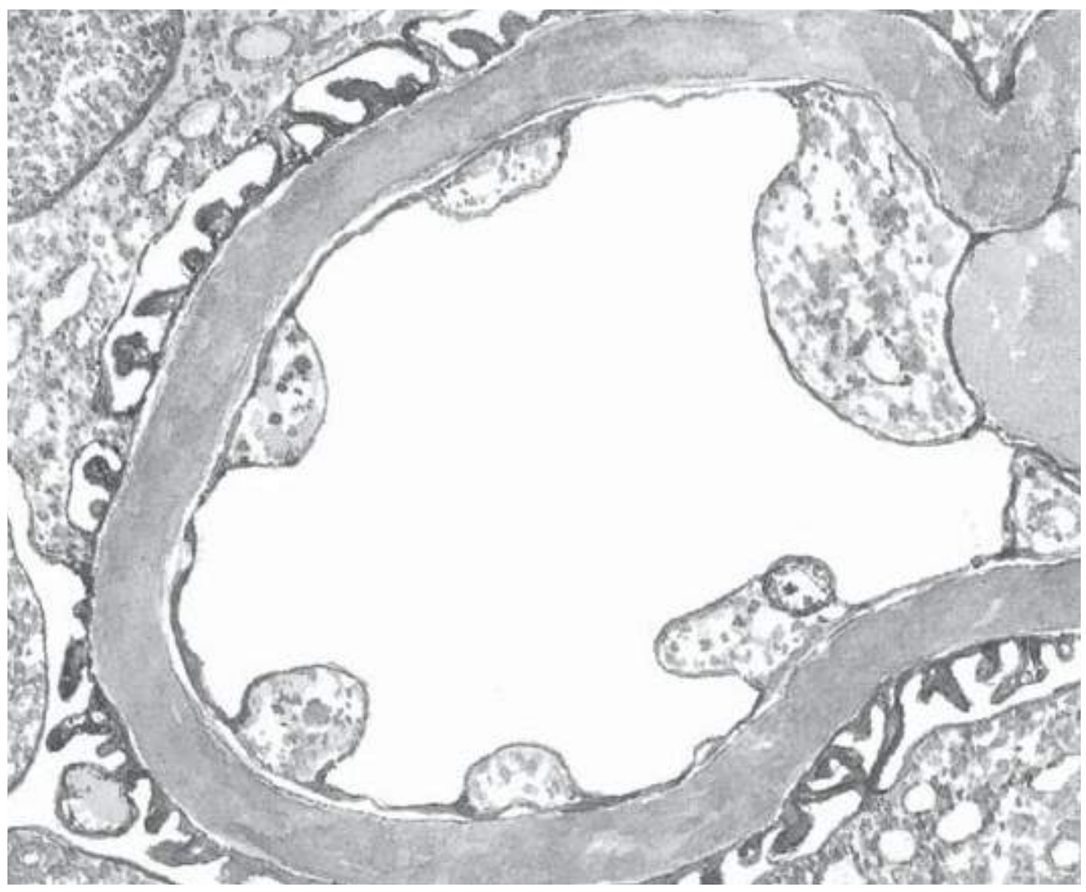


Fig. 39.10 Glomerulosclerosis as a consequence of long-term diabetes. Based on transmission electron micrographs. Podocyte foot processes are damaged, the basement membrane is thickened, and the endothelium is compromised.

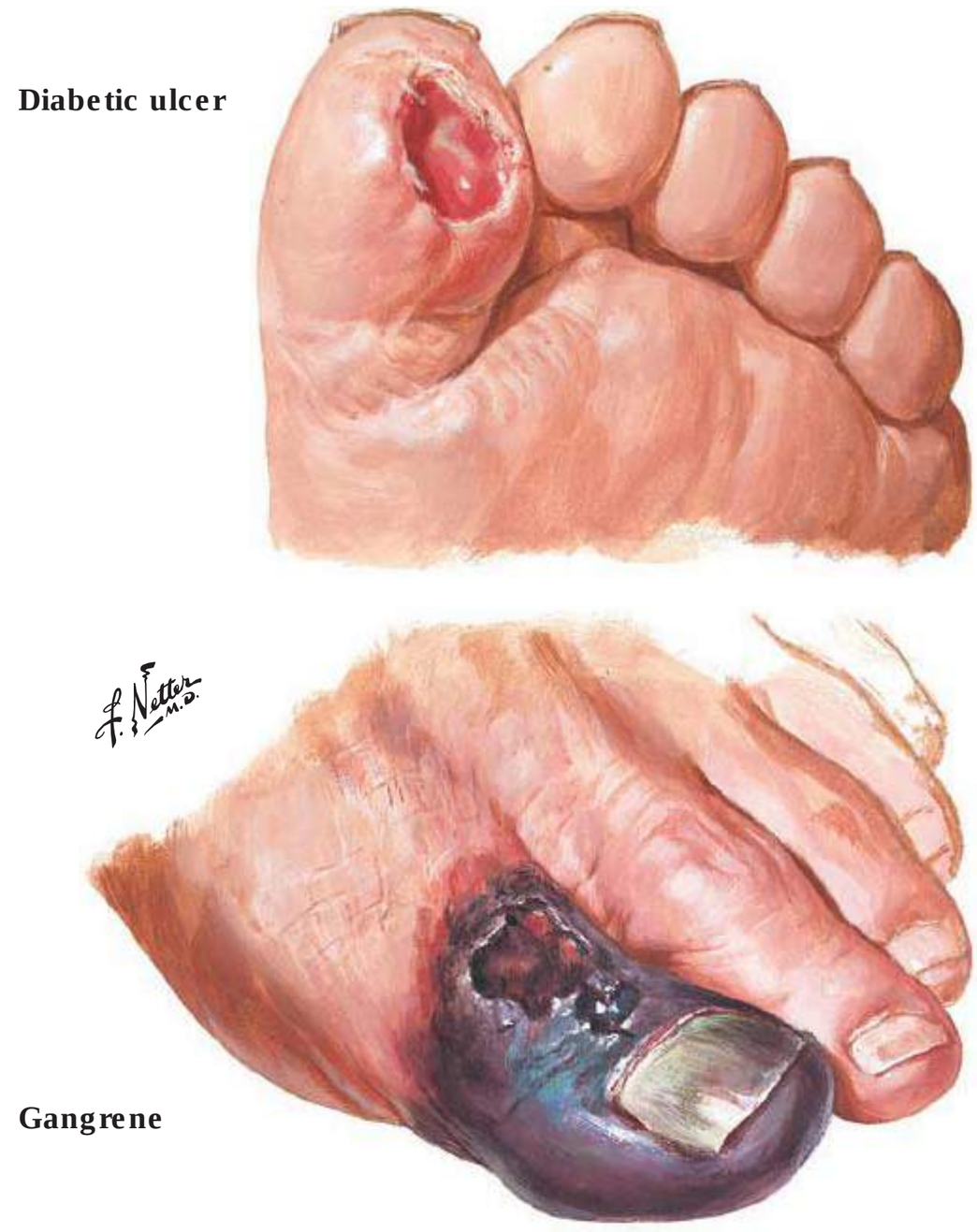


Fig. 39.11 Ulcer and gangrene as complications of diabetes. Peripheral neuropathy impairs a patient's ready detection of lesions. Hyperglycemia and poor tissue perfusion favor infections. When gangrene develops, an amputation becomes necessary.

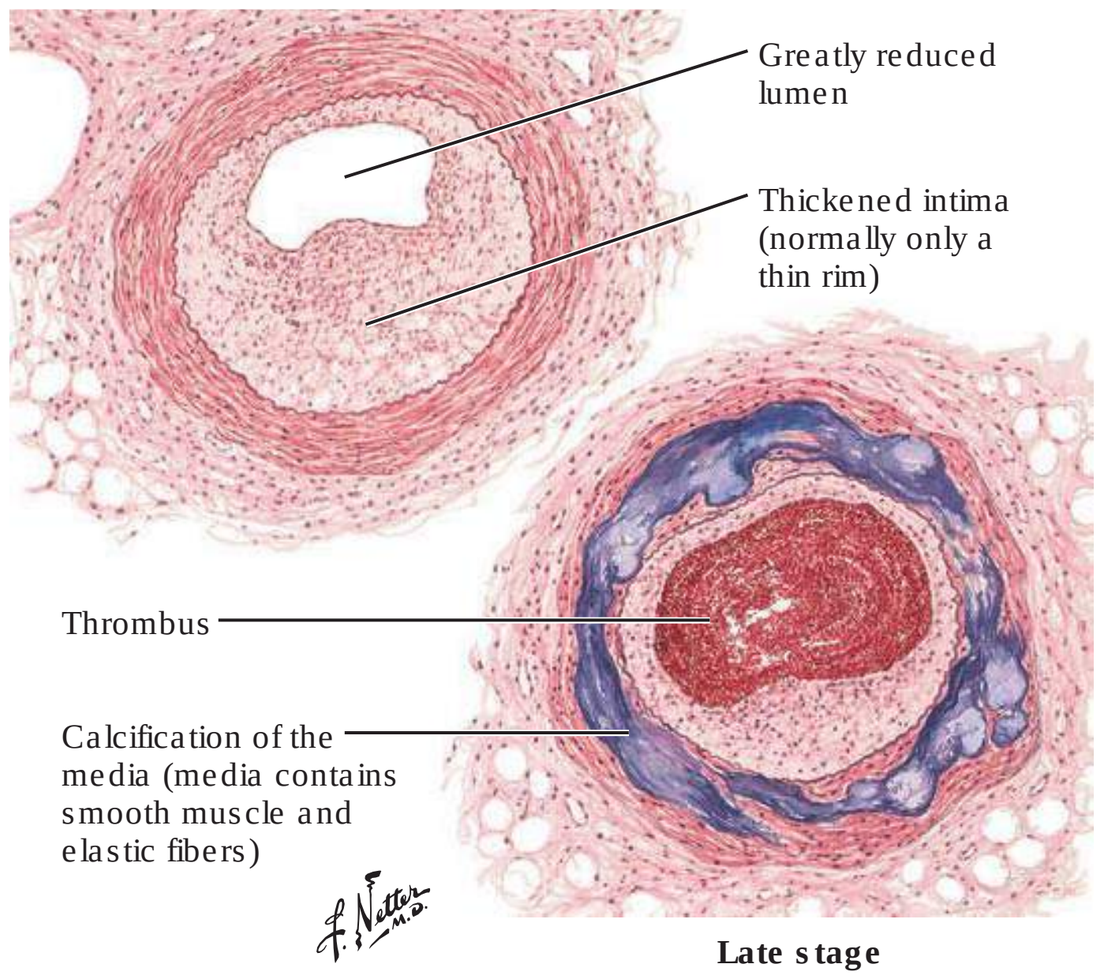


Fig. 39.12 Atherosclerosis in patients who have diabetes.

patients who have diabetes. T e neuropathy is thought to derive from inadequate blood flow and damage to neurons.

Patients who have diabetes and peripheral neuropathy, macrovascular disease, and marked hyperglycemia are at an increased risk of developing diabetic **ulcers**, especially on their feet and lower legs; the ulcers may progress to **gangrene** (Fig. 39.11).

Diabetic patients who have insulin resistance (e.g., due to obesity) and dyslipidemia are at an increased risk for **vascular disease** (Fig. 39.12).

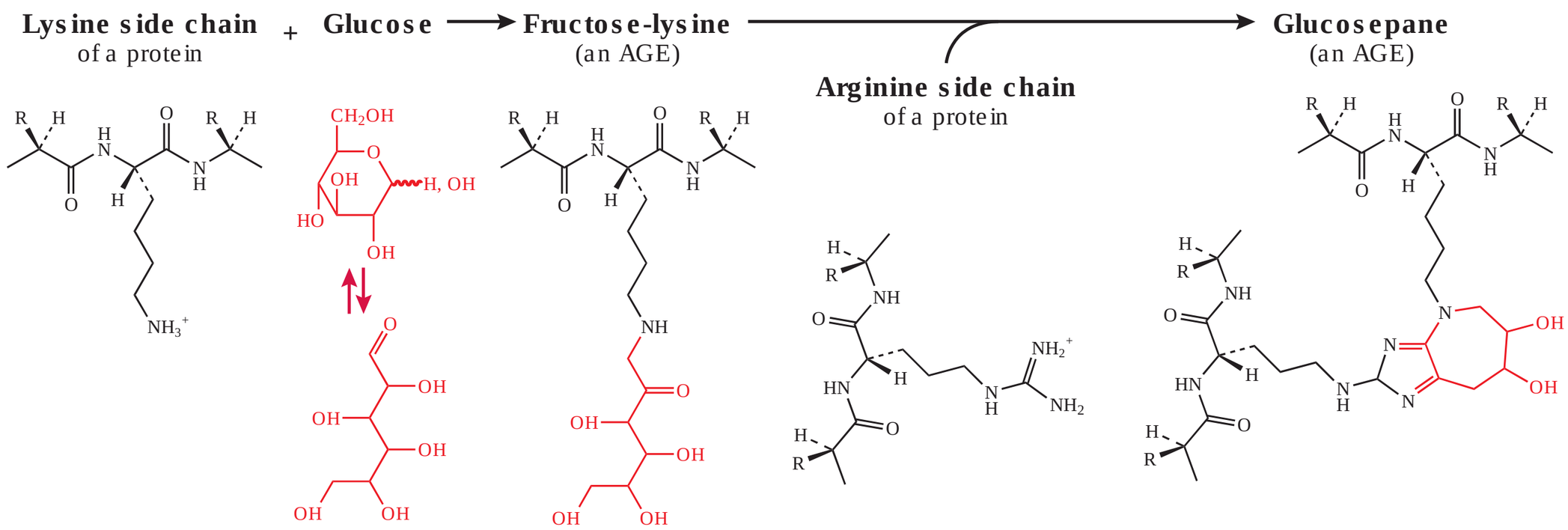


Fig. 39.13 **Nonenzymatic glycation of proteins by glucose gives rise to fructose-lysine and glucosepane.** AGE, advanced glycation end product.

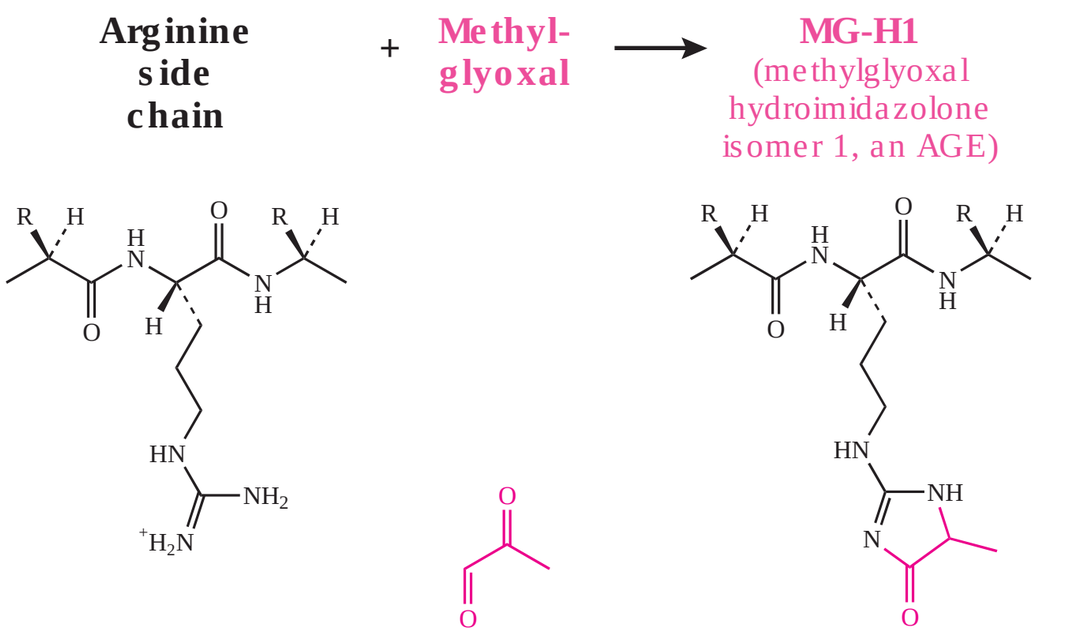


Fig. 39.14 **Methylglyoxal reacts with arginine side chains to form MG-H1, an advanced glycation end product.**

8.3. Potential Biochemical Causes of Complications of Diabetes

8.3.1. Nonenzymatic Glycation

When a patient is hyperglycemic, the concentration of glucose is not only elevated in blood vessels, but also in the extracellular space and inside all cells that do not regulate glucose uptake.

Sugars in general are quite reactive. Glucose has relatively low reactivity; perhaps this is why it evolved to its current position in mammalian metabolism. Fructose and galactose are considerably more reactive than glucose. Several metabolites of glycolysis are also quite reactive. The 3-carbon sugar **dihydroxyacetone** is even reactive enough that it is used as a topical **tanning** agent (dihydroxyacetone readily reacts with amino groups in the epidermis to form a brown product, melanoidin). Dihydroxyacetone is used cosmetically for patients who have **vitiligo** (see Fig. 35.18 and Section 4.4 in Chapter 35).

The term nonenzymatic **glycation** refers to the spontaneous reaction between a sugar and a protein (or peptide, nucleic acid, or lipid) that results in a bond other than a glycosyl linkage. A glycosyl linkage is a bond between the anomeric carbon of a sugar (this is C-1 for glucose, galactose, or ribose) and O, N, or S. In glycogen for instance, glucose residues are linked via O-glycosidic bonds (see Fig. 24.2). In ribonucleotides, a ribose is linked to a base via an N-glycosidic bond (see Fig. 1.1). In glycation, a sugar typically forms a Schif base with a free amino group, and the Schif base then undergoes an **Amadori rearrangement** that modifies the sugar such that it is no longer a glycosyl residue; that is, there is no longer a C linked to two O or one O and one N atom (Fig. 39.13; compare with Fig. 7.8). The formation of **HbA_{1c}** is an example of a glycation reaction. Glucose forms a Schif base with the N-terminal amino group of valine in β-globin. A subsequent Amadori rearrangement (see Fig. 39.13 for an analogous reaction) yields 1-deoxyfructosyl hemoglobin (i.e., HbA_{1c}). Glycation happens both inside and outside cells.

The term **advanced glycation end product** (**AGE**) refers to the product of a protein or peptide that was at one point linked to a sugar. In AGEs, the original sugar moiety is usually no longer a sugar (see below and Figs. 39.13 and 39.14).

Skin protein glycation parallels a type 1 diabetic patient's risk of microvascular and macrovascular complications. In skin, AGEs cross-link chiefly collagen. AGE content increases with age and with diabetes. The strongest associations between glycation products and risk of complications have been observed for **fructose-lysine**, **glucosepane**, and **methylglyoxal hydroimidazolone isomer 1** (**MG-H1**). Fructose-lysine is the product of glucose reacting with the free amino group of a lysine side chain (see Fig. 39.13). When fructose-lysine undergoes rearrangement and reacts with the side chain of an arginine residue, it gives rise to glucosepane. Methylglyoxal is thought to be chiefly formed from triose phosphates, such as dihydroxyacetone phosphate and glyceraldehyde 3-phosphate (see Fig. 19.2). A set of two ubiquitously expressed glyoxalases sequentially degrades methylglyoxal to lactate. Methylglyoxal readily reacts with the free guanidino group of an arginine side chain and thus gives rise to MG-H1 (see Fig. 39.14). There are many more AGEs, and the abundance of some of them also correlates with the risk for complications of diabetes.

Glycated proteins may have altered function, and they may activate AGE receptors (see below). Proteins of the extracellular matrix, such as collagens, elastin, and lens crystallins, have a relatively long lifetime and therefore accumulate an appreciable amount of AGEs. AGE–cross-linked collagens have altered physical properties and are also less susceptible to degradation. AGE-altered collagens especially af ect the properties of blood vessels, basement membranes in the kidney glomeruli, and skin. When proteins that contain AGEs are degraded, they give rise to AGE-modified amino acids, which are excreted into the urine.

Besides amino groups on amino acids, amino groups on **phospholipids** are also susceptible to glycation reactions. In phospholipids, amino groups are present in phosphatidylethanolamine and phosphatidylserine (see Fig. 11.2).

In addition to AGEs produced in the body, AGEs from the **diet** and **tobacco smoke** contribute to a person's AGE load. When heated (as during cooking or baking), sugars react more readily with amino groups, forming AGEs. A fraction of dietary AGE is taken up via the intestine, and it has a highly appreciable ef ect on the amount of AGEs in blood. Tobacco leaves accumulate AGEs during curing. A person who smokes inhales some of these AGEs and absorbs them via the lungs.

Many dif erent types of cells, especially endothelial cells, smooth muscle cells, and monocytes (which can become macrophages), have plasma membrane **receptors for advanced glycation end products**; **RAGE** is one such receptor. (RAGE also binds the Aβ-protein, as well as amyloid fibrils, which are rich in β-sheet structure; see Section 8 in Chapter 9). On binding an AGE, the RAGE signals across the membrane and, signaling via a variety of intracellular signaling pathways, alters the rate of transcription of certain genes. T is change leads to increased production of reactive oxygen species (**ROS**; see Section 8.3.2) and evokes the secretion of **cytokines**, which in turn elicit **inflammation**. ROS stem, in part, from NAD(P)H oxidase and, in part, from the electron transport chain in mitochondria. Other AGE receptors facilitate the uptake and intracellular degradation of AGEs.

8.3.2. Damage by Reactive Oxygen Species

NADPH oxidase and the electron transport chain in mitochondria give rise to **superoxide radicals** ($\bullet O_2^-$), which in turn can give rise to **hydrogen peroxide** (H_2O_2), **hydroxyl radicals** (•OH), **singlet oxygen**, or **peroxynitrite** ($ONOO^-$). NADPH oxidase is an enzyme that is present in phagocytic vacuoles and also on the cytoplasmic side of the plasma membrane, where it assembles in response to signaling from a variety of hormones, including cytokines. In the respiratory chain of mitochondria, it is mostly coenzyme Q interacting with complex III that gives rise to superoxide radicals (see Figs. 23.3 and 23.4). Radicals are discussed in Section 2.3 in Chapter 21. Singlet oxygen is a very reactive form of O_2 in which two electrons are in a high-energy electron configuration. Singlet oxygen is endogenously formed through various mechanisms. Peroxynitrite derives from the reaction of superoxide radicals ($\bullet O_2^-$) with the signaling molecule nitric oxide (NO; see Section 5.2 in Chapter 16). Compounds such as singlet oxygen, hydrogen peroxide, and oxygen-containing radicals are lumped together under the term **ROS**.

Cells have several mechanisms to remove ROS, as detailed in Section 2.3 in Chapter 21. T e extracellular space contains antioxidants that react spontaneously. T e major extracellular antioxidants are –SH groups on proteins, urate, ascorbate (vitamin C), and vitamin E. Inside cells, enzymes remove most ROS that are precursors of more reactive ROS. For instance, superoxide dismutase converts $\bullet O_2^-$ to H_2O_2, and glutathione peroxidase or catalase minimizes the formation of •OH by reducing H_2O_2 to water (see Section 2.3 in Chapter 21 and Fig. 21.6).

In hyperglycemic patients, the rate of ROS production is abnormally high. Damage to **protein** and **lipids** occurs when a significant amount of radicals reacts with these molecules rather than antioxidants or radical scavengers. When ROS such as •OH react with lipids, they form lipid radicals, lipid peroxyl radicals, and lipid hydroperoxides. T ese radicals are removed by reaction with vitamin E and glutathione, whereas the hydroperoxides are reduced by glutathione (see Section 2.3 in Chapter 21).

Tissues of patients who have pronounced hyperglycemia and a markedly increased concentration of free fatty acids are most likely to show damage from ROS.

ROSs also damage **DNA** (see Section 2.3 in Chapter 21). In the presence of Fe^{2+}, H_2O_2 can give rise to •OH, which is more reactive than either $\bullet O_2^-$ or H_2O_2. Hydroxyl radicals can react with deoxyguanosine and thereby damage DNA. Because a large amount of ROS is made in mitochondria, and because ROS react very rapidly, damage to mitochondrial DNA is presumably greater than damage to nuclear DNA. Both the nucleus and mitochondria have mechanisms to repair DNA. If damage to DNA is too extensive, apoptosis may be set into motion (see Chapters 2 and Chapter 8). Mitochondria can also be destroyed by autophagy (see Section 1.4 in Chapter 35).

Formation of ROSs potentiates the formation of **AGEs** (see Section 8.3.1) and vice versa. In fact, microvascular disease of the retinae, kidneys, and nerves appears to require both oxidative stress and hyperglycemia. An increased concentration of ROS leads to an increased rate of AGE formation. On the other hand, signaling by AGE receptors on the surface of cells induces the formation of ROS.

8.3.3. Deregulation of Metabolism

Most type 2 diabetic patients are **insulin resistant** and **hyperinsulinemic**. T e molecular mechanisms that underlie the insulin resistance syndrome have yet to be elucidated. Insulin resistance is defined by the potency with which insulin stimulates glucose uptake. However, insulin receptors signal via several pathways and af ect multiple pathways of metabolism. Analysis of tissues from diabetic patients shows that not all signaling pathways are similarly af ected. T e ef ects of altered insulin signaling on glucose and lipid metabolism in type 2 diabetic patients have yet to be elucidated.

Patients with type 2 diabetes typically have elevated concentrations of **glucose** and **fatty acids** in plasma. Uptake of glucose into muscle and adipose tissue is decreased. Endogenous glucose production, particularly gluconeogenesis, is excessive. Only a relatively small portion of glucose is stored as glycogen. The adipose tissue releases fatty acids at an abnormally high rate. In the liver, a high influx of fatty acids from the periphery and an elevated rate of fatty acid synthesis both contribute to increased production of triglycerides. This, together with insufficient clearance of very-low-density lipoprotein particles, leads to **hypertriglyceridemia**.

Many patients with type 2 diabetes have both altered metabolism and an increased risk of cardiovascular problems. The molecular links between these two pathologies are unclear; however, clinicians often use the term **metabolic syndrome** to define patients who are at risk of both diseases. A common definition of metabolic syndrome is the presence of three or more of the following five abnormalities: hypertension, hypertriglyceridemia, low high-density lipoprotein cholesterol, abdominal obesity, and impaired glucose homeostasis. In developed countries, ~10% to 30% of adults fulfill these criteria. The risk for cardiovascular disease seems to depend chiefly on the degree of a patient's hyperinsulinemia. By contrast, a patient's risk for retinopathy, nephropathy, and neuropathy mostly seems to parallel the degree of hyperglycemia.

During hyperglycemia, there is an appreciable increase in the synthesis of the sugar alcohol **sorbitol** from glucose. Sorbitol is synthesized via the **polyol pathway** (see Section 2 in Chapter 20 and Fig. 20.3). An overly active polyol pathway significantly reduces the availability of NADPH for the repair of oxidative damage (see Fig. 20.5). Hence, excessive sorbitol synthesis leads to impaired removal of **ROS** and increased damage of ROS to the lenses and to the microvasculature in the retinae, glomeruli, and peripheral nerves.

SUMMARY

- The autoimmune disease that leads to the destruction of pancreatic islet β-cells, typically before about age 40, is called type 1 diabetes. Patients with this disease usually require treatment with insulin, lest they develop life-threatening diabetic ketoacidosis.
- In type 1 diabetic patients, injections of recombinant mutant human insulins, which quickly reach the bloodstream (e.g., lispro insulin, insulin aspart, insulin glulisine), are used to mimic the secretion of insulin from the pancreas of nondiabetic individuals. Similarly, recombinant insulins (e.g., insulin detemir, insulin glargine) that slowly reach the bloodstream are used to provide a basal amount of insulin throughout a 24-hour day. As an alternative to several injections per day, pumps can be used that infuse insulin into the subcutaneous space both continuously and in boluses for meals. For such a pump, patients typically use a short-acting insulin.
- Patients who have had type 1 diabetes for many years gradually lose their glucagon and epinephrine secretion in response to hypoglycemia. These patients are therefore highly susceptible to an excess dose of insulin. Since hypoglycemia can be life threatening, these patients need to manage their blood glucose concentration accordingly. For these and all other diabetic patients, hypoglycemia limits the use of insulin for normalizing the concentration of glucose in the blood.
- Patients who have type 1 diabetes on average have a higher concentration of glucose in plasma than do nondiabetic individuals. The higher the average concentration of glucose in plasma, the more likely type 1 diabetic patients develop complications, such as microvascular disease, retinopathy, nephropathy, and peripheral neuropathy.
- All type 2 diabetic patients have a defect in insulin secretion. All obese type 2 diabetic and most of the lean type 2 diabetic patients are insulin resistant. How insulin resistance eventually gives rise to deficient insulin secretion is unknown.
- Type 2 diabetes shows a multifactorial pattern of inheritance. For most patients who are genetically predisposed, obesity is necessary for type 2 diabetes to develop.
- Newly diagnosed type 2 diabetic patients are treated with diet and exercise, and often also with metformin. Diet and exercise lead to increased insulin sensitivity and thus help improve blood glucose homeostasis. Metformin chiefly reduces excessive endogenous glucose production.
- Other oral antidiabetic drugs used in the treatment of type 2 diabetes include: α-glucosidase inhibitors, such as acarbose, which slow the digestion of starch in the intestine; inhibitors of ATP-sensitive K-channels (K_{ATP}-channels), such as sulfonylureas and glinides, which induce insulin secretion from pancreatic β-cells; inhibitors of dipeptidyl peptidase-4 (DPP-4; gliptins), which slow the degradation of glucagon-like peptide-1 (GLP-1), which in turn potentiates glucose-induced insulin secretion; and insulin sensitizers, such as thiazolidinediones (glitazones).
- Injectable drugs that are used in the treatment of type 2 diabetes include exenatide, which is an analog of GLP-1 that boosts glucose-induced insulin secretion and pramlintide, which is an analog of amylin that decreases food intake, slows gastric emptying, and inhibits glucagon secretion; short- and long-acting insulins.
- Maturity-onset diabetes of the young (MODY) is due to one of several mutations, which are inherited in an autosomal dominant manner and lead to defective insulin secretion before about 25 years of age even in the absence of obesity. MODY is 20 to 100 times less common than pure type 2 diabetes.
- Gestational diabetes affects about 5% of pregnant women. If untreated, the fetus acquires extra fat, which complicates delivery. Most pregnant women undergo an oral glucose tolerance test at 24 to 28 weeks of gestation. Currently, insulin is the mainstay of treatment of gestational diabetes. Women who have gestational diabetes typically become normoglycemic soon after delivery. However, a majority of these women develop type 2 diabetes within the ensuing 10 years.

- Complications of diabetes are thought to be due to hyperglycemia and hyperinsulinemia. Hyperglycemia increases the rate of spontaneous glycation of amino groups in proteins and phospholipids. Subsequent further reactions lead to advanced glycation end products (AGEs), such as glucosepane and fructose-lysine. AGEs damage the microvasculature and the renal glomeruli, leading to retinopathy, neuropathy, and nephropathy. Hyperglycemia also increases the production of reactive oxygen species (ROS) such as superoxide anion and hydroxyl radicals (•OH). AGE production increases ROS production and vice versa. AGEs and ROS together damage the microvasculature.

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Review Questions

1. A 14-year-old boy was sent to a children's hospital in the afternoon because he was feeling unwell and was suspected of having diabetes. During the past 3 months, he had felt increasingly thirsty, urinated frequently, and lost weight, even though he seemed to eat a good amount of food. On the day of admission, the patient ate breakfast. T e patient's height was 6 feet, 2 inches (1.88 m), and his weight was 143 lb (65 kg). Which of the following is most appropriate to test this patient for diabetes?

   A. Collect urine for 24 hours, then determine the amount of glucose lost via the urine during that time
   B. Fast the patient for at least 8 hours, give 75 g of glucose by mouth, and measure plasma glucose 2 hours later
   C. Fast the patient for at least 8 hours, then measure plasma glucose
   D. Give 75 g of glucose by mouth immediately, then measure plasma glucose 2 hours later
   E. Measure plasma glucose immediately

2. A 10-year-old boy who wrestles at school suddenly becomes disoriented, falls down, and becomes unresponsive. He wears a medical bracelet identifying him as having type 1 diabetes. T is patient would most likely benefit from which of the following?

   A. Infusion of saline solution
   B. Injection of an antihypertensive drug
   C. Injection of glucagon
   D. Injection of insulin lispro
   E. Water (given orally)

3. During the past 10 years, a 60-year-old male patient has become progressively more obese. His BMI is now 35 kg/m^2. Which one of the following tests is most appropriate to test for diabetes?

   A. Fast overnight, then measure plasma glucose in the fasting state
   B. Measure acetoacetate or β-hydroxybutyrate in the blood
   C. Measure autoantibodies to γ-aminobutyric acid decarboxylase
   D. Measure the concentration of insulin or C-peptide in plasma

4. Which one of the following drugs can be expected to have a side ef ect of hypoglycemia when used as monotherapy in the treatment of type 2 diabetes?

   A. An α-glucosidase inhibitor
   B. An analog of glucagon-like peptide I
   C. An inhibitor of dipeptidyl peptidase IV
   D. An inhibitor of K_{ATP}-channels

5. A researcher wants to assess the function of β-cells in a group of patients who have had type 1 diabetes for several years and receive near-optimal treatment. Measurement of the concentration in blood of which one of the following is most promising?

   A. C-peptide
   B. Glucagon
   C. GLP-1
   D. Insulin
   E. Somatostatin

6. A 13-year-old lean boy is found to have diabetes. At the time of diagnosis, the boy is hyperglycemic but not keto-acidotic. He receives treatment with metformin at a usual dose. However, despite adherence to the prescribed drug for 4 weeks, the concentration of glucose in plasma is still unacceptably high. Which of the following would be the most appropriate next step?

   A. Double the dose of metformin
   B. Prescribe an α-glucosidase inhibitor
   C. Prescribe a thiazolidinedione (an insulin sensitizer)
   D. Prescribe injections of insulin
   E. Test for MODY

Answers to Review Questions

CHAPTER 1

1. **A.** See Fig. 1.5.
2. **B.** DNA contains negatively charged phosphate groups (see Fig. 1.2).
3. **D.** Irinotecan inhibits topoisomerase I.
4. **D.** C and G together form 3 H-bonds and are therefore thermally more stable than A and T, which form only 2 H-bonds. Furthermore, according to Chargaf ′s rule, A and T, as well as C and G, occur equally frequently due to base pairing.
5. **B.** T e daughter is homozygous for the mutant allele. Incorrect: A (the daughter's amplicons appear fully cleaved), C (the parents are heterozygotes, and the pathogenic allele gives rise to 2 bands), D (there is no evidence of this, and 310 is the uncleaved normal allele).

CHAPTER 2

1. **C.** MSH2 and MSH6 are both part of mismatch repair, and Lynch syndrome is a hereditary cancer predisposition due to an allele for a nonfunctional mismatch repair protein. Incorrect: A (caused by deficient NER), B (caused by a mutant BRCA1 or BRCA2 allele), and D (primarily a disease of the colon; it is not associated with a loss of MSH2).
2. **D.** See Section 3.
3. **A.** See Section 1.
4. **E.** Adducts of DNA with polycyclic compounds usually distort the DNA helix; see Section 3.
5. **B.** See Section 4.2.

CHAPTER 3

1. **D.** See Fig. 3.3.
2. **B.** See Section 2.
3. **C.** See Fig. 3.10.

CHAPTER 4

1. **D.** T is is the only technique listed that can detect a point mutation.
2. **D.** T e forward primer has the same sequence as the 5′ end of the sequence that is shown and is to be amplified; the reverse primer has a sequence that is complementary to the 3′ end of the sequence shown, but the primer sequence has to be read from its 5′ end.
3. **B.** T is is the only technique listed that can detect a balanced translocation, and the translocation is large enough to be detected by this technique.
4. **B.** Incorrect: A (cannot detect inversions), C (inversion is too small to be recognized by G-banding), D (poorly suited to detecting chromosome rearrangements).

CHAPTER 5

1. **E.** T e same mutation leads to a variety of phenotypes. Incorrect: A-C (these refer to mutations, not phenotypes), D (this is not a question of individuals with the pathogenic genotype being positive for a disease).

CHAPTER 6

1. **B.** T e template strand is transcribed in the 3′→5′ direction, and the RNA is complementary to the template strand.
2. **E.** T e TATA box is located at about nucleotide –30. Incorrect: A (located at about nucleotide +30), B (never in the region of the core promoter), C (near the start site of transcription, at about nucleotide +1), D (a nuclear receptor is a transcription factor, a protein).
3. **D.** Rett syndrome is due to a deficiency in MECP2, a protein that binds to methylated CpGs.
4. **A.** Almost all transcription factors bind to both DNA strands.
5. **A.** Acetylation of histone tails leads to a more open chromatin structure that is more conducive to transcription.
6. **C.** Exon 1 + exon 2 + poly(A) tail + cap. T e UTRs are part of the exons; the intron is removed.

CHAPTER 7

1. **A.** See Section 3.
2. **B.** T e sequence from the coding strand can be used directly with the genetic code, except that U and T have to be exchanged.
3. **A.** Each codon is 3 nucleotides long, and only the exons minus the UTRs are translated (see Figs. 6.15 and 7.2).

CHAPTER 8

1. **B.** An increased concentration of cyclin D1 leads to increased phosphorylation of RB, which then frees E2Fs (see Fig. 8.2). Incorrect: A, C-E (all of these alterations decrease the chance of a neoplasm).

2. **D.** Lynch syndrome is due to an inherited loss-of-function mutation of a mismatch repair protein that is present in all somatic cells, including lymphocytes. Incorrect: A and B (patients with these findings more often have sporadic colorectal cancer), C (the grandfather's colon cancer occurred at an age typical of sporadic colorectal cancer).
3. **C.** See Section 1.1.
4. **C.** T is patient has polyposis, but the polyposis is not dominantly inherited as in FAP.

CHAPTER 9

1. **D.** See Section 3.3.
2. **A.** See Section 2.1.
3. **D.** T e amino group is definitely positively charged, and ALA has a net charge only if the carboxyl group is protonated.
4. **C.** See Section 4.2 and Fig. 9.8.

CHAPTER 10

1. **D.** T e enzyme from the cytoplasm most likely has lower activity at a pH of 5, and the protons act at a distance from the active site. Incorrect: A (there is most likely inhibition), B (there are no ionizable groups in the active site), C (the inhibition is most likely reversible), E (see B and C).
2. **C.** Half-maximal activity occurs at a higher concentration of DHF. Incorrect: A (the K_m increases; see C), B (there is a ~20-fold shift in K_m, and the methotrexate curve will reach as high as the control curve only at about 2,000 µM DHF), D (there is no evidence for this).
3. **B.** T e K_m is identical (arrow) and the V (product formed) can be compared directly. Incorrect: A and C (the K_m is identical [arrow], not dif erent), D (the opposite is true).

CHAPTER 11

1. **D.** Fig. 11.2 shows that it is negatively charged. Incorrect: A (both fatty acids are ester linked), B (a phospholipid does not contain isoprenes), C (it does not contain a sugar; it is a glycerophospholipid).
2. **C.** Incorrect: A (they flip phospholipids), B (it is a protein), D (it does not move aminophospholipids such as phosphatidylserine).

CHAPTER 12

1. **A.** T e parents both have thin basement membrane nephropathy due to heterozygosity for a mutation in the COL4A3 or COL4A4 gene, and each of their of spring has a 25% chance of being homozygous and thus having Alport syndrome.
2. **D.** Of the listed disorders, only Alport syndrome is associated with hematuria, and the father is asymptomatic.

CHAPTER 13

1. **D.** See Section 1.2.
2. **A.** See Section 1.4.
3. **D.** T is enzyme has insufficient activity in patients who have Hurler syndrome (see Section 2.4).

CHAPTER 14

1. **A.** Acute intermittent porphyria shows autosomal dominant inheritance. Incorrect: B (X-linked), C and E (require that the mother is a carrier), D (most often acquired, though it can be inherited, but this is less common than A).
2. **B.** An example of such an autoimmune disease is primary biliary cholangitis. Incorrect: A would not give rise to an elevated concentration of bilirubin; C and E would give rise to a more modest increase in bilirubin, and the direct bilirubin would be less than 15% of the total bilirubin. If D gave rise to liver damage, the direct bilirubin would be less than 15% of the total bilirubin.
3. **B.** Infusion of lipids displaces bilirubin from albumin, which increases the chance that the infant develops kernicterus. Hence, lipid is infused at a rate that is low but sufficient to prevent a deficiency of essential fatty acids. Incorrect: A and C would not favorably af ect bilirubin production, conjugation, or excretion.

CHAPTER 15

1. **C.** Phlebotomy is the preferred treatment for HFE hemochromatosis. Iron chelators, such as desferrioxamine or deferasirox, can be used, but they are reserved for patients who do not tolerate phlebotomy. Treatment with iron would worsen iron overload that is apparent from the patient's serum ferritin.
2. **D.** T e low hemoglobin, low MCV, low serum iron, somewhat high total iron binding capacity, low calculated transferrin saturation, and low serum ferritin all point to an iron deficiency (and thus speak against options A and C). T e normal serum folate makes a folate deficiency (B) unlikely, and the normal serum cobalamin makes pernicious anemia (E) unlikely.
3. **B.** Chronic inflammation stimulates hepcidin secretion. Juvenile and HFE hemochromatosis, as well as iron-deficiency anemia, are associated with a low rate of hepcidin secretion.

CHAPTER 16

1. **B.** T is is due to an excessive rate of hematopoiesis. A, C, and D are incorrect because erythrocytes are normal.
2. **B.** Nitrites induce the formation of methemoglobin, which binds cyanide temporarily. Incorrect: A and C (treated with oxygen, not nitrite), D (treated with methylene blue, except in G6PD deficient patients).

3. **D.** Increases in the concentration of H^+, CO_2, and 2,3-BPG all increase the P_{50} of hemoglobin for O_2.
4. **C.** The maximal saturation remains at 100%, but the P_{50} shifts to a higher pO_2.

CHAPTER 17

1. **C.** The woman's eggs have either no or one α-globin allele, and the man's sperm have either no or two α-globin alleles. The chance that an embryo has only one α-globin allele is $½ \times ½ = ¼$.
2. **A.** An HbA_2 + HbC + HbE of 5% can only indicate an elevated HbA_2; this, in turn, is a very strong indicator of β-thalassemia minor, to which a low hemoglobin and MCV fit well. Incorrect: B, C, and D (HbA_2 + HbC + HbE is much less than 50%), D and E (HbS is 0%).
3. **D.** The patient has HbA and HbS as the major hemoglobins; in a patient with sickle cell trait, HbS is typically somewhat less abundant than HbA. Incorrect: A and E (HbA_2 + HbC is normal), B (there is no indication of an abnormal HbA_{1c}), C and E (there is HbA).
4. **C.** The woman's eggs have either no or two α-globin alleles, and the man's sperm always have one α-globin allele, so the chance of the first-born having only one α-globin allele is $½ \times 1 = ½$.
5. **D.** The term sickle cell disease encompasses patients who have, for instance, hemoglobin SC disease or sickle cell/β-thalassemia. Incorrect: A is the genotype for β-thalassemia trait, B is for sickle cell trait, and C is for hemoglobin C disease.
6. **D.** The elevated bilirubin and LDH are indicative of a hemolytic anemia, and the low MCV can be due to a hemoglobinopathy. Incorrect: A (the fraction would be <15%), B (lab values are indicative of iron deficiency), C (lab values are indicative of polycythemia), E (MCHC is elevated, indicating a problem with DNA replication, not hemoglobin synthesis).

CHAPTER 18

1. **E.** Lactulose is only digested by bacteria; if a significant amount of H_2 is produced in response to lactulose, the patient's lactose tolerance test is truly negative; if lactulose does not induce H_2 production, the patient's gut flora likely does not produce a significant amount of H_2, and the lactose tolerance test therefore cannot be interpreted. Incorrect: A could reduce the number of bacteria in the intestine and thus lower H_2 production, but the result would not be helpful in making a diagnosis. B, C, and D are most likely taken up normally and are therefore very unlikely to give rise to increased H_2 production.
2. **A.** Adipose tissue and muscle contain insulin-sensitive GLUT-4 transporters. Incorrect: B-E do not contain insulin-sensitive glucose transporters.
3. **B.** Lactase is not an α-glucosidase. Incorrect: A, C, and E are hydrolyzed by α-glucosidases. D is not hydrolyzed by human enzymes, only by bacteria, which do not produce glucose.
4. **D.** Protein malnutrition impairs the synthesis of disaccharidases. Incorrect: A, B, and C have no direct effect on lactase activity and play no role in the digestion of milk.

CHAPTER 19

1. **F.** PFK1 is the rate-limiting enzyme and can be activated by fructose 2,6-bisphosphate in an insulin-dependent manner. Incorrect: A-E catalyze reversible reactions and can therefore not be effectively regulated. G, when activated, tends to decrease the concentration of intermediates between fructose 1,6-bisphosphate and phosphoenolpyruvate; this includes dihydroxyacetonephosphate.
2. **C.** The concentrations of all intermediates between fructose 1,6-bisphosphate and phosphoenolpyruvate are elevated, leading to an elevated concentration of 2,3-BPG. Incorrect: A is incorrect because a decrease in flux in one part of the pathway leads to a decrease in flux in the entire pathway; were it not for the 2,3-BPG bypass, both reactions would produce the same amount of ATP. B is incorrect because phosphoenolpyruvate cannot be transported across the plasma membrane. D is incorrect for the reason stated in A.
3. **C.** Hypophosphatemia inhibits the reaction from GAP to 1,3BPG. Incorrect: A and B are upstream of the deficiency and therefore would decrease; D and E are downstream of the deficiency and would increase.

CHAPTER 20

1. **B.** Most cells form galactose 1-phosphate, which they can metabolize only slowly. The accompanying low concentration of free phosphate impairs ATP synthesis.
2. **C.** Fructose and galactose are both metabolized by the liver.
3. **E.** Aldolase B deficiency causes hereditary fructose intolerance, and sucrose gives rise to fructose.

CHAPTER 21

1. **D.** The daughter of the affected male carries one G6PD-deficient X-chromosome, and there is a 50% chance that she will pass this on to her offspring. This applies to all X-linked disorders.
2. **B.** G6PD deficiency reduces the rate of NADPH production. Incorrect: A and D (primaquine is an oxidant), C (primaquine does not bind to G6PD), E (the sugar phosphate branch does not produce NADPH).

CHAPTER 22

1. **C.**
2. **D.** Hypoxia incapacitates oxidative phosphorylation, which leads to an accumulation of NADH.

CHAPTER 23

1. **B.** O_2 displaces CO. Incorrect: A, C, and D are inef ective.
2. **A.** Complex 1 deficiency is one cause of Leigh syndrome. Incorrect: B and C are associated with a deficiency of all complexes.

CHAPTER 24

1. **A.** See Fig. 24.7.
2. **D.** Pompe disease only impairs the turnover of glycogen particles in lysosomes, leading to muscle weakness. Incorrect: A is associated with enlarged glycogen particles, B with glycogen particles of abnormal structure, and C with abnormally small glycogen particles.
3. **B.** See Section 3.3. Incorrect: A does not explain any abnormalities, C would not impair the formation of glucose from galactose via glucose 6-phosphatase, and D would not give rise to a high glycogen content.

CHAPTER 25

1. **D.** Excess glucagon stimulates gluconeogenesis. Incorrect: A, B, C, and E are associated with a low rate of gluconeogenesis.
2. **D.** T e deficiency leads to decreased use of lactic acid. Incorrect: In A-C, the opposite occurs.

CHAPTER 26

1. **A.** See Section 3.3.2.
2. **C.** A mutant insulin can have normal immunoreactivity yet diminished biological activity (the receptor-binding region dif ers from the immunological epitope). Incorrect: A, B, D, and E (recombinant insulin had a normal ef ect).

CHAPTER 27

1. **A.** See Figs. 27.3 and 27.9. Incorrect: B (the acyl carrier protein is part of fatty acid synthase in the cytosol, but fatty acid oxidation takes place inside mitochondria [or peroxisomes]), C (there is no such transporter in the inner mitochondrial membrane), D (the enzyme mentioned plays a role in ketone body oxidation, not in fatty acid oxidation).
2. **A.** T e low bicarbonate is indicative of ketoacidosis, which is accompanied by ketonemia and ketonuria (see also Sections 5.4 and 7.3).
3. **B.** In the fasting state the liver synthesizes ketone bodies but not fatty acids, and it can never oxidize ketone bodies.

CHAPTER 28

1. **E.** T e liver synthesizes some fatty acids from glucose and receives others from the hydrolysis of infused triglycerides; the liver esterifies the fatty acids to triglycerides and exports them inside VLDL. Incorrect: A and B (without food, chylomicron production by the intestine is minuscule), C and D (IDL and LDL contain much less triglycerides than do VLDL).
2. **B.** Incorrect: A (de novo fatty acid synthesis occurs at only a low rate in the fed state, not the fasting state), C (VLDL give rise to LDL, not the reverse), D (the liver takes up chylomicron remnants, not chylomicrons; there are few chylomicrons in the fasting state; chylomicron remnants contain a relatively small fraction of triglycerides; and, in the fasting state, the triglycerides in the remnants are a minor source of triglycerides in VLDL).

CHAPTER 29

1. Increased expression of LDL receptors mediates the cholesterol-lowering ef ect of statins. At least one functional allele of the LDL receptor (as in heterozygous familial hypercholesterolemia) is required for such an ef ect.
2. **A.** See Figs. 29.1 and 29.3.
3. **D.** Hypertriglyceridemia leads to delipidation and loss of HDL (see Fig. 29.7). Incorrect: A, C, and E would favor an increase in HDL cholesterol, and B would not have a significant ef ect.
4. **B.** T is is a common cause of familial hypercholesterolemia (see Section 5.2). Incorrect: A and C-E do not cause high LDL cholesterol.

CHAPTER 30

1. **A.** Hemodialysis is used in cases of severe ethanol poisoning. Incorrect: B (disulfiram would lead to an accumulation of toxic acetaldehyde), C (fomepizole would inhibit metabolism of ethanol), D (the hyperglycemia is mild and not dangerous), and E (there is no need for this).
2. **C.** Glucagon normally stimulates glycogenolysis, but due to a low rate of gluconeogenesis, glycogen stores are low in alcohol-abusing patients, particularly those who also have diabetes. Incorrect: A (there is no clinical precedent for this), B and D (per se, these can be true statements, but they are not the most direct answers to the question).
3. **B.** See Section 2.1.

CHAPTER 31

1. **B.** See Fig. 31.17.
2. **B.** She produces too much aldosterone. Incorrect: A and C (the renin-angiotensin system is most likely functioning normally, and these drugs inhibit upstream signaling; aldosterone production in this patient is largely independent of the renin-angiotensin system), D and E (they do not reduce aldosterone synthesis or signaling).
3. **A.** Addison disease is due to destruction of the adrenal cortex; the low concentrations of cortisol and aldosterone lead to increased secretion of ACTH and renin. Incorrect: B (the opposite changes are occurring), C (these do not significantly af ect cortisol and aldosterone synthesis), and D (the concentration of CRH is high).
4. **E.** T is is the most likely reason the infant has a 46,XY disorder of sex development. Incorrect: A and D (would not cause development of female sex traits), B (would only af ect the synthesis of estrogens), C (would accentuate male sex traits).

CHAPTER 32

1. **B.** PGE_3 is made from EPA, and fish oil is the richest source of EPA. Incorrect: A (contains mostly DHA and very little EPA), C (contains mostly linolenic acid and virtually no EPA), D (is a poor source of ω-3 fatty acids).
2. **E.** T romboxane A_2 stimulates platelet aggregation.
3. **E.** Zileuton inhibits 5-lipoxygenase (see Fig. 32.3). Incorrect: A (a β_2-adrenergic agonist that has no ef ect on eicosanoid synthesis), B (inhibits COXs, which are essential for the synthesis of prostanoids), C (prostaglandin $F_{2\alpha}$), D (a CYSLTR1 antagonist that has no ef ect on eicosanoid synthesis).
4. **B.** Misoprostol is a PGE_1 analog that increases bicarbonate and mucus secretion to protect the mucosa of the stomach and duodenum. Incorrect: A (it further inhibits prostaglandin synthesis; see Fig. 32.2), C (a β_2-adrenergic agonist that has no ef ect on prostanoid synthesis), D (a CYSLTR1 antagonist that has no ef ect on prostanoid synthesis), E (a 5-lipoxygenase inhibitor that has no ef ect on prostanoid synthesis).

CHAPTER 33

1. **C.** See Section 2.
2. **C.** See Table 33.1.

CHAPTER 34

1. **A.** G-cells in the antrum secrete gastrin, which leads to HCl secretion from parietal cells; see Figs. 34.1 and 34.2). Incorrect: B and C (histamine- and somatostatin-secreting cells are largely absent from the antrum; these options would increase HCl secretion). D and E (parietal cells and chief cells are largely absent from the antrum).
2. **B.** Pentagastrin stimulates histamine secretion, which in turn stimulates HCl secretion from parietal cells. Incorrect: A (acetylcholine stimulates gastrin secretion, not the other way around); C (somatostatin inhibits histamine secretion); D and E (no causal relationship).
3. **D.** Proton pump inhibitors reduce HCl secretion, which reduces somatostatin secretion, which reduces the inhibitory ef ect of somatostatin on gastrin secretion. Incorrect: A (somatostatin inhibits gastrin secretion); B and C (these lead to a high concentration of somatostatin, which inhibits gastrin secretion).

CHAPTER 35

1. **E.** Ornithine carbamoyltransferase deficiency leads to an accumulation of ornithine and carbamoylphosphate that, in turn, leaks into the cytosol and gives rise to orotate. Incorrect: A and B lead to an elevated concentration of citrulline, and C and D do not give rise to a high excretion of orotate.
2. B. T ese drugs are commonly used to treat hyperammonemia in the hospital, and hemodialysis is indicated by the patient's state and the hyperammonemia. Incorrect: A (citrulline is only given for specific deficiencies of the urea cycle), C (essential amino acids would likely aggravate the hyperammonemia), D and E (neither addresses the life-threatening hyperammonemia).

CHAPTER 36

1. **C.** Incorrect: T e vitamins in A, B, D, and E play no role in the detoxification of methanol.
2. **D.** Incorrect: A and C are not specific for cobalamin deficiency, and B is not done (antibodies to intrinsic factor and/or parietal cells help in the diagnosis of pernicious anemia).
3. **C.** Incorrect: A is not specific for cobalamin deficiency, and B and D are not done.

CHAPTER 37

1. **D.** PRPP is synthesized only when a cell's phosphorylation state is good, a prerequisite for pyrimidine nucleotide de novo synthesis. By contrast, UTP (E) exerts feedback inhibition. A-C have no ef ect on pyrimidine nucleotide de novo synthesis.
2. **D.** Methotrexate inhibits dihydrofolate reductase (DHFR) and thereby depletes the pool of one-carbon loaded THFs. N^5-formyltetrahydrofolic acid (leucovorin, folinic acid) can replenish this pool without requiring DHFR. None of the other options (A-C, E and F) replenish the pool of one-carbon THFs. Gemcitabine (C) inhibits ribonucleotide reductase, and gimeracil (D) inhibits the degradation of 5-fluorouracil; neither drug relieves methotrexate toxicity.

CHAPTER 38

1. **B.** Rasburicase converts uric acid to allantoin. Allopurinol is also suitable to prevent tumor lysis syndrome, but rasburicase lowers existing hyperuricemia faster than allopurinol does. Incorrect: A and C have no hypouricemic effect. D and E would exacerbate the crystallization of uric acid in the kidneys.
2. **C.** Variant Lesch-Nyhan syndrome is due to reduced activity of HGPRT and therefore reduced salvage of hypoxanthine, resulting in increased urate production. Allopurinol inhibits urate production. Incorrect: A worsens the hyperuricemia by decreasing urate excretion. B is not a first-line treatment and is not sufficiently long-lived for the treatment of gout. D may cause uric acid nephrolithiasis.
3. **G.** The vast majority of patients who have gout are urate underexcretors. Incorrect: A-E all lead to urate overproduction, which is uncommon among patients who have gout. F is not thought to be common.

CHAPTER 39

1. **E.** His blood glucose will likely be higher than 200 mg/dL (11.1 mM).
2. **C.** The boy is most likely hypoglycemic.
3. **A.** This is the standard approach for someone who is not in acute distress; see Section 3.
4. **D.** K_{ATP} inhibitors stimulate insulin secretion even during hypoglycemia.
5. **A.** C-peptide is only secreted from the patient's own β-cells and parallels insulin secretion; injected insulin is free of C-peptide.
6. **E.** There is a good chance that he has MODY; a genetic diagnosis guides treatment.

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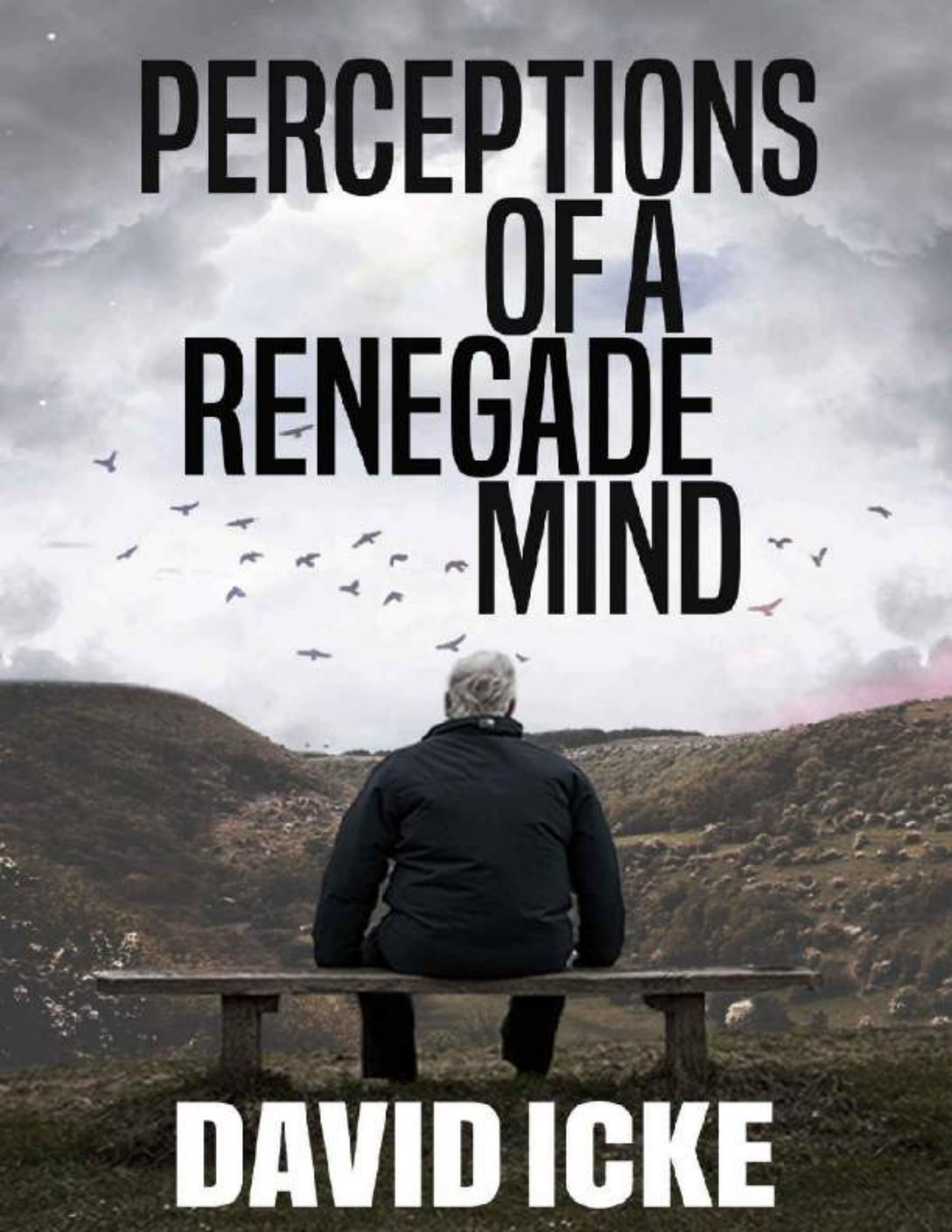
PERCEPTIONS OF A RENEGADE MIND



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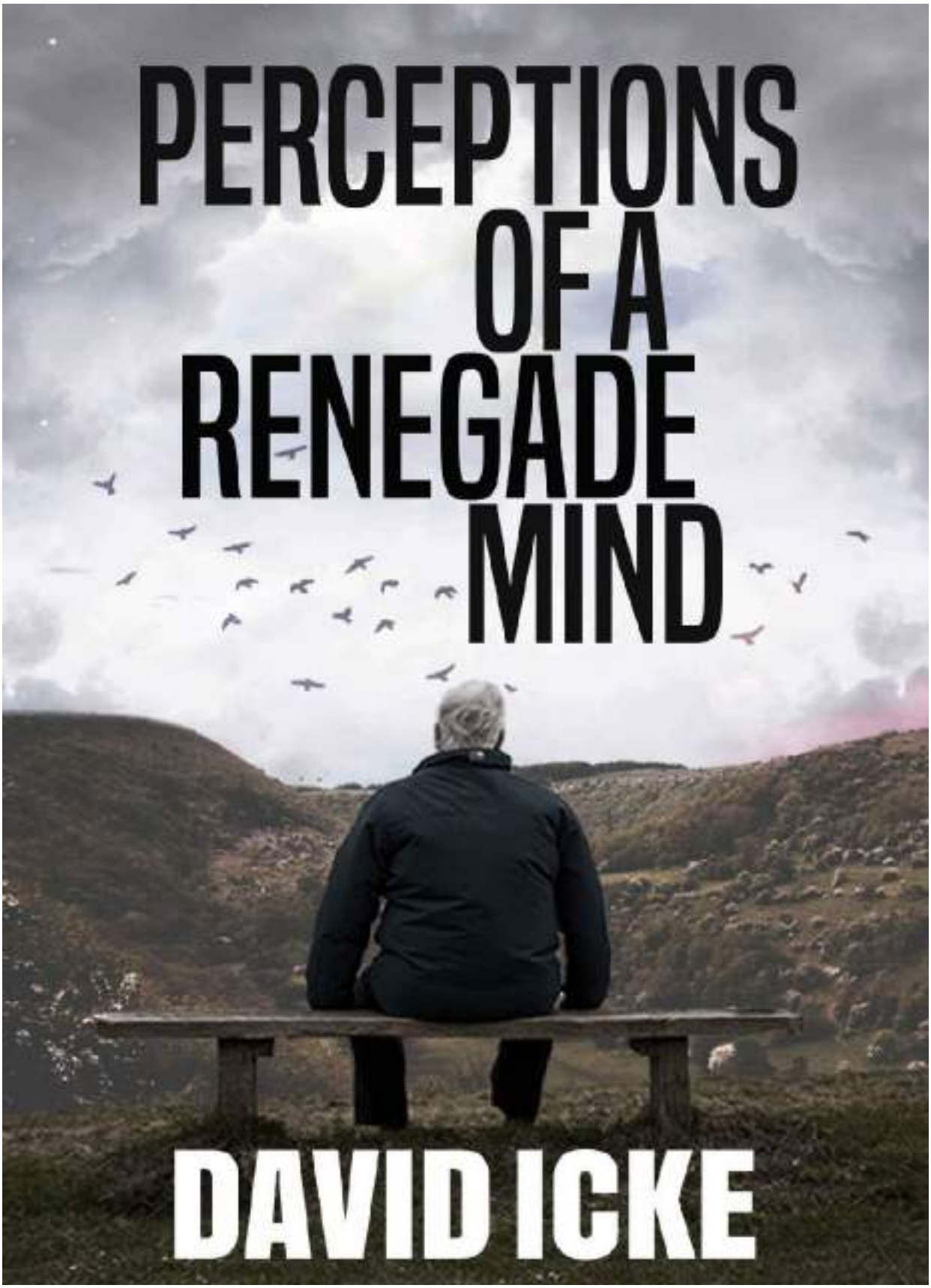
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PERCEPTIONS OF A RENEGADE MIND

DAVID ICKE

Dedication:

To *Freeeeeedom!*

Renegade:
Adjective
‘Having rejected tradition: Unconventional.’
Merriam-Webster Dictionary

Acquiescence to tyranny is the death of the spirit

You may be 38 years old, as I happen to be. And one day, some great opportunity stands before you and calls you to stand up for some great principle, some great issue, some great cause. And you refuse to do it because you are afraid … You refuse to do it because you want to live longer … You're afraid that you will lose your job, or you are afraid that you will be criticised or that you will lose your popularity, or you're afraid that somebody will stab you, or shoot at you or bomb your house; so you refuse to take the stand.

Well, you may go on and live until you are 90, but you're just as dead at 38 as you would be at 90. And the cessation of breathing in your life is but the belated announcement of an earlier death of the spirit.

Martin Luther King

How the few control the many and always have – the many do whatever they're told

'Forward, the Light Brigade!'
Was there a man dismayed?
Not though the soldier knew
Someone had blundered.
Theirs not to make reply,
Theirs not to reason why,
Theirs but to do and die.
Into the valley of Death
Rode the six hundred.

Cannon to right of them,
Cannon to left of them,
Cannon in front of them
Volleyed and thundered;
Stormed at with shot and shell,
Boldly they rode and well,
Into the jaws of Death,
Into the mouth of hell
Rode the six hundred

Alfred Lord Tennyson (1809-1892)

The mist is lifting slowly
I can see the way ahead
And I've left behind the empty streets
That once inspired my life
And the strength of the emotion
Is like thunder in the air
'Cos the promise that we made each other
Haunts me to the end

The secret of your beauty
And the mystery of your soul
I've been searching for in everyone I meet
And the times I've been mistaken
It's impossible to say
And the grass is growing
Underneath our feet

The words that I remember
From my childhood still are true
That there's none so blind
As those who will not see
And to those who lack the courage
And say it's dangerous to try
Well they just don't know
That love eternal will not be denied

I know you're out there somewhere
Somewhere, somewhere
I know you're out there somewhere

Somewhere you can hear my voice
I know I'll find you somehow
Somehow, somehow
I know I'll find you somehow
And somehow I'll return again to you

The Moody Blues

Are you a gutless wonder - or a Renegade Mind?

Monuments put from pen to paper,
Turns me into a gutless wonder,
And if you tolerate this,
Then your children will be next.
Gravity keeps my head down,
Or is it maybe shame ...

Manic Street Preachers

Rise like lions after slumber
In unvanquishable number.
Shake your chains to earth like dew
Which in sleep have fallen on you.
Ye are many – they are few.

Percy Shelley

Contents

CHAPTER ONE

I'm thinking' – Oh, but *are* you?

Think for yourself and let others enjoy the privilege of doing so too
Voltaire

French-born philosopher, mathematician and scientist René Descartes became famous for his statement in Latin in the 17th century which translates into English as: 'I think, therefore I am.'

On the face of it that is true. Thought reflects perception and perception leads to both behaviour and self-identity. In that sense 'we' are what we think. But who or what is doing the thinking and is thinking the only route to perception? Clearly, as we shall see, 'we' are not always the source of 'our' perception, indeed with regard to humanity as a whole this is rarely the case; and thinking is far from the only means of perception. Thought is the village idiot compared with other expressions of consciousness that we all have the potential to access and tap into. This has to be true when we *are* those other expressions of consciousness which are infinite in nature. We have forgotten this, or, more to the point, been manipulated to forget.

These are not just the esoteric musings of the navel. The whole foundation of human control and oppression is control of perception. Once perception is hijacked then so is behaviour which is dictated by perception. Collective perception becomes collective behaviour and collective behaviour is what we call human society. Perception is all and those behind human control know that which is

why perception is the target 24/7 of the psychopathic manipulators that I call the Global Cult. They know that if they dictate perception they will dictate behaviour and collectively dictate the nature of human society. They are further aware that perception is formed from information received and if they control the circulation of information they will to a vast extent direct human behaviour. Censorship of information and opinion has become globally Nazi-like in recent years and never more blatantly than since the illusory 'virus pandemic' was triggered out of China in 2019 and across the world in 2020. Why have billions submitted to house arrest and accepted fascistic societies in a way they would have never believed possible? Those controlling the information spewing from government, mainstream media and Silicon Valley (all controlled by the same Global Cult networks) told them they were in danger from a 'deadly virus' and only by submitting to house arrest and conceding their most basic of freedoms could they and their families be protected. This monumental and provable lie became the *perception* of the billions and therefore the *behaviour* of the billions. In those few words you have the whole structure and modus operandi of human control. Fear is a perception – **F**alse **E**motion **A**ppearing **R**eal – and fear is the currency of control. In short … get them by the balls (or give them the impression that you have) and their hearts and minds will follow. Nothing grips the dangly bits and freezes the rear-end more comprehensively than fear.

World number 1

There are two 'worlds' in what appears to be one 'world' and the prime difference between them is knowledge. First we have the mass of human society in which the population is maintained in coldly-calculated ignorance through control of information and the 'education' (indoctrination) system. That's all you really need to control to enslave billions in a perceptual delusion in which what are perceived to be *their* thoughts and opinions are ever-repeated mantras that the system has been downloading all their lives through 'education', media, science, medicine, politics and academia

in which the personnel and advocates are themselves overwhelmingly the perceptual products of the same repetition. Teachers and academics in general are processed by the same programming machine as everyone else, but unlike the great majority they never leave the 'education' program. It gripped them as students and continues to grip them as programmers of subsequent generations of students. The programmed become the programmers – the programmed programmers. The same can largely be said for scientists, doctors and politicians and not least because as the American writer Upton Sinclair said: 'It is difficult to get a man to understand something when his salary depends upon his not understanding it.' If your career and income depend on thinking the way the system demands then you will – bar a few free-minded exceptions – concede your mind to the Perceptual Mainframe that I call the Postage Stamp Consensus. This is a tiny band of perceived knowledge and possibility 'taught' (downloaded) in the schools and universities, pounded out by the mainstream media and on which all government policy is founded. Try thinking, and especially speaking and acting, outside of the 'box' of consensus and see what that does for your career in the Mainstream Everything which bullies, harasses, intimidates and ridicules the population into compliance. Here we have the simple structure which enslaves most of humanity in a perceptual prison cell for an entire lifetime and I'll go deeper into this process shortly. Most of what humanity is taught as fact is nothing more than programmed belief. American science fiction author Frank Herbert was right when he said: 'Belief can be manipulated. Only knowledge is dangerous.' In the 'Covid' age belief is promoted and knowledge is censored. It was always so, but never to the extreme of today.

World number 2

A 'number 2' is slang for 'doing a poo' and how appropriate that is when this other 'world' is doing just that on humanity every minute of every day. World number 2 is a global network of secret societies and semi-secret groups dictating the direction of society via

governments, corporations and authorities of every kind. I have spent more than 30 years uncovering and exposing this network that I call the Global Cult and knowing its agenda is what has made my books so accurate in predicting current and past events. Secret societies are secret for a reason. They want to keep their hoarded knowledge to themselves and their chosen initiates and to hide it from the population which they seek through ignorance to control and subdue. The whole foundation of the division between World 1 and World 2 is *knowledge*. What number 1 knows number 2 must not. Knowledge they have worked so hard to keep secret includes (a) the agenda to enslave humanity in a centrally-controlled global dictatorship, and (b) the nature of reality and life itself. The latter (b) must be suppressed to allow the former (a) to prevail as I shall be explaining. The way the Cult manipulates and interacts with the population can be likened to a spider's web. The 'spider' sits at the centre in the shadows and imposes its will through the web with each strand represented in World number 2 by a secret society, satanic or semi-secret group, and in World number 1 – the world of the seen – by governments, agencies of government, law enforcement, corporations, the banking system, media conglomerates and Silicon Valley (Fig 1 overleaf). The spider and the web connect and coordinate all these organisations to pursue the same global outcome while the population sees them as individual entities working randomly and independently. At the level of the web governments *are* the banking system *are* the corporations *are* the media *are* Silicon Valley *are* the World Health Organization working from their inner cores as one unit. Apparently unconnected countries, corporations, institutions, organisations and people are on the *same team* pursuing the same global outcome. Strands in the web immediately around the spider are the most secretive and exclusive secret societies and their membership is emphatically restricted to the Cult inner-circle emerging through the generations from particular bloodlines for reasons I will come to. At the core of the core you would get them in a single room. That's how many people are dictating the direction of human society and its transformation

through the ‘Covid’ hoax and other means. As the web expands out from the spider we meet the secret societies that many people will be aware of – the Freemasons, Knights Templar, Knights of Malta, Opus Dei, the inner sanctum of the Jesuit Order, and such like. Note how many are connected to the Church of Rome and there is a reason for that. The Roman Church was established as a revamp, a rebranding, of the relocated ‘Church’ of Babylon and the Cult imposing global tyranny today can be tracked back to Babylon and Sumer in what is now Iraq.

Figure 1: The global web through which the few control the many. (Image Neil Hague.)

Inner levels of the web operate in the unseen away from the public eye and then we have what I call the cusp organisations located at the point where the hidden meets the seen. They include a series of satellite organisations answering to a secret society founded in London in the late 19th century called the Round Table and among them are the Royal Institute of International Affairs (UK, founded in 1920); Council on Foreign Relations (US, 1921); Bilderberg Group (worldwide, 1954); Trilateral Commission (US/worldwide, 1972); and the Club of Rome (worldwide, 1968) which was created to exploit environmental concerns to justify the centralisation of global power to ‘save the planet’. The Club of Rome instigated with others the human-caused climate change hoax which has led to all the ‘green

new deals' demanding that very centralisation of control. Cusp organisations, which include endless 'think tanks' all over the world, are designed to coordinate a single global policy between political and business leaders, intelligence personnel, media organisations and anyone who can influence the direction of policy in their own sphere of operation. Major players and regular attenders will know what is happening – or some of it – while others come and go and are kept overwhelmingly in the dark about the big picture. I refer to these cusp groupings as semi-secret in that they can be publicly identified, but what goes on at the inner-core is kept very much 'in house' even from most of their members and participants through a fiercely-imposed system of compartmentalisation. Only let them know what they need to know to serve your interests and no more. The structure of secret societies serves as a perfect example of this principle. Most Freemasons never get higher than the bottom three levels of 'degree' (degree of knowledge) when there are 33 official degrees of the Scottish Rite. Initiates only qualify for the next higher 'compartment' or degree if those at that level choose to allow them. Knowledge can be carefully assigned only to those considered 'safe'. I went to my local Freemason's lodge a few years ago when they were having an 'open day' to show how cuddly they were and when I chatted to some of them I was astonished at how little the rank and file knew even about the most ubiquitous symbols they use. The mushroom technique – keep them in the dark and feed them bullshit – applies to most people in the web as well as the population as a whole. Sub-divisions of the web mirror in theme and structure transnational corporations which have a headquarters somewhere in the world dictating to all their subsidiaries in different countries. Subsidiaries operate in their methodology and branding to the same centrally-dictated plan and policy in pursuit of particular ends. The Cult web functions in the same way. Each country has its own web as a subsidiary of the global one. They consist of networks of secret societies, semi-secret groups and bloodline families and their job is to impose the will of the spider and the global web in their particular country. Subsidiary networks control and manipulate the national political system, finance, corporations, media, medicine, etc. to

ensure that they follow the globally-dictated Cult agenda. These networks were the means through which the 'Covid' hoax could be played out with almost every country responding in the same way.

The 'Yessir' pyramid

Compartmentalisation is the key to understanding how a tiny few can dictate the lives of billions when combined with a top-down sequence of imposition and acquiescence. The inner core of the Cult sits at the peak of the pyramidal hierarchy of human society (Fig 2 overleaf). It imposes its will – its agenda for the world – on the level immediately below which acquiesces to that imposition. This level then imposes the Cult will on the level below them which acquiesces and imposes on the next level. Very quickly we meet levels in the hierarchy that have no idea there even is a Cult, but the sequence of imposition and acquiescence continues down the pyramid in just the same way. 'I don't know why we are doing this but the order came from "on-high" and so we better just do it.' Alfred Lord Tennyson said of the cannon fodder levels in his poem *The Charge of the Light Brigade*: 'Theirs not to reason why; theirs but to do and die.' The next line says that 'into the valley of death rode the six hundred' and they died because they obeyed without question what their perceived 'superiors' told them to do. In the same way the population capitulated to 'Covid'. The whole hierarchical pyramid functions like this to allow the very few to direct the enormous many. Eventually imposition-acquiescence-imposition-acquiescence comes down to the mass of the population at the foot of the pyramid. If they acquiesce to those levels of the hierarchy imposing on them (governments/law enforcement/doctors/media) a circuit is completed between the population and the handful of super-psychopaths in the Cult inner core at the top of the pyramid. Without a circuit-breaking refusal to obey, the sequence of imposition and acquiescence allows a staggeringly few people to impose their will upon the entirety of humankind. We are looking at the very sequence that has subjugated billions since the start of 2020. Our freedom has not been taken from us. Humanity has given it

away. Fascists do not impose fascism because there are not enough of them. Fascism is imposed by the population acquiescing to fascism. Put another way allowing their perceptions to be programmed to the extent that leads to the population giving their freedom away by giving their perceptions – their mind – away. If this circuit is not broken by humanity ceasing to cooperate with their own enslavement then nothing can change. For that to happen people have to critically think and see through the lies and window dressing and then summon the backbone to act upon what they see. The Cult spends its days working to stop either happening and its methodology is systematic and highly detailed, but it can be overcome and that is what this book is all about.

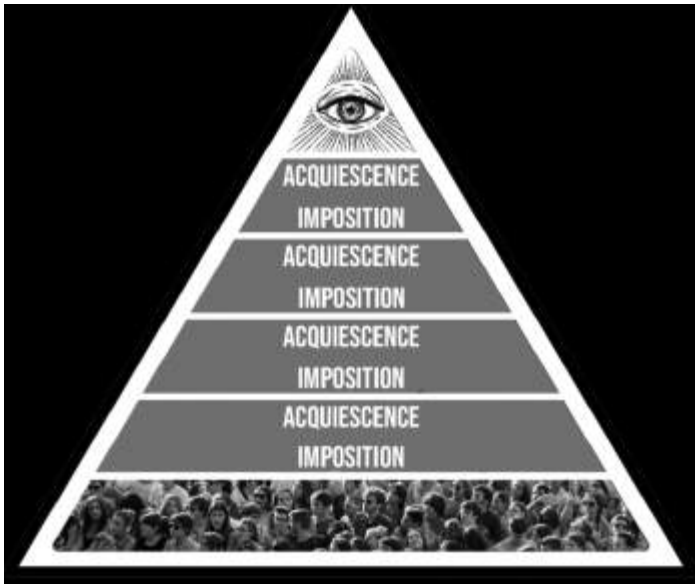

Figure 2: The simple sequence of imposition and compliance that allows a handful of people at the peak of the pyramid to dictate the lives of billions.

The Life Program

Okay, back to world number 1 or the world of the 'masses'. Observe the process of what we call 'life' and it is a perceptual download from cradle to grave. The Cult has created a global structure in which perception can be programmed and the program continually topped-up with what appears to be constant confirmation that the program is indeed true reality. The important word here is 'appears'.

This is the structure, the fly-trap, the Postage Stamp Consensus or Perceptual Mainframe, which represents that incredibly narrow band of perceived possibility delivered by the 'education' system, mainstream media, science and medicine. From the earliest age the download begins with parents who have themselves succumbed to the very programming their children are about to go through. Most parents don't do this out of malevolence and mostly it is quite the opposite. They do what they believe is best for their children and that is what the program has told them is best. Within three or four years comes the major transition from parental programming to full-blown state (Cult) programming in school, college and university where perceptually-programmed teachers and academics pass on their programming to the next generations. Teachers who resist are soon marginalised and their careers ended while children who resist are called a problem child for whom Ritalin may need to be prescribed. A few years after entering the 'world' children are under the control of authority figures representing the state telling them when they have to be there, when they can leave and when they can speak, eat, even go to the toilet. This is calculated preparation for a lifetime of obeying authority in all its forms. Reflex-action fear of authority is instilled by authority from the start. Children soon learn the carrot and stick consequences of obeying or defying authority which is underpinned daily for the rest of their life. Fortunately I daydreamed through this crap and never obeyed authority simply because it told me to. This approach to my alleged 'betters' continues to this day. There can be consequences of pursuing open-minded freedom in a world of closed-minded conformity. I spent a lot of time in school corridors after being ejected from the classroom for not taking some of it seriously and now I spend a lot of time being ejected from Facebook, YouTube and Twitter. But I can tell you that being true to yourself and not compromising your self-respect is far more exhilarating than bowing to authority for authority's sake. You don't have to be a sheep to the shepherd (authority) and the sheep dog (fear of not obeying authority).

The perceptual download continues throughout the formative years in school, college and university while script-reading 'teachers', 'academics' 'scientists', 'doctors' and 'journalists' insist that ongoing generations must be as programmed as they are. Accept the program or you will not pass your 'exams' which confirm your 'degree' of programming. It is tragic to think that many parents pressure their offspring to work hard at school to download the program and qualify for the next stage at college and university. The late, great, American comedian George Carlin said: 'Here's a bumper sticker I'd like to see: We are proud parents of a child who has resisted his teachers' attempts to break his spirit and bend him to the will of his corporate masters.' Well, the best of luck finding many of those, George. Then comes the moment to leave the formal programming years in academia and enter the 'adult' world of work. There you meet others in your chosen or prescribed arena who went through the same Postage Stamp Consensus program before you did. There is therefore overwhelming agreement between almost everyone on the basic foundations of Postage Stamp reality and the rejection, even contempt, of the few who have a mind of their own and are prepared to use it. This has two major effects. Firstly, the consensus confirms to the programmed that their download is really how things are. I mean, everyone knows that, right? Secondly, the arrogance and ignorance of Postage Stamp adherents ensure that anyone questioning the program will have unpleasant consequences for seeking their own truth and not picking their perceptions from the shelf marked: 'Things you must believe without question and if you don't you're a dangerous lunatic conspiracy theorist and a harebrained nutter'.

Every government, agency and corporation is founded on the same Postage Stamp prison cell and you can see why so many people believe the same thing while calling it their own 'opinion'. Fusion of governments and corporations in pursuit of the same agenda was the definition of fascism described by Italian dictator Benito Mussolini. The pressure to conform to perceptual norms downloaded for a lifetime is incessant and infiltrates society right

down to family groups that become censors and condemners of their own 'black sheep' for not, ironically, being sheep. We have seen an explosion of that in the 'Covid' era. Cult-owned global media unleashes its propaganda all day every day in support of the Postage Stamp and targets with abuse and ridicule anyone in the public eye who won't bend their mind to the will of the tyranny. Any response to this is denied (certainly in my case). They don't want to give a platform to expose official lies. Cult-owned-and-created Internet giants like Facebook, Google, YouTube and Twitter delete you for having an unapproved opinion. Facebook boasts that its AI censors delete 97-percent of 'hate speech' before anyone even reports it. Much of that 'hate speech' will simply be an opinion that Facebook and its masters don't want people to see. Such perceptual oppression is widely known as fascism. Even Facebook executive Benny Thomas, a 'CEO Global Planning Lead', said in comments secretly recorded by investigative journalism operation Project Veritas that Facebook is 'too powerful' and should be broken up:

I mean, no king in history has been the ruler of two billion people, but Mark Zuckerberg is … And he's 36. That's too much for a 36-year-old ... You should not have power over two billion people. I just think that's wrong.

Thomas said Facebook-owned platforms like Instagram, Oculus, and WhatsApp needed to be separate companies. 'It's too much power when they're all one together'. That's the way the Cult likes it, however. We have an executive of a Cult organisation in Benny Thomas that doesn't know there is a Cult such is the compartmentalisation. Thomas said that Facebook and Google 'are no longer companies, they're countries'. Actually they are more powerful than countries on the basis that if you control information you control perception and control human society.

I love my oppressor

Another expression of this psychological trickery is for those who realise they are being pressured into compliance to eventually

convince themselves to believe the official narratives to protect their self-respect from accepting the truth that they have succumbed to meek and subservient compliance. Such people become some of the most vehement defenders of the system. You can see them everywhere screaming abuse at those who prefer to think for themselves and by doing so reminding the compliers of their own capitulation to conformity. 'You are talking dangerous nonsense you Covidiot!!' Are you trying to convince me or yourself? It is a potent form of Stockholm syndrome which is defined as: 'A psychological condition that occurs when a victim of abuse identifies and attaches, or bonds, positively with their abuser.' An example is hostages bonding and even 'falling in love' with their kidnappers. The syndrome has been observed in domestic violence, abused children, concentration camp inmates, prisoners of war and many and various Satanic cults. These are some traits of Stockholm syndrome listed at goodtherapy.org:

- Positive regard towards perpetrators of abuse or captor [see 'Covid'].
- Failure to cooperate with police and other government authorities when it comes to holding perpetrators of abuse or kidnapping accountable [or in the case of 'Covid' cooperating with the police to enforce and defend their captors' demands].
- Little or no effort to escape [see 'Covid'].
- Belief in the goodness of the perpetrators or kidnappers [see 'Covid'].
- Appeasement of captors. This is a manipulative strategy for maintaining one's safety. As victims get rewarded – perhaps with less abuse or even with life itself – their appeasing behaviours are reinforced [see 'Covid'].
- Learned helplessness. This can be akin to 'if you can't beat 'em, join 'em'. As the victims fail to escape the abuse or captivity, they may start giving up and soon realize it's just easier for everyone if they acquiesce all their power to their captors [see 'Covid'].

- Feelings of pity toward the abusers, believing they are actually victims themselves. Because of this, victims may go on a crusade or mission to 'save' [protect] their abuser [see the venom unleashed on those challenging the official 'Covid' narrative].
- Unwillingness to learn to detach from their perpetrators and heal. In essence, victims may tend to be less loyal to themselves than to their abuser [*definitely* see 'Covid'].

Ponder on those traits and compare them with the behaviour of great swathes of the global population who have defended governments and authorities which have spent every minute destroying their lives and livelihoods and those of their children and grandchildren since early 2020 with fascistic lockdowns, house arrest and employment deletion to 'protect' them from a 'deadly virus' that their abusers' perceptually created to bring about this very outcome. We are looking at mass Stockholm syndrome. All those that agree to concede their freedom will believe those perceptions are originating in their own independent 'mind' when in fact by conceding their reality to Stockholm syndrome they have by definition conceded any independence of mind. Listen to the 'opinions' of the acquiescing masses in this 'Covid' era and what gushes forth is the repetition of the official version of everything delivered unprocessed, unfiltered and unquestioned. The whole programming dynamic works this way. I must be free because I'm told that I am and so I think that I am.

You can see what I mean with the chapter theme of 'I'm thinking – Oh, but *are* you?' The great majority are not thinking, let alone for themselves. They are repeating what authority has told them to believe which allows them to be controlled. Weaving through this mentality is the fear that the 'conspiracy theorists' are right and this again explains the often hysterical abuse that ensues when you dare to contest the official narrative of anything. Denial is the mechanism of hiding from yourself what you don't want to be true. Telling people what they want to hear is easy, but it's an infinitely greater challenge to tell them what they would rather not be happening.

One is akin to pushing against an open door while the other is met with vehement resistance no matter what the scale of evidence. I don't want it to be true so I'll convince myself that it's not. Examples are everywhere from the denial that a partner is cheating despite all the signs to the reflex-action rejection of any idea that world events in which country after country act in exactly the same way are centrally coordinated. To accept the latter is to accept that a force of unspeakable evil is working to destroy your life and the lives of your children with nothing too horrific to achieve that end. Who the heck wants that to be true? But if we don't face reality the end is duly achieved and the consequences are far worse and ongoing than breaking through the walls of denial today with the courage to make a stand against tyranny.

Connect the dots – but how?

A crucial aspect of perceptual programming is to portray a world in which everything is random and almost nothing is connected to anything else. Randomness cannot be coordinated by its very nature and once you perceive events as random the idea they could be connected is waved away as the rantings of the tinfoil-hat brigade. You can't plan and coordinate random you idiot! No, you can't, but you can hide the coldly-calculated and long-planned behind the *illusion* of randomness. A foundation manifestation of the Renegade Mind is to scan reality for patterns that connect the apparently random and turn pixels and dots into pictures. This is the way I work and have done so for more than 30 years. You look for similarities in people, modus operandi and desired outcomes and slowly, then ever quicker, the picture forms. For instance: There would seem to be no connection between the 'Covid pandemic' hoax and the human-caused global-warming hoax and yet they are masks (appropriately) on the same face seeking the same outcome. Those pushing the global warming myth through the Club of Rome and other Cult agencies are driving the lies about 'Covid' – Bill Gates is an obvious one, but they are endless. Why would the same people be involved in both when they are clearly not connected? Oh, but they

are. Common themes with personnel are matched by common goals. The 'solutions' to both 'problems' are centralisation of global power to impose the will of the few on the many to 'save' humanity from 'Covid' and save the planet from an 'existential threat' (we need 'zero Covid' and 'zero carbon emissions'). These, in turn, connect with the 'dot' of globalisation which was coined to describe the centralisation of global power in every area of life through incessant political and corporate expansion, trading blocks and superstates like the European Union. If you are the few and you want to control the many you have to centralise power and decision-making. The more you centralise power the more power the few at the centre will have over the many; and the more that power is centralised the more power those at the centre have to centralise even quicker. The momentum of centralisation gets faster and faster which is exactly the process we have witnessed. In this way the hoaxed 'pandemic' and the fakery of human-caused global warming serve the interests of globalisation and the seizure of global power in the hands of the Cult inner-circle which is behind 'Covid', 'climate change' *and* globalisation. At this point random 'dots' become a clear and obvious picture or pattern.

Klaus Schwab, the classic Bond villain who founded the Cult's Gates-funded World Economic Forum, published a book in 2020, *The Great Reset*, in which he used the 'problem' of 'Covid' to justify a total transformation of human society to 'save' humanity from 'climate change'. Schwab said: 'The pandemic represents a rare but narrow window of opportunity to reflect, reimagine, and reset our world.' What he didn't mention is that the Cult he serves is behind both hoaxes as I show in my book *The Answer*. He and the Cult don't have to reimagine the world. They know precisely what they want and that's why they destroyed human society with 'Covid' to 'build back better' in their grand design. Their job is not to imagine, but to get humanity to imagine and agree with their plans while believing it's all random. It must be pure coincidence that 'The Great Reset' has long been the Cult's code name for the global imposition of fascism and replaced previous code-names of the 'New World

Order' used by Cult frontmen like Father George Bush and the 'New Order of the Ages' which emerged from Freemasonry and much older secret societies. New Order of the Ages appears on the reverse of the Great Seal of the United States as 'Novus ordo seclorum' underneath the Cult symbol used since way back of the pyramid and all seeing-eye (Fig 3). The pyramid is the hierarchy of human control headed by the illuminated eye that symbolises the force behind the Cult which I will expose in later chapters. The term 'Annuit Coeptis' translates as 'He favours our undertaking'. We are told the 'He' is the Christian god, but 'He' is not as I will be explaining.

Figure 3: The all-seeing eye of the Cult 'god' on the Freemason-designed Great Seal of the United States and also on the dollar bill.

Having you on

Two major Cult techniques of perceptual manipulation that relate to all this are what I have called since the 1990s Problem-Reaction-Solution (PRS) and the Totalitarian Tiptoe (TT). They can be uncovered by the inquiring mind with a simple question: Who benefits? The answer usually identifies the perpetrators of a given action or happening through the concept of 'he who most benefits from a crime is the one most likely to have committed it'. The Latin 'Cue bono?' – Who benefits? – is widely attributed to the Roman orator and statesman Marcus Tullius Cicero. No wonder it goes back so far when the concept has been relevant to human behaviour since

history was recorded. Problem-Reaction-Solution is the technique used to manipulate us every day by covertly creating a problem (or the illusion of one) and offering the solution to the problem (or the illusion of one). In the first phase you create the problem and blame someone or something else for why it has happened. This may relate to a financial collapse, terrorist attack, war, global warming or pandemic, anything in fact that will allow you to impose the 'solution' to change society in the way you desire at that time. The 'problem' doesn't have to be real. PRS is manipulation of perception and all you need is the population to believe the problem is real. Human-caused global warming and the 'Covid pandemic' only have to be *perceived* to be real for the population to accept the 'solutions' of authority. I refer to this technique as NO-Problem-Reaction-Solution. Billions did not meekly accept house arrest from early 2020 because there was a real deadly 'Covid pandemic' but because they perceived – believed – that to be the case. The antidote to Problem-Reaction-Solution is to ask who benefits from the proposed solution. Invariably it will be anyone who wants to justify more control through deletion of freedom and centralisation of power and decision-making.

The two world wars were Problem-Reaction-Solutions that transformed and realigned global society. Both were manipulated into being by the Cult as I have detailed in books since the mid-1990s. They dramatically centralised global power, especially World War Two, which led to the United Nations and other global bodies thanks to the overt and covert manipulations of the Rockefeller family and other Cult bloodlines like the Rothschilds. The UN is a stalking horse for full-blown world government that I will come to shortly. The land on which the UN building stands in New York was donated by the Rockefellers and the same Cult family was behind Big Pharma scalpel and drug 'medicine' and the creation of the World Health Organization as part of the UN. They have been stalwarts of the eugenics movement and funded Hitler's race-purity expert' Ernst Rudin. The human-caused global warming hoax has been orchestrated by the Club of Rome through the UN which is

manufacturing both the 'problem' through its Intergovernmental Panel on Climate Change and imposing the 'solution' through its Agenda 21 and Agenda 2030 which demand the total centralisation of global power to 'save the world' from a climate hoax the United Nations is itself perpetrating. What a small world the Cult can be seen to be particularly among the inner circles. The bedfellow of Problem-Reaction-Solution is the Totalitarian Tiptoe which became the Totalitarian Sprint in 2020. The technique is fashioned to hide the carefully-coordinated behind the cover of apparently random events. You start the sequence at 'A' and you know you are heading for 'Z'. You don't want people to know that and each step on the journey is presented as a random happening while all the steps strung together lead in the same direction. The speed may have quickened dramatically in recent times, but you can still see the incremental approach of the Tiptoe in the case of 'Covid' as each new imposition takes us deeper into fascism. Tell people they have to do this or that to get back to 'normal', then this and this and this. With each new demand adding to the ones that went before the population's freedom is deleted until it disappears. The spider wraps its web around the flies more comprehensively with each new diktat. I'll highlight this in more detail when I get to the 'Covid' hoax and how it has been pulled off. Another prime example of the Totalitarian Tiptoe is how the Cult-created European Union went from a 'free-trade zone' to a centralised bureaucratic dictatorship through the Tiptoe of incremental centralisation of power until nations became mere administrative units for Cult-owned dark suits in Brussels.

The antidote to ignorance is knowledge which the Cult seeks vehemently to deny us, but despite the systematic censorship to that end the Renegade Mind can overcome this by vociferously seeking out the facts no matter the impediments put in the way. There is also a method of thinking and perceiving – *knowing* – that doesn't even need names, dates, place-type facts to identify the patterns that reveal the story. I'll get to that in the final chapter. All you need to know about the manipulation of human society and to what end is still out there – *at the time of writing* – in the form of books, videos

and websites for those that really want to breach the walls of programmed perception. To access this knowledge requires the abandonment of the mainstream media as a source of information in the awareness that this is owned and controlled by the Cult and therefore promotes mass perceptions that suit the Cult. Mainstream media lies all day, every day. That is its function and very reason for being. Where it does tell the truth, here and there, is only because the truth and the Cult agenda very occasionally coincide. If you look for fact and insight to the BBC, CNN and virtually all the rest of them you are asking to be conned and perceptually programmed.

Know the outcome and you'll see the journey

Events seem random when you have no idea where the world is being taken. Once you do the random becomes the carefully planned. Know the outcome and you'll see the journey is a phrase I have been using for a long time to give context to daily happenings that appear unconnected. Does a problem, or illusion of a problem, trigger a proposed 'solution' that further drives society in the direction of the outcome? Invariably the answer will be yes and the random – *abracadabra* – becomes the clearly coordinated. So what is this outcome that unlocks the door to a massively expanded understanding of daily events? I will summarise its major aspects – the fine detail is in my other books – and those new to this information will see that the world they thought they were living in is a very different place. The foundation of the Cult agenda is the incessant centralisation of power and all such centralisation is ultimately in pursuit of Cult control on a global level. I have described for a long time the planned world structure of top-down dictatorship as the Hunger Games Society. The term obviously comes from the movie series which portrayed a world in which a few living in military-protected hi-tech luxury were the overlords of a population condemned to abject poverty in isolated 'sectors' that were not allowed to interact. 'Covid' lockdowns and travel bans anyone? The 'Hunger Games' pyramid of structural control has the inner circle of the Cult at the top with pretty much the entire

population at the bottom under their control through dependency for survival on the Cult. The whole structure is planned to be protected and enforced by a military-police state (Fig 4).

Here you have the reason for the global lockdowns of the fake pandemic to coldly destroy independent incomes and livelihoods and make everyone dependent on the 'state' (the Cult that controls the 'states'). I have warned in my books for many years about the plan to introduce a 'guaranteed income' – a barely survivable pittance – designed to impose dependency when employment was destroyed by AI technology and now even more comprehensively at great speed by the 'Covid' scam. Once the pandemic was played and lockdown consequences began to delete independent income the authorities began to talk right on cue about the need for a guaranteed income and a 'Great Reset'. Guaranteed income will be presented as benevolent governments seeking to help a desperate people – desperate as a direct result of actions of the same governments. The truth is that such payments are a trap. You will only get them if you do exactly what the authorities demand including mass vaccination (genetic manipulation). We have seen this theme already in Australia where those dependent on government benefits have them reduced if parents don't agree to have their children vaccinated according to an insane health-destroying government-dictated schedule. Calculated economic collapse applies to governments as well as people. The Cult wants rid of countries through the creation of a world state with countries broken up into regions ruled by a world government and super states like the European Union. Countries must be bankrupted, too, to this end and it's being achieved by the trillions in 'rescue packages' and furlough payments, trillions in lost taxation, and money-no-object spending on 'Covid' including constant all-medium advertising (programming) which has made the media dependent on government for much of its income. The day of reckoning is coming – as planned – for government spending and given that it has been made possible by printing money and not by production/taxation there is inflation on the way that has the

potential to wipe out monetary value. In that case there will be no need for the Cult to steal your money. It just won't be worth anything (see the German Weimar Republic before the Nazis took over). Many have been okay with lockdowns while getting a percentage of their income from so-called furlough payments without having to work. Those payments are dependent, however, on people having at least a theoretical job with a business considered non-essential and ordered to close. As these business go under because they are closed by lockdown after lockdown the furlough stops and it will for everyone eventually. Then what? The 'then what?' is precisely the idea.

Figure 4: The Hunger Games Society structure I have long warned was planned and now the 'Covid' hoax has made it possible. This is the real reason for lockdowns.

Hired hands

Between the Hunger Games Cult elite and the dependent population is planned to be a vicious military-police state (a fusion of the two into one force). This has been in the making for a long time with police looking ever more like the military and carrying weapons to match. The pandemic scam has seen this process accelerate so fast as

lockdown house arrest is brutally enforced by carefully recruited fascist minds and gormless system-servers. The police and military are planned to merge into a centrally-directed world army in a global structure headed by a world government which wouldn't be elected even by the election fixes now in place. The world army is not planned even to be human and instead wars would be fought, primarily against the population, using robot technology controlled by artificial intelligence. I have been warning about this for decades and now militaries around the world are being transformed by this very AI technology. The global regime that I describe is a particular form of fascism known as a technocracy in which decisions are not made by clueless and co-opted politicians but by unelected technocrats – scientists, engineers, technologists and bureaucrats. Cult-owned-and-controlled Silicon Valley giants are examples of technocracy and they already have far more power to direct world events than governments. They are with their censorship *selecting* governments. I know that some are calling the 'Great Reset' a Marxist communist takeover, but fascism and Marxism are different labels for the same tyranny. Tell those who lived in fascist Germany and Stalinist Russia that there was a difference in the way their freedom was deleted and their lives controlled. I could call it a fascist technocracy or a Marxist technocracy and they would be equally accurate. The Hunger Games society with its world government structure would oversee a world army, world central bank and single world cashless currency imposing its will on a microchipped population (Fig 5). Scan its different elements and see how the illusory pandemic is forcing society in this very direction at great speed. Leaders of 23 countries and the World Health Organization (WHO) backed the idea in March, 2021, of a global treaty for 'international cooperation' in 'health emergencies' and nations should 'come together as a global community for peaceful cooperation that extends beyond this crisis'. Cut the Orwellian bullshit and this means another step towards global government. The plan includes a cashless digital money system that I first warned about in 1993. Right at the start of 'Covid' the deeply corrupt Tedros

Adhanom Ghebreyesus, the crooked and merely gofer 'head' of the World Health Organization, said it was possible to catch the 'virus' by touching cash and it was better to use cashless means. The claim was ridiculous nonsense and like the whole 'Covid' mind-trick it was nothing to do with 'health' and everything to do with pushing every aspect of the Cult agenda. As a result of the Tedros lie the use of cash has plummeted. The Cult script involves a single world digital currency that would eventually be technologically embedded in the body. China is a massive global centre for the Cult and if you watch what is happening there you will know what is planned for everywhere. The Chinese government is developing a digital currency which would allow fines to be deducted immediately via AI for anyone caught on camera breaking its fantastic list of laws and the money is going to be programmable with an expiry date to ensure that no one can accrue wealth except the Cult and its operatives.

Figure 5: The structure of global control the Cult has been working towards for so long and this has been enormously advanced by the 'Covid' illusion.

Serfdom is so smart

The Cult plan is far wider, extreme, and more comprehensive than even most conspiracy researchers appreciate and I will come to the true depths of deceit and control in the chapters 'Who controls the

Cult?' and 'Escaping Wetiko'. Even the world that we know is crazy enough. We are being deluged with ever more sophisticated and controlling technology under the heading of 'smart'. We have smart televisions, smart meters, smart cards, smart cars, smart driving, smart roads, smart pills, smart patches, smart watches, smart skin, smart borders, smart pavements, smart streets, smart cities, smart communities, smart environments, smart growth, smart planet ... smart *everything* around us. Smart technologies and methods of operation are designed to interlock to create a global Smart Grid connecting the entirety of human society including human minds to create a centrally-dictated 'hive' mind. 'Smart cities' is code for densely-occupied megacities of total surveillance and control through AI. Ever more destructive frequency communication systems like 5G have been rolled out without any official testing for health and psychological effects (colossal). 5G/6G/7G systems are needed to run the Smart Grid and each one becomes more destructive of body and mind. Deleting independent income is crucial to forcing people into these AI-policed prisons by ending private property ownership (except for the Cult elite). The Cult's Great Reset now openly foresees a global society in which no one will own any possessions and everything will be rented while the Cult would own literally everything under the guise of government and corporations. The aim has been to use the lockdowns to destroy sources of income on a mass scale and when the people are destitute and in unrepayable amounts of debt (problem) Cult assets come forward with the pledge to write-off debt in return for handing over all property and possessions (solution). Everything – literally everything including people – would be connected to the Internet via AI. I was warning years ago about the coming Internet of Things (IoT) in which all devices and technology from your car to your fridge would be plugged into the Internet and controlled by AI. Now we are already there with much more to come. The next stage is the Internet of Everything (IoE) which is planned to include the connection of AI to the human brain and body to replace the human mind with a centrally-controlled AI mind. Instead of perceptions

being manipulated through control of information and censorship those perceptions would come direct from the Cult through AI. What do you think? You think whatever AI decides that you think. In human terms there would be no individual 'think' any longer. Too incredible? The ravings of a lunatic? Not at all. Cult-owned crazies in Silicon Valley have been telling us the plan for years without explaining the real motivation and calculated implications. These include Google executive and 'futurist' Ray Kurzweil who highlights the year 2030 for when this would be underway. He said:

Our thinking ... will be a hybrid of biological and non-biological thinking ... humans will be able to extend their limitations and 'think in the cloud' ... We're going to put gateways to the cloud in our brains ... We're going to gradually merge and enhance ourselves ... In my view, that's the nature of being human – we transcend our limitations.

As the technology becomes vastly superior to what we are then the small proportion that is still human gets smaller and smaller and smaller until it's just utterly negligible.

The sales-pitch of Kurzweil and Cult-owned Silicon Valley is that this would make us 'super-human' when the real aim is to make us post-human and no longer 'human' in the sense that we have come to know. The entire global population would be connected to AI and become the centrally-controlled 'hive-mind' of externally-delivered perceptions. The Smart Grid being installed to impose the Cult's will on the world is being constructed to allow particular locations – even one location – to control the whole global system. From these prime control centres, which absolutely include China and Israel, anything connected to the Internet would be switched on or off and manipulated at will. Energy systems could be cut, communication via the Internet taken down, computer-controlled driverless autonomous vehicles driven off the road, medical devices switched off, the potential is limitless given how much AI and Internet connections now run human society. We have seen nothing yet if we allow this to continue. Autonomous vehicle makers are working with law enforcement to produce cars designed to automatically pull over if they detect a police or emergency vehicle flashing from up to 100 feet away. At a police stop the car would be unlocked and the

window rolled down automatically. Vehicles would only take you where the computer (the state) allowed. The end of petrol vehicles and speed limiters on all new cars in the UK and EU from 2022 are steps leading to electric computerised transport over which ultimately you have no control. The picture is far bigger even than the Cult global network or web and that will become clear when I get to the nature of the 'spider'. There is a connection between all these happenings and the instigation of DNA-manipulating 'vaccines' (which aren't 'vaccines') justified by the 'Covid' hoax. That connection is the unfolding plan to transform the human body from a biological to a synthetic biological state and this is why synthetic biology is such a fast-emerging discipline of mainstream science. 'Covid vaccines' are infusing self-replicating synthetic genetic material into the cells to cumulatively take us on the Totalitarian Tiptoe from Human 1.0 to the synthetic biological Human 2.0 which will be physically and perceptually attached to the Smart Grid to one hundred percent control every thought, perception and deed. Humanity needs to wake up and *fast*.

This is the barest explanation of where the 'outcome' is planned to go but it's enough to see the journey happening all around us. Those new to this information will already see 'Covid' in a whole new context. I will add much more detail as we go along, but for the minutiae evidence see my mega-works, *The Answer, The Trigger* and *Everything You Need to Know But Have Never Been Told*.

Now – how does a Renegade Mind see the 'world'?

CHAPTER TWO

Renegade Perception

It is one thing to be clever and another to be wise
George R.R. Martin

A simple definition of the difference between a programmed mind and a Renegade Mind would be that one sees only dots while the other connects them to see the picture. Reading reality with accuracy requires the observer to (a) know the planned outcome and (b) realise that everything, but *everything*, is connected.

The entirety of infinite reality is connected – that's its very nature – and with human society an expression of infinite reality the same must apply. Simple cause and effect is a connection. The effect is triggered by the cause and the effect then becomes the cause of another effect. Nothing happens in isolation because it *can't*. Life in whatever reality is simple choice and consequence. We make choices and these lead to consequences. If we don't like the consequences we can make different choices and get different consequences which lead to other choices and consequences. The choice and the consequence are not only connected they are indivisible. You can't have one without the other as an old song goes. A few cannot control the world unless those being controlled allow that to happen – cause and effect, choice and consequence. Control – who has it and who doesn't – is a two-way process, a symbiotic relationship, involving the controller and controlled. 'They took my freedom away!!' Well, yes, but you also gave it to them. Humanity is

subjected to mass control because humanity has acquiesced to that control. This is all cause and effect and literally a case of give and take. In the same way world events of every kind are connected and the Cult works incessantly to sell the illusion of the random and coincidental to maintain the essential (to them) perception of dots that hide the picture. Renegade Minds know this and constantly scan the world for patterns of connection. This is absolutely pivotal in understanding the happenings in the world and without that perspective clarity is impossible. First you know the planned outcome and then you identify the steps on the journey – the day-by-day apparently random which, when connected in relation to the outcome, no longer appear as individual events, but as the proverbial *chain* of events leading in the same direction. I'll give you some examples:

Political puppet show

We are told to believe that politics is 'adversarial' in that different parties with different beliefs engage in an endless tussle for power. There may have been some truth in that up to a point – and only a point – but today divisions between 'different' parties are rhetorical not ideological. Even the rhetorical is fusing into one-speak as the parties eject any remaining free thinkers while others succumb to the ever-gathering intimidation of anyone with the 'wrong' opinion. The Cult is not a new phenomenon and can be traced back thousands of years as my books have documented. Its intergenerational initiates have been manipulating events with increasing effect the more that global power has been centralised. In ancient times the Cult secured control through the system of monarchy in which 'special' bloodlines (of which more later) demanded the right to rule as kings and queens simply by birthright and by vanquishing others who claimed the same birthright. There came a time, however, when people had matured enough to see the unfairness of such tyranny and demanded a say in who governed them. Note the word – *governed* them. Not served them – *governed* them, hence government defined as 'the political direction and control exercised over the

actions of the members, citizens, or inhabitants of communities, societies, and states; direction of the affairs of a state, community, etc.' Governments exercise control over rather than serve just like the monarchies before them. Bizarrely there are still countries like the United Kingdom which are ruled by a monarch *and* a government that officially answers to the monarch. The UK head of state and that of Commonwealth countries such as Canada, Australia and New Zealand is 'selected' by who in a *single family* had unprotected sex with whom and in what order. Pinch me it can't be true. Ouch! Shit, it is. The demise of monarchies in most countries offered a potential vacuum in which some form of free and fair society could arise and the Cult had that base covered. Monarchies had served its interests but they couldn't continue in the face of such widespread opposition and, anyway, replacing a 'royal' dictatorship that people could see with a dictatorship 'of the people' hiding behind the concept of 'democracy' presented far greater manipulative possibilities and ways of hiding coordinated tyranny behind the illusion of 'freedom'.

Democracy is quite wrongly defined as government selected by the population. This is not the case at all. It is government selected by *some* of the population (and then only in theory). This 'some' doesn't even have to be the majority as we have seen so often in first-past-the-post elections in which the so-called majority party wins fewer votes than the 'losing' parties combined. Democracy can give total power to a party in government from a minority of the votes cast. It's a sleight of hand to sell tyranny as freedom. Seventy-four million Trump-supporting Americans didn't vote for the 'Democratic' Party of Joe Biden in the distinctly dodgy election in 2020 and yet far from acknowledging the wishes and feelings of that great percentage of American society the Cult-owned Biden government set out from day one to destroy them and their right to a voice and opinion. Empty shell Biden and his Cult handlers said they were doing this to 'protect democracy'. Such is the level of lunacy and sickness to which politics has descended. Connect the dots and relate them to the desired outcome – a world government run by self-appointed technocrats and no longer even elected

politicians. While operating through its political agents in government the Cult is at the same time encouraging public distain for politicians by putting idiots and incompetents in theoretical power on the road to deleting them. The idea is to instil a public reaction that says of the technocrats: 'Well, they couldn't do any worse than the pathetic politicians.' It's all about controlling perception and Renegade Minds can see through that while programmed minds cannot when they are ignorant of both the planned outcome and the manipulation techniques employed to secure that end. This knowledge can be learned, however, and fast if people choose to get informed.

Politics may at first sight appear very difficult to control from a central point. I mean look at the 'different' parties and how would you be able to oversee them all and their constituent parts? In truth, it's very straightforward because of their structure. We are back to the pyramid of imposition and acquiescence. Organisations are structured in the same way as the system as a whole. Political parties are not open forums of free expression. They are hierarchies. I was a national spokesman for the British Green Party which claimed to be a different kind of politics in which influence and power was devolved; but I can tell you from direct experience – and it's far worse now – that Green parties are run as hierarchies like all the others however much they may try to hide that fact or kid themselves that it's not true. A very few at the top of all political parties are directing policy and personnel. They decide if you are elevated in the party or serve as a government minister and to do that you have to be a yes man or woman. Look at all the maverick political thinkers who never ascended the greasy pole. If you want to progress within the party or reach 'high-office' you need to fall into line and conform. Exceptions to this are rare indeed. Should you want to run for parliament or Congress you have to persuade the local or state level of the party to select you and for that you need to play the game as dictated by the hierarchy. If you secure election and wish to progress within the greater structure you need to go on conforming to what is acceptable to those running the hierarchy

from the peak of the pyramid. Political parties are perceptual gulags and the very fact that there are party 'Whips' appointed to 'whip' politicians into voting the way the hierarchy demands exposes the ridiculous idea that politicians are elected to serve the people they are supposed to represent. Cult operatives and manipulation has long seized control of major parties that have any chance of forming a government and at least most of those that haven't. A new party forms and the Cult goes to work to infiltrate and direct. This has reached such a level today that you see video compilations of 'leaders' of all parties whether Democrats, Republicans, Conservative, Labour and Green parroting the same Cult mantra of 'Build Back Better' and the 'Great Reset' which are straight off the Cult song-sheet to describe the transformation of global society in response to the Cult-instigated hoaxes of the 'Covid pandemic' and human-caused 'climate change'. To see Caroline Lucas, the Green Party MP that I knew when I was in the party in the 1980s, speaking in support of plans proposed by Cult operative Klaus Schwab representing the billionaire global elite is a real head-shaker.

Many parties – one master

The party system is another mind-trick and was instigated to change the nature of the dictatorship by swapping 'royalty' for dark suits that people believed – though now ever less so – represented their interests. Understanding this trick is to realise that a single force (the Cult) controls all parties either directly in terms of the major ones or through manipulation of perception and ideology with others. You don't need to manipulate Green parties to demand your transformation of society in the name of 'climate change' when they are obsessed with the lie that this is essential to 'save the planet'. You just give them a platform and away they go serving your interests while believing they are being environmentally virtuous. America's political structure is a perfect blueprint for how the two or multi-party system is really a one-party state. The Republican Party is controlled from one step back in the shadows by a group made up of billionaires and their gofers known as neoconservatives or Neocons.

I have exposed them in fine detail in my books and they were the driving force behind the policies of the imbecilic presidency of Boy George Bush which included 9/11 (see *The Trigger* for a comprehensive demolition of the official story), the subsequent 'war on terror' (war *of* terror) and the invasions of Afghanistan and Iraq. The latter was a No-Problem-Reaction-Solution based on claims by Cult operatives, including Bush and British Prime Minister Tony Blair, about Saddam Hussein's 'weapons of mass destruction' which did not exist as war criminals Bush and Blair well knew.

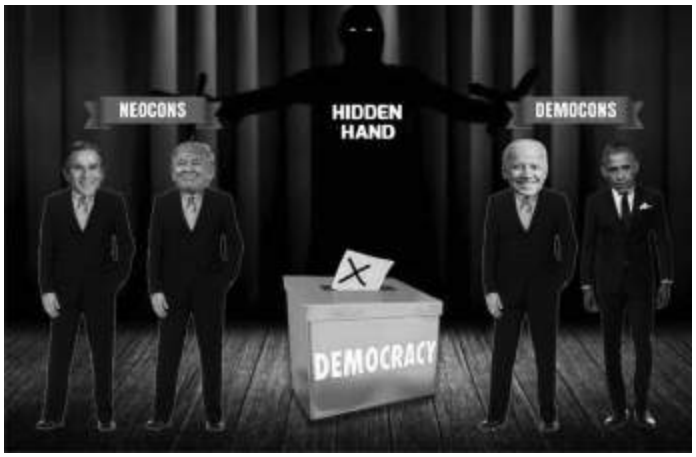

Figure 6: Different front people, different parties – same control system.

The Democratic Party has its own 'Neocon' group controlling from the background which I call the 'Democons' and here's the penny-drop – the Neocons and Democons answer to the same masters one step further back into the shadows (Fig 6). At that level of the Cult the Republican and Democrat parties are controlled by the same people and no matter which is in power the Cult is in power. This is how it works in almost every country and certainly in Britain with Conservative, Labour, Liberal Democrat and Green parties now all on the same page whatever the rhetoric may be in their feeble attempts to appear different. Neocons operated at the time of Bush through a think tank called The Project for the New American Century which in September, 2000, published a document entitled *Rebuilding America's Defenses: Strategies, Forces, and Resources*

For a New Century demanding that America fight 'multiple, simultaneous major theatre wars' as a 'core mission' to force regime-change in countries including Iraq, Libya and Syria. Neocons arranged for Bush ('Republican') and Blair ('Labour Party') to front-up the invasion of Iraq and when they departed the Democons orchestrated the targeting of Libya and Syria through Barack Obama ('Democrat') and British Prime Minister David Cameron ('Conservative Party'). We have 'different' parties and 'different' people, but the same unfolding script. The more the Cult has seized the reigns of parties and personnel the more their policies have transparently pursued the same agenda to the point where the fascist 'Covid' impositions of the Conservative junta of Jackboot Johnson in Britain were opposed by the Labour Party because they were not fascist enough. The Labour Party is likened to the US Democrats while the Conservative Party is akin to a British version of the Republicans and on both sides of the Atlantic they all speak the same language and support the direction demanded by the Cult although some more enthusiastically than others. It's a similar story in country after country because it's all centrally controlled. Oh, but what about Trump? I'll come to him shortly. Political 'choice' in the 'party' system goes like this: You vote for Party A and they get into government. You don't like what they do so next time you vote for Party B and they get into government. You don't like what they do when it's pretty much the same as Party A and why wouldn't that be with both controlled by the same force? Given that only two, sometimes three, parties have any chance of forming a government to get rid of Party B that you don't like you have to vote again for Party A which … you don't like. This, ladies and gentlemen, is what they call 'democracy' which we are told – wrongly – is a term interchangeable with 'freedom'.

The cult of cults

At this point I need to introduce a major expression of the Global Cult known as Sabbatian-Frankism. Sabbatian is also spelt as Sabbatean. I will summarise here. I have published major exposés

and detailed background in other works. Sabbatian-Frankism combines the names of two frauds posing as 'Jewish' men, Sabbatai Zevi (1626-1676), a rabbi, black magician and occultist who proclaimed he was the Jewish messiah; and Jacob Frank (1726-1791), the Polish 'Jew', black magician and occultist who said he was the reincarnation of 'messiah' Zevi and biblical patriarch Jacob. They worked across two centuries to establish the Sabbatian-Frankist cult that plays a major, indeed central, role in the manipulation of human society by the Global Cult which has its origins much further back in history than Sabbatai Zevi. I should emphasise two points here in response to the shrill voices that will scream 'anti-Semitism': (1) Sabbatian-Frankists are NOT Jewish and only pose as such to hide their cult behind a Jewish façade; and (2) my information about this cult has come from Jewish sources who have long realised that their society and community has been infiltrated and taken over by interloper Sabbatian-Frankists. Infiltration has been the foundation technique of Sabbatian-Frankism from its official origin in the 17th century. Zevi's Sabbatian sect attracted a massive following described as the biggest messianic movement in Jewish history, spreading as far as Africa and Asia, and he promised a return for the Jews to the 'Promised Land' of Israel. Sabbatianism was not Judaism but an inversion of everything that mainstream Judaism stood for. So much so that this sinister cult would have a feast day when Judaism had a fast day and whatever was forbidden in Judaism the Sabbatians were encouraged and even commanded to do. This included incest and what would be today called Satanism. Members were forbidden to marry outside the sect and there was a system of keeping their children ignorant of what they were part of until they were old enough to be trusted not to unknowingly reveal anything to outsiders. The same system is employed to this day by the Global Cult in general which Sabbatian-Frankism has enormously influenced and now largely controls.

Zevi and his Sabbatians suffered a setback with the intervention by the Sultan of the Islamic Ottoman Empire in the Middle East and what is now the Republic of Turkey where Zevi was located. The

Sultan gave him the choice of proving his 'divinity', converting to Islam or facing torture and death. Funnily enough Zevi chose to convert or at least appear to. Some of his supporters were disillusioned and drifted away, but many did not with 300 families also converting – only in theory – to Islam. They continued behind this Islamic smokescreen to follow the goals, rules and rituals of Sabbatianism and became known as 'crypto-Jews' or the 'Dönmeh' which means 'to turn'. This is rather ironic because they didn't 'turn' and instead hid behind a fake Islamic persona. The process of appearing to be one thing while being very much another would become the calling card of Sabbatianism especially after Zevi's death and the arrival of the Satanist Jacob Frank in the 18th century when the cult became Sabbatian-Frankism and plumbed still new depths of depravity and infiltration which included – still includes – human sacrifice and sex with children. Wherever Sabbatians go paedophilia and Satanism follow and is it really a surprise that Hollywood is so infested with child abuse and Satanism when it was established by Sabbatian-Frankists and is still controlled by them? Hollywood has been one of the prime vehicles for global perceptual programming and manipulation. How many believe the version of 'history' portrayed in movies when it is a travesty and inversion (again) of the truth? Rabbi Marvin Antelman describes Frankism in his book, *To Eliminate the Opiate*, as 'a movement of complete evil' while Jewish professor Gershom Scholem said of Frank in *The Messianic Idea in Judaism*: 'In all his actions [he was] a truly corrupt and degenerate individual ... one of the most frightening phenomena in the whole of Jewish history.' Frank was excommunicated by traditional rabbis, as was Zevi, but Frank was undeterred and enjoyed vital support from the House of Rothschild, the infamous banking dynasty whose inner-core are Sabbatian-Frankists and not Jews. Infiltration of the Roman Church and Vatican was instigated by Frank with many Dönmeh 'turning' again to convert to Roman Catholicism with a view to hijacking the reins of power. This was the ever-repeating modus operandi and continues to be so. Pose as an advocate of the religion, culture or country that you want to control and then

manipulate your people into the positions of authority and influence largely as advisers, administrators and Svengalis for those that appear to be in power. They did this with Judaism, Christianity (Christian Zionism is part of this), Islam and other religions and nations until Sabbatian-Frankism spanned the world as it does today.

Sabbatian Saudis and the terror network

One expression of the Sabbatian-Frankist Dönmeh within Islam is the ruling family of Saudi Arabia, the House of Saud, through which came the vile distortion of Islam known as Wahhabism. This is the violent creed followed by terrorist groups like Al-Qaeda and ISIS or Islamic State. Wahhabism is the hand-chopping, head-chopping 'religion' of Saudi Arabia which is used to keep the people in a constant state of fear so the interloper House of Saud can continue to rule. Al-Qaeda and Islamic State were lavishly funded by the House of Saud while being created and directed by the Sabbatian-Frankist network in the United States that operates through the Pentagon, CIA and the government in general of whichever 'party'. The front man for the establishment of Wahhabism in the middle of the 18th century was a Sabbatian-Frankist 'crypto-Jew' posing as Islamic called Muhammad ibn Abd al-Wahhab. His daughter would marry the son of Muhammad bin Saud who established the first Saudi state before his death in 1765 with support from the British Empire. Bin Saud's successors would establish modern Saudi Arabia in league with the British and Americans in 1932 which allowed them to seize control of Islam's major shrines in Mecca and Medina. They have dictated the direction of Sunni Islam ever since while Iran is the major centre of the Shiite version and here we have the source of at least the public conflict between them. The Sabbatian network has used its Wahhabi extremists to carry out Problem-Reaction-Solution terrorist attacks in the name of 'Al-Qaeda' and 'Islamic State' to justify a devastating 'war on terror', ever-increasing surveillance of the population and to terrify people into compliance. Another insight of the Renegade Mind is the streetwise understanding that

just because a country, location or people are attacked doesn't mean that those apparently representing that country, location or people are not behind the attackers. Often they are *orchestrating* the attacks because of the societal changes that can be then justified in the name of 'saving the population from terrorists'.

I show in great detail in *The Trigger* how Sabbatian-Frankists were the real perpetrators of 9/11 and not '19 Arab hijackers' who were blamed for what happened. Observe what was justified in the name of 9/11 alone in terms of Middle East invasions, mass surveillance and control that fulfilled the demands of the Project for the New American Century document published by the Sabbatian Neocons. What appear to be enemies are on the deep inside players on the same Sabbatian team. Israel and Arab 'royal' dictatorships are all ruled by Sabbatians and the recent peace agreements between Israel and Saudi Arabia, the United Arab Emirates (UAE) and others are only making formal what has always been the case behind the scenes. Palestinians who have been subjected to grotesque tyranny since Israel was bombed and terrorised into existence in 1948 have never stood a chance. Sabbatian-Frankists have controlled Israel (so the constant theme of violence and war which Sabbatians love) and they have controlled the Arab countries that Palestinians have looked to for real support that never comes. 'Royal families' of the Arab world in Saudi Arabia, Bahrain, UAE, etc., are all Sabbatians with allegiance to the aims of the cult and not what is best for their Arabic populations. They have stolen the oil and financial resources from their people by false claims to be 'royal dynasties' with a genetic right to rule and by employing vicious militaries to impose their will.

Satanic 'illumination'

The Satanist Jacob Frank formed an alliance in 1773 with two other Sabbatians, Mayer Amschel Rothschild (1744-1812), founder of the Rothschild banking dynasty, and Jesuit-educated fraudulent Jew, Adam Weishaupt, and this led to the formation of the Bavarian Illuminati, firstly under another name, in 1776. The Illuminati would

be the manipulating force behind the French Revolution (1789-1799) and was also involved in the American Revolution (1775-1783) before and after the Illuminati's official creation. Weishaupt would later become (in public) a Protestant Christian in archetypal Sabbatian style. I read that his name can be decoded as Adam-Weishaupt or 'the first man to lead those who know'. He wasn't a leader in the sense that he was a subordinate, but he did lead those below him in a crusade of transforming human society that still continues today. The theme was confirmed as early as 1785 when a horseman courier called Lanz was reported to be struck by lighting and extensive Illuminati documents were found in his saddlebags. They made the link to Weishaupt and detailed the plan for world takeover. Current events with 'Covid' fascism have been in the making for a very long time. Jacob Frank was jailed for 13 years by the Catholic Inquisition after his arrest in 1760 and on his release he headed for Frankfurt, Germany, home city and headquarters of the House of Rothschild where the alliance was struck with Mayer Amschel Rothschild and Weishaupt. Rothschild arranged for Frank to be given the title of Baron and he became a wealthy nobleman with a big following of Jews in Germany, the Austro-Hungarian Empire and other European countries. Most of them would have believed he was on their side.

The name 'Illuminati' came from the Zohar which is a body of works in the Jewish mystical 'bible' called the Kabbalah. 'Zohar' is the foundation of Sabbatian-Frankist belief and in Hebrew 'Zohar' means 'splendour', 'radiance', 'illuminated', and so we have 'Illuminati'. They claim to be the 'Illuminated Ones' from their knowledge systematically hidden from the human population and passed on through generations of carefully-chosen initiates in the global secret society network or Cult. Hidden knowledge includes an awareness of the Cult agenda for the world and the nature of our collective reality that I will explore later. Cult 'illumination' is symbolised by the torch held by the Statue of Liberty which was gifted to New York by French Freemasons in Paris who knew exactly what it represents. 'Liberty' symbolises the goddess worshipped in

Babylon as Queen Semiramis or Ishtar. The significance of this will become clear. Notice again the ubiquitous theme of inversion with the Statue of 'Liberty' really symbolising mass control (Fig 7). A mirror-image statute stands on an island in the River Seine in Paris from where New York Liberty originated (Fig 8). A large replica of the Liberty flame stands on top of the Pont de l'Alma tunnel in Paris where Princess Diana died in a Cult ritual described in *The Biggest Secret*. Lucifer 'the light bringer' is related to all this (and much more as we'll see) and 'Lucifer' is a central figure in Sabbatian-Frankism and its associated Satanism. Sabbatians reject the Jewish Torah, or Pentateuch, the 'five books of Moses' in the Old Testament known as Genesis, Exodus, Leviticus, Numbers, and Deuteronomy which are claimed by Judaism and Christianity to have been dictated by 'God' to Moses on Mount Sinai. Sabbatians say these do not apply to them and they seek to replace them with the Zohar to absorb Judaism and its followers into their inversion which is an expression of a much greater global inversion. They want to delete all religions and force humanity to worship a one-world religion – Sabbatian Satanism that also includes worship of the Earth goddess. Satanic themes are being more and more introduced into mainstream society and while Christianity is currently the foremost target for destruction the others are planned to follow.

Figure 7: The Cult goddess of Babylon disguised as the Statue of Liberty holding the flame of Lucifer the 'light bringer'.

Figure 8: Liberty's mirror image in Paris where the New York version originated.

Marx brothers

Rabbi Marvin Antelman connects the Illuminati to the Jacobins in *To Eliminate the Opiate* and Jacobins were the force behind the French Revolution. He links both to the Bund der Gerechten, or League of the Just, which was the network that inflicted communism/Marxism on the world. Antelman wrote:

> The original inner circle of the Bund der Gerechten consisted of born Catholics, Protestants and Jews [Sabbatian-Frankist infiltrators], and those representatives of respective subdivisions formulated schemes for the ultimate destruction of their faiths. The heretical Catholics laid plans which they felt would take a century or more for the ultimate destruction of the church; the apostate Jews for the ultimate destruction of the Jewish religion.

Sabbatian-created communism connects into this anti-religion agenda in that communism does not allow for the free practice of religion. The Sabbatian 'Bund' became the International Communist Party and Communist League and in 1848 'Marxism' was born with the Communist Manifesto of Sabbatian assets Karl Marx and Friedrich Engels. It is absolutely no coincidence that Marxism, just a different name for fascist and other centrally-controlled tyrannies, is being imposed worldwide as a result of the 'Covid' hoax and nor that Marxist/fascist China was the place where the hoax originated. The reason for this will become very clear in the chapter 'Covid: The calculated catastrophe'. The so-called 'Woke' mentality has hijacked

traditional beliefs of the political left and replaced them with far-right make-believe 'social justice' better known as Marxism. Woke will, however, be swallowed by its own perceived 'revolution' which is really the work of billionaires and billionaire corporations feigning being 'Woke'. Marxism is being touted by Wokers as a replacement for 'capitalism' when we don't have 'capitalism'. We have cartelism in which the market is stitched up by the very Cult billionaires and corporations bankrolling Woke. Billionaires love Marxism which keeps the people in servitude while they control from the top. Terminally naïve Wokers think they are 'changing the world' when it's the Cult that is doing the changing and when they have played their vital part and become surplus to requirements they, too, will be targeted. The Illuminati-Jacobins were behind the period known as 'The Terror' in the French Revolution in 1793 and 1794 when Jacobin Maximillian de Robespierre and his Orwellian 'Committee of Public Safety' killed 17,000 'enemies of the Revolution' who had once been 'friends of the Revolution'. Karl Marx (1818-1883), whose Sabbatian creed of Marxism has cost the lives of at least 100 million people, is a hero once again to Wokers who have been systematically kept ignorant of real history by their 'education' programming. As a result they now promote a Sabbatian 'Marxist' abomination destined at some point to consume them. Rabbi Antelman, who spent decades researching the Sabbatian plot, said of the League of the Just and Karl Marx:

> Contrary to popular opinion Karl Marx did not originate the Communist Manifesto. He was paid for his services by the League of the Just, which was known in its country of origin, Germany, as the Bund der Geaechteten.

Antelman said the text attributed to Marx was the work of other people and Marx 'was only repeating what others already said'. Marx was 'a hired hack – lackey of the wealthy Illuminists'. Marx famously said that religion was the 'opium of the people' (part of the Sabbatian plan to demonise religion) and Antelman called his books, *To Eliminate the Opiate*. Marx was born Jewish, but his family converted to Christianity (Sabbatian modus operandi) and he

attacked Jews, not least in his book, *A World Without Jews*. In doing so he supported the Sabbatian plan to destroy traditional Jewishness and Judaism which we are clearly seeing today with the vindictive targeting of orthodox Jews by the Sabbatian government of Israel over 'Covid' laws. I don't follow any religion and it has done much damage to the world over centuries and acted as a perceptual straightjacket. Renegade Minds, however, are always asking *why* something is being done. It doesn't matter if they agree or disagree with what is happening – *why* is it happening is the question. The 'why?' can be answered with regard to religion in that religions create interacting communities of believers when the Cult wants to dismantle all discourse, unity and interaction (see 'Covid' lockdowns) and the ultimate goal is to delete all religions for a one-world religion of Cult Satanism worshipping their 'god' of which more later. We see the same 'why?' with gun control in America. I don't have guns and don't want them, but why is the Cult seeking to disarm the population at the same time that law enforcement agencies are armed to their molars and why has every tyrant in history sought to disarm people before launching the final takeover? They include Hitler, Stalin, Pol Pot and Mao who followed confiscation with violent seizing of power. You know it's a Cult agenda by the people who immediately race to the microphones to exploit dead people in multiple shootings. Ultra-Zionist Cult lackey Senator Chuck Schumer was straight on the case after ten people were killed in Boulder, Colorado in March, 2121. Simple rule … if Schumer wants it the Cult wants it and the same with his ultra-Zionist mate the wild-eyed Senator Adam Schiff. At the same time they were calling for the disarmament of Americans, many of whom live a long way from a police response, Schumer, Schiff and the rest of these pampered clowns were sitting on Capitol Hill behind a razor-wired security fence protected by thousands of armed troops in addition to their own armed bodyguards. Mom and pop in an isolated home? They're just potential mass shooters.

Zion Mainframe

Sabbatian-Frankists and most importantly the Rothschilds were behind the creation of 'Zionism', a political movement that demanded a Jewish homeland in Israel as promised by Sabbatai Zevi. The very symbol of Israel comes from the German meaning of the name Rothschild. Dynasty founder Mayer Amschel Rothschild changed the family name from Bauer to Rothschild, or 'Red-Shield' in German, in deference to the six-pointed 'Star of David' hexagram displayed on the family's home in Frankfurt. The symbol later appeared on the flag of Israel after the Rothschilds were centrally involved in its creation. Hexagrams are not a uniquely Jewish symbol and are widely used in occult ('hidden') networks often as a symbol for Saturn (see my other books for why). Neither are Zionism and Jewishness interchangeable. Zionism is a political movement and philosophy and not a 'race' or a people. Many Jews oppose Zionism and many non-Jews, including US President Joe Biden, call themselves Zionists as does Israel-centric Donald Trump. America's support for the Israel government is pretty much a gimme with ultra-Zionist billionaires and corporations providing fantastic and dominant funding for both political parties. Former Congresswoman Cynthia McKinney has told how she was approached immediately she ran for office to 'sign the pledge' to Israel and confirm that she would always vote in that country's best interests. All American politicians are approached in this way. Anyone who refuses will get no support or funding from the enormous and all-powerful Zionist lobby that includes organisations like mega-lobby group AIPAC, the American Israel Public Affairs Committee. Trump's biggest funder was ultra-Zionist casino and media billionaire Sheldon Adelson while major funders of the Democratic Party include ultra-Zionist George Soros and ultra-Zionist financial and media mogul, Haim Saban. Some may reel back at the suggestion that Soros is an Israel-firster (Sabbatian-controlled Israel-firster), but Renegade Minds watch the actions not the words and everywhere Soros donates his billions the Sabbatian agenda benefits. In the spirit of Sabbatian inversion Soros pledged $1 billion for a new university network to promote 'liberal values and tackle intolerance'. He made the announcement during his annual speech

at the Cult-owned World Economic Forum in Davos, Switzerland, in January, 2020, after his 'harsh criticism' of 'authoritarian rulers' around the world. You can only laugh at such brazen mendacity. How *he* doesn't laugh is the mystery. Translated from the Orwellian 'liberal values and tackle intolerance' means teaching non-white people to hate white people and for white people to loathe themselves for being born white. The reason for that will become clear.

The 'Anti-Semitism' fraud

Zionists support the Jewish homeland in the land of Palestine which has been the Sabbatian-Rothschild goal for so long, but not for the benefit of Jews. Sabbatians and their global Anti-Semitism Industry have skewed public and political opinion to equate opposing the violent extremes of Zionism to be a blanket attack and condemnation of all Jewish people. Sabbatians and their global Anti-Semitism Industry have skewed public and political opinion to equate opposing the violent extremes of Zionism to be a blanket attack and condemnation of all Jewish people. This is nothing more than a Sabbatian protection racket to stop legitimate investigation and exposure of their agendas and activities. The official definition of 'anti-Semitism' has more recently been expanded to include criticism of Zionism – a *political movement* – and this was done to further stop exposure of Sabbatian infiltrators who created Zionism as we know it today in the 19th century. Renegade Minds will talk about these subjects when they know the shit that will come their way. People must decide if they want to know the truth or just cower in the corner in fear of what others will say. Sabbatians have been trying to label me as 'anti-Semitic' since the 1990s as I have uncovered more and more about their background and agendas. Useless, gutless, fraudulent 'journalists' then just repeat the smears without question and on the day I was writing this section a pair of unquestioning repeaters called Ben Quinn and Archie Bland (how appropriate) outright called me an 'anti-Semite' in the establishment propaganda sheet, the London *Guardian*, with no supporting evidence. The

Sabbatian Anti-Semitism Industry said so and who are they to question that? They wouldn't dare. Ironically 'Semitic' refers to a group of languages in the Middle East that are almost entirely Arabic. 'Anti-Semitism' becomes 'anti-Arab' which if the consequences of this misunderstanding were not so grave would be hilarious. Don't bother telling Quinn and Bland. I don't want to confuse them, bless 'em. One reason I am dubbed 'anti-Semitic' is that I wrote in the 1990s that Jewish operatives (Sabbatians) were heavily involved in the Russian Revolution when Sabbatians overthrew the Romanov dynasty. This apparently made me 'anti-Semitic'. Oh, really? Here is a section from *The Trigger*:

British journalist Robert Wilton confirmed these themes in his 1920 book *The Last Days of the Romanovs* when he studied official documents from the Russian government to identify the members of the Bolshevik ruling elite between 1917 and 1919. The Central Committee included 41 Jews among 62 members; the Council of the People's Commissars had 17 Jews out of 22 members; and 458 of the 556 most important Bolshevik positions between 1918 and 1919 were occupied by Jewish people. Only 17 were Russian. Then there were the 23 Jews among the 36 members of the vicious Cheka Soviet secret police established in 1917 who would soon appear all across the country.

Professor Robert Service of Oxford University, an expert on 20th century Russian history, found evidence that ['Jewish'] Leon Trotsky had sought to make sure that Jews were enrolled in the Red Army and were disproportionately represented in the Soviet civil bureaucracy that included the Cheka which performed mass arrests, imprisonment and executions of 'enemies of the people'. A US State Department Decimal File (861.00/5339) dated November 13th, 1918, names [Rothschild banking agent in America] Jacob Schiff and a list of ultra-Zionists as funders of the Russian Revolution leading to claims of a 'Jewish plot', but the key point missed by all is they were not 'Jews' – they were Sabbatian-Frankists.

Britain's Winston Churchill made the same error by mistake or otherwise. He wrote in a 1920 edition of the *Illustrated Sunday Herald* that those behind the Russian revolution were part of a 'worldwide conspiracy for the overthrow of civilisation and for the reconstitution of society on the basis of arrested development, of envious malevolence, and impossible equality' (see 'Woke' today because that has been created by the same network). Churchill said there was no need to exaggerate the part played in the creation of Bolshevism and in the actual bringing about of the Russian

Revolution 'by these international and for the most part atheistical Jews' ['atheistical Jews' = Sabbatians]. Churchill said it is certainly a very great one and probably outweighs all others: 'With the notable exception of Lenin, the majority of the leading figures are Jews.' He went on to describe, knowingly or not, the Sabbatian modus operandi of placing puppet leaders nominally in power while they control from the background:

> Moreover, the principal inspiration and driving power comes from the Jewish leaders. Thus Tchitcherin, a pure Russian, is eclipsed by his nominal subordinate, Litvinoff, and the influence of Russians like Bukharin or Lunacharski cannot be compared with the power of Trotsky, or of Zinovieff, the Dictator of the Red Citadel (Petrograd), or of Krassin or Radek – all Jews. In the Soviet institutions the predominance of Jews is even more astonishing. And the prominent, if not indeed the principal, part in the system of terrorism applied by the Extraordinary Commissions for Combatting Counter-Revolution has been taken by Jews, and in some notable cases by Jewesses.

What I said about seriously disproportionate involvement in the Russian Revolution by Jewish 'revolutionaries' (Sabbatians) is provable fact, but truth is no defence against the Sabbatian Anti-Semitism Industry, its repeater parrots like Quinn and Bland, and the now breathtaking network of so-called 'Woke' 'anti-hate' groups with interlocking leaderships and funding which have the role of discrediting and silencing anyone who gets too close to exposing the Sabbatians. We have seen 'truth is no defence' confirmed in legal judgements with the Saskatchewan Human Rights Commission in Canada decreeing this: 'Truthful statements can be presented in a manner that would meet the definition of hate speech, and not all truthful statements must be free from restriction.' Most 'anti-hate' activists, who are themselves consumed by hatred, are too stupid and ignorant of the world to know how they are being used. They are far too far up their own virtue-signalling arses and it's far too dark for them to see anything.

The 'revolution' game

The background and methods of the 'Russian' Revolution are straight from the Sabbatian playbook seen in the French Revolution

and endless others around the world that appear to start as a revolution of the people against tyrannical rule and end up with a regime change to more tyrannical rule overtly or covertly. Wars, terror attacks and regime overthrows follow the Sabbatian cult through history with its agents creating them as Problem-Reaction-Solutions to remove opposition on the road to world domination. Sabbatian dots connect the Rothschilds with the Illuminati, Jacobins of the French Revolution, the 'Bund' or League of the Just, the International Communist Party, Communist League and the Communist Manifesto of Karl Marx and Friedrich Engels that would lead to the Rothschild-funded Russian Revolution. The sequence comes under the heading of 'creative destruction' when you advance to your global goal by continually destroying the status quo to install a new status quo which you then also destroy. The two world wars come to mind. With each new status quo you move closer to your planned outcome. Wars and mass murder are to Sabbatians a collective blood sacrifice ritual. They are obsessed with death for many reasons and one is that death is an inversion of life. Satanists and Sabbatians are obsessed with death and often target churches and churchyards for their rituals. Inversion-obsessed Sabbatians explain the use of inverted symbolism including the *inverted* pentagram and *inverted* cross. The inversion of the cross has been related to targeting Christianity, but the cross was a religious symbol long before Christianity and its inversion is a statement about the Sabbatian mentality and goals more than any single religion.

Sabbatians operating in Germany were behind the rise of the occult-obsessed Nazis and the subsequent Jewish exodus from Germany and Europe to Palestine and the United States after World War Two. The Rothschild dynasty was at the forefront of this both as political manipulators and by funding the operation. Why would Sabbatians help to orchestrate the horrors inflicted on Jews by the Nazis and by Stalin after they organised the Russian Revolution? Sabbatians hate Jews and their religion, that's why. They pose as Jews and secure positions of control within Jewish society and play the 'anti-Semitism' card to protect themselves from exposure

through a global network of organisations answering to the Sabbatian-created-and-controlled globe-spanning intelligence network that involves a stunning web of military-intelligence operatives and operations for a tiny country of just nine million. Among them are Jewish assets who are not Sabbatians but have been convinced by them that what they are doing is for the good of Israel and the Jewish community to protect them from what they have been programmed since childhood to believe is a Jew-hating hostile world. The Jewish community is just a highly convenient cover to hide the true nature of Sabbatians. Anyone getting close to exposing their game is accused by Sabbatian place-people and gofers of 'anti-Semitism' and claiming that all Jews are part of a plot to take over the world. I am not saying that. I am saying that Sabbatians – the *real* Jew-haters – have infiltrated the Jewish community to use them both as a cover and an 'anti-Semitic' defence against exposure. Thus we have the Anti-Semitism Industry targeted researchers in this way and most Jewish people think this is justified and genuine. They don't know that their 'Jewish' leaders and institutions of state, intelligence and military are not controlled by Jews at all, but cultists and stooges of Sabbatian-Frankism. I once added my name to a pro-Jewish freedom petition online and the next time I looked my name was gone and text had been added to the petition blurb to attack me as an 'anti-Semite' such is the scale of perceptual programming.

Moving on America

I tell the story in *The Trigger* and a chapter called 'Atlantic Crossing' how particularly after Israel was established the Sabbatians moved in on the United States and eventually grasped control of government administration, the political system via both Democrats and Republicans, the intelligence community like the CIA and National Security Agency (NSA), the Pentagon and mass media. Through this seriously compartmentalised network Sabbatians and their operatives in Mossad, Israeli Defense Forces (IDF) and US agencies pulled off 9/11 and blamed it on 19 'Al-Qaeda hijackers' dominated by men from, or connected to, Sabbatian-ruled Saudi

Arabia. The '19' were not even on the planes let alone flew those big passenger jets into buildings while being largely incompetent at piloting one-engine light aircraft. 'Hijacker' Hani Hanjour who is said to have flown American Airlines Flight 77 into the Pentagon with a turn and manoeuvre most professional pilots said they would have struggled to do was banned from renting a small plane by instructors at the Freeway Airport in Bowie, Maryland, just *six weeks* earlier on the grounds that he was an incompetent pilot. The Jewish population of the world is just 0.2 percent with even that almost entirely concentrated in Israel (75 percent Jewish) and the United States (around two percent). This two percent and globally 0.2 percent refers to *Jewish* people and not Sabbatian interlopers who are a fraction of that fraction. What a sobering thought when you think of the fantastic influence on world affairs of tiny Israel and that the Project for the New America Century (PNAC) which laid out the blueprint in September, 2000, for America's war on terror and regime change wars in Iraq, Libya and Syria was founded and dominated by Sabbatians known as 'Neocons'. The document conceded that this plan would not be supported politically or publicly without a major attack on American soil and a Problem-Reaction-Solution excuse to send troops to war across the Middle East. Sabbatian Neocons said:

> ... [The] process of transformation ... [war and regime change] ... is likely to be a long one, absent some catastrophic and catalysing event – like a new Pearl Harbor.

Four months later many of those who produced that document came to power with their inane puppet George Bush from the long-time Sabbatian Bush family. They included Sabbatian Dick Cheney who was officially vice-president, but really de-facto president for the entirety of the 'Bush' government. Nine months after the 'Bush' inauguration came what Bush called at the time 'the Pearl Harbor of the 21st century' and with typical Sabbatian timing and symbolism 2001 was the 60th anniversary of the attack in 1941 by the Japanese Air Force on Pearl Harbor, Hawaii, which allowed President Franklin Delano Roosevelt to take the United States into a Sabbatian-

instigated Second World War that he said in his election campaign that he never would. The evidence is overwhelming that Roosevelt and his military and intelligence networks knew the attack was coming and did nothing to stop it, but they did make sure that America's most essential naval ships were not in Hawaii at the time. Three thousand Americans died in the Pearl Harbor attacks as they did on September 11th. By the 9/11 year of 2001 Sabbatians had widely infiltrated the US government, military and intelligence operations and used their compartmentalised assets to pull off the 'Al-Qaeda' attacks. If you read *The Trigger* it will blow your mind to see the utterly staggering concentration of 'Jewish' operatives (Sabbatian infiltrators) in essential positions of political, security, legal, law enforcement, financial and business power before, during, and after the attacks to make them happen, carry them out, and then cover their tracks – and I do mean *staggering* when you think of that 0.2 percent of the world population and two percent of Americans which are Jewish while Sabbatian infiltrators are a fraction of that. A central foundation of the 9/11 conspiracy was the hijacking of government, military, Air Force and intelligence computer systems in real time through 'back-door' access made possible by Israeli (Sabbatian) 'cyber security' software. Sabbatian-controlled Israel is on the way to rivalling Silicon Valley for domination of cyberspace and is becoming the dominant force in cyber-security which gives them access to entire computer systems and their passcodes across the world. Then add to this that Zionists head (officially) Silicon Valley giants like Google (Larry Page and Sergey Brin), Google-owned YouTube (Susan Wojcicki), Facebook (Mark Zuckerberg and Sheryl Sandberg), and Apple (Chairman Arthur D. Levinson), and that ultra-Zionist hedge fund billionaire Paul Singer has a $1 billion stake in Twitter which is only nominally headed by 'CEO' pothead Jack Dorsey. As cable news host Tucker Carlson said of Dorsey: 'There used to be debate in the medical community whether dropping a ton of acid had permanent effects and I think that debate has now ended.' Carlson made the comment after Dorsey told a hearing on Capitol Hill (if you cut through his bullshit) that he

believed in free speech so long as he got to decide what you can hear and see. These 'big names' of Silicon Valley are only front men and women for the Global Cult, not least the Sabbatians, who are the true controllers of these corporations. Does anyone still wonder why these same people and companies have been ferociously censoring and banning people (like me) for exposing any aspect of the Cult agenda and especially the truth about the 'Covid' hoax which Sabbatians have orchestrated?

The Jeffrey Epstein paedophile ring was a Sabbatian operation. He was officially 'Jewish' but he was a Sabbatian and women abused by the ring have told me about the high number of 'Jewish' people involved. The Epstein horror has Sabbatian written all over it and matches perfectly their modus operandi and obsession with sex and ritual. Epstein was running a Sabbatian blackmail ring in which famous people with political and other influence were provided with young girls for sex while everything was being filmed and recorded on hidden cameras and microphones at his New York house, Caribbean island and other properties. Epstein survivors have described this surveillance system to me and some have gone public. Once the famous politician or other figure knew he or she was on video they tended to do whatever they were told. Here we go again …when you've got them by the balls their hearts and minds will follow. Sabbatians use this blackmail technique on a wide scale across the world to entrap politicians and others they need to act as demanded. Epstein's private plane, the infamous 'Lolita Express', had many well-known passengers including Bill Clinton while Bill Gates has flown on an Epstein plane and met with him four years after Epstein had been jailed for paedophilia. They subsequently met many times at Epstein's home in New York according to a witness who was there. Epstein's infamous side-kick was Ghislaine Maxwell, daughter of Mossad agent and ultra-Zionist mega-crooked British businessman, Bob Maxwell, who at one time owned the *Daily Mirror* newspaper. Maxwell was murdered at sea on his boat in 1991 by Sabbatian-controlled Mossad when he became a liability with his

business empire collapsing as a former Mossad operative has confirmed (see *The Trigger*).

Money, money, money, funny money …

Before I come to the Sabbatian connection with the last three US presidents I will lay out the crucial importance to Sabbatians of controlling banking and finance. Sabbatian Mayer Amschel Rothschild set out to dominate this arena in his family's quest for total global control. What is freedom? It is, in effect, choice. The more choices you have the freer you are and the fewer your choices the more you are enslaved. In the global structure created over centuries by Sabbatians the biggest decider and restrictor of choice is … money. Across the world if you ask people what they would like to do with their lives and why they are not doing that they will reply 'I don't have the money'. This is the idea. A global elite of multi-billionaires are described as 'greedy' and that is true on one level; but control of money – who has it and who doesn't – is not primarily about greed. It's about control. Sabbatians have seized ever more control of finance and sucked the wealth of the world out of the hands of the population. We talk now, after all, about the 'One-percent' and even then the wealthiest are a lot fewer even than that. This has been made possible by a money scam so outrageous and so vast it could rightly be called the scam of scams founded on creating 'money' out of nothing and 'loaning' that with interest to the population. Money out of nothing is called 'credit'. Sabbatians have asserted control over governments and banking ever more completely through the centuries and secured financial laws that allow banks to lend hugely more than they have on deposit in a confidence trick known as fractional reserve lending. Imagine if you could lend money that doesn't exist and charge the recipient interest for doing so. You would end up in jail. Bankers by contrast end up in mansions, private jets, Malibu and Monaco.

Banks are only required to keep a fraction of their deposits and wealth in their vaults and they are allowed to lend 'money' they don't have called 'credit. Go into a bank for a loan and if you succeed

the banker will not move any real wealth into your account. They will type into your account the amount of the agreed 'loan' – say £100,000. This is not wealth that really exists; it is non-existent, fresh-air, created-out-of-nothing 'credit' which has never, does not, and will never exist except in theory. Credit is backed by nothing except wind and only has buying power because people think that it has buying power and accept it in return for property, goods and services. I have described this situation as like those cartoon characters you see chasing each other and when they run over the edge of a cliff they keep running forward on fresh air until one of them looks down, realises what's happened, and they all crash into the ravine. The whole foundation of the Sabbatian financial system is to stop people looking down except for periodic moments when they want to crash the system (as in 2008 and 2020 ongoing) and reap the rewards from all the property, businesses and wealth their borrowers had signed over as 'collateral' in return for a 'loan' of fresh air. Most people think that money is somehow created by governments when it comes into existence from the start as a debt through banks 'lending' illusory money called credit. Yes, the very currency of exchange is a *debt* from day one issued as an interest-bearing loan. Why don't governments create money interest-free and lend it to their people interest-free? Governments are controlled by Sabbatians and the financial system is controlled by Sabbatians for whom interest-free money would be a nightmare come true. Sabbatians underpin their financial domination through their global network of central banks, including the privately-owned US Federal Reserve and Britain's Bank of England, and this is orchestrated by a privately-owned central bank coordination body called the Bank for International Settlements in Basle, Switzerland, created by the usual suspects including the Rockefellers and Rothschilds. Central bank chiefs don't answer to governments or the people. They answer to the Bank for International Settlements or, in other words, the Global Cult which is dominated today by Sabbatians.

Built-in disaster

There are so many constituent scams within the overall banking scam. When you take out a loan of thin-air credit only the amount of that loan is theoretically brought into circulation to add to the amount in circulation; but you are paying back the principle plus interest. The additional interest is not created and this means that with every 'loan' there is a shortfall in the money in circulation between what is borrowed and what has to be paid back. There is never even close to enough money in circulation to repay all outstanding public and private debt including interest. Coldly weaved in the very fabric of the system is the certainty that some will lose their homes, businesses and possessions to the banking 'lender'. This is less obvious in times of 'boom' when the amount of money in circulation (and the debt) is expanding through more people wanting and getting loans. When a downturn comes and the money supply contracts it becomes painfully obvious that there is not enough money to service all debt and interest. This is less obvious in times of 'boom' when the amount of money in circulation (and the debt) is expanding through more people wanting and getting loans. When a downturn comes and the money supply contracts and it becomes painfully obvious – as in 2008 and currently – that there is not enough money to service all debt and interest. Sabbatian banksters have been leading the human population through a calculated series of booms (more debt incurred) and busts (when the debt can't be repaid and the banks get the debtor's tangible wealth in exchange for non-existent 'credit'). With each 'bust' Sabbatian bankers have absorbed more of the world's tangible wealth and we end up with the One-percent. Governments are in bankruptcy levels of debt to the same system and are therefore owned by a system they do not control. The Federal Reserve, 'America's central bank', is privately-owned and American presidents only nominally appoint its chairman or woman to maintain the illusion that it's an arm of government. It's not. The 'Fed' is a cartel of private banks which handed billions to its associates and friends after the crash of 2008 and has been Sabbatian-controlled since it was manipulated into being in 1913 through the covert trickery of Rothschild banking agents Jacob Schiff and Paul

Warburg, and the Sabbatian Rockefeller family. Somehow from a Jewish population of two-percent and globally 0.2 percent (Sabbatian interlopers remember are far smaller) ultra-Zionists headed the Federal Reserve for 31 years between 1987 and 2018 in the form of Alan Greenspan, Bernard Bernanke and Janet Yellen (now Biden's Treasury Secretary) with Yellen's deputy chairman a Israeli-American duel citizen and ultra-Zionist Stanley Fischer, a former governor of the Bank of Israel. Ultra-Zionist Fed chiefs spanned the presidencies of Ronald Reagan ('Republican'), Father George Bush ('Republican'), Bill Clinton ('Democrat'), Boy George Bush ('Republican') and Barack Obama ('Democrat'). We should really add the pre-Greenspan chairman, Paul Adolph Volcker, 'appointed' by Jimmy Carter ('Democrat') who ran the Fed between 1979 and 1987 during the Carter and Reagan administrations before Greenspan took over. Volcker was a long-time associate and business partner of the Rothschilds. No matter what the 'party' officially in power the United States economy was directed by the same force. Here are members of the Obama, Trump and Biden administrations and see if you can make out a common theme.

Barack Obama ('Democrat')

Ultra-Zionists Robert Rubin, Larry Summers, and Timothy Geithner ran the US Treasury in the Clinton administration and two of them reappeared with Obama. Ultra-Zionist Fed chairman Alan Greenspan had manipulated the crash of 2008 through deregulation and jumped ship just before the disaster to make way for ultra-Zionist Bernard Bernanke to hand out trillions to Sabbatian 'too big to fail' banks and businesses, including the ubiquitous ultra-Zionist Goldman Sachs which has an ongoing staff revolving door operation between itself and major financial positions in government worldwide. Obama inherited the fallout of the crash when he took office in January, 2009, and fortunately he had the support of his ultra-Zionist White House Chief of Staff Rahm Emmanuel, son of a terrorist who helped to bomb Israel into being in 1948, and his ultra-Zionist senior adviser David Axelrod, chief strategist in Obama's two

successful presidential campaigns. Emmanuel, later mayor of Chicago and former senior fundraiser and strategist for Bill Clinton, is an example of the Sabbatian policy after Israel was established of migrating insider families to America so their children would be born American citizens. 'Obama' chose this financial team throughout his administration to respond to the Sabbatian-instigated crisis:

Timothy Geithner (ultra-Zionist) Treasury Secretary; Jacob J. Lew, Treasury Secretary; Larry Summers (ultra-Zionist), director of the White House National Economic Council; Paul Adolph Volcker (Rothschild business partner), chairman of the Economic Recovery Advisory Board; Peter Orszag (ultra-Zionist), director of the Office of Management and Budget overseeing all government spending; Penny Pritzker (ultra-Zionist), Commerce Secretary; Jared Bernstein (ultra-Zionist), chief economist and economic policy adviser to Vice President Joe Biden; Mary Schapiro (ultra-Zionist), chair of the Securities and Exchange Commission (SEC); Gary Gensler (ultra-Zionist), chairman of the Commodity Futures Trading Commission (CFTC); Sheila Bair (ultra-Zionist), chair of the Federal Deposit Insurance Corporation (FDIC); Karen Mills (ultra-Zionist), head of the Small Business Administration (SBA); Kenneth Feinberg (ultra-Zionist), Special Master for Executive [bail-out] Compensation. Feinberg would be appointed to oversee compensation (with strings) to 9/11 victims and families in a campaign to stop them having their day in court to question the official story. At the same time ultra-Zionist Bernard Bernanke was chairman of the Federal Reserve and these are only some of the ultra-Zionists with allegiance to Sabbatian-controlled Israel in the Obama government. Obama's biggest corporate donor was ultra-Zionist Goldman Sachs which had employed many in his administration.

Donald Trump ('Republican')

Trump claimed to be an outsider (he wasn't) who had come to 'drain the swamp'. He embarked on this goal by immediately appointing ultra-Zionist Steve Mnuchin, a Goldman Sachs employee for 17

years, as his Treasury Secretary. Others included Gary Cohn (ultra-Zionist), chief operating officer of Goldman Sachs, his first Director of the National Economic Council and chief economic adviser, who was later replaced by Larry Kudlow (ultra-Zionist). Trump's senior adviser throughout his four years in the White House was his sinister son-in-law Jared Kushner, a life-long friend of Israel Prime Minister Benjamin Netanyahu. Kushner is the son of a convicted crook who was pardoned by Trump in his last days in office. Other ultra-Zionists in the Trump administration included: Stephen Miller, Senior Policy Adviser; Avrahm Berkowitz, Deputy Adviser to Trump and his Senior Adviser Jared Kushner; Ivanka Trump, Adviser to the President, who converted to Judaism when she married Jared Kushner; David Friedman, Trump lawyer and Ambassador to Israel; Jason Greenblatt, Trump Organization executive vice president and chief legal officer, who was made Special Representative for International Negotiations and the Israeli-Palestinian Conflict; Rod Rosenstein, Deputy Attorney General; Elliot Abrams, Special Representative for Venezuela, then Iran; John Eisenberg, National Security Council Legal Adviser and Deputy Council to the President for National Security Affairs; Anne Neuberger, Deputy National Manager, National Security Agency; Ezra Cohen-Watnick, Acting Under Secretary of Defense for Intelligence; Elan Carr, Special Envoy to monitor and combat anti-Semitism; Len Khodorkovsky, Deputy Special Envoy to monitor and combat anti-Semitism; Reed Cordish, Assistant to the President, Intragovernmental and Technology Initiatives. Trump Vice President Mike Pence and Secretary of State Mike Pompeo, both Christian Zionists, were also vehement supporters of Israel and its goals and ambitions.

Donald 'free-speech believer' Trump pardoned a number of financial and violent criminals while ignoring calls to pardon Julian Assange and Edward Snowden whose crimes are revealing highly relevant information about government manipulation and corruption and the widespread illegal surveillance of the American people by US 'security' agencies. It's so good to know that Trump is on the side of freedom and justice and not mega-criminals with

allegiance to Sabbatian-controlled Israel. These included a pardon for Israeli spy Jonathan Pollard who was jailed for life in 1987 under the Espionage Act. Aviem Sella, the Mossad agent who recruited Pollard, was also pardoned by Trump while Assange sat in jail and Snowden remained in exile in Russia. Sella had 'fled' (was helped to escape) to Israel in 1987 and was never extradited despite being charged under the Espionage Act. A Trump White House statement said that Sella's clemency had been 'supported by Benjamin Netanyahu, Ron Dermer, Israel's US Ambassador, David Friedman, US Ambassador to Israel and Miriam Adelson, wife of leading Trump donor Sheldon Adelson who died shortly before. Other friends of Jared Kushner were pardoned along with Sholom Weiss who was believed to be serving the longest-ever white-collar prison sentence of more than 800 years in 2000. The sentence was commuted of Ponzi-schemer Eliyahu Weinstein who defrauded Jews and others out of $200 million. I did mention that Assange and Snowden were ignored, right? Trump gave Sabbatians almost everything they asked for in military and political support, moving the US Embassy from Tel Aviv to Jerusalem with its critical symbolic and literal implications for Palestinian statehood, and the 'deal of the Century' designed by Jared Kushner and David Friedman which gave the Sabbatian Israeli government the green light to substantially expand its already widespread program of building illegal Jewish-only settlements in the occupied land of the West Bank. This made a two-state 'solution' impossible by seizing all the land of a potential Palestinian homeland and that had been the plan since 1948 and then 1967 when the Arab-controlled Gaza Strip, West Bank, Sinai Peninsula and Syrian Golan Heights were occupied by Israel. All the talks about talks and road maps and delays have been buying time until the West Bank was physically occupied by Israeli real estate. Trump would have to be a monumentally ill-informed idiot not to see that this was the plan he was helping to complete. The Trump administration was in so many ways the Kushner administration which means the Netanyahu administration which means the Sabbatian administration. I understand why many opposing Cult fascism in all its forms gravitated to Trump, but he

was a crucial part of the Sabbatian plan and I will deal with this in the next chapter.

Joe Biden ('Democrat')

A barely cognitive Joe Biden took over the presidency in January, 2021, along with his fellow empty shell, Vice-President Kamala Harris, as the latest Sabbatian gofers to enter the White House. Names on the door may have changed and the 'party' – the force behind them remained the same as Zionists were appointed to a stream of pivotal areas relating to Sabbatian plans and policy. They included: Janet Yellen, Treasury Secretary, former head of the Federal Reserve, and still another ultra-Zionist running the US Treasury after Mnuchin (Trump), Lew and Geithner (Obama), and Summers and Rubin (Clinton); Anthony Blinken, Secretary of State; Wendy Sherman, Deputy Secretary of State (so that's 'Biden's' Sabbatian foreign policy sorted); Jeff Zients, White House coronavirus coordinator; Rochelle Walensky, head of the Centers for Disease Control; Rachel Levine, transgender deputy health secretary (that's 'Covid' hoax policy under control); Merrick Garland, Attorney General; Alejandro Mayorkas, Secretary of Homeland Security; Cass Sunstein, Homeland Security with responsibility for new immigration laws; Avril Haines, Director of National Intelligence; Anne Neuberger, National Security Agency cybersecurity director (note, cybersecurity); David Cohen, CIA Deputy Director; Ronald Klain, Biden's Chief of Staff (see Rahm Emanuel); Eric Lander, a 'leading geneticist', Office of Science and Technology Policy director (see Smart Grid, synthetic biology agenda); Jessica Rosenworcel, acting head of the Federal Communications Commission (FCC) which controls Smart Grid technology policy and electromagnetic communication systems including 5G. How can it be that so many pivotal positions are held by two-percent of the American population and 0.2 percent of the world population administration after administration no matter who is the president and what is the party? It's a coincidence? Of course it's not and this is why Sabbatians have built their colossal global web of interlocking 'anti-

hate' hate groups to condemn anyone who asks these glaring questions as an 'anti-Semite'. The way that Jewish people horrifically abused in Sabbatian-backed Nazi Germany are exploited to this end is stomach-turning and disgusting beyond words.

Political fusion

Sabbatian manipulation has reversed the roles of Republicans and Democrats and the same has happened in Britain with the Conservative and Labour Parties. Republicans and Conservatives were always labelled the 'right' and Democrats and Labour the 'left', but look at the policy positions now and the Democrat-Labour 'left' has moved further to the 'right' than Republicans and Conservatives under the banner of 'Woke', the Cult-created far-right tyranny. Where once the Democrat-Labour 'left' defended free speech and human rights they now seek to delete them and as I said earlier despite the 'Covid' fascism of the Jackboot Johnson Conservative government in the UK the Labour Party of leader Keir Starmer demanded even more extreme measures. The Labour Party has been very publicly absorbed by Sabbatians after a political and media onslaught against the previous leader, the weak and inept Jeremy Corbyn, over made-up allegations of 'anti-Semitism' both by him and his party. The plan was clear with this 'anti-Semite' propaganda and what was required in response was a swift and decisive 'fuck off' from Corbyn and a statement to expose the Anti-Semitism Industry (Sabbatian) attempt to silence Labour criticism of the Israeli government (Sabbatians) and purge the party of all dissent against the extremes of ultra-Zionism (Sabbatians). Instead Corbyn and his party fell to their knees and appeased the abusers which, by definition, is impossible. Appeasing one demand leads only to a new demand to be appeased until takeover is complete. Like I say – 'fuck off' would have been a much more effective policy and I have used it myself with great effect over the years when Sabbatians are on my case which is most of the time. I consider that fact a great compliment, by the way. The outcome of the Labour Party capitulation is that we now have a Sabbatian-controlled

Conservative Party 'opposed' by a Sabbatian-controlled Labour Party in a one-party Sabbatian state that hurtles towards the extremes of tyranny (the Sabbatian cult agenda). In America the situation is the same. Labour's Keir Starmer spends his days on his knees with his tongue out pointing to Tel Aviv, or I guess now Jerusalem, while Boris Johnson has an 'anti-Semitism czar' in the form of former Labour MP John Mann who keeps Starmer company on his prayer mat.

Sabbatian influence can be seen in Jewish members of the Labour Party who have been ejected for criticism of Israel including those from families that suffered in Nazi Germany. Sabbatians despise real Jewish people and target them even more harshly because it is so much more difficult to dub them 'anti-Semitic' although in their desperation they do try.

CHAPTER THREE

The Pushbacker sting

Until you realize how easy it is for your mind to be manipulated, you remain the puppet of someone else's game
Evita Ochel

I will use the presidencies of Trump and Biden to show how the manipulation of the one-party state plays out behind the illusion of political choice across the world. No two presidencies could – on the face of it – be more different and apparently at odds in terms of direction and policy.

A Renegade Mind sees beyond the obvious and focuses on outcomes and consequences and not image, words and waffle. The Cult embarked on a campaign to divide America between those who blindly support its agenda (the mentality known as 'Woke') and those who are pushing back on where the Cult and its Sabbatians want to go. This presents infinite possibilities for dividing and ruling the population by setting them at war with each other and allows a perceptual ring fence of demonisation to encircle the Pushbackers in a modern version of the Little Big Horn in 1876 when American cavalry led by Lieutenant Colonel George Custer were drawn into a trap, surrounded and killed by Native American tribes defending their land of thousands of years from being seized by the government. In this modern version the roles are reversed and it's those defending themselves from the Sabbatian government who are surrounded and the government that's seeking to destroy them. This trap was set years ago and to explain how we must return to 2016

and the emergence of Donald Trump as a candidate to be President of the United States. He set out to overcome the best part of 20 other candidates in the Republican Party before and during the primaries and was not considered by many in those early stages to have a prayer of living in the White House. The Republican Party was said to have great reservations about Trump and yet somehow he won the nomination. When you know how American politics works – politics in general – there is no way that Trump could have become the party's candidate unless the Sabbatian-controlled 'Neocons' that run the Republican Party wanted that to happen. We saw the proof in emails and documents made public by WikiLeaks that the Democratic Party hierarchy, or Democons, systematically undermined the campaign of Bernie Sanders to make sure that Sabbatian gofer Hillary Clinton won the nomination to be their presidential candidate. If the Democons could do that then the Neocons in the Republican Party could have derailed Trump in the same way. But they didn't and at that stage I began to conclude that Trump could well be the one chosen to be president. If that was the case the 'why' was pretty clear to see – the goal of dividing America between Cult agenda-supporting Wokers and Pushbackers who gravitated to Trump because he was telling them what they wanted to hear. His constituency of support had been increasingly ignored and voiceless for decades and profoundly through the eight years of Sabbatian puppet Barack Obama. Now here was someone speaking their language of pulling back from the incessant globalisation of political and economic power, the exporting of American jobs to China and elsewhere by 'American' (Sabbatian) corporations, the deletion of free speech, and the mass immigration policies that had further devastated job opportunities for the urban working class of all races and the once American heartlands of the Midwest.

Beware the forked tongue

Those people collectively sighed with relief that at last a political leader was apparently on their side, but another trait of the Renegade Mind is that you look even harder at people telling you

what you want to hear than those who are telling you otherwise. Obviously as I said earlier people wish what they want to hear to be true and genuine and they are much more likely to believe that than someone saying what they don't want to here and don't want to be true. Sales people are taught to be skilled in eliciting by calculated questioning what their customers want to hear and repeating that back to them as their own opinion to get their targets to like and trust them. Assets of the Cult are also sales people in the sense of selling perception. To read Cult manipulation you have to play the long and expanded game and not fall for the Vaudeville show of party politics. Both American parties are vehicles for the Cult and they exploit them in different ways depending on what the agenda requires at that moment. Trump and the Republicans were used to be the focus of dividing America and isolating Pushbackers to open the way for a Biden presidency to become the most extreme in American history by advancing the full-blown Woke (Cult) agenda with the aim of destroying and silencing Pushbackers now labelled Nazi Trump supporters and white supremacists.

Sabbatians wanted Trump in office for the reasons described by ultra-Zionist Saul Alinsky (1909-1972) who was promoting the Woke philosophy through 'community organising' long before anyone had heard of it. In those days it still went by its traditional name of Marxism. The reason for the manipulated Trump phenomenon was laid out in Alinsky's 1971 book, *Rules for Radicals*, which was his blueprint for overthrowing democratic and other regimes and replacing them with Sabbatian Marxism. Not surprisingly his to-do list was evident in the Sabbatian French and Russian 'Revolutions' and that in China which will become very relevant in the next chapter about the 'Covid' hoax. Among Alinsky's followers have been the deeply corrupt Barack Obama, House Speaker Nancy Pelosi and Hillary Clinton who described him as a 'hero'. All three are Sabbatian stooges with Pelosi personifying the arrogant corrupt idiocy that so widely fronts up for the Cult inner core. Predictably as a Sabbatian advocate of the 'light-bringer' Alinsky features Lucifer on the dedication page of his book as the original radical who gained

his own kingdom ('Earth' as we shall see). One of Alinsky's golden radical rules was to pick an individual and focus all attention, hatred and blame on them and not to target faceless bureaucracies and corporations. *Rules for Radicals* is really a Sabbatian handbook with its contents repeatedly employed all over the world for centuries and why wouldn't Sabbatians bring to power their designer-villain to be used as the individual on which all attention, hatred and blame was bestowed? This is what they did and the only question for me is how much Trump knew that and how much he was manipulated. A bit of both, I suspect. This was Alinsky's Trump technique from a man who died in 1972. The technique has spanned history:

Pick the target, freeze it, personalize it, polarize it. Don't try to attack abstract corporations or bureaucracies. Identify a responsible individual. Ignore attempts to shift or spread the blame.

From the moment Trump came to illusory power everything was about him. It wasn't about Republican policy or opinion, but all about Trump. Everything he did was presented in negative, derogatory and abusive terms by the Sabbatian-dominated media led by Cult operations such as CNN, MSNBC, *The New York Times* and the Jeff Bezos-owned *Washington Post* – 'Pick the target, freeze it, personalize it, polarize it.' Trump was turned into a demon to be vilified by those who hated him and a demi-god loved by those who worshipped him. This, in turn, had his supporters, too, presented as equally demonic in preparation for the punchline later down the line when Biden was about to take office. It was here's a Trump, there's a Trump, everywhere a Trump, Trump. Virtually every news story or happening was filtered through the lens of 'The Donald'. You loved him or hated him and which one you chose was said to define you as Satan's spawn or a paragon of virtue. Even supporting some Trump policies or statements and not others was enough for an assault on your character. No shades of grey were or are allowed. Everything is black and white (literally and figuratively). A Californian I knew had her head utterly scrambled by her hatred for Trump while telling people they should love each other. She was so totally consumed by

Trump Derangement Syndrome as it became to be known that this glaring contradiction would never have occurred to her. By definition anyone who criticised Trump or praised his opponents was a hero and this lady described Joe Biden as 'a kind, honest gentleman' when he's a provable liar, mega-crook and vicious piece of work to boot. Sabbatians had indeed divided America using Trump as the fall-guy and all along the clock was ticking on the consequences for his supporters.

In hock to his masters

Trump gave Sabbatians via Israel almost everything they wanted in his four years. Ask and you shall receive was the dynamic between himself and Benjamin Netanyahu orchestrated by Trump's ultra-Zionist son-in-law Jared Kushner, his ultra-Zionist Ambassador to Israel, David Friedman, and ultra-Zionist 'Israel adviser', Jason Greenblatt. The last two were central to the running and protecting from collapse of his business empire, the Trump Organisation, and colossal business failures made him forever beholding to Sabbatian networks that bailed him out. By the start of the 1990s Trump owed $4 billion to banks that he couldn't pay and almost $1billion of that was down to him personally and not his companies. This mega-disaster was the result of building two new casinos in Atlantic City and buying the enormous Taj Mahal operation which led to crippling debt payments. He had borrowed fantastic sums from 72 banks with major Sabbatian connections and although the scale of debt should have had him living in a tent alongside the highway they never foreclosed. A plan was devised to lift Trump from the mire by BT Securities Corporation and Rothschild Inc. and the case was handled by Wilber Ross who had worked for the Rothschilds for 27 years. Ross would be named US Commerce Secretary after Trump's election. Another crucial figure in saving Trump was ultra-Zionist 'investor' Carl Icahn who bought the Taj Mahal casino. Icahn was made special economic adviser on financial regulation in the Trump administration. He didn't stay long but still managed to find time to make a tidy sum of a reported $31.3 million when he sold his

holdings affected by the price of steel three days before Trump imposed a 235 percent tariff on steel imports. What amazing bits of luck these people have. Trump and Sabbatian operatives have long had a close association and his mentor and legal adviser from the early 1970s until 1986 was the dark and genetically corrupt ultra-Zionist Roy Cohn who was chief counsel to Senator Joseph McCarthy's 'communist' witch-hunt in the 1950s. *Esquire* magazine published an article about Cohn with the headline 'Don't mess with Roy Cohn'. He was described as the most feared lawyer in New York and 'a ruthless master of dirty tricks ... [with] ... more than one Mafia Don on speed dial'. Cohn's influence, contacts, support and protection made Trump a front man for Sabbatians in New York with their connections to one of Cohn's many criminal employers, the 'Russian' Sabbatian Mafia. Israel-centric media mogul Rupert Murdoch was introduced to Trump by Cohn and they started a long friendship. Cohn died in 1986 weeks after being disbarred for unethical conduct by the Appellate Division of the New York State Supreme Court. The wheels of justice do indeed run slow given the length of Cohn's crooked career.

QAnon-sense

We are asked to believe that Donald Trump with his fundamental connections to Sabbatian networks and operatives has been leading the fight to stop the Sabbatian agenda for the fascistic control of America and the world. Sure he has. A man entrapped during his years in the White House by Sabbatian operatives and whose biggest financial donor was casino billionaire Sheldon Adelson who was Sabbatian to his DNA?? Oh, do come on. Trump has been used to divide America and isolate Pushbackers on the Cult agenda under the heading of 'Trump supporters', 'insurrectionists' and 'white supremacists'. The US Intelligence/Mossad Psyop or psychological operation known as QAnon emerged during the Trump years as a central pillar in the Sabbatian campaign to lead Pushbackers into the trap set by those that wished to destroy them. I knew from the start that QAnon was a scam because I had seen the same scenario many

times before over 30 years under different names and I had written about one in particular in the books. 'Not again' was my reaction when QAnon came to the fore. The same script is pulled out every few years and a new name added to the letterhead. The story always takes the same form: 'Insiders' or 'the good guys' in the government-intelligence-military 'Deep State' apparatus were going to instigate mass arrests of the 'bad guys' which would include the Rockefellers, Rothschilds, Barack Obama, Hillary Clinton, George Soros, etc., etc. Dates are given for when the 'good guys' are going to move in, but the dates pass without incident and new dates are given which pass without incident. The central message to Pushbackers in each case is that they don't have to do anything because there is 'a plan' and it is all going to be sorted by the 'good guys' on the inside. 'Trust the plan' was a QAnon mantra when the only plan was to misdirect Pushbackers into putting their trust in a Psyop they believed to be real. Beware, beware, those who tell you what you want to hear and always check it out. Right up to Biden's inauguration QAnon was still claiming that 'the Storm' was coming and Trump would stay on as president when Biden and his cronies were arrested and jailed. It was never going to happen and of course it didn't, but what did happen as a result provided that punchline to the Sabbatian Trump/QAnon Psyop.

On January 6th, 2021, a very big crowd of Trump supporters gathered in the National Mall in Washington DC down from the Capitol Building to protest at what they believed to be widespread corruption and vote fraud that stopped Trump being re-elected for a second term as president in November, 2020. I say as someone that does not support Trump or Biden that the evidence is clear that major vote-fixing went on to favour Biden, a man with cognitive problems so advanced he can often hardly string a sentence together without reading the words written for him on the Teleprompter. Glaring ballot discrepancies included serious questions about electronic voting machines that make vote rigging a comparative cinch and hundreds of thousands of paper votes that suddenly appeared during already advanced vote counts and virtually all of

them for Biden. Early Trump leads in crucial swing states suddenly began to close and disappear. The pandemic hoax was used as the excuse to issue almost limitless numbers of mail-in ballots with no checks to establish that the recipients were still alive or lived at that address. They were sent to streams of people who had not even asked for them. Private organisations were employed to gather these ballots and who knows what they did with them before they turned up at the counts. The American election system has been manipulated over decades to become a sick joke with more holes than a Swiss cheese for the express purpose of dictating the results. Then there was the criminal manipulation of information by Sabbatian tech giants like Facebook, Twitter and Google-owned YouTube which deleted pro-Trump, anti-Biden accounts and posts while everything in support of Biden was left alone. Sabbatians wanted Biden to win because after the dividing of America it was time for full-on Woke and every aspect of the Cult agenda to be unleashed.

Hunter gatherer

Extreme Silicon Valley bias included blocking information by the *New York Post* exposing a Biden scandal that should have ended his bid for president in the final weeks of the campaign. Hunter Biden, his monumentally corrupt son, is reported to have sent a laptop to be repaired at a local store and failed to return for it. Time passed until the laptop became the property of the store for non-payment of the bill. When the owner saw what was on the hard drive he gave a copy to the FBI who did nothing even though it confirmed widespread corruption in which the Joe Biden family were using his political position, especially when he was vice president to Obama, to make multiple millions in countries around the world and most notably Ukraine and China. Hunter Biden's one-time business partner Tony Bobulinski went public when the story broke in the *New York Post* to confirm the corruption he saw and that Joe Biden not only knew what was going on he also profited from the spoils. Millions were handed over by a Chinese company with close

connections – like all major businesses in China – to the Chinese communist party of President Xi Jinping. Joe Biden even boasted at a meeting of the Cult's World Economic Forum that as vice president he had ordered the government of Ukraine to fire a prosecutor. What he didn't mention was that the same man just happened to be investigating an energy company which was part of Hunter Biden's corrupt portfolio. The company was paying him big bucks for no other reason than the influence his father had. Overnight Biden's presidential campaign should have been over given that he had lied publicly about not knowing what his son was doing. Instead almost the entire Sabbatian-owned mainstream media and Sabbatian-owned Silicon Valley suppressed circulation of the story. This alone went a mighty way to rigging the election of 2020. Cult assets like Mark Zuckerberg at Facebook also spent hundreds of millions to be used in support of Biden and vote 'administration'.

The Cult had used Trump as the focus to divide America and was now desperate to bring in moronic, pliable, corrupt Biden to complete the double-whammy. No way were they going to let little things like the will of the people thwart their plan. Silicon Valley widely censored claims that the election was rigged because it *was* rigged. For the same reason anyone claiming it was rigged was denounced as a 'white supremacist' including the pathetically few Republican politicians willing to say so. Right across the media where the claim was mentioned it was described as a 'false claim' even though these excuses for 'journalists' would have done no research into the subject whatsoever. Trump won seven million more votes than any sitting president had ever achieved while somehow a cognitively-challenged soon to be 78-year-old who was hidden away from the public for most of the campaign managed to win more votes than any presidential candidate in history. It makes no sense. You only had to see election rallies for both candidates to witness the enthusiasm for Trump and the apathy for Biden. Tens of thousands would attend Trump events while Biden was speaking in empty car parks with often only television crews attending and framing their shots to hide the fact that no one was there. It was pathetic to see

footage come to light of Biden standing at a podium making speeches only to TV crews and party fixers while reading the words written for him on massive Teleprompter screens. So, yes, those protestors on January 6th had a point about election rigging, but some were about to walk into a trap laid for them in Washington by the Cult Deep State and its QAnon Psyop. This was the Capitol Hill riot ludicrously dubbed an 'insurrection'.

The spider and the fly

Renegade Minds know there are not two 'sides' in politics, only one side, the Cult, working through all 'sides'. It's a stage show, a puppet show, to direct the perceptions of the population into focusing on diversions like parties and candidates while missing the puppeteers with their hands holding all the strings. The Capitol Hill 'insurrection' brings us back to the Little Big Horn. Having created two distinct opposing groupings – Woke and Pushbackers – the trap was about to be sprung. Pushbackers were to be encircled and isolated by associating them all in the public mind with Trump and then labelling Trump as some sort of Confederate leader. I knew immediately that the Capitol riot was a set-up because of two things. One was how easy the rioters got into the building with virtually no credible resistance and secondly I could see – as with the 'Covid' hoax in the West at the start of 2020 – how the Cult could exploit the situation to move its agenda forward with great speed. My experience of Cult techniques and activities over more than 30 years has showed me that while they do exploit situations they haven't themselves created this never happens with events of fundamental agenda significance. Every time major events giving cultists the excuse to rapidly advance their plan you find they are manipulated into being for the specific reason of providing that excuse – Problem-Reaction-Solution. Only a tiny minority of the huge crowd of Washington protestors sought to gain entry to the Capitol by smashing windows and breaching doors. That didn't matter. The whole crowd and all Pushbackers, even if they did not support Trump, were going to be lumped together as dangerous

insurrectionists and conspiracy theorists. The latter term came into widespread use through a CIA memo in the 1960s aimed at discrediting those questioning the nonsensical official story of the Kennedy assassination and it subsequently became widely employed by the media. It's still being used by inept 'journalists' with no idea of its origin to discredit anyone questioning anything that authority claims to be true. When you are perpetrating a conspiracy you need to discredit the very word itself even though the dictionary definition of conspiracy is merely 'the activity of secretly planning with other people to do something bad or illegal' and 'a general agreement to keep silent about a subject for the purpose of keeping it secret'. On that basis there are conspiracies almost wherever you look. For obvious reasons the Cult and its lapdog media have to claim there are no conspiracies even though the word appears in state laws as with conspiracy to defraud, to murder, and to corrupt public morals.

Agent provocateurs are widely used by the Cult Deep State to manipulate genuine people into acting in ways that suit the desired outcome. By genuine in this case I mean protestors genuinely supporting Trump and claims that the election was stolen. In among them, however, were agents of the state wearing the garb of Trump supporters and QAnon to pump-prime the Capital riot which some genuine Trump supporters naively fell for. I described the situation as 'Come into my parlour said the spider to the fly'. Leaflets appeared through the Woke paramilitary arm Antifa, the anti-fascist fascists, calling on supporters to turn up in Washington looking like Trump supporters even though they hated him. Some of those arrested for breaching the Capitol Building were sourced to Antifa and its stable mate Black Lives Matter. Both organisations are funded by Cult billionaires and corporations. One man charged for the riot was according to his lawyer a former FBI agent who had held top secret security clearance for 40 years. Attorney Thomas Plofchan said of his client, 66-year-old Thomas Edward Caldwell:

He has held a Top Secret Security Clearance since 1979 and has undergone multiple Special Background Investigations in support of his clearances. After retiring from the Navy, he

worked as a section chief for the Federal Bureau of Investigation from 2009-2010 as a GS-12 [mid-level employee].

He also formed and operated a consulting firm performing work, often classified, for U.S government customers including the US. Drug Enforcement Agency, Department of Housing and Urban Development, the US Coast Guard, and the US Army Personnel Command.

A judge later released Caldwell pending trial in the absence of evidence about a conspiracy or that he tried to force his way into the building. *The New York Post* reported a 'law enforcement source' as saying that 'at least two known Antifa members were spotted' on camera among Trump supporters during the riot while one of the rioters arrested was John Earle Sullivan, a seriously extreme Black Lives Matter Trump-hater from Utah who was previously arrested and charged in July, 2020, over a BLM-Antifa riot in which drivers were threatened and one was shot. Sullivan is the founder of Utah-based Insurgence USA which is an affiliate of the Cult-created-and-funded Black Lives Matter movement. Footage appeared and was then deleted by Twitter of Trump supporters calling out Antifa infiltrators and a group was filmed changing into pro-Trump clothing before the riot. Security at the building was *pathetic* – as planned. Colonel Leroy Fletcher Prouty, a man with long experience in covert operations working with the US security apparatus, once described the tell-tale sign to identify who is involved in an assassination. He said:

No one has to direct an assassination – it happens. The active role is played secretly by permitting it to happen. This is the greatest single clue. Who has the power to call off or reduce the usual security precautions?

This principle applies to many other situations and certainly to the Capitol riot of January 6th, 2021.

The sting

With such a big and potentially angry crowd known to be gathering near the Capitol the security apparatus would have had a major police detail to defend the building with National Guard troops on

standby given the strength of feeling among people arriving from all over America encouraged by the QAnon Psyop and statements by Donald Trump. Instead Capitol Police 'security' was flimsy, weak, and easily breached. The same number of officers was deployed as on a regular day and that is a blatant red flag. They were not staffed or equipped for a possible riot that had been an obvious possibility in the circumstances. No protective and effective fencing worth the name was put in place and there were no contingency plans. The whole thing was basically a case of standing aside and waving people in. Once inside police mostly backed off apart from one Capitol police officer who ridiculously shot dead unarmed Air Force veteran protestor Ashli Babbitt without a warning as she climbed through a broken window. The 'investigation' refused to name or charge the officer after what must surely be considered a murder in the circumstances. They just lifted a carpet and swept. The story was endlessly repeated about five people dying in the 'armed insurrection' when there was no report of rioters using weapons. Apart from Babbitt the other four died from a heart attack, strokes and apparently a drug overdose. Capitol police officer Brian Sicknick was reported to have died after being bludgeoned with a fire extinguisher when he was alive after the riot was over and died later of what the Washington Medical Examiner's Office said was a stroke. Sicknick had no external injuries. The lies were delivered like rapid fire. There was a narrative to build with incessant repetition of the lie until the lie became the accepted 'everybody knows that' truth. The 'Big Lie' technique of Nazi Propaganda Minister Joseph Goebbels is constantly used by the Cult which was behind the Nazis and is today behind the 'Covid' and 'climate change' hoaxes. Goebbels said:

If you tell a lie big enough and keep repeating it, people will eventually come to believe it. The lie can be maintained only for such time as the State can shield the people from the political, economic and/or military consequences of the lie. It thus becomes vitally important for the State to use all of its powers to repress dissent, for the truth is the mortal enemy of the lie, and thus by extension, the truth is the greatest enemy of the State.

Most protestors had a free run of the Capitol Building. This allowed pictures to be taken of rioters in iconic parts of the building including the Senate chamber which could be used as propaganda images against all Pushbackers. One Congresswoman described the scene as 'the worst kind of non-security anybody could ever imagine'. Well, the first part was true, but someone obviously did imagine it and made sure it happened. Some photographs most widely circulated featured people wearing QAnon symbols and now the Psyop would be used to dub all QAnon followers with the ubiquitous fit-all label of 'white supremacist' and 'insurrectionists'. When a Muslim extremist called Noah Green drove his car at two police officers at the Capitol Building killing one in April, 2021, there was no such political and media hysteria. They were just disappointed he wasn't white.

The witch-hunt

Government prosecutor Michael Sherwin, an aggressive, dark-eyed, professional Rottweiler led the 'investigation' and to call it over the top would be to understate reality a thousand fold. Hundreds were tracked down and arrested for the crime of having the wrong political views and people were jailed who had done nothing more than walk in the building, committed no violence or damage to property, took a few pictures and left. They were labelled a 'threat to the Republic' while Biden sat in the White House signing executive orders written for him that were dismantling 'the Republic'. Even when judges ruled that a mother and son should not be in jail the government kept them there. Some of those arrested have been badly beaten by prison guards in Washington and lawyers for one man said he suffered a fractured skull and was made blind in one eye. Meanwhile a woman is shot dead for no reason by a Capitol Police officer and we are not allowed to know who he is never mind what has happened to him although that will be *nothing*. The Cult's QAnon/Trump sting to identify and isolate Pushbackers and then target them on the road to crushing and deleting them was a resounding success. You would have thought the Russians had

invaded the building at gunpoint and lined up senators for a firing squad to see the political and media reaction. Congresswoman Alexandria Ocasio-Cortez is a child in a woman's body, a terrible-twos, me, me, me, Woker narcissist of such proportions that words have no meaning. She said she thought she was going to die when 'insurrectionists' banged on her office door. It turned out she wasn't even in the Capitol Building when the riot was happening and the 'banging' was a Capitol Police officer. She referred to herself as a 'survivor' which is an insult to all those true survivors of violent and sexual abuse while she lives her pampered and privileged life talking drivel for a living. Her Woke colleague and fellow mega-narcissist Rashida Tlaib broke down describing the devastating effect on her, too, of *not being* in the building when the rioters were there. Ocasio-Cortez and Tlaib are members of a fully-Woke group of Congresswomen known as 'The Squad' along with Ilhan Omar and Ayanna Pressley. The Squad from what I can see can be identified by its vehement anti-white racism, anti-white men agenda, and, as always in these cases, the absence of brain cells on active duty.

The usual suspects were on the riot case immediately in the form of Democrat ultra-Zionist senators and operatives Chuck Schumer and Adam Schiff demanding that Trump be impeached for 'his part in the insurrection'. The same pair of prats had led the failed impeachment of Trump over the invented 'Russia collusion' nonsense which claimed Russia had helped Trump win the 2016 election. I didn't realise that Tel Aviv had been relocated just outside Moscow. I must find an up-to-date map. The Russia hoax was a Sabbatian operation to keep Trump occupied and impotent and to stop any rapport with Russia which the Cult wants to retain as a perceptual enemy to be pulled out at will. Puppet Biden began attacking Russia when he came to office as the Cult seeks more upheaval, division and war across the world. A two-year stage show 'Russia collusion inquiry' headed by the not-very-bright former 9/11 FBI chief Robert Mueller, with support from 19 lawyers, 40 FBI agents plus intelligence analysts, forensic accountants and other

staff, devoured tens of millions of dollars and found no evidence of Russia collusion which a ten-year-old could have told them on day one. Now the same moronic Schumer and Schiff wanted a second impeachment of Trump over the Capitol 'insurrection' (riot) which the arrested development of Schumer called another 'Pearl Harbor' while others compared it with 9/11 in which 3,000 died and, in the case of CNN, with the Rwandan genocide in the 1990s in which an estimated 500,000 to 600,000 were murdered, between 250, 000 and 500,000 women were raped, and populations of whole towns were hacked to death with machetes. To make those comparisons purely for Cult political reasons is beyond insulting to those that suffered and lost their lives and confirms yet again the callous inhumanity that we are dealing with. Schumer is a monumental idiot and so is Schiff, but they serve the Cult agenda and do whatever they're told so they get looked after. Talking of idiots – another inane man who spanned the Russia and Capitol impeachment attempts was Senator Eric Swalwell who had the nerve to accuse Trump of collusion with the Russians while sleeping with a Chinese spy called Christine Fang or 'Fang Fang' which is straight out of a Bond film no doubt starring Klaus Schwab as the bloke living on a secret island and controlling laser weapons positioned in space and pointing at world capitals. Fang Fang plays the part of Bond's infiltrator girlfriend which I'm sure she would enjoy rather more than sharing a bed with the brainless Swalwell, lying back and thinking of China. The FBI eventually warned Swalwell about Fang Fang which gave her time to escape back to the Chinese dictatorship. How very thoughtful of them. The second Trump impeachment also failed and hardly surprising when an impeachment is supposed to remove a sitting president and by the time it happened Trump was no longer president. These people are running your country America, well, officially anyway. Terrifying isn't it?

Outcomes tell the story - always

The outcome of all this – and it's the *outcome* on which Renegade Minds focus, not the words – was that a vicious, hysterical and

obviously pre-planned assault was launched on Pushbackers to censor, silence and discredit them and even targeted their right to earn a living. They have since been condemned as 'domestic terrorists' that need to be treated like Al-Qaeda and Islamic State. 'Domestic terrorists' is a label the Cult has been trying to make stick since the period of the Oklahoma bombing in 1995 which was blamed on 'far-right domestic terrorists'. If you read *The Trigger* you will see that the bombing was clearly a Problem-Reaction-Solution carried out by the Deep State during a Bill Clinton administration so corrupt that no dictionary definition of the term would even nearly suffice. Nearly 30, 000 troops were deployed from all over America to the empty streets of Washington for Biden's inauguration. Ten thousand of them stayed on with the pretext of protecting the capital from insurrectionists when it was more psychological programming to normalise the use of the military in domestic law enforcement in support of the Cult plan for a police-military state. Biden's fascist administration began a purge of 'wrong-thinkers' in the military which means anyone that is not on board with Woke. The Capitol Building was surrounded by a fence with razor wire and the Land of the Free was further symbolically and literally dismantled. The circle was completed with the installation of Biden and the exploitation of the QAnon Psyop.

America had never been so divided since the civil war of the 19th century, Pushbackers were isolated and dubbed terrorists and now, as was always going to happen, the Cult immediately set about deleting what little was left of freedom and transforming American society through a swish of the hand of the most controlled 'president' in American history leading (officially at least) the most extreme regime since the country was declared an independent state on July 4th, 1776. Biden issued undebated, dictatorial executive orders almost by the hour in his opening days in office across the whole spectrum of the Cult wish-list including diluting controls on the border with Mexico allowing thousands of migrants to illegally enter the United States to transform the demographics of America and import an election-changing number of perceived Democrat

voters. Then there were Biden deportation amnesties for the already illegally resident (estimated to be as high as 20 or even 30 million). A bill before Congress awarded American citizenship to anyone who could prove they had worked in agriculture for just 180 days in the previous two years as 'Big Ag' secured its slave labour long-term. There were the plans to add new states to the union such as Puerto Rico and making Washington DC a state. They are all parts of a plan to ensure that the Cult-owned Woke Democrats would be permanently in power.

Border – what border?

I have exposed in detail in other books how mass immigration into the United States and Europe is the work of Cult networks fuelled by the tens of billions spent to this and other ends by George Soros and his global Open Society (open borders) Foundations. The impact can be seen in America alone where the population has increased by *100 million* in little more than 30 years mostly through immigration. I wrote in *The Answer* that the plan was to have so many people crossing the southern border that the numbers become unstoppable and we are now there under Cult-owned Biden. El Salvador in Central America puts the scale of what is happening into context. A third of the population now lives in the United States, much of it illegally, and many more are on the way. The methodology is to crush Central and South American countries economically and spread violence through machete-wielding psychopathic gangs like MS-13 based in El Salvador and now operating in many American cities. Biden-imposed lax security at the southern border means that it is all but open. He said before his 'election' that he wanted to see a surge towards the border if he became president and that was the green light for people to do just that after election day to create the human disaster that followed for both America and the migrants. When that surge came the imbecilic Alexandria Ocasio-Cortez said it wasn't a 'surge' because they are 'children, not insurgents' and the term 'surge' (used by Biden) was a claim of 'white supremacists'.

This disingenuous lady may one day enter the realm of the most basic intelligence, but it won't be any time soon.

Sabbatians and the Cult are in the process of destroying America by importing violent people and gangs in among the genuine to terrorise American cities and by overwhelming services that cannot cope with the sheer volume of new arrivals. Something similar is happening in Europe as Western society in general is targeted for demographic and cultural transformation and upheaval. The plan demands violence and crime to create an environment of intimidation, fear and division and Soros has been funding the election of district attorneys across America who then stop prosecuting many crimes, reduce sentences for violent crimes and free as many violent criminals as they can. Sabbatians are creating the chaos from which order – their order – can respond in a classic Problem-Reaction-Solution. A Freemasonic moto says 'Ordo Ab Chao' (Order out of Chaos) and this is why the Cult is constantly creating chaos to impose a new 'order'. Here you have the reason the Cult is constantly creating chaos. The 'Covid' hoax can be seen with those entering the United States by plane being forced to take a 'Covid' test while migrants flooding through southern border processing facilities do not. Nothing is put in the way of mass migration and if that means ignoring the government's own 'Covid' rules then so be it. They know it's all bullshit anyway. Any pushback on this is denounced as 'racist' by Wokers and Sabbatian fronts like the ultra-Zionist Anti-Defamation League headed by the appalling Jonathan Greenblatt which at the same time argues that Israel should not give citizenship and voting rights to more Palestinian Arabs or the 'Jewish population' (in truth the Sabbatian network) will lose control of the country.

Society-changing numbers

Biden's masters have declared that countries like El Salvador are so dangerous that their people must be allowed into the United States for humanitarian reasons when there are fewer murders in large parts of many Central American countries than in US cities like

Baltimore. That is not to say Central America cannot be a dangerous place and Cult-controlled American governments have been making it so since way back, along with the dismantling of economies, in a long-term plan to drive people north into the United States. Parts of Central America are very dangerous, but in other areas the story is being greatly exaggerated to justify relaxing immigration criteria. Migrants are being offered free healthcare and education in the United States as another incentive to head for the border and there is no requirement to be financially independent before you can enter to prevent the resources of America being drained. You can't blame migrants for seeking what they believe will be a better life, but they are being played by the Cult for dark and nefarious ends. The numbers since Biden took office are huge. In February, 2021, more than 100,000 people were known to have tried to enter the US illegally through the southern border (it was 34,000 in the same month in 2020) and in March it was 170,000 – a 418 percent increase on March, 2020. These numbers are only known people, not the ones who get in unseen. The true figure for migrants illegally crossing the border in a single month was estimated by one congressman at 250,000 and that number will only rise under Biden's current policy. Gangs of murdering drug-running thugs that control the Mexican side of the border demand money – thousands of dollars – to let migrants cross the Rio Grande into America. At the same time gun battles are breaking out on the border several times a week between rival Mexican drug gangs (which now operate globally) who are equipped with sophisticated military-grade weapons, grenades and armoured vehicles. While the Capitol Building was being 'protected' from a non-existent 'threat' by thousands of troops, and others were still deployed at the time in the Cult Neocon war in Afghanistan, the southern border of America was left to its fate. This is not incompetence, it is cold calculation.

By March, 2021, there were 17,000 unaccompanied children held at border facilities and many of them are ensnared by people traffickers for paedophile rings and raped on their journey north to America. This is not conjecture – this is fact. Many of those designated

children are in reality teenage boys or older. Meanwhile Wokers posture their self-purity for encouraging poor and tragic people to come to America and face this nightmare both on the journey and at the border with the disgusting figure of House Speaker Nancy Pelosi giving disingenuous speeches about caring for migrants. The woman's evil. Wokers condemned Trump for having children in cages at the border (so did Obama, *Shhhh*), but now they are sleeping on the floor without access to a shower with one border facility 729 percent over capacity. The Biden insanity even proposed flying migrants from the southern border to the northern border with Canada for 'processing'. The whole shambles is being overseen by ultra-Zionist Secretary of Homeland Security, the moronic liar Alejandro Mayorkas, who banned news cameras at border facilities to stop Americans seeing what was happening. Mayorkas said there was not a ban on news crews; it was just that they were not allowed to film. Alongside him at Homeland Security is another ultra-Zionist Cass Sunstein appointed by Biden to oversee new immigration laws. Sunstein despises conspiracy researchers to the point where he suggests they should be banned or *taxed* for having such views. The man is not bonkers or anything. He's perfectly well-adjusted, but adjusted to what is the question. Criticise what is happening and you are a 'white supremacist' when earlier non-white immigrants also oppose the numbers which effect their lives and opportunities. Black people in poor areas are particularly damaged by uncontrolled immigration and the increased competition for work opportunities with those who will work for less. They are also losing voting power as Hispanics become more dominant in former black areas. It's a downward spiral for them while the billionaires behind the policy drone on about how much they care about black people and 'racism'. None of this is about compassion for migrants or black people – that's just wind and air. Migrants are instead being mercilessly exploited to transform America while the countries they leave are losing their future and the same is true in Europe. Mass immigration may now be the work of Woke Democrats, but it can be traced back to the 1986 Immigration Reform and Control Act (it

wasn't) signed into law by Republican hero President Ronald Reagan which gave amnesty to millions living in the United States illegally and other incentives for people to head for the southern border. Here we have the one-party state at work again.

Save me syndrome

Almost every aspect of what I have been exposing as the Cult agenda was on display in even the first days of 'Biden' with silencing of Pushbackers at the forefront of everything. A Renegade Mind will view the Trump years and QAnon in a very different light to their supporters and advocates as the dots are connected. The QAnon/Trump Psyop has given the Cult all it was looking for. We may not know how much, or little, that Trump realised he was being used, but that's a side issue. This pincer movement produced the desired outcome of dividing America and having Pushbackers isolated. To turn this around we have to look at new routes to empowerment which do not include handing our power to other people and groups through what I will call the 'Save Me Syndrome' – 'I want someone else to do it so that I don't have to'. We have seen this at work throughout human history and the QAnon/Trump Psyop is only the latest incarnation alongside all the others. Religion is an obvious expression of this when people look to a 'god' or priest to save them or tell them how to be saved and then there are 'save me' politicians like Trump. Politics is a diversion and not a 'saviour'. It is a means to block positive change, not make it possible.

Save Me Syndrome always comes with the same repeating theme of handing your power to whom or what you believe will save you while your real 'saviour' stares back from the mirror every morning. Renegade Minds are constantly vigilant in this regard and always asking the question 'What can I do?' rather than 'What can someone else do for me?' Gandhi was right when he said: 'You must be the change you want to see in the world.' We are indeed the people we have been waiting for. We are presented with a constant raft of reasons to concede that power to others and forget where the real power is. Humanity has the numbers and the Cult does not. It has to

use diversion and division to target the unstoppable power that comes from unity. Religions, governments, politicians, corporations, media, QAnon, are all different manifestations of this power-diversion and dilution. Refusing to give your power to governments and instead handing it to Trump and QAnon is not to take a new direction, but merely to recycle the old one with new names on the posters. I will explore this phenomenon as we proceed and how to break the cycles and recycles that got us here through the mists of repeating perception and so repeating history.

For now we shall turn to the most potent example in the entire human story of the consequences that follow when you give your power away. I am talking, of course, of the 'Covid' hoax.

CHAPTER FOUR

'Covid': Calculated catastrophe

Facts are threatening to those invested in fraud
DaShanne Stokes

We can easily unravel the real reason for the 'Covid pandemic' hoax by employing the Renegade Mind methodology that I have outlined this far. We'll start by comparing the long-planned Cult outcome with the 'Covid pandemic' outcome. Know the outcome and you'll see the journey.

I have highlighted the plan for the Hunger Games Society which has been in my books for so many years with the very few controlling the very many through ongoing dependency. To create this dependency it is essential to destroy independent livelihoods, businesses and employment to make the population reliant on the state (the Cult) for even the basics of life through a guaranteed pittance income. While independence of income remained these Cult ambitions would be thwarted. With this knowledge it was easy to see where the 'pandemic' hoax was going once talk of 'lockdowns' began and the closing of all but perceived 'essential' businesses to 'save' us from an alleged 'deadly virus'. Cult corporations like Amazon and Walmart were naturally considered 'essential' while mom and pop shops and stores had their doors closed by fascist decree. As a result with every new lockdown and new regulation more small and medium, even large businesses not owned by the Cult, went to the wall while Cult giants and their frontmen and women grew financially fatter by the second. Mom and pop were

denied an income and the right to earn a living and the wealth of people like Jeff Bezos (Amazon), Mark Zuckerberg (Facebook) and Sergei Brin and Larry Page (Google/Alphabet) have reached record levels. The Cult was increasing its own power through further dramatic concentrations of wealth while the competition was being destroyed and brought into a state of dependency. Lockdowns have been instigated to secure that very end and were never anything to do with health. My brother Paul spent 45 years building up a bus repair business, but lockdowns meant buses were running at a fraction of normal levels for months on end. Similar stories can told in their hundreds of millions worldwide. Efforts of a lifetime coldly destroyed by Cult multi-billionaires and their lackeys in government and law enforcement who continued to earn their living from the taxation of the people while denying the right of the same people to earn theirs. How different it would have been if those making and enforcing these decisions had to face the same financial hardships of those they affected, but they never do.

Gates of Hell

Behind it all in the full knowledge of what he is doing and why is the psychopathic figure of Cult operative Bill Gates. His puppet Tedros at the World Health Organization declared 'Covid' a pandemic in March, 2020. The WHO had changed the definition of a 'pandemic' in 2009 just a month before declaring the 'swine flu pandemic' which would not have been so under the previous definition. The same applies to 'Covid'. The definition had included… 'an infection by an infectious agent, occurring simultaneously in different countries, with a significant mortality rate relative to the proportion of the population infected'. The new definition removed the need for 'significant mortality'. The 'pandemic' has been fraudulent even down to the definition, but Gates demanded economy-destroying lockdowns, school closures, social distancing, mandatory masks, a 'vaccination' for every man, woman and child on the planet and severe consequences and restrictions for those that refused. Who gave him this power? The

Cult did which he serves like a little boy in short trousers doing what his daddy tells him. He and his psychopathic missus even smiled when they said that much worse was to come (what they knew was planned to come). Gates responded in the matter-of-fact way of all psychopaths to a question about the effect on the world economy of what he was doing:

> Well, it won't go to zero but it will shrink. Global GDP is probably going to take the biggest hit ever [Gates was smiling as he said this] … in my lifetime this will be the greatest economic hit. But you don't have a choice. People act as if you have a choice. People don't feel like going to the stadium when they might get infected … People are deeply affected by seeing these stats, by knowing they could be part of the transmission chain, old people, their parents and grandparents, could be affected by this, and so you don't get to say ignore what is going on here.
>
> There will be the ability to open up, particularly in rich countries, if things are done well over the next few months, but for the world at large normalcy only returns when we have largely vaccinated the entire population.

The man has no compassion or empathy. How could he when he's a psychopath like all Cult players? My own view is that even beyond that he is very seriously mentally ill. Look in his eyes and you can see this along with his crazy flailing arms. You don't do what he has done to the world population since the start of 2020 unless you are mentally ill and at the most extreme end of psychopathic. You especially don't do it when to you know, as we shall see, that cases and deaths from 'Covid' are fakery and a product of monumental figure massaging. 'These stats' that Gates referred to are based on a 'test' that's not testing for the 'virus' as he has known all along. He made his fortune with big Cult support as an infamously ruthless software salesman and now buys global control of 'health' (death) policy without the population he affects having any say. It's a breathtaking outrage. Gates talked about people being deeply affected by fear of 'Covid' when that was because of *him* and his global network lying to them minute-by-minute supported by a lying media that he seriously influences and funds to the tune of hundreds of millions. He's handed big sums to media operations including the BBC, NBC, Al Jazeera, Univision, *PBS NewsHour*,

ProPublica, National Journal, The Guardian, The Financial Times, The Atlantic, Texas Tribune, USA Today publisher Gannett, *Washington Monthly, Le Monde,* Center for Investigative Reporting, Pulitzer Center on Crisis Reporting, National Press Foundation, International Center for Journalists, Solutions Journalism Network, the Poynter Institute for Media Studies, and many more. Gates is everywhere in the 'Covid' hoax and the man must go to prison – or a mental facility – for the rest of his life and his money distributed to those he has taken such enormous psychopathic pleasure in crushing.

The Muscle

The Hunger Games global structure demands a police-military state – a fusion of the two into one force – which viciously imposes the will of the Cult on the population and protects the Cult from public rebellion. In that regard, too, the 'Covid' hoax just keeps on giving. Often unlawful, ridiculous and contradictory 'Covid' rules and regulations have been policed across the world by moronic automatons and psychopaths made faceless by face-nappy masks and acting like the Nazi SS and fascist blackshirts and brownshirts of Hitler and Mussolini. The smallest departure from the rules decreed by the psychos in government and their clueless gofers were jumped upon by the face-nappy fascists. Brutality against public protestors soon became commonplace even on girls, women and old people as the brave men with the batons – the Face-Nappies as I call them – broke up peaceful protests and handed out fines like confetti to people who couldn't earn a living let alone pay hundreds of pounds for what was once an accepted human right. Robot Face-Nappies of Nottingham police in the English East Midlands fined one group £11,000 for attending a child's birthday party. For decades I charted the transformation of law enforcement as genuine, decent officers were replaced with psychopaths and the brain dead who would happily and brutally do whatever their masters told them. Now they were let loose on the public and I would emphasise the point that none of this just happened. The step-by-step change in the dynamic between police and public was orchestrated from the shadows by

those who knew where this was all going and the same with the perceptual reframing of those in all levels of authority and official administration through 'training courses' by organisations such as Common Purpose which was created in the late 1980s and given a massive boost in Blair era Britain until it became a global phenomenon. Supposed public 'servants' began to view the population as the enemy and the same was true of the police. This was the start of the explosion of behaviour manipulation organisations and networks preparing for the all-war on the human psyche unleashed with the dawn of 2020. I will go into more detail about this later in the book because it is a core part of what is happening.

Police desecrated beauty spots to deter people gathering and arrested women for walking in the countryside alone 'too far' from their homes. We had arrogant, clueless sergeants in the Isle of Wight police where I live posting on Facebook what they insisted the population must do or else. A schoolmaster sergeant called Radford looked young enough for me to ask if his mother knew he was out, but he was posting what he *expected* people to do while a Sergeant Wilkinson boasted about fining lads for meeting in a McDonald's car park where they went to get a lockdown takeaway. Wilkinson added that he had even cancelled their order. What a pair of prats these people are and yet they have increasingly become the norm among Jackboot Johnson's Yellowshirts once known as the British police. This was the theme all over the world with police savagery common during lockdown protests in the United States, the Netherlands, and the fascist state of Victoria in Australia under its tyrannical and again moronic premier Daniel Andrews. Amazing how tyrannical and moronic tend to work as a team and the same combination could be seen across America as arrogant, narcissistic Woke governors and mayors such as Gavin Newsom (California), Andrew Cuomo (New York), Gretchen Whitmer (Michigan), Lori Lightfoot (Chicago) and Eric Garcetti (Los Angeles) did their Nazi and Stalin impressions with the full support of the compliant brutality of their enforcers in uniform as they arrested small business owners defying

fascist shutdown orders and took them to jail in ankle shackles and handcuffs. This happened to bistro owner Marlena Pavlos-Hackney in Gretchen Whitmer's fascist state of Michigan when police arrived to enforce an order by a state-owned judge for 'putting the community at risk' at a time when other states like Texas were dropping restrictions and migrants were pouring across the southern border without any 'Covid' questions at all. I'm sure there are many officers appalled by what they are ordered to do, but not nearly enough of them. If they were truly appalled they would not do it. As the months passed every opportunity was taken to have the military involved to make their presence on the streets ever more familiar and 'normal' for the longer-term goal of police-military fusion.

Another crucial element to the Hunger Games enforcement network has been encouraging the public to report neighbours and others for 'breaking the lockdown rules'. The group faced with £11,000 in fines at the child's birthday party would have been dobbed-in by a neighbour with a brain the size of a pea. The technique was most famously employed by the Stasi secret police in communist East Germany who had public informants placed throughout the population. A police chief in the UK says his force doesn't need to carry out 'Covid' patrols when they are flooded with so many calls from the public reporting other people for visiting the beach. Dorset police chief James Vaughan said people were so enthusiastic about snitching on their fellow humans they were now operating as an auxiliary arm of the police: 'We are still getting around 400 reports a week from the public, so we will respond to reports …We won't need to be doing hotspot patrols because people are very quick to pick the phone up and tell us.' Vaughan didn't say that this is a pillar of all tyrannies of whatever complexion and the means to hugely extend the reach of enforcement while spreading distrust among the people and making them wary of doing anything that might get them reported. Those narcissistic Isle of Wight sergeants Radford and Wilkinson never fail to add a link to their Facebook posts where the public can inform on their fellow slaves.

Neither would be self-aware enough to realise they were imitating the Stasi which they might well never have heard of. Government psychologists that I will expose later laid out a policy to turn communities against each other in the same way.

A coincidence? Yep, and I can knit fog

I knew from the start of the alleged pandemic that this was a Cult operation. It presented limitless potential to rapidly advance the Cult agenda and exploit manipulated fear to demand that every man, woman and child on the planet was 'vaccinated' in a process never used on humans before which infuses self-replicating *synthetic* material into human cells. Remember the plan to transform the human body from a biological to a synthetic biological state. I'll deal with the 'vaccine' (that's not actually a vaccine) when I focus on the genetic agenda. Enough to say here that mass global 'vaccination' justified by this 'new virus' set alarms ringing after 30 years of tracking these people and their methods. The 'Covid' hoax officially beginning in China was also a big red flag for reasons I will be explaining. The agenda potential was so enormous that I could dismiss any idea that the 'virus' appeared naturally. Major happenings with major agenda implications never occur without Cult involvement in making them happen. My questions were twofold in early 2020 as the media began its campaign to induce global fear and hysteria: Was this alleged infectious agent released on purpose by the Cult or did it even exist at all? I then did what I always do in these situations. I sat, observed and waited to see where the evidence and information would take me. By March and early April synchronicity was strongly – and ever more so since then – pointing me in the direction of *there is no 'virus'*. I went public on that with derision even from swathes of the alternative media that voiced a scenario that the Chinese government released the 'virus' in league with Deep State elements in the United States from a top-level bio-lab in Wuhan where the 'virus' is said to have first appeared. I looked at that possibility, but I didn't buy it for several reasons. Deaths from the 'virus' did not in any way match what they

would have been with a 'deadly bioweapon' and it is much more effective if you sell the *illusion* of an infectious agent rather than having a real one unless you can control through injection who has it and who doesn't. Otherwise you lose control of events. A made-up 'virus' gives you a blank sheet of paper on which you can make it do whatever you like and have any symptoms or mutant 'variants' you choose to add while a real infectious agent would limit you to what it actually does. A phantom disease allows you to have endless ludicrous 'studies' on the 'Covid' dollar to widen the perceived impact by inventing ever more 'at risk' groups including one study which said those who walk slowly may be almost four times more likely to die from the 'virus'. People are in psychiatric wards for less.

A real 'deadly bioweapon' can take out people in the hierarchy that are not part of the Cult, but essential to its operation. Obviously they don't want that. Releasing a real disease means you immediately lose control of it. Releasing an illusory one means you don't. Again it's vital that people are extra careful when dealing with what they want to hear. A bioweapon unleashed from a Chinese laboratory in collusion with the American Deep State may fit a conspiracy narrative, but is it true? Would it not be far more effective to use the excuse of a 'virus' to justify the real bioweapon – the 'vaccine'? That way your disease agent does not have to be transmitted and arrives directly through a syringe. I saw a French virologist Luc Montagnier quoted in the alternative media as saying he had discovered that the alleged 'new' severe acute respiratory syndrome coronavirus , or SARS-CoV-2, was made artificially and included elements of the human immunodeficiency 'virus' (HIV) and a parasite that causes malaria. SARS-CoV-2 is alleged to trigger an alleged illness called Covid-19. I remembered Montagnier's name from my research years before into claims that an HIV 'retrovirus' causes AIDs – claims that were demolished by Berkeley virologist Peter Duesberg who showed that no one had ever proved that HIV causes acquired immunodeficiency syndrome or AIDS. Claims that become accepted as fact, publicly and medically, with no proof whatsoever are an ever-recurring story that profoundly applies to

'Covid'. Nevertheless, despite the lack of proof, Montagnier's team at the Pasteur Institute in Paris had a long dispute with American researcher Robert Gallo over which of them discovered and isolated the HIV 'virus' and with *no evidence* found it to cause AIDS. You will see later that there is also no evidence that any 'virus' causes any disease or that there is even such a thing as a 'virus' in the way it is said to exist. The claim to have 'isolated' the HIV 'virus' will be presented in its real context as we come to the shocking story – and it is a story – of SARS-CoV-2 and so will Montagnier's assertion that he identified the full SARS-CoV-2 genome.

Hoax in the making

We can pick up the 'Covid' story in 2010 and the publication by the Rockefeller Foundation of a document called 'Scenarios for the Future of Technology and International Development'. The inner circle of the Rockefeller family has been serving the Cult since John D. Rockefeller (1839-1937) made his fortune with Standard Oil. It is less well known that the same Rockefeller – the Bill Gates of his day – was responsible for establishing what is now referred to as 'Big Pharma', the global network of pharmaceutical companies that make outrageous profits dispensing scalpel and drug 'medicine' and are obsessed with pumping vaccines in ever-increasing number into as many human arms and backsides as possible. John D. Rockefeller was the driving force behind the creation of the 'education' system in the United States and elsewhere specifically designed to program the perceptions of generations thereafter. The Rockefeller family donated exceptionally valuable land in New York for the United Nations building and were central in establishing the World Health Organization in 1948 as an agency of the UN which was created from the start as a Trojan horse and stalking horse for world government. Now enter Bill Gates. His family and the Rockefellers have long been extremely close and I have seen genealogy which claims that if you go back far enough the two families fuse into the same bloodline. Gates has said that the Bill and Melinda Gates Foundation was inspired by the Rockefeller Foundation and why not

when both are serving the same Cult? Major tax-exempt foundations are overwhelmingly criminal enterprises in which Cult assets fund the Cult agenda in the guise of 'philanthropy' while avoiding tax in the process. Cult operatives can become mega-rich in their role of front men and women for the psychopaths at the inner core and they, too, have to be psychopaths to knowingly serve such evil. Part of the deal is that a big percentage of the wealth gleaned from representing the Cult has to be spent advancing the ambitions of the Cult and hence you have the Rockefeller Foundation, Bill and Melinda Gates Foundation (and *so* many more) and people like George Soros with his global Open Society Foundations spending their billions in pursuit of global Cult control. Gates is a global public face of the Cult with his interventions in world affairs including Big Tech influence; a central role in the 'Covid' and 'vaccine' scam; promotion of the climate change shakedown; manipulation of education; geoengineering of the skies; and his food-control agenda as the biggest owner of farmland in America, his GMO promotion and through other means. As one writer said: 'Gates monopolizes or wields disproportionate influence over the tech industry, global health and vaccines, agriculture and food policy (including biopiracy and fake food), weather modification and other climate technologies, surveillance, education and media.' The almost limitless wealth secured through Microsoft and other not-allowed-to-fail ventures (including vaccines) has been ploughed into a long, long list of Cult projects designed to enslave the entire human race. Gates and the Rockefellers have been working as one unit with the Rockefeller-established World Health Organization leading global 'Covid' policy controlled by Gates through his mouth-piece Tedros. Gates became the WHO's biggest funder when Trump announced that the American government would cease its donations, but Biden immediately said he would restore the money when he took office in January, 2021. The Gates Foundation (the Cult) owns through limitless funding the world health system and the major players across the globe in the 'Covid' hoax.

Okay, with that background we return to that Rockefeller Foundation document of 2010 headed 'Scenarios for the Future of Technology and International Development' and its 'imaginary' epidemic of a virulent and deadly influenza strain which infected 20 percent of the global population and killed eight million in seven months. The Rockefeller scenario was that the epidemic destroyed economies, closed shops, offices and other businesses and led to governments imposing fierce rules and restrictions that included mandatory wearing of face masks and body-temperature checks to enter communal spaces like railway stations and supermarkets. The document predicted that even after the height of the Rockefeller-envisaged epidemic the authoritarian rule would continue to deal with further pandemics, transnational terrorism, environmental crises and rising poverty. Now you may think that the Rockefellers are our modern-day seers or alternatively, and rather more likely, that they well knew what was planned a few years further on. Fascism had to be imposed, you see, to 'protect citizens from risk and exposure'. The Rockefeller scenario document said:

During the pandemic, national leaders around the world flexed their authority and imposed airtight rules and restrictions, from the mandatory wearing of face masks to body-temperature checks at the entries to communal spaces like train stations and supermarkets. Even after the pandemic faded, this more authoritarian control and oversight of citizens and their activities stuck and even intensified. In order to protect themselves from the spread of increasingly global problems – from pandemics and transnational terrorism to environmental crises and rising poverty – leaders around the world took a firmer grip on power.

At first, the notion of a more controlled world gained wide acceptance and approval. Citizens willingly gave up some of their sovereignty – and their privacy – to more paternalistic states in exchange for greater safety and stability. Citizens were more tolerant, and even eager, for top-down direction and oversight, and national leaders had more latitude to impose order in the ways they saw fit.

In developed countries, this heightened oversight took many forms: biometric IDs for all citizens, for example, and tighter regulation of key industries whose stability was deemed vital to national interests. In many developed countries, enforced cooperation with a suite of new regulations and agreements slowly but steadily restored both order and, importantly, economic growth.

There we have the prophetic Rockefellers in 2010 and three years later came their paper for the Global Health Summit in Beijing, China, when government representatives, the private sector, international organisations and groups met to discuss the next 100 years of 'global health'. The Rockefeller Foundation-funded paper was called 'Dreaming the Future of Health for the Next 100 Years and more prophecy ensued as it described a dystopian future: 'The abundance of data, digitally tracking and linking people may mean the 'death of privacy' and may replace physical interaction with transient, virtual connection, generating isolation and raising questions of how values are shaped in virtual networks.' Next in the 'Covid' hoax preparation sequence came a 'table top' simulation in 2018 for another 'imaginary' pandemic of a disease called Clade X which was said to kill 900 million people. The exercise was organised by the Gates-funded Johns Hopkins University's Center for Health Security in the United States and this is the very same university that has been compiling the disgustingly and systematically erroneous global figures for 'Covid' cases and deaths. Similar Johns Hopkins health crisis scenarios have included the Dark Winter exercise in 2001 and Atlantic Storm in 2005.

Nostradamus 201

For sheer predictive genius look no further prophecy-watchers than the Bill Gates-funded Event 201 held only six weeks before the 'coronavirus pandemic' is supposed to have broken out in China and Event 201 was based on a scenario of a global 'coronavirus pandemic'. Melinda Gates, the great man's missus, told the BBC that he had 'prepared for years' for a coronavirus pandemic which told us what we already knew. Nostradamugates had predicted in a TED talk in 2015 that a pandemic was coming that would kill a lot of people and demolish the world economy. My god, the man is a machine – possibly even literally. Now here he was only weeks before the real thing funding just such a simulated scenario and involving his friends and associates at Johns Hopkins, the World Economic Forum Cult-front of Klaus Schwab, the United Nations,

Johnson & Johnson, major banks, and officials from China and the Centers for Disease Control in the United States. What synchronicity – Johns Hopkins would go on to compile the fraudulent 'Covid' figures, the World Economic Forum and Schwab would push the 'Great Reset' in response to 'Covid', the Centers for Disease Control would be at the forefront of 'Covid' policy in the United States, Johnson & Johnson would produce a 'Covid vaccine', and everything would officially start just weeks later in China. Spooky, eh? They were even accurate in creating a simulation of a 'virus' pandemic because the 'real thing' would also be a simulation. Event 201 was not an exercise preparing for something that might happen; it was a rehearsal for what those in control knew was *going* to happen and very shortly. Hours of this simulation were posted on the Internet and the various themes and responses mirrored what would soon be imposed to transform human society. News stories were inserted and what they said would be commonplace a few weeks later with still more prophecy perfection. Much discussion focused on the need to deal with misinformation and the 'anti-vax movement' which is exactly what happened when the 'virus' arrived – was said to have arrived – in the West.

Cult-owned social media banned criticism and exposure of the official 'virus' narrative and when I said there *was* no 'virus' in early April, 2020, I was banned by one platform after another including YouTube, Facebook and later Twitter. The mainstream broadcast media in Britain was in effect banned from interviewing me by the Tony-Blair-created government broadcasting censor Ofcom headed by career government bureaucrat Melanie Dawes who was appointed just as the 'virus' hoax was about to play out in January, 2020. At the same time the Ickonic media platform was using Vimeo, another ultra-Zionist-owned operation, while our own player was being created and they deleted in an instant hundreds of videos, documentaries, series and shows to confirm their unbelievable vindictiveness. We had copies, of course, and they had to be restored one by one when our player was ready. These people have no class. Sabbatian Facebook promised free advertisements for the Gates-

controlled World Health Organization narrative while deleting 'false claims and conspiracy theories' to stop 'misinformation' about the alleged coronavirus. All these responses could be seen just a short while earlier in the scenarios of Event 201. Extreme censorship was absolutely crucial for the Cult because the official story was so ridiculous and unsupportable by the evidence that it could never survive open debate and the free-flow of information and opinion. If you can't win a debate then don't have one is the Cult's approach throughout history. Facebook's little boy front man – front boy – Mark Zuckerberg equated 'credible and accurate information' with official sources and exposing their lies with 'misinformation'.

Silencing those that can see

The censorship dynamic of Event 201 is now the norm with an army of narrative-supporting 'fact-checker' organisations whose entire reason for being is to tell the public that official narratives are true and those exposing them are lying. One of the most appalling of these 'fact-checkers' is called NewsGuard founded by ultra-Zionist Americans Gordon Crovitz and Steven Brill. Crovitz is a former publisher of *The Wall Street Journal*, former Executive Vice President of Dow Jones, a member of the Council on Foreign Relations (CFR), and on the board of the American Association of Rhodes Scholars. The CFR and Rhodes Scholarships, named after Rothschild agent Cecil Rhodes who plundered the gold and diamonds of South Africa for his masters and the Cult, have featured widely in my books. NewsGuard don't seem to like me for some reason – I really can't think why – and they have done all they can to have me censored and discredited which is, to quote an old British politician, like being savaged by a dead sheep. They are, however, like all in the censorship network, very well connected and funded by organisations themselves funded by, or connected to, Bill Gates. As you would expect with anything associated with Gates NewsGuard has an offshoot called HealthGuard which 'fights online health care hoaxes'. How very kind. Somehow the NewsGuard European Managing Director Anna-Sophie Harling, a remarkably young-

looking woman with no broadcasting experience and little hands-on work in journalism, has somehow secured a position on the 'Content Board' of UK government broadcast censor Ofcom. An executive of an organisation seeking to discredit dissidents of the official narratives is making decisions for the government broadcast 'regulator' about content?? Another appalling 'fact-checker' is Full Fact funded by George Soros and global censors Google and Facebook.

It's amazing how many activists in the 'fact-checking', 'anti-hate', arena turn up in government-related positions – people like UK Labour Party activist Imran Ahmed who heads the Center for Countering Digital Hate founded by people like Morgan McSweeney, now chief of staff to the Labour Party's hapless and useless 'leader' Keir Starmer. Digital Hate – which is what it really is – uses the American spelling of Center to betray its connection to a transatlantic network of similar organisations which in 2020 shapeshifted from attacking people for 'hate' to attacking them for questioning the 'Covid' hoax and the dangers of the 'Covid vaccine'. It's just a coincidence, you understand. This is one of Imran Ahmed's hysterical statements: 'I would go beyond calling anti-vaxxers conspiracy theorists to say they are an extremist group that pose a national security risk.' No one could ever accuse this prat of understatement and he's including in that those parents who are now against vaccines after their children were damaged for life or killed by them. He's such a nice man. Ahmed does the rounds of the Woke media getting soft-ball questions from spineless 'journalists' who never ask what right he has to campaign to destroy the freedom of speech of others while he demands it for himself. There also seems to be an overrepresentation in Ofcom of people connected to the narrative-worshipping BBC. This incredible global network of narrative-support was super-vital when the 'Covid' hoax was played in the light of the mega-whopper lies that have to be defended from the spotlight cast by the most basic intelligence.

Setting the scene

The Cult plays the long game and proceeds step-by-step ensuring that everything is in place before major cards are played and they don't come any bigger than the 'Covid' hoax. The psychopaths can't handle events where the outcome isn't certain and as little as possible – preferably nothing – is left to chance. Politicians, government and medical officials who would follow direction were brought to illusory power in advance by the Cult web whether on the national stage or others like state governors and mayors of America. For decades the dynamic between officialdom, law enforcement and the public was changed from one of service to one of control and dictatorship. Behaviour manipulation networks established within government were waiting to impose the coming 'Covid' rules and regulations specifically designed to subdue and rewire the psyche of the people in the guise of protecting health. These included in the UK the Behavioural Insights Team part-owned by the British government Cabinet Office; the Scientific Pandemic Insights Group on Behaviours (SPI-B); and a whole web of intelligence and military groups seeking to direct the conversation on social media and control the narrative. Among them are the cyberwarfare (on the people) 77th Brigade of the British military which is also coordinated through the Cabinet Office as civilian and military leadership continues to combine in what they call the Fusion Doctrine. The 77th Brigade is a British equivalent of the infamous Israeli (Sabbatian) military cyberwarfare and Internet manipulation operation Unit 8200 which I expose at length in *The Trigger*. Also carefully in place were the medical and science advisers to government – many on the payroll past or present of Bill Gates – and a whole alternative structure of unelected government stood by to take control when elected parliaments were effectively closed down once the 'Covid' card was slammed on the table. The structure I have described here and so much more was installed in every major country through the Cult networks. The top-down control hierarchy looks like this: The Cult – Cult-owned Gates – the World Health Organization and Tedros – Gates-funded or controlled chief medical officers and science 'advisers' (dictators) in each country –

political 'leaders'– law enforcement – The People. Through this simple global communication and enforcement structure the policy of the Cult could be imposed on virtually the entire human population so long as they acquiesced to the fascism. With everything in place it was time for the button to be pressed in late 2019/early 2020.

These were the prime goals the Cult had to secure for its will to prevail:

1) Locking down economies, closing all but designated 'essential' businesses (Cult-owned corporations were 'essential'), and putting the population under house arrest was an imperative to destroy independent income and employment and ensure dependency on the Cult-controlled state in the Hunger Games Society. Lockdowns had to be established as the global blueprint from the start to respond to the 'virus' and followed by pretty much the entire world.

2) The global population had to be terrified into believing in a deadly 'virus' that didn't actually exist so they would unquestioningly obey authority in the belief that authority must know how best to protect them and their families. Software salesman Gates would suddenly morph into the world's health expert and be promoted as such by the Cult-owned media.

3) A method of testing that wasn't testing for the 'virus', but was only claimed to be, had to be in place to provide the illusion of 'cases' and subsequent 'deaths' that had a very different cause to the 'Covid-19' that would be scribbled on the death certificate.

4) Because there was no 'virus' and the great majority testing positive with a test not testing for the 'virus' would have no symptoms of anything the lie had to be sold that people without symptoms (without the 'virus') could still pass it on to others. This was crucial to justify for the first time quarantining – house arresting – healthy people. Without this the economy-destroying lockdown of *everybody* could not have been credibly sold.

5) The 'saviour' had to be seen as a vaccine which beyond evil drug companies were working like angels of mercy to develop as quickly as possible, with all corners cut, to save the day. The public must absolutely not know that the 'vaccine' had nothing to do with a 'virus' or that the contents were ready and waiting with a very different motive long before the 'Covid' card was even lifted from the pack.

I said in March, 2020, that the 'vaccine' would have been created way ahead of the 'Covid' hoax which justified its use and the following December an article in the New York *Intelligencer* magazine said the Moderna 'vaccine' had been 'designed' by

January, 2020. This was 'before China had even acknowledged that the disease could be transmitted from human to human, more than a week before the first confirmed coronavirus case in the United States'. The article said that by the time the first American death was announced a month later 'the vaccine had already been manufactured and shipped to the National Institutes of Health for the beginning of its Phase I clinical trial'. The 'vaccine' was actually 'designed' long before that although even with this timescale you would expect the article to ask how on earth it could have been done that quickly. Instead it asked why the 'vaccine' had not been rolled out then and not months later. Journalism in the mainstream is truly dead. I am going to detail in the next chapter why the 'virus' has never existed and how a hoax on that scale was possible, but first the foundation on which the Big Lie of 'Covid' was built.

The test that doesn't test

Fraudulent 'testing' is the bottom line of the whole 'Covid' hoax and was the means by which a 'virus' that did not exist *appeared* to exist. They could only achieve this magic trick by using a test not testing for the 'virus'. To use a test that *was* testing for the 'virus' would mean that every test would come back negative given there was no 'virus'. They chose to exploit something called the RT-PCR test invented by American biochemist Kary Mullis in the 1980s who said publicly that his PCR test … *cannot detect infectious disease*. Yes, the 'test' used worldwide to detect infectious 'Covid' to produce all the illusory 'cases' and 'deaths' compiled by Johns Hopkins and others *cannot detect infectious disease*. This fact came from the mouth of the man who invented PCR and was awarded the Nobel Prize in Chemistry in 1993 for doing so. Sadly, and incredibly conveniently for the Cult, Mullis died in August, 2019, at the age of 74 just before his test would be fraudulently used to unleash fascism on the world. He was said to have died from pneumonia which was an irony in itself. A few months later he would have had 'Covid-19' on his death certificate. I say the timing of his death was convenient because had he lived Mullis, a brilliant, honest and decent man, would have been

vociferously speaking out against the use of his test to detect 'Covid' when it was never designed, or able, to do that. I know that to be true given that Mullis made the same point when his test was used to 'detect' – not detect – HIV. He had been seriously critical of the Gallo/Montagnier claim to have isolated the HIV 'virus' and shown it to cause AIDS for which Mullis said there was no evidence. AIDS is actually not a disease but a series of diseases from which people die all the time. When they die from those *same diseases* after a positive 'test' for HIV then AIDS goes on their death certificate. I think I've heard that before somewhere. Countries instigated a policy with 'Covid' that anyone who tested positive with a test not testing for the 'virus' and died of any other cause within 28 days and even longer 'Covid-19' had to go on the death certificate. Cases have come from the test that can't test for infectious disease and the deaths are those who have died of *anything* after testing positive with a test not testing for the 'virus'. I'll have much more later about the death certificate scandal.

Mullis was deeply dismissive of the now US 'Covid' star Anthony Fauci who he said was a liar who didn't know anything about anything – 'and I would say that to his face – nothing.' He said of Fauci: 'The man thinks he can take a blood sample, put it in an electron microscope and if it's got a virus in there you'll know it – he doesn't understand electron microscopy and he doesn't understand medicine and shouldn't be in a position like he's in.' That position, terrifyingly, has made him the decider of 'Covid' fascism policy on behalf of the Cult in his role as director since 1984 of the National Institute of Allergy and Infectious Diseases (NIAID) while his record of being wrong is laughable; but being wrong, so long as it's the *right kind* of wrong, is why the Cult loves him. He'll say anything the Cult tells him to say. Fauci was made Chief Medical Adviser to the President immediately Biden took office. Biden was installed in the White House by Cult manipulation and one of his first decisions was to elevate Fauci to a position of even more control. This is a coincidence? Yes, and I identify as a flamenco dancer called Lola. How does such an incompetent criminal like Fauci remain in that

pivotal position in American health since *the 1980s*? When you serve the Cult it looks after you until you are surplus to requirements. Kary Mullis said prophetically of Fauci and his like: 'Those guys have an agenda and it's not an agenda we would like them to have … they make their own rules, they change them when they want to, and Tony Fauci does not mind going on television in front of the people who pay his salary and lie directly into the camera.' Fauci has done that almost daily since the 'Covid' hoax began. Lying is in Fauci's DNA. To make the situation crystal clear about the PCR test this is a direct quote from its inventor Kary Mullis:

> It [the PCR test] doesn't tell you that you're sick and doesn't tell you that the thing you ended up with was really going to hurt you ...'

Ask yourself why governments and medical systems the world over have been using this very test to decide who is 'infected' with the SARS-CoV-2 'virus' and the alleged disease it allegedly causes, 'Covid-19'. The answer to that question will tell you what has been going on. By the way, here's a little show-stopper – the 'new' SARS-CoV-2 'virus' was 'identified' as such right from the start using … *the PCR test not testing for the 'virus'*. If you are new to this and find that shocking then stick around. I have hardly started yet. Even worse, other 'tests', like the 'Lateral Flow Device' (LFD), are considered so useless that they have to be *confirmed* by the PCR test! Leaked emails written by Ben Dyson, adviser to UK 'Health' Secretary Matt Hancock, said they were 'dangerously unreliable'. Dyson, executive director of strategy at the Department of Health, wrote: 'As of today, someone who gets a positive LFD result in (say) London has at best a 25 per cent chance of it being a true positive, but if it is a self-reported test potentially as low as 10 per cent (on an optimistic assumption about specificity) or as low as 2 per cent (on a more pessimistic assumption).' These are the 'tests' that schoolchildren and the public are being urged to have twice a week or more and have to isolate if they get a positive. Each fake positive goes in the statistics as a 'case' no matter how ludicrously inaccurate and the

'cases' drive lockdown, masks and the pressure to 'vaccinate'. The government said in response to the email leak that the 'tests' were accurate which confirmed yet again what shocking bloody liars they are. The real false positive rate is *100 percent* as we'll see. In another 'you couldn't make it up' the UK government agreed to pay £2.8 billion to California's Innova Medical Group to supply the irrelevant lateral flow tests. The company's primary test-making centre is in China. Innova Medical Group, established in March, 2020, is owned by Pasaca Capital Inc, chaired by Chinese-American millionaire Charles Huang who was born in Wuhan.

How it works – and how it doesn't

The RT-PCR test, known by its full title of Polymerase chain reaction, is used across the world to make millions, even billions, of copies of a DNA/RNA genetic information sample. The process is called 'amplification' and means that a tiny sample of genetic material is amplified to bring out the detailed content. I stress that it is not testing for an infectious disease. It is simply amplifying a sample of genetic material. In the words of Kary Mullis: 'PCR is … just a process that's used to make a whole lot of something out of something.' To emphasise the point companies that make the PCR tests circulated around the world to 'test' for 'Covid' warn on the box that it can't be used to detect 'Covid' or infectious disease and is for research purposes only. It's okay, rest for a minute and you'll be fine. This is the test that produces the 'cases' and 'deaths' that have been used to destroy human society. All those global and national medical and scientific 'experts' demanding this destruction to 'save us' *KNOW* that the test is not testing for the 'virus' and the cases and deaths they claim to be real are an almost unimaginable fraud. Every one of them and so many others including politicians and psychopaths like Gates and Tedros must be brought before Nuremburg-type trials and jailed for the rest of their lives. The more the genetic sample is amplified by PCR the more elements of that material become sensitive to the test and by that I don't mean sensitive for a 'virus' but for elements of the genetic material which

is *naturally* in the body or relates to remnants of old conditions of various kinds lying dormant and causing no disease. Once the amplification of the PCR reaches a certain level *everyone* will test positive. So much of the material has been made sensitive to the test that everyone will have some part of it in their body. Even lying criminals like Fauci have said that once PCR amplifications pass 35 cycles everything will be a false positive that cannot be trusted for the reasons I have described. I say, like many proper doctors and scientists, that 100 percent of the 'positives' are false, but let's just go with Fauci for a moment.

He says that any amplification over 35 cycles will produce false positives and yet the US Centers for Disease Control (CDC) and Food and Drug Administration (FDA) have recommended up to *40 cycles* and the National Health Service (NHS) in Britain admitted in an internal document for staff that it was using *45 cycles* of amplification. A long list of other countries has been doing the same and at least one 'testing' laboratory has been using *50 cycles*. Have you ever heard a doctor, medical 'expert' or the media ask what level of amplification has been used to claim a 'positive'. The 'test' comes back 'positive' and so you have the 'virus', end of story. Now we can see how the government in Tanzania could send off samples from a goat and a pawpaw fruit under human names and both came back positive for 'Covid-19'. Tanzania president John Magufuli mocked the 'Covid' hysteria, the PCR test and masks and refused to import the DNA-manipulating 'vaccine'. The Cult hated him and an article sponsored by the Bill Gates Foundation appeared in the London *Guardian* in February, 2021, headed 'It's time for Africa to rein in Tanzania's anti-vaxxer president'. Well, 'reined in' he shortly was. Magufuli appeared in good health, but then, in March, 2021, he was dead at 61 from 'heart failure'. He was replaced by Samia Hassan Suhulu who is connected to Klaus Schwab's World Economic Forum and she immediately reversed Magufuli's 'Covid' policy. A sample of cola tested positive for 'Covid' with the PCR test in Germany while American actress and singer-songwriter Erykah Badu tested positive in one nostril and negative in the other. Footballer Ronaldo called

the PCR test 'bullshit' after testing positive three times and being forced to quarantine and miss matches when there was nothing wrong with him. The mantra from Tedros at the World Health Organization and national governments (same thing) has been test, test, test. They know that the more tests they can generate the more fake 'cases' they have which go on to become 'deaths' in ways I am coming to. The UK government has its Operation Moonshot planned to test multiple millions every day in workplaces and schools with free tests for everyone to use twice a week at home in line with the Cult plan from the start to make testing part of life. A government advertisement for an 'Interim Head of Asymptomatic Testing Communication' said the job included responsibility for delivering a 'communications strategy' (propaganda) 'to support the expansion of asymptomatic testing that *'normalises testing as part of everyday life*'. More tests means more fake 'cases', 'deaths' and fascism. I have heard of, and from, many people who booked a test, couldn't turn up, and yet got a positive result through the post for a test they'd never even had. The whole thing is crazy, but for the Cult there's method in the madness. Controlling and manipulating the level of amplification of the test means the authorities can control whenever they want the number of apparent 'cases' and 'deaths'. If they want to justify more fascist lockdown and destruction of livelihoods they keep the amplification high. If they want to give the illusion that lockdowns and the 'vaccine' are working then they lower the amplification and 'cases' and 'deaths' will appear to fall. In January, 2021, the Cult-owned World Health Organization suddenly warned laboratories about over-amplification of the test and to lower the threshold. Suddenly headlines began appearing such as: 'Why ARE "Covid" cases plummeting?' This was just when the vaccine rollout was underway and I had predicted months before they would make cases appear to fall through amplification tampering when the 'vaccine' came. These people are so predictable.

Cow vaccines?

The question must be asked of what is on the test swabs being poked far up the nose of the population to the base of the brain? A nasal swab punctured one woman's brain and caused it to leak fluid. Most of these procedures are being done by people with little training or medical knowledge. Dr Lorraine Day, former orthopaedic trauma surgeon and Chief of Orthopaedic Surgery at San Francisco General Hospital, says the tests are really a *'vaccine'*. Cows have long been vaccinated this way. She points out that masks have to cover the nose and the mouth where it is claimed the 'virus' exists in saliva. Why then don't they take saliva from the mouth as they do with a DNA test instead of pushing a long swab up the nose towards the brain? The ethmoid bone separates the nasal cavity from the brain and within that bone is the cribriform plate. Dr Day says that when the swab is pushed up against this plate and twisted the procedure is 'depositing things back there'. She claims that among these 'things' are nanoparticles that can enter the brain. Researchers have noted that a team at the Gates-funded Johns Hopkins have designed tiny, star-shaped micro-devices that can latch onto intestinal mucosa and release drugs into the body. Mucosa is the thin skin that covers the inside surface of parts of the body such as *the nose* and mouth and produces mucus to protect them. The Johns Hopkins micro-devices are called 'theragrippers' and were 'inspired' by a parasitic worm that digs its sharp teeth into a host's intestines. Nasal swabs are also coated in the sterilisation agent ethylene oxide. The US National Cancer Institute posts this explanation on its website:

> At room temperature, ethylene oxide is a flammable colorless gas with a sweet odor. It is used primarily to produce other chemicals, including antifreeze. In smaller amounts, ethylene oxide is used as a pesticide and a sterilizing agent. The ability of ethylene oxide to damage DNA makes it an effective sterilizing agent but also accounts for its cancer-causing activity.

The Institute mentions lymphoma and leukaemia as cancers most frequently reported to be associated with occupational exposure to ethylene oxide along with stomach and breast cancers. How does anyone think this is going to work out with the constant testing

regime being inflicted on adults and children at home and at school that will accumulate in the body anything that's on the swab?

Doctors know best

It is vital for people to realise that 'hero' doctors 'know' only what the Big Pharma-dominated medical authorities tell them to 'know' and if they refuse to 'know' what they are told to 'know' they are out the door. They are mostly not physicians or healers, but repeaters of the official narrative – or else. I have seen alleged professional doctors on British television make shocking statements that we are supposed to take seriously. One called 'Dr' Amir Khan, who is actually telling patients how to respond to illness, said that men could take the birth pill to 'help slow down the effects of Covid-19'. In March, 2021, another ridiculous 'Covid study' by an American doctor proposed injecting men with the female sex hormone progesterone as a 'Covid' treatment. British doctor Nighat Arif told the BBC that face coverings were now going to be part of ongoing normal. Yes, the vaccine protects you, she said (evidence?) … but the way to deal with viruses in the community was always going to come down to hand washing, face covering and keeping a physical distance. That's not what we were told before the 'vaccine' was circulating. Arif said she couldn't imagine ever again going on the underground or in a lift without a mask. I was just thanking my good luck that she was not my doctor when she said – in March, 2021 – that if 'we are *behaving* and we are doing all the right things' she thought we could 'have our nearest and dearest around us at home … around *Christmas* and N*ew Year!* Her patronising delivery was the usual school teacher talking to six-year-olds as she repeated every government talking point and probably believed them all. If we have learned anything from the 'Covid' experience surely it must be that humanity's perception of doctors needs a fundamental rethink. NHS 'doctor' Sara Kayat told her television audience that the 'Covid vaccine' would '100 percent prevent hospitalisation and death'. Not even Big Pharma claimed that. We have to stop taking 'experts' at their word without question when so many of them are

clueless and only repeating the party line on which their careers depend. That is not to say there are not brilliants doctors – there are and I have spoken to many of them since all this began – but you won't see them in the mainstream media or quoted by the psychopaths and yes-people in government.

Remember the name – Christian Drosten

German virologist Christian Drosten, Director of Charité Institute of Virology in Berlin, became a national star after the pandemic hoax began. He was feted on television and advised the German government on 'Covid' policy. Most importantly to the wider world Drosten led a group that produced the 'Covid' testing protocol for the PCR test. What a remarkable feat given the PCR cannot test for infectious disease and even more so when you think that Drosten said that his method of testing for SARS-CoV-2 was developed 'without having virus material available'. *He developed a test for a 'virus' that he didn't have and had never seen*. Let that sink in as you survey the global devastation that came from what he did. The whole catastrophe of Drosten's 'test' was based on the alleged genetic sequence published by Chinese scientists on the Internet. We will see in the next chapter that this alleged 'genetic sequence' has never been produced by China or anyone and cannot be when there *is no* SARS-CoV-2. Drosten, however, doesn't seem to let little details like that get in the way. He was the lead author with Victor Corman from the same Charité Hospital of the paper 'Detection of 2019 novel coronavirus (2019-nCoV) by real-time PCR' published in a magazine called *Eurosurveillance*. This became known as the Corman-Drosten paper. In November, 2020, with human society devastated by the effects of the Corman-Drosten test baloney, the protocol was publicly challenged by 22 international scientists and independent researchers from Europe, the United States, and Japan. Among them were senior molecular geneticists, biochemists, immunologists, and microbiologists. They produced a document headed 'External peer review of the RTPCR test to detect SARS-Cov-2 Reveals 10 Major Flaws At The Molecular and Methodological Level: Consequences

For False-Positive Results'. The flaws in the Corman-Drosten test included the following:

- The test is non-specific because of erroneous design
- Results are enormously variable
- The test is unable to discriminate between the whole 'virus' and viral fragments
- It doesn't have positive or negative controls
- The test lacks a standard operating procedure
- It is unsupported by proper peer view

The scientists said the PCR 'Covid' testing protocol was not founded on science and they demanded the Corman-Drosten paper be retracted by *Eurosurveillance*. They said all present and previous Covid deaths, cases, and 'infection rates' should be subject to a massive retroactive inquiry. Lockdowns and travel restrictions should be reviewed and relaxed and those diagnosed through PCR to have 'Covid-19' should not be forced to isolate. Dr Kevin Corbett, a health researcher and nurse educator with a long academic career producing a stream of peer-reviewed publications at many UK universities, made the same point about the PCR test debacle. He said of the scientists' conclusions: 'Every scientific rationale for the development of that test has been totally destroyed by this paper. It's like Hiroshima/Nagasaki to the Covid test.' He said that China hadn't given them an isolated 'virus' when Drosten developed the test. Instead they had developed the test from *a sequence in a gene bank*.' Put another way … *they made it up*! The scientists were supported in this contention by a Portuguese appeals court which ruled in November, 2020, that PCR tests are unreliable and it is unlawful to quarantine people based solely on a PCR test. The point about China not providing an isolated virus must be true when the 'virus' has never been isolated to this day and the consequences of that will become clear. Drosten and company produced this useless 'protocol' right on cue in January, 2020, just as the 'virus' was said to

be moving westward and it somehow managed to successfully pass a peer-review in 24 hours. In other words there was no peer-review for a test that would be used to decide who had 'Covid' and who didn't across the world. The Cult-created, Gates-controlled World Health Organization immediately recommended all its nearly 200 member countries to use the Drosten PCR protocol to detect 'cases' and 'deaths'. The sting was underway and it continues to this day.

So who is this Christian Drosten that produced the means through which death, destruction and economic catastrophe would be justified? His education background, including his doctoral thesis, would appear to be somewhat shrouded in mystery and his track record is dire as with another essential player in the 'Covid' hoax, the Gates-funded Professor Neil Ferguson at the Gates-funded Imperial College in London of whom more shortly. Drosten predicted in 2003 that the alleged original SARS 'virus' (SARS-1') was an epidemic that could have serious effects on economies and an effective vaccine would take at least two years to produce. Drosten's answer to every alleged 'outbreak' is a vaccine which you won't be shocked to know. What followed were just 774 official deaths worldwide and none in Germany where there were only nine cases. That is even if you believe there ever was a SARS 'virus' when the evidence is zilch and I will expand on this in the next chapter. Drosten claims to be co-discoverer of 'SARS-1' and developed a test for it in 2003. He was screaming warnings about 'swine flu' in 2009 and how it was a widespread infection far more severe than any dangers from a vaccine could be and people should get vaccinated. It would be helpful for Drosten's vocal chords if he simply recorded the words 'the virus is deadly and you need to get vaccinated' and copies could be handed out whenever the latest made-up threat comes along. Drosten's swine flu epidemic never happened, but Big Pharma didn't mind with governments spending hundreds of millions on vaccines that hardly anyone bothered to use and many who did wished they hadn't. A study in 2010 revealed that the risk of dying from swine flu, or H1N1, was no higher than that of the annual seasonal flu which is what at least most of 'it' really was as in

the case of 'Covid-19'. A media investigation into Drosten asked how with such a record of inaccuracy he could be *the* government adviser on these issues. The answer to that question is the same with Drosten, Ferguson and Fauci – they keep on giving the authorities the 'conclusions' and 'advice' they want to hear. Drosten certainly produced the goods for them in January, 2020, with his PCR protocol garbage and provided the foundation of what German internal medicine specialist Dr Claus Köhnlein, co-author of *Virus Mania*, called the 'test pandemic'. The 22 scientists in the *Eurosurveillance* challenge called out conflicts of interest within the Drosten 'protocol' group and with good reason. Olfert Landt, a regular co-author of Drosten 'studies', owns the biotech company TIB Molbiol Syntheselabor GmbH in Berlin which manufactures and sells the tests that Drosten and his mates come up with. They have done this with SARS, Enterotoxigenic E. coli (ETEC), MERS, Zika 'virus', yellow fever, and now 'Covid'. Landt told the *Berliner Zeitung* newspaper:

> The testing, design and development came from the Charité [Drosten and Corman]. We simply implemented it immediately in the form of a kit. And if we don't have the virus, which originally only existed in Wuhan, we can make a synthetic gene to simulate the genome of the virus. That's what we did very quickly.

This is more confirmation that the Drosten test was designed without access to the 'virus' and only a synthetic simulation which is what SARS-CoV-2 really is – a computer-generated synthetic fiction. It's quite an enterprise they have going here. A Drosten team decides what the test for something should be and Landt's biotech company flogs it to governments and medical systems across the world. His company must have made an absolute fortune since the 'Covid' hoax began. Dr Reiner Fuellmich, a prominent German consumer protection trial lawyer in Germany and California, is on Drosten's case and that of Tedros at the World Health Organization for crimes against humanity with a class-action lawsuit being prepared in the United States and other legal action in Germany.

Why China?

Scamming the world with a 'virus' that doesn't exist would seem impossible on the face of it, but not if you have control of the relatively few people that make policy decisions and the great majority of the global media. Remember it's not about changing 'real' reality it's about controlling *perception* of reality. You don't have to make something happen you only have make people *believe* that it's happening. Renegade Minds understand this and are therefore much harder to swindle. 'Covid-19' is not a 'real' 'virus'. It's a mind virus, like a computer virus, which has infected the minds, not the bodies, of billions. It all started, publically at least, in China and that alone is of central significance. The Cult was behind the revolution led by its asset Mao Zedong, or Chairman Mao, which established the People's Republic of China on October 1st, 1949. It should have been called The Cult's Republic of China, but the name had to reflect the recurring illusion that vicious dictatorships are run by and for the people (see all the 'Democratic Republics' controlled by tyrants). In the same way we have the 'Biden' Democratic Republic of America officially ruled by a puppet tyrant (at least temporarily) on behalf of Cult tyrants. The creation of Mao's merciless communist/fascist dictatorship was part of a frenzy of activity by the Cult at the conclusion of World War Two which, like the First World War, it had instigated through its assets in Germany, Britain, France, the United States and elsewhere. Israel was formed in 1948; the Soviet Union expanded its 'Iron Curtain' control, influence and military power with the Warsaw Pact communist alliance in 1955; the United Nations was formed in 1945 as a Cult precursor to world government; and a long list of world bodies would be established including the World Health Organization (1948), World Trade Organization (1948 under another name until 1995), International Monetary Fund (1945) and World Bank (1944). Human society was redrawn and hugely centralised in the global Problem-Reaction-Solution that was World War Two. All these changes were significant. Israel would become the headquarters of the Sabbatians

and the revolution in China would prepare the ground and control system for the events of 2019/2020.

Renegade Minds know there are no borders except for public consumption. The Cult is a seamless, borderless global entity and to understand the game we need to put aside labels like borders, nations, countries, communism, fascism and democracy. These delude the population into believing that countries are ruled within their borders by a government of whatever shade when these are mere agencies of a global power. America's illusion of democracy and China's communism/fascism are subsidiaries – vehicles – for the same agenda. We may hear about conflict and competition between America and China and on the lower levels that will be true; but at the Cult level they are branches of the same company in the way of the McDonald's example I gave earlier. I have tracked in the books over the years support by US governments of both parties for Chinese Communist Party infiltration of American society through allowing the sale of land, even military facilities, and the acquisition of American business and university influence. All this is underpinned by the infamous stealing of intellectual property and technological know-how. Cult-owned Silicon Valley corporations waive their fraudulent 'morality' to do business with human-rights-free China; Cult-controlled Disney has become China's PR department; and China in effect owns 'American' sports such as basketball which depends for much of its income on Chinese audiences. As a result any sports player, coach or official speaking out against China's horrific human rights record is immediately condemned or fired by the China-worshipping National Basketball Association. One of the first acts of China-controlled Biden was to issue an executive order telling federal agencies to stop making references to the 'virus' by the 'geographic location of its origin'. Long-time Congressman Jerry Nadler warned that criticising China, America's biggest rival, leads to hate crimes against Asian people in the United States. So shut up you bigot. China is fast closing in on Israel as a country that must not be criticised which is apt, really, given that Sabbatians control them both. The two countries have

developed close economic, military, technological and strategic ties which include involvement in China's 'Silk Road' transport and economic initiative to connect China with Europe. Israel was the first country in the Middle East to recognise the establishment of Mao's tyranny in 1950 months after it was established.

Project Wuhan – the 'Covid' Psyop

I emphasise again that the Cult plays the long game and what is happening to the world today is the result of centuries of calculated manipulation following a script to take control step-by-step of every aspect of human society. I will discuss later the common force behind all this that has spanned those centuries and thousands of years if the truth be told. Instigating the Mao revolution in China in 1949 with a 2020 'pandemic' in mind is not only how they work – the 71 years between them is really quite short by the Cult's standards of manipulation preparation. The reason for the Cult's Chinese revolution was to create a fiercely-controlled environment within which an extreme structure for human control could be incubated to eventually be unleashed across the world. We have seen this happen since the 'pandemic' emerged from China with the Chinese control-structure founded on AI technology and tyrannical enforcement sweep across the West. Until the moment when the Cult went for broke in the West and put its fascism on public display Western governments had to pay some lip-service to freedom and democracy to not alert too many people to the tyranny-in-the-making. Freedoms were more subtly eroded and power centralised with covert government structures put in place waiting for the arrival of 2020 when that smokescreen of 'freedom' could be dispensed with. The West was not able to move towards tyranny before 2020 anything like as fast as China which was created as a tyranny and had no limits on how fast it could construct the Cult's blueprint for global control. When the time came to impose that structure on the world it was the same Cult-owned Chinese communist/fascist government that provided the excuse – the 'Covid pandemic'. It was absolutely crucial to the Cult plan for the Chinese response to the 'pandemic' –

draconian lockdowns of the entire population – to become the blueprint that Western countries would follow to destroy the livelihoods and freedom of their people. This is why the Cult-owned, Gates-owned, WHO Director-General Tedros said early on:

The Chinese government is to be congratulated for the extraordinary measures it has taken to contain the outbreak. China is actually setting a new standard for outbreak response and it is not an exaggeration.

Forbes magazine said of China: '… those measures protected untold millions from getting the disease'. The Rockefeller Foundation 'epidemic scenario' document in 2010 said 'prophetically':

However, a few countries did fare better – China in particular. The Chinese government's quick imposition and enforcement of mandatory quarantine for all citizens, as well as its instant and near-hermetic sealing off of all borders, saved millions of lives, stopping the spread of the virus far earlier than in other countries and enabling a swifter post-pandemic recovery.

Once again – *spooky*.

The first official story was the 'bat theory' or rather the bat diversion. The source of the 'virus outbreak' we were told was a ''wet market' in Wuhan where bats and other animals are bought and eaten in horrifically unhygienic conditions. Then another story emerged through the alternative media that the 'virus' had been released on purpose or by accident from a BSL-4 (biosafety level 4) laboratory in Wuhan not far from the wet market. The lab was reported to create and work with lethal concoctions and bioweapons. Biosafety level 4 is the highest in the World Health Organization system of safety and containment. Renegade Minds are aware of what I call designer manipulation. The ideal for the Cult is for people to buy its prime narrative which in the opening salvoes of the 'pandemic' was the wet market story. It knows, however, that there is now a considerable worldwide alternative media of researchers sceptical of anything governments say and they are often given a version of events in a form they can perceive as credible while misdirecting them from the real truth. In this case let them

think that the conspiracy involved is a 'bioweapon virus' released from the Wuhan lab to keep them from the real conspiracy – *there is no 'virus'*. The WHO's current position on the source of the outbreak at the time of writing appears to be: 'We haven't got a clue, mate.' This is a good position to maintain mystery and bewilderment. The inner circle will know where the 'virus' came from – *nowhere*. The bottom line was to ensure the public believed there *was* a 'virus' and it didn't much matter if they thought it was natural or had been released from a lab. The belief that there was a 'deadly virus' was all that was needed to trigger global panic and fear. The population was terrified into handing their power to authority and doing what they were told. They had to or they were 'all gonna die'.

In March, 2020, information began to come my way from real doctors and scientists and my own additional research which had my intuition screaming: 'Yes, that's it! *There is no virus*.' The 'bioweapon' was not the 'virus'; it was the *'vaccine'* already being talked about that would be the bioweapon. My conclusion was further enhanced by happenings in Wuhan. The 'virus' was said to be sweeping the city and news footage circulated of people collapsing in the street (which they've never done in the West with the same 'virus'). The Chinese government was building 'new hospitals' in a matter of ten days to 'cope with demand' such was the virulent nature of the 'virus'. Yet in what seemed like no time the 'new hospitals' closed – even if they even opened – and China declared itself 'virus-free'. It was back to business as usual. This was more propaganda to promote the Chinese draconian lockdowns in the West as the way to 'beat the virus'. Trouble was that we subsequently had lockdown after lockdown, but never business as usual. As the people of the West and most of the rest of the world were caught in an ever-worsening spiral of lockdown, social distancing, masks, isolated old people, families forced apart, and livelihood destruction, it was party-time in Wuhan. Pictures emerged of thousands of people enjoying pool parties and concerts. It made no sense until you realised there never was a 'virus' and the

whole thing was a Cult set-up to transform human society out of one its major global strongholds – China.

How is it possible to deceive virtually the entire world population into believing there is a deadly virus when there is not even a 'virus' let alone a deadly one? It's nothing like as difficult as you would think and that's clearly true because it happened.

Postscript: See end of book Postscript for more on the 'Wuhan lab virus release' story which the authorities and media were pushing heavily in the summer of 2021 to divert attention from the truth that the 'Covid virus' is pure invention.

CHAPTER FIVE

There *is no* 'virus'

You can fool some of the people all of the time, and all of the people some of the time, but you cannot fool all of the people all of the time
Abraham Lincoln

The greatest form of mind control is repetition. The more you repeat the same mantra of alleged 'facts' the more will accept them to be true. It becomes an 'everyone knows that, mate'. If you can also censor any other version or alternative to your alleged 'facts' you are pretty much home and cooking.

By the start of 2020 the Cult owned the global mainstream media almost in its entirety to spew out its 'Covid' propaganda and ignore or discredit any other information and view. Cult-owned social media platforms in Cult-owned Silicon Valley were poised and ready to unleash a campaign of ferocious censorship to obliterate all but the official narrative. To complete the circle many demands for censorship by Silicon Valley were led by the mainstream media as 'journalists' became full-out enforcers for the Cult both as propagandists and censors. Part of this has been the influx of young people straight out of university who have become 'journalists' in significant positions. They have no experience and a headful of programmed perceptions from their years at school and university at a time when today's young are the most perceptually-targeted generations in known human history given the insidious impact of technology. They enter the media perceptually prepared and ready to repeat the narratives of the system that programmed them to

repeat its narratives. The BBC has a truly pathetic 'specialist disinformation reporter' called Marianna Spring who fits this bill perfectly. She is clueless about the world, how it works and what is really going on. Her role is to discredit anyone doing the job that a proper journalist would do and system-serving hacks like Spring wouldn't dare to do or even see the need to do. They are too busy licking the arse of authority which can never be wrong and, in the case of the BBC propaganda programme, *Panorama*, contacting payments systems such as PayPal to have a donations page taken down for a film company making documentaries questioning vaccines. Even the BBC soap opera *EastEnders* included a disgracefully biased scene in which an inarticulate white working class woman was made to look foolish for questioning the 'vaccine' while a well-spoken black man and Asian woman promoted the government narrative. It ticked every BBC box and the fact that the black and minority community was resisting the 'vaccine' had nothing to do with the way the scene was written. The BBC has become a disgusting tyrannical propaganda and censorship operation that should be defunded and disbanded and a free media take its place with a brief to stop censorship instead of demanding it. A BBC 'interview' with Gates goes something like: 'Mr Gates, sir, if I can call you sir, would you like to tell our audience why you are such a great man, a wonderful humanitarian philanthropist, and why you should absolutely be allowed as a software salesman to decide health policy for approaching eight billion people? Thank you, sir, please sir.' Propaganda programming has been incessant and merciless and when all you hear is the same story from the media, repeated by those around you who have only heard the same story, is it any wonder that people on a grand scale believe absolute mendacious garbage to be true? You are about to see, too, why this level of information control is necessary when the official 'Covid' narrative is so nonsensical and unsupportable by the evidence.

Structure of Deceit

The pyramid structure through which the 'Covid' hoax has been manifested is very simple and has to be to work. As few people as possible have to be involved with full knowledge of what they are doing – and why – or the real story would get out. At the top of the pyramid are the inner core of the Cult which controls Bill Gates who, in turn, controls the World Health Organization through his pivotal funding and his puppet Director-General mouthpiece, Tedros. Before he was appointed Tedros was chair of the Gates-founded Global Fund to 'fight against AIDS, tuberculosis and malaria', a board member of the Gates-funded 'vaccine alliance' GAVI, and on the board of another Gates-funded organisation. Gates owns him and picked him for a specific reason – Tedros is a crook and worse. 'Dr' Tedros (he's not a medical doctor, the first WHO chief not to be) was a member of the tyrannical Marxist government of Ethiopia for decades with all its human rights abuses. He has faced allegations of corruption and misappropriation of funds and was exposed three times for covering up cholera epidemics while Ethiopia's health minister. Tedros appointed the mass-murdering genocidal Zimbabwe dictator Robert Mugabe as a WHO goodwill ambassador for public health which, as with Tedros, is like appointing a psychopath to run a peace and love campaign. The move was so ridiculous that he had to drop Mugabe in the face of widespread condemnation. American economist David Steinman, a Nobel peace prize nominee, lodged a complaint with the International Criminal Court in The Hague over alleged genocide by Tedros when he was Ethiopia's foreign minister. Steinman says Tedros was a 'crucial decision maker' who directed the actions of Ethiopia's security forces from 2013 to 2015 and one of three officials in charge when those security services embarked on the 'killing' and 'torturing' of Ethiopians. You can see where Tedros is coming from and it's sobering to think that he has been the vehicle for Gates and the Cult to direct the global response to 'Covid'. Think about that. A psychopathic Cult dictates to psychopath Gates who dictates to psychopath Tedros who dictates how countries of the world must respond to a 'Covid virus' never scientifically shown to exist. At the same time psychopathic Cult-owned Silicon Valley information

giants like Google, YouTube, Facebook and Twitter announced very early on that they would give the Cult/Gates/Tedros/WHO version of the narrative free advertising and censor those who challenged their intelligence-insulting, mendacious story.

The next layer in the global 'medical' structure below the Cult, Gates and Tedros are the chief medical officers and science 'advisers' in each of the WHO member countries which means virtually all of them. Medical officers and arbiters of science (they're not) then take the WHO policy and recommended responses and impose them on their country's population while the political 'leaders' say they are deciding policy (they're clearly not) by 'following the science' on the advice of the 'experts' – the same medical officers and science 'advisers' (dictators). In this way with the rarest of exceptions the entire world followed the same policy of lockdown, people distancing, masks and 'vaccines' dictated by the psychopathic Cult, psychopathic Gates and psychopathic Tedros who we are supposed to believe give a damn about the health of the world population they are seeking to enslave. That, amazingly, is all there is to it in terms of crucial decision-making. Medical staff in each country then follow like sheep the dictates of the shepherds at the top of the national medical hierarchies – chief medical officers and science 'advisers' who themselves follow like sheep the shepherds of the World Health Organization and the Cult. Shepherds at the national level often have major funding and other connections to Gates and his Bill and Melinda Gates Foundation which carefully hands out money like confetti at a wedding to control the entire global medical system from the WHO down.

Follow the money

Christopher Whitty, Chief Medical Adviser to the UK Government at the centre of 'virus' policy, a senior adviser to the government's Scientific Advisory Group for Emergencies (SAGE), and Executive Board member of the World Health Organization, was gifted a grant of $40 million by the Bill and Melinda Gates Foundation for malaria research in Africa. The BBC described the unelected Whitty as 'the

official who will probably have the greatest impact on our everyday lives of any individual policymaker in modern times' and so it turned out. What Gates and Tedros have said Whitty has done like his equivalents around the world. Patrick Vallance, co-chair of SAGE and the government's Chief Scientific Adviser, is a former executive of Big Pharma giant GlaxoSmithKline with its fundamental financial and business connections to Bill Gates. In September, 2020, it was revealed that Vallance owned a deferred bonus of shares in GlaxoSmithKline worth £600,000 while the company was 'developing' a 'Covid vaccine'. Move along now – nothing to see here – what could possibly be wrong with that? Imperial College in London, a major player in 'Covid' policy in Britain and elsewhere with its 'Covid-19' Response Team, is funded by Gates and has big connections to China while the now infamous Professor Neil Ferguson, the useless 'computer modeller' at Imperial College is also funded by Gates. Ferguson delivered the dramatically inaccurate excuse for the first lockdowns (much more in the next chapter). The Institute for Health Metrics and Evaluation (IHME) in the United States, another source of outrageously false 'Covid' computer models to justify lockdowns, is bankrolled by Gates who is a vehement promotor of lockdowns. America's version of Whitty and Vallance, the again now infamous Anthony Fauci, has connections to 'Covid vaccine' maker Moderna as does Bill Gates through funding from the Bill and Melinda Gates Foundation. Fauci is director of the National Institute of Allergy and Infectious Diseases (NIAID), a major recipient of Gates money, and they are very close. Deborah Birx who was appointed White House Coronavirus Response Coordinator in February, 2020, is yet another with ties to Gates. Everywhere you look at the different elements around the world behind the coordination and decision making of the 'Covid' hoax there is Bill Gates and his money. They include the World Health Organization; Centers for Disease Control (CDC) in the United States; National Institutes of Health (NIH) of Anthony Fauci; Imperial College and Neil Ferguson; the London School of Hygiene where Chris Whitty worked; Regulatory agencies like the UK Medicines & Healthcare products Regulatory Agency (MHRA)

which gave emergency approval for 'Covid vaccines'; Wellcome Trust; GAVI, the Vaccine Alliance; the Coalition for Epidemic Preparedness Innovations (CEPI); Johns Hopkins University which has compiled the false 'Covid' figures; and the World Economic Forum. A Nationalfile.com article said:

> Gates has a lot of pull in the medical world, he has a multi-million dollar relationship with Dr. Fauci, and Fauci originally took the Gates line supporting vaccines and casting doubt on [the drug hydroxychloroquine]. Coronavirus response team member Dr. Deborah Birx, appointed by former president Obama to serve as United States Global AIDS Coordinator, also sits on the board of a group that has received billions from Gates' foundation, and Birx reportedly used a disputed Bill Gates-funded model for the White House's Coronavirus effort. Gates is a big proponent for a population lockdown scenario for the Coronavirus outbreak.

Another funder of Moderna is the Defense Advanced Research Projects Agency (DARPA), the technology-development arm of the Pentagon and one of the most sinister organisations on earth. DARPA had a major role with the CIA covert technology-funding operation In-Q-Tel in the development of Google and social media which is now at the centre of global censorship. Fauci and Gates are extremely close and openly admit to talking regularly about 'Covid' policy, but then why wouldn't Gates have a seat at every national 'Covid' table after his Foundation committed $1.75 billion to the 'fight against Covid-19'. When passed through our Orwellian Translation Unit this means that he has bought and paid for the Cult-driven 'Covid' response worldwide. Research the major 'Covid' response personnel in your own country and you will find the same Gates funding and other connections again and again. Medical and science chiefs following World Health Organization 'policy' sit atop a medical hierarchy in their country of administrators, doctors and nursing staff. These 'subordinates' are told they must work and behave in accordance with the policy delivered from the 'top' of the national 'health' pyramid which is largely the policy delivered by the WHO which is the policy delivered by Gates and the Cult. The whole 'Covid' narrative has been imposed on medical staff by a climate of fear although great numbers don't even need that to comply. They do so through breathtaking levels of ignorance and

include doctors who go through life simply repeating what Big Pharma and their hierarchical masters tell them to say and believe. No wonder Big Pharma 'medicine' is one of the biggest killers on Planet Earth.

The same top-down system of intimidation operates with regard to the Cult Big Pharma cartel which also dictates policy through national and global medical systems in this way. The Cult and Big Pharma agendas are the same because the former controls and owns the latter. 'Health' administrators, doctors, and nursing staff are told to support and parrot the dictated policy or they will face consequences which can include being fired. How sad it's been to see medical staff meekly repeating and imposing Cult policy without question and most of those who can see through the deceit are only willing to speak anonymously off the record. They know what will happen if their identity is known. This has left the courageous few to expose the lies about the 'virus', face masks, overwhelmed hospitals that aren't, and the dangers of the 'vaccine' that isn't a vaccine. When these medical professionals and scientists, some renowned in their field, have taken to the Internet to expose the truth their articles, comments and videos have been deleted by Cult-owned Facebook, Twitter and YouTube. What a real head-shaker to see YouTube videos with leading world scientists and highly qualified medical specialists with an added link underneath to the notorious Cult propaganda website *Wikipedia* to find the 'facts' about the same subject.

HIV – the 'Covid' trial-run

I'll give you an example of the consequences for health and truth that come from censorship and unquestioning belief in official narratives. The story was told by PCR inventor Kary Mullis in his book *Dancing Naked in the Mind Field*. He said that in 1984 he accepted as just another scientific fact that Luc Montagnier of France's Pasteur Institute and Robert Gallo of America's National Institutes of Health had independently discovered that a 'retrovirus' dubbed HIV (human immunodeficiency virus) caused AIDS. They

were, after all, Mullis writes, specialists in retroviruses. This is how the medical and science pyramids work. Something is announced or *assumed* and then becomes an everybody-knows-that purely through repetition of the assumption as if it is fact. Complete crap becomes accepted truth with no supporting evidence and only repetition of the crap. This is how a 'virus' that doesn't exist became the 'virus' that changed the world. The HIV-AIDS fairy story became a multi-billion pound industry and the media poured out propaganda terrifying the world about the deadly HIV 'virus' that caused the lethal AIDS. By then Mullis was working at a lab in Santa Monica, California, to detect retroviruses with his PCR test in blood donations received by the Red Cross. In doing so he asked a virologist where he could find a reference for HIV being the cause of AIDS. 'You don't need a reference,' the virologist said … *'Everybody knows it.'* Mullis said he wanted to quote a reference in the report he was doing and he said he felt a little funny about not knowing the source of such an important discovery when everyone else seemed to. The virologist suggested he cite a report by the Centers for Disease Control and Prevention (CDC) on morbidity and mortality. Mullis read the report, but it only said that an organism had been identified and did not say how. The report did not identify the original scientific work. Physicians, however, *assumed* (key recurring theme) that if the CDC was convinced that HIV caused AIDS then proof must exist. Mullis continues:

I did computer searches. Neither Montagnier, Gallo, nor anyone else had published papers describing experiments which led to the conclusion that HIV probably caused AIDS. I read the papers in Science for which they had become well known as AIDS doctors, but all they had said there was that they had found evidence of a past infection by something which was probably HIV in some AIDS patients.

They found antibodies. Antibodies to viruses had always been considered evidence of past disease, not present disease. Antibodies signaled that the virus had been defeated. The patient had saved himself. There was no indication in these papers that this virus caused a disease. They didn't show that everybody with the antibodies had the disease. In fact they found some healthy people with antibodies.

Mullis asked why their work had been published if Montagnier and Gallo hadn't really found this evidence, and why had they been fighting so hard to get credit for the discovery? He says he was hesitant to write 'HIV is the probable cause of AIDS' until he found published evidence to support that. 'Tens of thousands of scientists and researchers were spending billions of dollars a year doing research based on this idea,' Mullis writes. 'The reason had to be there somewhere; otherwise these people would not have allowed their research to settle into one narrow channel of investigation.' He said he lectured about PCR at numerous meetings where people were always talking about HIV and he asked them how they knew that HIV was the cause of AIDS:

> Everyone said something. Everyone had the answer at home, in the office, in some drawer. They all knew, and they would send me the papers as soon as they got back. But I never got any papers. Nobody ever sent me the news about how AIDS was caused by HIV.

Eventually Mullis was able to ask Montagnier himself about the reference proof when he lectured in San Diego at the grand opening of the University of California AIDS Research Center. Mullis says this was the last time he would ask his question without showing anger. Montagnier said he should reference the CDC report. 'I read it', Mullis said, and it didn't answer the question. 'If Montagnier didn't know the answer who the hell did?' Then one night Mullis was driving when an interview came on National Public Radio with Peter Duesberg, a prominent virologist at Berkeley and a California Scientist of the Year. Mullis says he finally understood why he could not find references that connected HIV to AIDS – *there weren't any!* No one had ever proved that HIV causes AIDS even though it had spawned a multi-billion pound global industry and the media was repeating this as fact every day in their articles and broadcasts terrifying the shit out of people about AIDS and giving the impression that a positive test for HIV (see 'Covid') was a death sentence. Duesberg was a threat to the AIDS gravy train and the agenda that underpinned it. He was therefore abused and castigated after he told the Proceedings of the National Academy of Sciences

there was no good evidence implicating the new 'virus'. Editors rejected his manuscripts and his research funds were deleted. Mullis points out that the CDC has defined AIDS as one of more than 30 diseases *if accompanied* by a positive result on a test that detects antibodies to HIV; but those same diseases are not defined as AIDS cases when antibodies are not detected:

> If an HIV-positive woman develops uterine cancer, for example, she is considered to have AIDS. If she is not HIV positive, she simply has uterine cancer. An HIV-positive man with tuberculosis has AIDS; if he tests negative he simply has tuberculosis. If he lives in Kenya or Colombia, where the test for HIV antibodies is too expensive, he is simply presumed to have the antibodies and therefore AIDS, and therefore he can be treated in the World Health Organization's clinic. It's the only medical help available in some places. And it's free, because the countries that support WHO are worried about AIDS.

Mullis accuses the CDC of continually adding new diseases (see ever more 'Covid symptoms') to the grand AIDS definition and of virtually doctoring the books to make it appear as if the disease continued to spread. He cites how in 1993 the CDC enormously broadened its AIDS definition and county health authorities were delighted because they received $2,500 per year from the Federal government for every reported AIDS case. Ladies and gentlemen, I have just described, via Kary Mullis, the 'Covid pandemic' of 2020 and beyond. Every element is the same and it's been pulled off in the same way by the same networks.

The 'Covid virus' exists? Okay – prove it. Er … still waiting

What Kary Mullis described with regard to 'HIV' has been repeated with 'Covid'. A claim is made that a new, or 'novel', infection has been found and the entire medical system of the world repeats that as fact exactly as they did with HIV and AIDS. No one in the mainstream asks rather relevant questions such as 'How do you know?' and 'Where is your proof?' The SARS-Cov-2 'virus' and the 'Covid-19 disease' became an overnight 'everybody-knows-that'. The origin could be debated and mulled over, but what you could not suggest was that 'SARS-Cov-2' didn't exist. That would be

ridiculous. 'Everybody knows' the 'virus' exists. Well, I didn't for one along with American proper doctors like Andrew Kaufman and Tom Cowan and long-time American proper journalist Jon Rappaport. We dared to pursue the obvious and simple question: 'Where's the evidence?' The overwhelming majority in medicine, journalism and the general public did not think to ask that. After all, *everyone knew* there was a new 'virus'. Everyone was saying so and I heard it on the BBC. Some would eventually argue that the 'deadly virus' was nothing like as deadly as claimed, but few would venture into the realms of its very existence. Had they done so they would have found that the evidence for that claim had gone AWOL as with HIV causes AIDS. In fact, not even that. For something to go AWOL it has to exist in the first place and scientific proof for a 'SARS-Cov-2' can be filed under nothing, nowhere and zilch.

Dr Andrew Kaufman is a board-certified forensic psychiatrist in New York State, a Doctor of Medicine and former Assistant Professor and Medical Director of Psychiatry at SUNY Upstate Medical University, and Medical Instructor of Hematology and Oncology at the Medical School of South Carolina. He also studied biology at the Massachusetts Institute of Technology (MIT) and trained in Psychiatry at Duke University. Kaufman is retired from allopathic medicine, but remains a consultant and educator on natural healing, I saw a video of his very early on in the 'Covid' hoax in which he questioned claims about the 'virus' in the absence of any supporting evidence and with plenty pointing the other way. I did everything I could to circulate his work which I felt was asking the pivotal questions that needed an answer. I can recommend an excellent pull-together interview he did with the website The Last Vagabond entitled *Dr Andrew Kaufman: Virus Isolation, Terrain Theory and Covid-19* and his website is andrewkaufmanmd.com. Kaufman is not only a forensic psychiatrist; he is forensic in all that he does. He always reads original scientific papers, experiments and studies instead of second-third-fourth-hand reports about the 'virus' in the media which are repeating the repeated repetition of the narrative. When he did so with the original Chinese 'virus' papers Kaufman

realised that there was no evidence of a 'SARS-Cov-2'. They had never – from the start – shown it to exist and every repeat of this claim worldwide was based on the accepted existence of proof that was nowhere to be found – see Kary Mullis and HIV. Here we go again.

Let's postulate

Kaufman discovered that the Chinese authorities immediately concluded that the cause of an illness that broke out among about 200 initial patients in Wuhan was a 'new virus' when there were no grounds to make that conclusion. The alleged 'virus' was not isolated from other genetic material in their samples and then shown through a system known as Koch's postulates to be the causative agent of the illness. The world was told that the SARS-Cov-2 'virus' caused a disease they called 'Covid-19' which had 'flu-like' symptoms and could lead to respiratory problems and pneumonia. If it wasn't so tragic it would almost be funny. *'Flu-like' symptoms'? Pneumonia? Respiratory disease?* What in *CHINA* and particularly in *Wuhan*, one of the most polluted cities in the world with a resulting epidemic of respiratory disease?? Three hundred thousand people get pneumonia in China every year and there are nearly a billion cases worldwide of 'flu-like symptoms'. These have a whole range of causes – including pollution in Wuhan – but no other possibility was credibly considered in late 2019 when the world was told there was a new and deadly 'virus'. The global prevalence of pneumonia and 'flu-like systems' gave the Cult networks unlimited potential to re-diagnose these other causes as the mythical 'Covid-19' and that is what they did from the very start. Kaufman revealed how Chinese medical and science authorities (all subordinates to the Cult-owned communist government) took genetic material from the lungs of only a few of the first patients. The material contained their own cells, bacteria, fungi and other microorganisms living in their bodies. The only way you could prove the existence of the 'virus' and its responsibility for the alleged 'Covid-19' was to isolate the virus from all the other material – a process also known as 'purification' – and

then follow the postulates sequence developed in the late 19th century by German physician and bacteriologist Robert Koch which became the 'gold standard' for connecting an alleged causation agent to a disease:

1. The microorganism (bacteria, fungus, virus, etc.) must be present in every case of the disease and all patients must have the same symptoms. It must also *not be present in healthy individuals*.

2. The microorganism must be isolated from the host with the disease. If the microorganism is a bacteria or fungus it must be grown in a pure culture. If it is a virus, it must be purified (i.e. containing no other material except the virus particles) from a clinical sample.

3. The specific disease, with all of its characteristics, must be reproduced when the infectious agent (the purified virus or a pure culture of bacteria or fungi) is inoculated into a healthy, susceptible host.

4. The microorganism must be recoverable from the experimentally infected host as in step 2.

Not one of these criteria has been met in the case of 'SARS-Cov-2' and 'Covid-19'. Not ONE. *EVER*. Robert Koch refers to bacteria and not viruses. What are called 'viral particles' are so minute (hence masks are useless by any definition) that they could only be seen after the invention of the electron microscope in the 1930s and can still only be observed through that means. American bacteriologist and virologist Thomas Milton Rivers, the so-called 'Father of Modern Virology' who was very significantly director of the Rockefeller Institute for Medical Research in the 1930s, developed a less stringent version of Koch's postulates to identify 'virus' causation known as 'Rivers criteria'. 'Covid' did not pass that process either. Some even doubt whether any 'virus' can be isolated from other particles containing genetic material in the Koch method. Freedom of Information requests in many countries asking for scientific proof that the 'Covid virus' has been purified and isolated and shown to exist have all come back with a 'we don't have that' and when this happened with a request to the UK Department of Health they added this comment:

However, outside of the scope of the [Freedom of Information Act] and on a discretionary basis, the following information has been advised to us, which may be of interest. Most infectious diseases are caused by viruses, bacteria or fungi. Some bacteria or fungi have the capacity to grow on their own in isolation, for example in colonies on a petri dish. Viruses are different in that they are what we call 'obligate pathogens' – that is, they cannot survive or reproduce without infecting a host ...

… For some diseases, it is possible to establish causation between a microorganism and a disease by isolating the pathogen from a patient, growing it in pure culture and reintroducing it to a healthy organism. These are known as 'Koch's postulates' and were developed in 1882. However, as our understanding of disease and different disease-causing agents has advanced, these are no longer the method for determining causation [Andrew Kaufman asks why in that case are there two published articles falsely claiming to satisfy Koch's postulates].

It has long been known that viral diseases cannot be identified in this way as viruses cannot be grown in 'pure culture'. When a patient is tested for a viral illness, this is normally done by looking for the presence of antigens, or viral genetic code in a host with molecular biology techniques [Kaufman asks how you could know the origin of these chemicals without having a pure culture for comparison].

For the record 'antigens' are defined so:

Invading microorganisms have antigens on their surface that the human body can recognise as being foreign – meaning not belonging to it. When the body recognises a foreign antigen, lymphocytes (white blood cells) produce antibodies, which are complementary in shape to the antigen.

Notwithstanding that this is open to question in relation to 'SARS-Cov-2' the presence of 'antibodies' can have many causes and they are found in people that are perfectly well. Kary Mullis said: 'Antibodies … had always been considered evidence of past disease, not present disease.'

'Covid' really is a *computer* 'virus'

Where the UK Department of Health statement says 'viruses' are now 'diagnosed' through a 'viral genetic code in a host with molecular biology techniques', they mean … *the PCR test* which its inventor said cannot test for infectious disease. They have no credible method of connecting a 'virus' to a disease and we will see that there is no scientific proof that any 'virus' causes any disease or there is any such thing as a 'virus' in the way that it is described. Tenacious Canadian researcher Christine Massey and her team made

some 40 Freedom of Information requests to national public health agencies in different countries asking for proof that SARS-CoV-2 has been isolated and not one of them could supply that information. Massey said of her request in Canada: 'Freedom of Information reveals Public Health Agency of Canada has no record of 'SARS-COV-2' isolation performed by anyone, anywhere, ever.' If you accept the comment from the UK Department of Health it's because they can't isolate a 'virus'. Even so many 'science' papers claimed to have isolated the 'Covid virus' until they were questioned and had to admit they hadn't. A reply from the Robert Koch Institute in Germany was typical: 'I am not aware of a paper which purified isolated SARS-CoV-2.' So what the hell was Christian Drosten and his gang using to design the 'Covid' testing protocol that has produced all the illusory Covid' cases and 'Covid' deaths when the head of the Chinese version of the CDC admitted there was a problem right from the start in that the 'virus' had never been isolated/purified? Breathe deeply: What they are calling 'Covid' is actually created by a *computer program* i.e. *they made it up* – er, that's it. They took lung fluid, with many sources of genetic material, from one single person alleged to be infected with Covid-19 by a PCR test which they *claimed*, without clear evidence, contained a 'virus'. They used several computer programs to create a model of a theoretical virus genome sequence from more than fifty-six million small sequences of RNA, each of an unknown source, assembling them like a puzzle with no known solution. The computer filled in the gaps with sequences from bits in the gene bank to make it look like a bat SARS-like coronavirus! A wave of the magic wand and poof, an in silico (computer-generated) genome, a scientific fantasy, was created. UK health researcher Dr Kevin Corbett made the same point with this analogy:

> … It's like giving you a few bones and saying that's your fish. It could be any fish. Not even a skeleton. Here's a few fragments of bones. That's your fish … It's all from gene bank and the bits of the virus sequence that weren't there they made up.
>
> They synthetically created them to fill in the blanks. That's what genetics is; it's a code. So it's ABBBCCDDD and you're missing some what you think is EEE so you put it in. It's all

synthetic. You just manufacture the bits that are missing. This is the end result of the geneticization of virology. This is basically a computer virus.

Further confirmation came in an email exchange between British citizen journalist Frances Leader and the government's Medicines & Healthcare Products Regulatory Agency (the Gates-funded MHRA) which gave emergency permission for untested 'Covid vaccines' to be used. The agency admitted that the 'vaccine' is not based on an isolated 'virus', but comes from a *computer-generated model*. Frances Leader was naturally banned from Cult-owned fascist Twitter for making this exchange public. The process of creating computer-generated alleged 'viruses' is called 'in silico' or 'in silicon' – computer chips – and the term 'in silico' is believed to originate with biological experiments using only a computer in 1989. 'Vaccines' involved with 'Covid' are also produced 'in silico' or by computer not a natural process. If the original 'virus' is nothing more than a made-up computer model how can there be 'new variants' of something that never existed in the first place? They are not new 'variants'; they are new *computer models* only minutely different to the original program and designed to further terrify the population into having the 'vaccine' and submitting to fascism. You want a 'new variant'? Click, click, enter – there you go. Tell the medical profession that you have discovered a 'South African variant', 'UK variants' or a 'Brazilian variant' and in the usual HIV-causes-AIDS manner they will unquestioningly repeat it with no evidence whatsoever to support these claims. They will go on television and warn about the dangers of 'new variants' while doing nothing more than repeating what they have been told to be true and knowing that any deviation from that would be career suicide. Big-time insiders will know it's a hoax, but much of the medical community is clueless about the way they are being played and themselves play the public without even being aware they are doing so. What an interesting 'coincidence' that AstraZeneca and Oxford University were conducting 'Covid vaccine trials' in the three countries – the UK, South Africa and Brazil – where the first three 'variants' were claimed to have 'broken out'.

Here's your 'virus' – it's a unicorn

Dr Andrew Kaufman presented a brilliant analysis describing how the 'virus' was imagined into fake existence when he dissected an article published by *Nature* and written by 19 authors detailing *alleged* 'sequencing of a complete viral genome' of the 'new SARS-CoV-2 virus'. This computer-modelled *in silico* genome was used as a template for all subsequent genome sequencing experiments that resulted in the so-called variants which he said now number more than *6,000*. The fake genome was constructed from more than 56 million individual short strands of RNA. Those little pieces were assembled into longer pieces by finding areas of overlapping sequences. The computer programs created over two million possible combinations from which the authors simply chose the longest one. They then compared this to a 'bat virus' and the computer 'alignment' rearranged the sequence and filled in the gaps! They called this computer-generated abomination the 'complete genome'. Dr Tom Cowan, a fellow medical author and collaborator with Kaufman, said such computer-generation constitutes scientific fraud and he makes this superb analogy:

> Here is an equivalency: A group of researchers claim to have found a unicorn because they found a piece of a hoof, a hair from a tail, and a snippet of a horn. They then add that information into a computer and program it to re-create the unicorn, and they then claim this computer re-creation is the real unicorn. Of course, they had never actually seen a unicorn so could not possibly have examined its genetic makeup to compare their samples with the actual unicorn's hair, hooves and horn.
>
> The researchers claim they decided which is the real genome of SARS-CoV-2 by 'consensus', sort of like a vote. Again, different computer programs will come up with different versions of the imaginary 'unicorn', so they come together as a group and decide which is the real imaginary unicorn.

This is how the 'virus' that has transformed the world was brought into fraudulent 'existence'. Extraordinary, yes, but as the Nazis said the bigger the lie the more will believe it. Cowan, however, wasn't finished and he went on to identify what he called the real blockbuster in the paper. He quotes this section from a paper written

by virologists and published by the CDC and then explains what it means:

Therefore, we examined the capacity of SARS-CoV-2 to infect and replicate in several common primate and human cell lines, including human adenocarcinoma cells (A549), human liver cells (HUH 7.0), and human embryonic kidney cells (HEK-293T). In addition to Vero E6 and Vero CCL81 cells. ... Each cell line was inoculated at high multiplicity of infection and examined 24h post-infection.

No CPE was observed in any of the cell lines except in Vero cells, which grew to greater than 10 to the 7th power at 24 h post-infection. In contrast, HUH 7.0 and 293T showed only modest viral replication, and A549 cells were incompatible with SARS CoV-2 infection.

Cowan explains that when virologists attempt to prove infection they have three possible 'hosts' or models on which they can test. The first was humans. Exposure to humans was generally not done for ethical reasons and has never been done with SARS-CoV-2 or any coronavirus. The second possible host was animals. Cowan said that forgetting for a moment that they never actually use purified virus when exposing animals they do use solutions that they *claim* contain the virus. Exposure to animals has been done with SARS-CoV-2 in an experiment involving mice and this is what they found: *None of the wild (normal) mice got sick*. In a group of genetically-modified mice, a statistically insignificant number lost weight and had slightly bristled fur, but they experienced nothing like the illness called 'Covid-19'. Cowan said the third method – the one they mostly rely on – is to inoculate solutions they *say* contain the virus onto a variety of tissue cultures. This process had never been shown to kill tissue *unless* the sample material was starved of nutrients and poisoned as *part of the process*. Yes, incredibly, in tissue experiments designed to show the 'virus' is responsible for killing the tissue they starve the tissue of nutrients and add toxic drugs including antibiotics and they do not have control studies to see if it's the starvation and poisoning that is degrading the tissue rather than the 'virus' they allege to be in there somewhere. You want me to pinch you? Yep, I understand. Tom Cowan said this about the whole nonsensical farce as he explains what that quote from the CDC paper really means:

The shocking thing about the above quote is that using their own methods, the virologists found that solutions containing SARS-CoV-2 – even in high amounts – were NOT, I repeat NOT, infective to any of the three human tissue cultures they tested. In plain English, this means they proved, on their terms, that this 'new coronavirus' is not infectious to human beings. It is ONLY infective to monkey kidney cells, and only then when you add two potent drugs (gentamicin and amphotericin), known to be toxic to kidneys, to the mix.

My friends, read this again and again. These virologists, published by the CDC, performed a clear proof, on their terms, showing that the SARS-CoV-2 virus is harmless to human beings. That is the only possible conclusion, but, unfortunately, this result is not even mentioned in their conclusion. They simply say they can provide virus stocks cultured only on monkey Vero cells, thanks for coming.

Cowan concluded: 'If people really understood how this "science" was done, I would hope they would storm the gates and demand honesty, transparency and truth.' Dr Michael Yeadon, former Vice President and Chief Scientific Adviser at drug giant Pfizer has been a vocal critic of the 'Covid vaccine' and its potential for multiple harm. He said in an interview in April, 2021, that 'not one [vaccine] has the virus. He was asked why vaccines normally using a 'dead' version of a disease to activate the immune system were not used for 'Covid' and instead we had the synthetic methods of the 'mRNA Covid vaccine'. Yeadon said that to do the former 'you'd have to have some of [the virus] wouldn't you?' He added: 'No-one's got any – seriously.' Yeadon said that surely they couldn't have fooled the whole world for a year without having a virus, 'but oddly enough ask around – no one's got it'. He didn't know why with all the 'great labs' around the world that the virus had not been isolated – 'Maybe they've been too busy running bad PCR tests and vaccines that people don't need.' What is today called 'science' is not 'science' at all. Science is no longer what is, but whatever people can be manipulated to *believe* that it is. Real science has been hijacked by the Cult to dispense and produce the 'expert scientists' and contentions that suit the agenda of the Cult. How big-time this has happened with the 'Covid' hoax which is entirely based on fake science delivered by fake 'scientists' and fake 'doctors'. The human-caused climate change hoax is also entirely based on fake science delivered by fake 'scientists' and fake 'climate experts'. In both cases real

scientists, climate experts and doctors have their views suppressed and deleted by the Cult-owned science establishment, media and Silicon Valley. This is the 'science' that politicians claim to be 'following' and a common denominator of 'Covid' and climate are Cult psychopaths Bill Gates and his mate Klaus Schwab at the Gates-funded World Economic Forum. But, don't worry, it's all just a coincidence and absolutely nothing to worry about. *Zzzzzzzz*.

What is a 'virus' REALLY?

Dr Tom Cowan is one of many contesting the very existence of viruses let alone that they cause disease. This is understandable when there is no scientific evidence for a disease-causing 'virus'. German virologist Dr Stefan Lanka won a landmark case in 2017 in the German Supreme Court over his contention that there is no such thing as a measles virus. He had offered a big prize for anyone who could prove there is and Lanka won his case when someone sought to claim the money. There is currently a prize of more than 225,000 euros on offer from an Isolate Truth Fund for anyone who can prove the isolation of SARS-CoV-2 and its genetic substance. Lanka wrote in an article headed 'The Misconception Called Virus' that scientists think a 'virus' is causing tissue to become diseased and degraded when in fact it is the *processes they are using* which do that – not a 'virus'. Lanka has done an important job in making this point clear as Cowan did in his analysis of the CDC paper. Lanka says that all claims about viruses as disease-causing pathogens are wrong and based on 'easily recognisable, understandable and verifiable misinterpretations.' Scientists believed they were working with 'viruses' in their laboratories when they were really working with 'typical particles of specific dying tissues or cells …' Lanka said that the tissue decaying process claimed to be caused by a 'virus' still happens when no alleged 'virus' is involved. It's the *process* that does the damage and not a 'virus'. The genetic sample is deprived of nutrients, removed from its energy supply through removal from the body and then doused in toxic antibiotics to remove any bacteria. He confirms again that establishment scientists do not (pinch me)

conduct control experiments to see if this is the case and if they did they would see the claims that 'viruses' are doing the damage is nonsense. He adds that during the measles 'virus' court case he commissioned an independent laboratory to perform just such a control experiment and the result was that the tissues and cells died in the exact same way as with alleged 'infected' material. This is supported by a gathering number of scientists, doctors and researchers who reject what is called 'germ theory' or the belief in the body being infected by contagious sources emitted by other people. Researchers Dawn Lester and David Parker take the same stance in their highly-detailed and sourced book *What Really Makes You Ill – Why everything you thought you knew about disease is wrong* which was recommended to me by a number of medical professionals genuinely seeking the truth. Lester and Parker say there is no provable scientific evidence to show that a 'virus' can be transmitted between people or people and animals or animals and people:

> The definition also claims that viruses are the cause of many diseases, as if this has been definitively proven. But this is not the case; there is no original scientific evidence that definitively demonstrates that any virus is the cause of any disease. The burden of proof for any theory lies with those who proposed it; but none of the existing documents provides 'proof' that supports the claim that 'viruses' are pathogens.

Dr Tom Cowan employs one of his clever analogies to describe the process by which a 'virus' is named as the culprit for a disease when what is called a 'virus' is only material released by cells detoxing themselves from infiltration by chemical or radiation poisoning. The tidal wave of technologically-generated radiation in the 'smart' modern world plus all the toxic food and drink are causing this to happen more than ever. Deluded 'scientists' misread this as a gathering impact of what they wrongly label 'viruses'.

Paper can infect houses

Cowan said in an article for davidicke.com – with his tongue only mildly in his cheek – that he believed he had made a tremendous

discovery that may revolutionise science. He had discovered that small bits of paper are alive, 'well alive-ish', can 'infect' houses, and then reproduce themselves inside the house. The result was that this explosion of growth in the paper inside the house causes the house to explode, blowing it to smithereens. His evidence for this new theory is that in the past months he had carefully examined many of the houses in his neighbourhood and found almost no scraps of paper on the lawns and surrounds of the house. There was an occasional stray label, but nothing more. Then he would return to these same houses a week or so later and with a few, not all of them, particularly the old and decrepit ones, he found to his shock and surprise they were littered with stray bits of paper. He knew then that the paper had infected these houses, made copies of itself, and blew up the house. A young boy on a bicycle at one of the sites told him he had seen a demolition crew using dynamite to explode the house the previous week, but Cowan dismissed this as the idle thoughts of silly boys because 'I was on to something big'. He was on to how 'scientists' mistake genetic material in the detoxifying process for something they call a 'virus'. Cowan said of his house and paper story:

> If this sounds crazy to you, it's because it should. This scenario is obviously nuts. But consider this admittedly embellished, for effect, current viral theory that all scientists, medical doctors and virologists currently believe.

He takes the example of the 'novel SARS-Cov2' virus to prove the point. First they take someone with an undefined illness called 'Covid-19' and don't even attempt to find any virus in their sputum. Never mind the scientists still describe how this 'virus', which they have not located attaches to a cell receptor, injects its genetic material, in 'Covid's' case, RNA, into the cell. The RNA once inserted exploits the cell to reproduce itself and makes 'thousands, nay millions, of copies of itself … Then it emerges victorious to claim its next victim':

If you were to look in the scientific literature for proof, actual scientific proof, that uniform SARS-CoV2 viruses have been properly isolated from the sputum of a sick person, that actual spike proteins could be seen protruding from the virus (which has not been found), you would find that such evidence doesn't exist.

If you go looking in the published scientific literature for actual pictures, proof, that these spike proteins or any viral proteins are ever attached to any receptor embedded in any cell membrane, you would also find that no such evidence exists. If you were to look for a video or documented evidence of the intact virus injecting its genetic material into the body of the cell, reproducing itself and then emerging victorious by budding off the cell membrane, you would find that no such evidence exists.

The closest thing you would find is electron micrograph pictures of cellular particles, possibly attached to cell debris, both of which to be seen were stained by heavy metals, a process that completely distorts their architecture within the living organism. This is like finding bits of paper stuck to the blown-up bricks, thereby proving the paper emerged by taking pieces of the bricks on its way out.

The Enders baloney

Cowan describes the 'Covid' story as being just as make-believe as his paper story and he charts back this fantasy to a Nobel Prize winner called John Enders (1897-1985), an American biomedical scientist who has been dubbed 'The Father of Modern Vaccines'. Enders is claimed to have 'discovered' the process of the viral culture which 'proved' that a 'virus' caused measles. Cowan explains how Enders did this 'by using the EXACT same procedure that has been followed by every virologist to find and characterize every new virus since 1954'. Enders took throat swabs from children with measles and immersed them in 2ml of milk. Penicillin (100u/ml) and the antibiotic streptomycin (50,g/ml) were added and the whole mix was centrifuged – rotated at high speed to separate large cellular debris from small particles and molecules as with milk and cream, for example. Cowan says that if the aim is to find little particles of genetic material ('viruses') in the snot from children with measles it would seem that the last thing you would do is mix the snot with other material – milk –that also has genetic material. 'How are you ever going to know whether whatever you found came from the snot or the milk?' He points out that streptomycin is a 'nephrotoxic' or poisonous-to-the-kidney drug. You will see the relevance of that

shortly. Cowan says that it gets worse, much worse, when Enders describes the culture medium upon which the virus 'grows': 'The culture medium consisted of bovine amniotic fluid (90%), beef embryo extract (5%), horse serum (5%), antibiotics and phenol red as an indicator of cell metabolism.' Cowan asks incredulously: 'Did he just say that the culture medium also contained fluids and tissues that are themselves rich sources of genetic material?' The genetic cocktail, or 'medium', is inoculated onto tissue and cells from rhesus monkey *kidney* tissue. This is where the importance of streptomycin comes in and currently-used antimicrobials and other drugs that are *poisonous to kidneys* and used in ALL modern viral cultures (e.g. gentamicin, streptomycin, and amphotericin). Cowan asks: 'How are you ever going to know from this witch's brew where any genetic material comes from as we now have five different sources of rich genetic material in our mix?' Remember, he says, that all genetic material, whether from monkey kidney tissues, bovine serum, milk, etc., is made from the exact same components. The same central question returns: 'How are you possibly going to know that it was the virus that killed the kidney tissue and not the toxic antibiotic and starvation rations on which you are growing the tissue?' John Enders answered the question himself – *you can't*:

A second agent was obtained from an uninoculated culture of monkey kidney cells. The cytopathic changes [death of the cells] it induced in the unstained preparations could not be distinguished with confidence from the viruses isolated from measles.

The death of the cells ('cytopathic changes') happened in exactly the same manner, whether they inoculated the kidney tissue with the measles snot or not, Cowan says. 'This is evidence that the destruction of the tissue, the very proof of viral causation of illness, was not caused by anything in the snot because they saw the same destructive effect when the snot was not even used … the cytopathic, i.e., cell-killing, changes come from the process of the culture itself, not from any virus in any snot, period.' Enders quotes in his 1957 paper a virologist called Ruckle as reporting similar findings 'and in addition has isolated an agent from monkey kidney tissue that is so

far indistinguishable from human measles virus'. In other words, Cowan says, these particles called 'measles viruses' are simply and clearly breakdown products of the starved and poisoned tissue. For measles 'virus' see all 'viruses' including the so-called 'Covid virus'. Enders, the 'Father of Modern Vaccines', also said:

There is a potential risk in employing cultures of primate cells for the production of vaccines composed of attenuated virus, since the presence of other agents possibly latent in primate tissues cannot be definitely excluded by any known method.

Cowan further quotes from a paper published in the journal *Viruses* in May, 2020, while the 'Covid pandemic' was well underway in the media if not in reality. 'EVs' here refers to particles of genetic debris from our own tissues, such as exosomes of which more in a moment: 'The remarkable resemblance between EVs and viruses has caused quite a few problems in the studies focused on the analysis of EVs released during viral infections.' Later the paper adds that to date a reliable method that can actually guarantee a complete separation (of EVs from viruses) DOES NOT EXIST. This was published at a time when a fairy tale 'virus' was claimed in total certainty to be causing a fairy tale 'viral disease' called 'Covid-19' – a fairy tale that was already well on the way to transforming human society in the image that the Cult has worked to achieve for so long. Cowan concludes his article:

To summarize, there is no scientific evidence that pathogenic viruses exist. What we think of as 'viruses' are simply the normal breakdown products of dead and dying tissues and cells. When we are well, we make fewer of these particles; when we are starved, poisoned, suffocated by wearing masks, or afraid, we make more.

There is no engineered virus circulating and making people sick. People in laboratories all over the world are making genetically modified products to make people sick. These are called vaccines. There is no virome, no 'ecosystem' of viruses, viruses are not 8%, 50% or 100 % of our genetic material. These are all simply erroneous ideas based on the misconception called a virus.

What is 'Covid'? Load of bollocks

The background described here by Cowan and Lanka was emphasised in the first video presentation that I saw by Dr Andrew Kaufman when he asked whether the 'Covid virus' was in truth a natural defence mechanism of the body called 'exosomes'. These are released by cells when in states of toxicity – see the same themes returning over and over. They are released ever more profusely as chemical and radiation toxicity increases and think of the potential effect therefore of 5G alone as its destructive frequencies infest the human energetic information field with a gathering pace (5G went online in Wuhan in 2019 as the 'virus' emerged). I'll have more about this later. Exosomes transmit a warning to the rest of the body that 'Houston, we have a problem'. Kaufman presented images of exosomes and compared them with 'Covid' under an electron microscope and the similarity was remarkable. They both attach to the same cell receptors (*claimed* in the case of 'Covid'), contain the same genetic material in the form of RNA or ribonucleic acid, and both are found in 'viral cell cultures' with damaged or dying cells. James Hildreth MD, President and Chief Executive Officer of the Meharry Medical College at Johns Hopkins, said: 'The virus is fully an exosome in every sense of the word.' Kaufman's conclusion was that there is no 'virus': 'This entire pandemic is a completely manufactured crisis … there is no evidence of anyone dying from [this] illness.' Dr Tom Cowan and Sally Fallon Morell, authors of *The Contagion Myth*, published a statement with Dr Kaufman in February, 2021, explaining why the 'virus' does not exist and you can read it that in full in the Appendix.

'Virus' theory can be traced to the 'cell theory' in 1858 of German physician Rudolf Virchow (1821-1920) who contended that disease originates from a single cell infiltrated by a 'virus'. Dr Stefan Lanka said that findings and insights with respect to the structure, function and central importance of tissues in the creation of life, which were already known in 1858, comprehensively refute the cell theory. Virchow ignored them. We have seen the part later played by John Enders in the 1950s and Lanka notes that infection theories were only established as a global dogma through the policies and

eugenics of the Third Reich in Nazi Germany (creation of the same Sabbatian cult behind the 'Covid' hoax). Lanka said: 'Before 1933, scientists dared to contradict this theory; after 1933, these critical scientists were silenced'. Dr Tom Cowan's view is that ill-heath is caused by too much of something, too little of something, or toxification from chemicals and radiation – not contagion. We must also highlight as a major source of the 'virus' theology a man still called the 'Father of Modern Virology' – Thomas Milton Rivers (1888-1962). There is no way given the Cult's long game policy that it was a coincidence for the 'Father of Modern Virology' to be director of the Rockefeller Institute for Medical Research from 1937 to 1956 when he is credited with making the Rockefeller Institute a leader in 'viral research'. Cult Rockefellers were the force behind the creation of Big Pharma 'medicine', established the World Health Organisation in 1948, and have long and close associations with the Gates family that now runs the WHO during the pandemic hoax through mega-rich Cult gofer and psychopath Bill Gates.

Only a Renegade Mind can see through all this bullshit by asking the questions that need to be answered, not taking 'no' or prevarication for an answer, and certainly not hiding from the truth in fear of speaking it. Renegade Minds have always changed the world for the better and they will change this one no matter how bleak it may currently appear to be.

CHAPTER SIX

Sequence of deceit

If you tell the truth, you don't have to remember anything
Mark Twain

Against the background that I have laid out this far the sequence that took us from an invented 'virus' in Cult-owned China in late 2019 to the fascist transformation of human society can be seen and understood in a whole new context.

We were told that a deadly disease had broken out in Wuhan and the world media began its campaign (coordinated by behavioural psychologists as we shall see) to terrify the population into unquestioning compliance. We were shown images of Chinese people collapsing in the street which never happened in the West with what was supposed to be the same condition. In the earliest days when alleged cases and deaths were few the fear register was hysterical in many areas of the media and this would expand into the common media narrative across the world. The real story was rather different, but we were never told that. The Chinese government, one of the Cult's biggest centres of global operation, said they had discovered a new illness with flu-like and pneumonia-type symptoms in a city with such toxic air that it is overwhelmed with flu-like symptoms, pneumonia and respiratory disease. Chinese scientists said it was a new – 'novel' – coronavirus which they called Sars-Cov-2 and that it caused a disease they labelled 'Covid-19'. There was no evidence for this and the 'virus' has never to this day been isolated, purified and its genetic code established from that. It

was from the beginning a computer-generated fiction. Stories of Chinese whistleblowers saying the number of deaths was being supressed or that the 'new disease' was related to the Wuhan bio-lab misdirected mainstream and alternative media into cul-de-sacs to obscure the real truth – there was no 'virus'.

Chinese scientists took genetic material from the lung fluid of just a few people and said they had found a 'new' disease when this material had a wide range of content. There was no evidence for a 'virus' for the very reasons explained in the last two chapters. The 'virus' has never been shown to (a) exist and (b) cause any disease. People were diagnosed on symptoms that are so widespread in Wuhan and polluted China and with a PCR test that can't detect infectious disease. On this farce the whole global scam was sold to the rest of the world which would also diagnose respiratory disease as 'Covid-19' from symptoms alone or with a PCR test not testing for a 'virus'. Flu miraculously disappeared *worldwide* in 2020 and into 2021 as it was redesignated 'Covid-19'. It was really the same old flu with its 'flu-like' symptoms attributed to 'flu-like' 'Covid-19'. At the same time with very few exceptions the Chinese response of draconian lockdown and fascism was the chosen weapon to respond across the West as recommended by the Cult-owned Tedros at the Cult-owned World Health Organization run by the Cult-owned Gates. All was going according to plan. Chinese scientists – everything in China is controlled by the Cult-owned government – compared their contaminated RNA lung-fluid material with other RNA sequences and said it appeared to be just under 80 percent identical to the SARS-CoV-1 'virus' claimed to be the cause of the SARS (severe acute respiratory syndrome) 'outbreak' in 2003. They decreed that because of this the 'new virus' had to be related and they called it SARS-CoV-2. There are some serious problems with this assumption and *assumption* was all it was. Most 'factual' science turns out to be assumptions repeated into everyone-knows-that. A match of under 80-percent is meaningless. Dr Kaufman makes the point that there's a *96 percent* genetic correlation between humans and chimpanzees, but 'no one would say our genetic material is part

of the chimpanzee family'. Yet the Chinese authorities were claiming that a much lower percentage, less than 80 percent, proved the existence of a new 'coronavirus'. For goodness sake human DNA is 60 percent similar to a *banana*.

You are feeling sleepy

The entire 'Covid' hoax is a global Psyop, a psychological operation to program the human mind into believing and fearing a complete fantasy. A crucial aspect of this was what *appeared* to happen in Italy. It was all very well streaming out daily images of an alleged catastrophe in Wuhan, but to the Western mind it was still on the other side of the world in a very different culture and setting. A reaction of 'this could happen to me and my family' was still nothing like as intense enough for the mind-doctors. The Cult needed a Western example to push people over that edge and it chose Italy, one of its major global locations going back to the Roman Empire. An Italian 'Covid' crisis was manufactured in a particular area called Lombardy which just happens to be notorious for its toxic air and therefore respiratory disease. Wuhan, China, déjà vu. An hysterical media told horror stories of Italians dying from 'Covid' in their droves and how Lombardy hospitals were being overrun by a tidal wave of desperately ill people needing treatment after being struck down by the 'deadly virus'. Here was the psychological turning point the Cult had planned. Wow, if this is happening in Italy, the Western mind concluded, this indeed could happen to me and my family. Another point is that Italian authorities responded by following the Chinese blueprint so vehemently recommended by the Cult-owned World Health Organization. They imposed fascistic lockdowns on the whole country viciously policed with the help of surveillance drones sweeping through the streets seeking out anyone who escaped from mass house arrest. Livelihoods were destroyed and psychology unravelled in the way we have witnessed since in all lockdown countries. Crucial to the plan was that Italy responded in this way to set the precedent of suspending freedom and imposing fascism in a 'Western liberal democracy'. I emphasised in an

animated video explanation on davidicke.com posted in the summer of 2020 how important it was to the Cult to expand the Chinese lockdown model across the West. Without this, and the bare-faced lie that non-symptomatic people could still transmit a 'disease' they didn't have, there was no way locking down the whole population, sick and not sick, could be pulled off. At just the right time and with no evidence Cult operatives and gofers claimed that people without symptoms could pass on the 'disease'. In the name of protecting the 'vulnerable' like elderly people, who lockdowns would kill by the tens of thousands, we had for the first time healthy people told to isolate as well as the sick. The great majority of people who tested positive had no symptoms because there was nothing wrong with them. It was just a trick made possible by a test not testing for the 'virus'.

Months after my animated video the Gates-funded Professor Neil Ferguson at the Gates-funded Imperial College confirmed that I was right. He didn't say it in those terms, naturally, but he did say it. Ferguson will enter the story shortly for his outrageously crazy 'computer models' that led to Britain, the United States and many other countries following the Chinese and now Italian methods of response. Put another way, following the Cult script. Ferguson said that SAGE, the UK government's scientific advisory group which has controlled 'Covid' policy from the start, wanted to follow the Chinese lockdown model (while they all continued to work and be paid), but they wondered if they could possibly, in Ferguson's words, 'get away with it in Europe'. 'Get away with it'? Who the hell do these moronic, arrogant people think they are? This appalling man Ferguson said that once Italy went into national lockdown they realised they, too, could mimic China:

> It's a communist one-party state, we said. We couldn't get away with it in Europe, we thought … and then Italy did it. And we realised we could. Behind this garbage from Ferguson is a simple fact: Doing the same as China in every country was the plan from the start and Ferguson's 'models' would play a central role in achieving that. It's just a coincidence, of course, and absolutely nothing to worry your little head about.

Oops, sorry, our mistake

Once the Italian segment of the Psyop had done the job it was designed to do a very different story emerged. Italian authorities revealed that 99 percent of those who had 'died from Covid-19' in Italy had one, two, three, or more 'co-morbidities' or illnesses and health problems that could have ended their life. The US Centers for Disease Control and Prevention (CDC) published a figure of 94 percent for Americans dying of 'Covid' while having other serious medical conditions – on average two to three (some five or six) other potential causes of death. In terms of death from an unproven 'virus' I say it is 100 percent. The other one percent in Italy and six percent in the US would presumably have died from 'Covid's' flu-like symptoms with a range of other possible causes in conjunction with a test not testing for the 'virus'. Fox News reported that even more startling figures had emerged in one US county in which 410 of 422 deaths attributed to 'Covid-19' had other potentially deadly health conditions. The Italian National Health Institute said later that the average age of people dying with a 'Covid-19' diagnosis in Italy was about 81. Ninety percent were over 70 with ten percent over 90. In terms of other reasons to die some 80 percent had two or more chronic diseases with half having three or more including cardiovascular problems, diabetes, respiratory problems and cancer. Why is the phantom 'Covid-19' said to kill overwhelmingly old people and hardly affect the young? Old people continually die of many causes and especially respiratory disease which you can re-diagnose 'Covid-19' while young people die in tiny numbers by comparison and rarely of respiratory disease. Old people 'die of Covid' because they die of other things that can be redesignated 'Covid' and it really is that simple.

Flu has flown

The blueprint was in place. Get your illusory 'cases' from a test not testing for the 'virus' and redesignate other causes of death as 'Covid-19'. You have an instant 'pandemic' from something that is nothing more than a computer-generated fiction. With near-on a

billion people having 'flu-like' symptoms every year the potential was limitless and we can see why flu quickly and apparently miraculously disappeared *worldwide* by being diagnosed 'Covid-19'. The painfully bloody obvious was explained away by the childlike media in headlines like this in the UK '*Independent*': 'Not a single case of flu detected by Public Health England this year as Covid restrictions suppress virus'. I kid you not. The masking, social distancing and house arrest that did not make the 'Covid virus' disappear somehow did so with the 'flu virus'. Even worse the article, by a bloke called Samuel Lovett, suggested that maybe the masking, sanitising and other 'Covid' measures should continue to keep the flu away. With a ridiculousness that disturbs your breathing (it's 'Covid-19') the said Lovett wrote: 'With widespread social distancing and mask-wearing measures in place throughout the UK, the usual routes of transmission for influenza have been blocked.' He had absolutely no evidence to support that statement, but look at the consequences of him acknowledging the obvious. With flu not disappearing at all and only being relabelled 'Covid-19' he would have to contemplate that 'Covid' was a hoax on a scale that is hard to imagine. You need guts and commitment to truth to even go there and that's clearly something Samuel Lovett does not have in abundance. He would never have got it through the editors anyway.

Tens of thousands die in the United States alone every winter from flu including many with pneumonia complications. CDC figures record *45 million* Americans diagnosed with flu in 2017-2018 of which 61,000 died and some reports claim 80,000. Where was the same hysteria then that we have seen with 'Covid-19'? Some 250,000 Americans are admitted to hospital with pneumonia every year with about 50,000 cases proving fatal. About 65 million suffer respiratory disease every year and three million deaths makes this the third biggest cause of death worldwide. You only have to redesignate a portion of all these people 'Covid-19' and you have an instant global pandemic or the *appearance* of one. Why would doctors do this? They are told to do this and all but a few dare not refuse those who must be obeyed. Doctors in general are not researching their own

knowledge and instead take it direct and unquestioned from the authorities that own them and their careers. The authorities say they must now diagnose these symptoms 'Covid-19' and not flu, or whatever, and they do it. Dark suits say put 'Covid-19' on death certificates no matter what the cause of death and the doctors do it. Renegade Minds don't fall for the illusion that doctors and medical staff are all highly-intelligent, highly-principled, seekers of medical truth. *Some are*, but not the majority. They are repeaters, gofers, and yes sir, no sir, purveyors of what the system demands they purvey. The 'Covid' con is not merely confined to diseases of the lungs. Instructions to doctors to put 'Covid-19' on death certificates for anyone dying of *anything* within 28 days (or much more) of a positive test not testing for the 'virus' opened the floodgates. The term dying *with* 'Covid' and not *of* 'Covid' was coined to cover the truth. Whether it was a *with* or an *of* they were all added to the death numbers attributed to the 'deadly virus' compiled by national governments and globally by the Gates-funded Johns Hopkins operation in the United States that was so involved in those 'pandemic' simulations. Fraudulent deaths were added to the ever-growing list of fraudulent 'cases' from false positives from a false test. No wonder Professor Walter Ricciardi, scientific advisor to the Italian minister of health, said after the Lombardy hysteria had done its job that 'Covid' death rates were due to Italy having the second oldest population in the world and to *how hospitals record deaths*:

The way in which we code deaths in our country is very generous in the sense that all the people who die in hospitals with the coronavirus are deemed to be dying of the coronavirus. On re-evaluation by the National Institute of Health, only 12 per cent of death certificates have shown a direct causality from coronavirus, while 88 per cent of patients who have died have at least one pre-morbidity – many had two or three.

This is extraordinary enough when you consider the propaganda campaign to use Italy to terrify the world, but how can they even say twelve percent were genuine when the 'virus' has not been shown to exist, its 'code' is a computer program, and diagnosis comes from a test not testing for it? As in China, and soon the world, 'Covid-19' in

Italy was a redesignation of diagnosis. Lies and corruption were to become the real 'pandemic' fuelled by a pathetically-compliant medical system taking its orders from the tiny few at the top of their national hierarchy who answered to the World Health Organization which answers to Gates and the Cult. Doctors were told – ordered – to diagnose a particular set of symptoms 'Covid-19' and put that on the death certificate for any cause of death if the patient had tested positive with a test not testing for the virus or had 'Covid' symptoms like the flu. The United States even introduced big financial incentives to manipulate the figures with hospitals receiving £4,600 from the Medicare system for diagnosing someone with regular pneumonia, $13,000 if they made the diagnosis from the same symptoms 'Covid-19' pneumonia, and $39, 000 if they put a 'Covid' diagnosed patient on a ventilator that would almost certainly kill them. A few – painfully and pathetically few – medical whistleblowers revealed (before Cult-owned YouTube deleted their videos) that they had been instructed to 'let the patient crash' and put them straight on a ventilator instead of going through a series of far less intrusive and dangerous methods as they would have done before the pandemic hoax began and the financial incentives kicked in. We are talking cold-blooded murder given that ventilators are so damaging to respiratory systems they are usually the last step before heaven awaits. Renegade Minds never fall for the belief that people in white coats are all angels of mercy and cannot be full-on psychopaths. I have explained in detail in *The Answer* how what I am describing here played out across the world coordinated by the World Health Organization through the medical hierarchies in almost every country.

Medical scientist calls it

Information about the non-existence of the 'virus' began to emerge for me in late March, 2020, and mushroomed after that. I was sent an email by Sir Julian Rose, a writer, researcher, and organic farming promotor, from a medical scientist friend of his in the United States. Even at that early stage in March the scientist was able to explain

how the 'Covid' hoax was being manipulated. He said there were no reliable tests for a specific 'Covid-19 virus' and nor were there any reliable agencies or media outlets for reporting numbers of actual 'Covid-19' cases. We have seen in the long period since then that he was absolutely right. 'Every action and reaction to Covid-19 is based on totally flawed data and we simply cannot make accurate assessments,' he said. Most people diagnosed with 'Covid-19' were showing nothing more than cold and flu-like symptoms 'because most coronavirus strains *are* nothing more than cold/flu-like symptoms'. We had farcical situations like an 84-year-old German man testing positive for 'Covid-19' and his nursing home ordered to quarantine only for him to be found to have a common cold. The scientist described back then why PCR tests and what he called the 'Mickey Mouse test kits' were useless for what they were claimed to be identifying. 'The idea these kits can isolate a specific virus like Covid-19 is nonsense,' he said. Significantly, he pointed out that 'if you want to create a totally false panic about a totally false pandemic – pick a coronavirus'. This is exactly what the Cult-owned Gates, World Economic Forum and Johns Hopkins University did with their Event 201 'simulation' followed by their real-life simulation called the 'pandemic'. The scientist said that all you had to do was select the sickest of people with respiratory-type diseases in a single location – 'say Wuhan' – and administer PCR tests to them. You can then claim that anyone showing 'viral sequences' similar to a coronavirus 'which will inevitably be quite a few' is suffering from a 'new' disease:

> Since you already selected the sickest flu cases a fairly high proportion of your sample will go on to die. You can then say this 'new' virus has a CFR [case fatality rate] higher than the flu and use this to infuse more concern and do more tests which will of course produce more 'cases', which expands the testing, which produces yet more 'cases' and so on and so on. Before long you have your 'pandemic', and all you have done is use a simple test kit trick to convert the worst flu and pneumonia cases into something new that doesn't ACTUALLY EXIST [my emphasis].

He said that you then 'just run the same scam in other countries' and make sure to keep the fear message running high 'so that people

will feel panicky and less able to think critically'. The only problem to overcome was the fact *there is no* actual new deadly pathogen and only regular sick people. This meant that deaths from the 'new deadly pathogen' were going to be way too low for a real new deadly virus pandemic, but he said this could be overcome in the following ways – all of which would go on to happen:

1. You can claim this is just the beginning and more deaths are imminent [you underpin this with fantasy 'computer projections']. Use this as an excuse to quarantine everyone and then claim the quarantine prevented the expected millions of dead.

2. You can [say that people] 'minimizing' the dangers are irresponsible and bully them into not talking about numbers.

3. You can talk crap about made up numbers hoping to blind people with pseudoscience.

4. You can start testing well people (who, of course, will also likely have shreds of coronavirus [RNA] in them) and thus inflate your 'case figures' with 'asymptomatic carriers' (you will of course have to spin that to sound deadly even though any virologist knows the more symptom-less cases you have the less deadly is your pathogen).

The scientist said that if you take these simple steps 'you can have your own entirely manufactured pandemic up and running in weeks'. His analysis made so early in the hoax was brilliantly prophetic of what would actually unfold. Pulling all the information together in these recent chapters we have this is simple 1, 2, 3, of how you can delude virtually the entire human population into believing in a 'virus' that doesn't exist:

- A 'Covid case' is someone who tests positive with a test not testing for the 'virus'.

- A 'Covid death' is someone who dies of *any cause* within 28 days (or much longer) of testing positive with a test not testing for the 'virus.

- Asymptomatic means there is nothing wrong with you, but they claim you can pass on what you don't have to justify locking

down (quarantining) healthy people in totality.

The foundations of the hoax are that simple. A study involving ten million people in Wuhan, published in November, 2020, demolished the whole lie about those without symptoms passing on the 'virus'. They found '300 asymptomatic cases' and traced their contacts to find that not one of them was detected with the 'virus'. 'Asymptomatic' patients and their contacts were isolated for no less than two weeks and nothing changed. I know it's all crap, but if you are going to claim that those without symptoms can transmit 'the virus' then you must produce evidence for that and they never have. Even World Health Organization official Dr Maria Van Kerkhove, head of the emerging diseases and zoonosis unit, said as early as June, 2020, that she doubted the validity of asymptomatic transmission. She said that 'from the data we have, it still seems to be rare that an asymptomatic person actually transmits onward to a secondary individual' and by 'rare' she meant that she couldn't cite any case of asymptomatic transmission.

The Ferguson factor

The problem for the Cult as it headed into March, 2020, when the script had lockdown due to start, was that despite all the manipulation of the case and death figures they still did not have enough people alleged to have died from 'Covid' to justify mass house arrest. This was overcome in the way the scientist described: 'You can claim this is just the beginning and more deaths are imminent … Use this as an excuse to quarantine everyone and then claim the quarantine prevented the expected millions of dead.' Enter one Professor Neil Ferguson, the Gates-funded 'epidemiologist' at the Gates-funded Imperial College in London. Ferguson is Britain's Christian Drosten in that he has a dire record of predicting health outcomes, but is still called upon to advise government on the next health outcome when another 'crisis' comes along. This may seem to be a strange and ridiculous thing to do. Why would you keep turning for policy guidance to people who have a history of being

monumentally wrong? Ah, but it makes sense from the Cult point of view. These 'experts' keep on producing predictions that suit the Cult agenda for societal transformation and so it was with Neil Ferguson as he revealed his horrific (and clearly insane) computer model predictions that allowed lockdowns to be imposed in Britain, the United States and many other countries. Ferguson does not have even an A-level in biology and would appear to have no formal training in computer modelling, medicine or epidemiology, according to Derek Winton, an MSc in Computational Intelligence. He wrote an article somewhat aghast at what Ferguson did which included taking no account of respiratory disease 'seasonality' which means it is far worse in the winter months. Who would have thought that respiratory disease could be worse in the winter? Well, certainly not Ferguson.

The massively China-connected Imperial College and its bizarre professor provided the excuse for the long-incubated Chinese model of human control to travel westward at lightning speed. Imperial College confirms on its website that it collaborates with the Chinese Research Institute; publishes more than 600 research papers every year with Chinese research institutions; has 225 Chinese staff; 2,600 Chinese students – the biggest international group; 7,000 former students living in China which is the largest group outside the UK; and was selected for a tour by China's President Xi Jinping during his state visit to the UK in 2015. The college takes major donations from China and describes itself as the UK's number one university collaborator with Chinese research institutions. The China communist/fascist government did not appear phased by the woeful predictions of Ferguson and Imperial when during the lockdown that Ferguson induced the college signed a five-year collaboration deal with China tech giant Huawei that will have Huawei's indoor 5G network equipment installed at the college's West London tech campus along with an 'AI cloud platform'. The deal includes Chinese sponsorship of Imperial's Venture Catalyst entrepreneurship competition. Imperial is an example of the enormous influence the Chinese government has within British and North American

universities and research centres – and further afield. Up to 200 academics from more than a dozen UK universities are being investigated on suspicion of 'unintentionally' helping the Chinese government build weapons of mass destruction by 'transferring world-leading research in advanced military technology such as aircraft, missile designs and cyberweapons'. Similar scandals have broken in the United States, but it's all a coincidence. Imperial College serves the agenda in many other ways including the promotion of every aspect of the United Nations Agenda 21/2030 (the Great Reset) and produced computer models to show that human-caused 'climate change' is happening when in the real world it isn't. Imperial College is driving the climate agenda as it drives the 'Covid' agenda (both Cult hoaxes) while Patrick Vallance, the UK government's Chief Scientific Adviser on 'Covid', was named Chief Scientific Adviser to the UN 'climate change' conference known as COP26 hosted by the government in Glasgow, Scotland. 'Covid' and 'climate' are fundamentally connected.

Professor Woeful

From Imperial's bosom came Neil Ferguson still advising government despite his previous disasters and it was announced early on that he and other key people like UK Chief Medical Adviser Chris Whitty had caught the 'virus' as the propaganda story was being sold. Somehow they managed to survive and we had Prime Minister Boris Johnson admitted to hospital with what was said to be a severe version of the 'virus' in this same period. His whole policy and demeanour changed when he returned to Downing Street. It's a small world with these government advisors – especially in their communal connections to Gates – and Ferguson had partnered with Whitty to write a paper called 'Infectious disease: Tough choices to reduce Ebola transmission' which involved another scare-story that didn't happen. Ferguson's 'models' predicted that up to150, 000 could die from 'mad cow disease', or BSE, and its version in sheep if it was transmitted to humans. BSE was not transmitted and instead triggered by an organophosphate pesticide used to treat a pest on

cows. Fewer than 200 deaths followed from the human form. Models by Ferguson and his fellow incompetents led to the unnecessary culling of millions of pigs, cattle and sheep in the foot and mouth outbreak in 2001 which destroyed the lives and livelihoods of farmers and their families who had often spent decades building their herds and flocks. Vast numbers of these animals did not have foot and mouth and had no contact with the infection. Another 'expert' behind the cull was Professor Roy Anderson, a computer modeller at Imperial College specialising in the epidemiology of *human*, not animal, disease. Anderson has served on the Bill and Melinda Gates Grand Challenges in Global Health advisory board and chairs another Gates-funded organisation. Gates is everywhere.

In a precursor to the 'Covid' script Ferguson backed closing schools 'for prolonged periods' over the swine flu 'pandemic' in 2009 and said it would affect a third of the world population if it continued to spread at the speed he claimed to be happening. His mates at Imperial College said much the same and a news report said: 'One of the authors, the epidemiologist and disease modeller Neil Ferguson, who sits on the World Health Organisation's emergency committee for the outbreak, said the virus had "full pandemic potential".' Professor Liam Donaldson, the Chris Whitty of his day as Chief Medical Officer, said the worst case could see 30 percent of the British people infected by swine flu with 65,000 dying. Ferguson and Donaldson were indeed proved correct when at the end of the year the number of deaths attributed to swine flu was 392. The term 'expert' is rather liberally applied unfortunately, not least to complete idiots. Swine flu 'projections' were great for GlaxoSmithKline (GSK) as millions rolled in for its Pandemrix influenza vaccine which led to brain damage with children most affected. The British government (taxpayers) paid out more than £60 million in compensation after GSK was given immunity from prosecution. Yet another 'Covid' déjà vu. Swine flu was supposed to have broken out in Mexico, but Dr Wolfgang Wodarg, a German doctor, former member of parliament and critic of the 'Covid' hoax, observed 'the spread of swine flu' in Mexico City at the time. He

said: 'What we experienced in Mexico City was a very mild flu which did not kill more than usual – which killed even fewer people than usual.' Hyping the fear against all the facts is not unique to 'Covid' and has happened many times before. Ferguson is reported to have over-estimated the projected death toll of bird flu (H5N1) by some three million-fold, but bird flu vaccine makers again made a killing from the scare. This is some of the background to the Neil Ferguson who produced the perfectly-timed computer models in early 2020 predicting that half a million people would die in Britain without draconian lockdown and 2.2 million in the United States. Politicians panicked, people panicked, and lockdowns of alleged short duration were instigated to 'flatten the curve' of cases gleaned from a test not testing for the 'virus'. I said at the time that the public could forget the 'short duration' bit. This was an agenda to destroy the livelihoods of the population and force them into mass control through dependency and there was going to be nothing 'short' about it. American researcher Daniel Horowitz described the consequences of the 'models' spewed out by Gates-funded Ferguson and Imperial College:

> What led our government and the governments of many other countries into panic was a single Imperial College of UK study, funded by global warming activists, that predicted 2.2 million deaths if we didn't lock down the country. In addition, the reported 8-9% death rate in Italy scared us into thinking there was some other mutation of this virus that they got, which might have come here.
>
> Together with the fact that we were finally testing and had the ability to actually report new cases, we thought we were headed for a death spiral. But again … we can't flatten a curve if we don't know when the curve started.

How about it *never* started?

Giving them what they want

An investigation by German news outlet *Welt Am Sonntag (World on Sunday)* revealed how in March, 2020, the German government gathered together 'leading scientists from several research institutes and universities' and 'together, they were to produce a [modelling]

paper that would serve as legitimization for further tough political measures'. The Cult agenda was justified by computer modelling not based on evidence or reality; it was specifically constructed to justify the Cult demand for lockdowns all over the world to destroy the independent livelihoods of the global population. All these modellers and everyone responsible for the 'Covid' hoax have a date with a trial like those in Nuremberg after World War Two when Nazis faced the consequences of their war crimes. These corrupt-beyond-belief 'modellers' wrote the paper according to government instructions and it said that that if lockdown measures were lifted then up to one million Germans would die from 'Covid-19' adding that some would die 'agonizingly at home, gasping for breath' unable to be treated by hospitals that couldn't cope. All lies. No matter – it gave the Cult all that it wanted. What did long-time government 'modeller' Neil Ferguson say? If the UK and the United States didn't lockdown half a million would die in Britain and 2.2 million Americans. Anyone see a theme here? 'Modellers' are such a crucial part of the lockdown strategy that we should look into their background and follow the money. Researcher Rosemary Frei produced an excellent article headlined 'The Modelling-paper Mafiosi'. She highlights a guy called John Edmunds, a British epidemiologist, and professor in the Faculty of Epidemiology and Population Health at the London School of Hygiene & Tropical Medicine. He studied at Imperial College. Edmunds is a member of government 'Covid' advisory bodies which have been dictating policy, the New and Emerging Respiratory Virus Threats Advisory Group (NERVTAG) and the Scientific Advisory Group for Emergencies (SAGE).

Ferguson, another member of NERVTAG and SAGE, led the way with the original 'virus' and Edmunds has followed in the 'variant' stage and especially the so-called UK or Kent variant known as the 'Variant of Concern' (VOC) B.1.1.7. He said in a co-written report for the Centre for Mathematical modelling of Infectious Diseases at the London School of Hygiene and Tropical Medicine, with input from the Centre's 'Covid-19' Working Group, that there was 'a realistic

possibility that VOC B.1.1.7 is associated with an increased risk of death compared to non-VOC viruses'. Fear, fear, fear, get the vaccine, fear, fear, fear, get the vaccine. Rosemary Frei reveals that almost all the paper's authors and members of the modelling centre's 'Covid-19' Working Group receive funding from the Bill and Melinda Gates Foundation and/or the associated Gates-funded Wellcome Trust. The paper was published by e-journal *Medr χiv* which only publishes papers not peer-reviewed and the journal was established by an organisation headed by Facebook's Mark Zuckerberg and his missus. What a small world it is. Frei discovered that Edmunds is on the Scientific Advisory Board of the Coalition for Epidemic Preparedness Innovations (CEPI) which was established by the Bill and Melinda Gates Foundation, Klaus Schwab's Davos World Economic Forum and Big Pharma giant Wellcome. CEPI was 'launched in Davos [in 2017] to develop vaccines to stop future epidemics', according to its website. 'Our mission is to accelerate the development of vaccines against emerging infectious diseases and enable equitable access to these vaccines for people during outbreaks.' What kind people they are. Rosemary Frei reveals that Public Health England (PHE) director Susan Hopkins is an author of her organisation's non-peer-reviewed reports on 'new variants'. Hopkins is a professor of infectious diseases at London's Imperial College which is gifted tens of millions of dollars a year by the Bill and Melinda Gates Foundation. Gates-funded modelling disaster Neil Ferguson also co-authors Public Health England reports and he spoke in December, 2020, about the potential danger of the B.1.1.7. 'UK variant' promoted by Gates-funded modeller John Edmunds. When I come to the 'Covid vaccines' the 'new variants' will be shown for what they are – bollocks.

Connections, connections

All these people and modellers are lockdown-obsessed or, put another way, they demand what the Cult demands. Edmunds said in January, 2021, that to ease lockdowns too soon would be a disaster and they had to 'vaccinate much, much, much more widely than the

elderly'. Rosemary Frei highlights that Edmunds is married to Jeanne Pimenta who is described in a LinkedIn profile as director of epidemiology at GlaxoSmithKline (GSK) and she held shares in the company. Patrick Vallance, co-chair of SAGE and the government's Chief Scientific Adviser, is a former executive of GSK and has a deferred bonus of shares in the company worth £600,000. GSK has serious business connections with Bill Gates and is collaborating with mRNA-'vaccine' company CureVac to make 'vaccines' for the new variants that Edmunds is talking about. GSK is planning a 'Covid vaccine' with drug giant Sanofi. Puppet Prime Minister Boris Johnson announced in the spring of 2021 that up to 60 million vaccine doses were to be made at the GSK facility at Barnard Castle in the English North East. Barnard Castle, with a population of just 6,000, was famously visited in breach of lockdown rules in April, 2020, by Johnson aide Dominic Cummings who said that he drove there 'to test his eyesight' before driving back to London. Cummings would be better advised to test his integrity – not that it would take long. The GSK facility had nothing to do with his visit then although I'm sure Patrick Vallance would have been happy to arrange an introduction and some tea and biscuits. Ruthless psychopath Gates has made yet another fortune from vaccines in collaboration with Big Pharma companies and gushes at the phenomenal profits to be made from vaccines – more than a 20-to-1 return as he told one interviewer. Gates also tweeted in December, 2019, with the foreknowledge of what was coming: 'What's next for our foundation? I'm particularly excited about what the next year could mean for one of the best buys in global health: vaccines.'

Modeller John Edmunds is a big promotor of vaccines as all these people appear to be. He's the dean of the London School of Hygiene & Tropical Medicine's Faculty of Epidemiology and Population Health which is primarily funded by the Bill and Melinda Gates Foundation and the Gates-established and funded GAVI vaccine alliance which is the Gates vehicle to vaccinate the world. The organisation Doctors Without Borders has described GAVI as being 'aimed more at supporting drug-industry desires to promote new

products than at finding the most efficient and sustainable means for fighting the diseases of poverty'. But then that's why the psychopath Gates created it. John Edmunds said in a video that the London School of Hygiene & Tropical Medicine is involved in every aspect of vaccine development including large-scale clinical trials. He contends that mathematical modelling can show that vaccines protect individuals and society. That's on the basis of shit in and shit out, I take it. Edmunds serves on the UK Vaccine Network as does Ferguson and the government's foremost 'Covid' adviser, the grim-faced, dark-eyed Chris Whitty. The Vaccine Network says it works 'to support the government to identify and shortlist targeted investment opportunities for the most promising vaccines and vaccine technologies that will help combat infectious diseases with epidemic potential, and to address structural issues related to the UK's broader vaccine infrastructure'. Ferguson is acting Director of the Imperial College Vaccine Impact Modelling Consortium which has funding from the Bill and Melina Gates Foundation and the Gates-created GAVI 'vaccine alliance'. Anyone wonder why these characters see vaccines as the answer to every problem? Ferguson is wildly enthusiastic in his support for GAVI's campaign to vaccine children en masse in poor countries. You would expect someone like Gates who has constantly talked about the need to reduce the population to want to fund vaccines to keep more people alive. I'm sure that's why he does it. The John Edmunds London School of Hygiene & Tropical Medicine (LSHTM) has a Vaccines Manufacturing Innovation Centre which develops, tests and commercialises vaccines. Rosemary Frei writes:

> The vaccines centre also performs affiliated activities like combating 'vaccine hesitancy'. The latter includes the Vaccine Confidence Project. The project's stated purpose is, among other things, 'to provide analysis and guidance for early response and engagement with the public to ensure sustained confidence in vaccines and immunisation'. The Vaccine Confidence Project's director is LSHTM professor Heidi Larson. For more than a decade she's been researching how to combat vaccine hesitancy.

How the bloody hell can blokes like John Edmunds and Neil Ferguson with those connections and financial ties model 'virus' case

and death projections for the government and especially in a way that gives their paymasters like Gates exactly what they want? It's insane, but this is what you find throughout the world.

'Covid' is not dangerous, oops, wait, yes it is

Only days before Ferguson's nightmare scenario made Jackboot Johnson take Britain into a China-style lockdown to save us from a deadly 'virus' the UK government website gov.uk was reporting something very different to Ferguson on a page of official government guidance for 'high consequence infectious diseases (HCID)'. It said this about 'Covid-19':

As of 19 March 2020, COVID-19 *is no longer considered to be a high consequence infectious diseases (HCID) in the UK* [my emphasis]. The 4 nations public health HCID group made an interim recommendation in January 2020 to classify COVID-19 as an HCID. This was based on consideration of the UK HCID criteria about the virus and the disease with information available during the early stages of the outbreak.

Now that more is known about COVID-19, the public health bodies in the UK have reviewed the most up to date information about COVID-19 against the UK HCID criteria. They have determined that several features have now changed; in particular, more information is available about mortality rates (low overall), and there is now greater clinical awareness and a specific and sensitive laboratory test, the availability of which continues to increase. The Advisory Committee on Dangerous Pathogens (ACDP) is also of the opinion that COVID-19 should no longer be classified as an HCID.

Soon after the government had been exposed for downgrading the risk they upgraded it again and everyone was back to singing from the same Cult hymn book. Ferguson and his fellow Gates clones indicated that lockdowns and restrictions would have to continue until a Gates-funded vaccine was developed. Gates said the same because Ferguson and his like were repeating the Gates script which is the Cult script. 'Flatten the curve' became an ongoing nightmare of continuing lockdowns with periods in between of severe restrictions in pursuit of destroying independent incomes and had nothing to do with protecting health about which the Cult gives not a shit. Why wouldn't Ferguson be pushing a vaccine 'solution' when he's owned by vaccine-obsessive Gates who makes a fortune from them and

when Ferguson heads the Vaccine Impact Modelling Consortium at Imperial College funded by the Gates Foundation and GAVI, the 'vaccine alliance', created by Gates as his personal vaccine promotion operation? To compound the human catastrophe that Ferguson's 'models' did so much to create he was later exposed for breaking his own lockdown rules by having sexual liaisons with his married girlfriend Antonia Staats at his home while she was living at another location with her husband and children. Staats was a 'climate' activist and senior campaigner at the Soros-funded Avaaz which I wouldn't trust to tell me that grass is green. Ferguson had to resign as a government advisor over this hypocrisy in May, 2020, but after a period of quiet he was back being quoted by the ridiculous media on the need for more lockdowns and a vaccine rollout. Other government-advising 'scientists' from Imperial College' held the fort in his absence and said lockdown could be indefinite until a vaccine was found. The Cult script was being sung by the payrolled choir. I said there was no intention of going back to 'normal' when the 'vaccine' came because the 'vaccine' is part of a very different agenda that I will discuss in Human 2.0. Why would the Cult want to let the world go back to normal when destroying that normal forever was the whole point of what was happening? House arrest, closing businesses and schools through lockdown, (un)social distancing and masks all followed the Ferguson fantasy models. Again as I predicted (these people are so predictable) when the 'vaccine' arrived we were told that house arrest, lockdown, (un)social distancing and masks would still have to continue. I will deal with the masks in the next chapter because they are of fundamental importance.

Where's the 'pandemic'?

Any mildly in-depth assessment of the figures revealed what was really going on. Cult-funded and controlled organisations still have genuine people working within them such is the number involved. So it is with Genevieve Briand, assistant program director of the Applied Economics master's degree program at Johns Hopkins

University. She analysed the impact that 'Covid-19' had on deaths from *all* causes in the United States using official data from the CDC for the period from early February to early September, 2020. She found that allegedly 'Covid' *related*-deaths exceeded those from heart disease which she found strange with heart disease always the biggest cause of fatalities. Her research became even more significant when she noted the sudden decline in 2020 of *all* non-'Covid' deaths: 'This trend is completely contrary to the pattern observed in all previous years … the total decrease in deaths by other causes almost exactly equals the increase in deaths by Covid-19.' This was such a game, set and match in terms of what was happening that Johns Hopkins University deleted the article on the grounds that it 'was being used to support false and dangerous inaccuracies about the impact of the pandemic'. No – because it exposed the scam from official CDC figures and this was confirmed when those figures were published in January, 2021. Here we can see the effect of people dying from heart attacks, cancer, road accidents and gunshot wounds – *anything* – having 'Covid-19' on the death certificate along with those diagnosed from 'symptoms' who had even not tested positive with a test not testing for the 'virus'. I am not kidding with the gunshot wounds, by the way. Brenda Bock, coroner in Grand County, Colorado, revealed that two gunshot victims tested positive for the 'virus' within the previous 30 days and were therefore classified as 'Covid deaths'. Bock said: 'These two people had tested positive for Covid, but that's not what killed them. A gunshot wound is what killed them.' She said she had not even finished her investigation when the state listed the gunshot victims as deaths due to the 'virus'. The death and case figures for 'Covid-19' are an absolute joke and yet they are repeated like parrots by the media, politicians and alleged medical 'experts'. The official Cult narrative is the only show in town.

Genevieve Briand found that deaths from all causes were not exceptional in 2020 compared with previous years and a Spanish magazine published figures that said the same about Spain which was a 'Covid' propaganda hotspot at one point. *Discovery Salud*, a

health and medicine magazine, quoted government figures which showed how 17,000 *fewer* people died in Spain in 2020 than in 2019 and more than 26,000 fewer than in 2018. The age-standardised mortality rate for England and Wales when age distribution is taken into account was significantly lower in 2020 than the 1970s, 80s and 90s, and was only the ninth highest since 2000. Where is the 'pandemic'?

Post mortems and autopsies virtually disappeared for 'Covid' deaths amid claims that 'virus-infected' bodily fluids posed a risk to those carrying out the autopsy. This was rejected by renowned German pathologist and forensic doctor Klaus Püschel who said that he and his staff had by then done 150 autopsies on 'Covid' patients with no problems at all. He said they were needed to know why some 'Covid' patients suffered blood clots and not severe respiratory infections. The 'virus' is, after all, called SARS or 'severe acute respiratory syndrome'. I highlighted in the spring of 2020 this phenomenon and quoted New York intensive care doctor Cameron Kyle-Sidell who posted a soon deleted YouTube video to say that they had been told to prepare to treat an infectious disease called 'Covid-19', but that was not what they were dealing with. Instead he likened the lung condition of the most severely ill patients to what you would expect with cabin depressurisation in a plane at 30,000 feet or someone dropped on the top of Everest without oxygen or acclimatisation. I have never said this is not happening to a small minority of alleged 'Covid' patients – I am saying this is not caused by a phantom 'contagious virus'. Indeed Kyle-Sidell said that 'Covid-19' was not the disease they were told was coming their way. 'We are operating under a medical paradigm that is untrue,' he said, and he believed they were treating the wrong disease: 'These people are being slowly starved of oxygen.' Patients would take off their oxygen masks in a state of fear and stress and while they were blue in the face on the brink of death. They did not look like patients dying of pneumonia. You can see why they don't want autopsies when their virus doesn't exist and there is another condition in some people that they don't wish to be uncovered. I should add here that

the 5G system of millimetre waves was being rapidly introduced around the world in 2020 and even more so now as they fire 5G at the Earth from satellites. At 60 gigahertz within the 5G range that frequency interacts with the oxygen molecule and stops people breathing in sufficient oxygen to be absorbed into the bloodstream. They are installing 5G in schools and hospitals. The world is not mad or anything. 5G can cause major changes to the lungs and blood as I detail in *The Answer* and these consequences are labelled 'Covid-19', the alleged symptoms of which can be caused by 5G and other electromagnetic frequencies as cells respond to radiation poisoning.

The 'Covid death' scam

Dr Scott Jensen, a Minnesota state senator and medical doctor, exposed 'Covid' Medicare payment incentives to hospitals and death certificate manipulation. He said he was sent a seven-page document by the US Department of Health 'coaching' him on how to fill out death certificates which had never happened before. The document said that he didn't need to have a laboratory test for 'Covid-19' to put that on the death certificate and that shocked him when death certificates are supposed to be about facts. Jensen described how doctors had been 'encouraged, if not pressured' to make a diagnosis of 'Covid-19' if they thought it was probable or *'presumed*'. No positive test was necessary – not that this would have mattered anyway. He said doctors were told to diagnose 'Covid' by symptoms when these were the same as colds, allergies, other respiratory problems, and certainly with influenza which 'disappeared' in the 'Covid' era. A common sniffle was enough to get the dreaded verdict. Ontario authorities decreed that a single care home resident with *one* symptom from a long list must lead to the isolation of the entire home. Other courageous doctors like Jensen made the same point about death figure manipulation and how deaths by other causes were falling while 'Covid-19 deaths' were rising at the same rate due to re-diagnosis. Their videos rarely survive long on YouTube with its Cult-supporting algorithms courtesy of CEO Susan Wojcicki and her bosses at Google. Figure-tampering was so glaring

and ubiquitous that even officials were letting it slip or outright saying it. UK chief scientific adviser Patrick Vallance said on one occasion that 'Covid' on the death certificate doesn't mean 'Covid' was the cause of death (so why the hell is it there?) and we had the rare sight of a BBC reporter telling the truth when she said: 'Someone could be successfully treated for Covid, in say April, discharged, and then in June, get run over by a bus and die … That person would still be counted as a Covid death in England.' Yet the BBC and the rest of the world media went on repeating the case and death figures as if they were real. Illinois Public Health Director Dr Ngozi Ezike revealed the deceit while her bosses must have been clenching their buttocks:

> If you were in a hospice and given a few weeks to live and you were then found to have Covid that would be counted as a Covid death. [There might be] a clear alternate cause, but it is still listed as a Covid death. So everyone listed as a Covid death doesn't mean that was the cause of the death, but that they had Covid at the time of death.

Yes, a 'Covid virus' never shown to exist and tested for with a test not testing for the 'virus'. In the first period of the pandemic hoax through the spring of 2020 the process began of designating almost everything a 'Covid' death and this has continued ever since. I sat in a restaurant one night listening to a loud conversation on the next table where a family was discussing in bewilderment how a relative who had no symptoms of 'Covid', and had died of a long-term problem, could have been diagnosed a death by the 'virus'. I could understand their bewilderment. If they read this book they will know why this medical fraud has been perpetrated the world over.

Some media truth shock

The media ignored the evidence of death certificate fraud until eventually one columnist did speak out when she saw it first-hand. Bel Mooney is a long-time national newspaper journalist in Britain currently working for the *Daily Mail*. Her article on February 19th, 2021, carried this headline: 'My dad Ted passed three Covid tests

and died of a chronic illness yet he's officially one of Britain's 120,000 victims of the virus and is far from alone ... so how many more are there?' She told how her 99-year-old father was in a care home with a long-standing chronic obstructive pulmonary disease and vascular dementia. Maybe, but he was still aware enough to tell her from the start that there was no 'virus' and he refused the 'vaccine' for that reason. His death was not unexpected given his chronic health problems and Mooney said she was shocked to find that 'Covid-19' was declared the cause of death on his death certificate. She said this was a 'bizarre and unacceptable untruth' for a man with long-time health problems who had tested negative twice at the home for the 'virus'. I was also shocked by this story although not by what she said. I had been highlighting the death certificate manipulation for ten months. It was the confirmation that a professional full-time journalist only realised this was going on when it affected her directly and neither did she know that whether her dad tested positive or negative was irrelevant with the test not testing for the 'virus'. Where had she been? She said she did not believe in 'conspiracy theories' without knowing I'm sure that this and 'conspiracy theorists' were terms put into widespread circulation by the CIA in the 1960s to discredit those who did not accept the ridiculous official story of the Kennedy assassination. A blanket statement of 'I don't believe in conspiracy theories' is always bizarre. The dictionary definition of the term alone means the world is drowning in conspiracies. What she said was even more daft when her dad had just been affected by the 'Covid' conspiracy. Why else does she think that 'Covid-19' was going on the death certificates of people who died of something else?

To be fair once she saw from personal experience what was happening she didn't mince words. Mooney was called by the care home on the morning of February 9th to be told her father had died in his sleep. When she asked for the official cause of death what came back was 'Covid-19'. Mooney challenged this and was told there had been deaths from Covid on the dementia floor (confirmed by a test not testing for the 'virus') so they considered it 'reasonable

to assume'. 'But doctor,' Mooney rightly protested, 'an assumption isn't a diagnosis.' She said she didn't blame the perfectly decent and sympathetic doctor – 'he was just doing his job'. Sorry, but that's *bullshit*. He wasn't doing his job at all. He was putting a false cause of death on the death certificate and that is a criminal offence for which he should be brought to account and the same with the millions of doctors worldwide who have done the same. They were not doing their job they were following orders and that must not wash at new Nuremberg trials any more than it did at the first ones. Mooney's doctor was 'assuming' (presuming) as he was told to, but 'just following orders' makes no difference to his actions. A doctor's job is to serve the patient and the truth, not follow orders, but that's what they have done all over the world and played a central part in making the 'Covid' hoax possible with all its catastrophic consequences for humanity. Shame on them and they must answer for their actions. Mooney said her disquiet worsened when she registered her father's death by telephone and was told by the registrar there had been very many other cases like hers where 'the deceased' had not tested positive for 'Covid' yet it was recorded as the cause of death. The test may not matter, but those involved at their level *think* it matters and it shows a callous disregard for accurate diagnosis. The pressure to do this is coming from the top of the national 'health' pyramids which in turn obey the World Health Organization which obeys Gates and the Cult. Mooney said the registrar agreed that this must distort the national figures adding that 'the strangest thing is that every winter we record countless deaths from flu, and this winter there have been none. Not one!' She asked if the registrar thought deaths from flu were being misdiagnosed and lumped together with 'Covid' deaths. The answer was a 'puzzled yes'. Mooney said that the funeral director said the same about 'Covid' deaths which had nothing to do with 'Covid'. They had lost count of the number of families upset by this and other funeral companies in different countries have had the same experience. Mooney wrote:

The nightly shroud-waving and shocking close-ups of pain imposed on us by the TV news bewildered and terrified the population into eager compliance with lockdowns. We were invited to 'save the NHS' and to grieve for strangers – the real-life loved ones behind those shocking death counts. Why would the public imagine what I now fear, namely that the way Covid-19 death statistics are compiled might make the numbers seem greater than they are?

Oh, just a little bit – like 100 percent.

Do the maths

Mooney asked why a country would wish to skew its mortality figures by wrongly certifying deaths? What had been going on? Well, if you don't believe in conspiracies you will never find the answer which is that *it's a conspiracy*. She did, however, describe what she had discovered as a 'national scandal'. In reality it's a global scandal and happening everywhere. Pillars of this conspiracy were all put into place before the button was pressed with the Drosten PCR protocol and high amplifications to produce the cases and death certificate changes to secure illusory 'Covid' deaths. Mooney notes that normally two doctors were needed to certify a death, with one having to know the patient, and how the rules were changed in the spring of 2020 to allow one doctor to do this. In the same period 'Covid deaths' were decreed to be all cases where Covid-19 was put on the death certificate even without a positive test or any symptoms. Mooney asked: 'How many of the 30,851 (as of January 15) care home resident deaths with Covid-19 on the certificate (32.4 per cent of all deaths so far) were based on an assumption, like that of my father? And what has that done to our national psyche?'All of them is the answer to the first question and it has devastated and dismantled the national psyche, actually the global psyche, on a colossal scale. In the UK case and death data is compiled by organisations like Public Health England (PHE) and the Office for National Statistics (ONS). Mooney highlights the insane policy of counting a death from any cause as 'Covid-19' if this happens within 28 days of a positive test (with a test not testing for the 'virus') and she points out that ONS statistics reflect deaths 'involving Covid' 'or due to Covid' which meant in practice any

death where 'Covid-19' was mentioned on the death certificate. She described the consequences of this fraud:

Most people will accept the narrative they are fed, so panicky governments here and in Europe witnessed the harsh measures enacted in totalitarian China and jumped into lockdown. Headlines about Covid deaths tolled like the knell that would bring doomsday to us all. Fear stalked our empty streets. Politicians parroted the frankly ridiculous aim of 'zero Covid' and shut down the economy, while most British people agreed that lockdown was essential and (astonishingly to me, as a patriotic Brit) even wanted more restrictions.

For what? Lies on death certificates? Never mind the grim toll of lives ruined, suicides, schools closed, rising inequality, depression, cancelled hospital treatments, cancer patients in a torture of waiting, poverty, economic devastation, loneliness, families kept apart, and so on. How many lives have been lost as a direct result of lockdown?

She said that we could join in a national chorus of shock and horror at reaching the 120,000 death toll which was surely certain to have been totally skewed all along, but what about the human cost of lockdown justified by these 'death figures'? *The British Medical Journal* had reported a 1,493 percent increase in cases of children taken to Great Ormond Street Hospital with abusive head injuries alone and then there was the effect on families:

Perhaps the most shocking thing about all this is that families have been kept apart – and obeyed the most irrational, changing rules at the whim of government – because they believed in the statistics. They succumbed to fear, which his generation rejected in that war fought for freedom. Dad (God rest his soul) would be angry. And so am I.

Another theme to watch is that in the winter months when there are more deaths from all causes they focus on 'Covid' deaths and in the summer when the British Lung Foundation says respiratory disease plummets by 80 percent they rage on about 'cases'. Either way fascism on population is always the answer.

Nazi eugenics in the 21st century

Elderly people in care homes have been isolated from their families month after lonely month with no contact with relatives and grandchildren who were banned from seeing them. We were told

that lockdown fascism was to 'protect the vulnerable' like elderly people. At the same time Do Not Resuscitate (DNR) orders were placed on their medical files so that if they needed resuscitation it wasn't done and 'Covid-19' went on their death certificates. Old people were not being 'protected' they were being culled – murdered in truth. DNR orders were being decreed for disabled and young people with learning difficulties or psychological problems. The UK Care Quality Commission, a non-departmental body of the Department of Health and Social Care, found that 34 percent of those working in health and social care were pressured into placing 'do not attempt cardiopulmonary resuscitation' orders on 'Covid' patients who suffered from disabilities and learning difficulties without involving the patient or their families in the decision. UK judges ruled that an elderly woman with dementia should have the DNA-manipulating 'Covid vaccine' against her son's wishes and that a man with severe learning difficulties should have the jab despite his family's objections. Never mind that many had already died. The judiciary always supports doctors and government in fascist dictatorships. They wouldn't dare do otherwise. A horrific video was posted showing fascist officers from Los Angeles police forcibly giving the 'Covid' shot to women with special needs who were screaming that they didn't want it. The same fascists are seen giving the jab to a sleeping elderly woman in a care home. This is straight out of the Nazi playbook. Hitler's Nazis committed mass murder of the mentally ill and physically disabled throughout Germany and occupied territories in the programme that became known as Aktion T4, or just T4. Sabbatian-controlled Hitler and his grotesque crazies set out to kill those they considered useless and unnecessary. The Reich Committee for the Scientific Registering of Hereditary and Congenital Illnesses registered the births of babies identified by physicians to have 'defects'. By 1941 alone more than 5,000 children were murdered by the state and it is estimated that in total the number of innocent people killed in Aktion T4 was between 275,000 and 300,000. Parents were told their children had been sent away for 'special treatment' never to return. It is rather pathetic to see claims about plans for new extermination camps being dismissed today

when the same force behind current events did precisely that 80 years ago. Margaret Sanger was a Cult operative who used 'birth control' to sanitise her programme of eugenics. Organisations she founded became what is now Planned Parenthood. Sanger proposed that 'the whole dysgenic population would have its choice of segregation or sterilization'. These included epileptics, 'feeble-minded', and prostitutes. Sanger opposed charity because it perpetuated 'human waste'. She reveals the Cult mentality and if anyone thinks that extermination camps are a 'conspiracy theory' their naivety is touching if breathtakingly stupid.

If you don't believe that doctors can act with callous disregard for their patients it is worth considering that doctors and medical staff agreed to put government-decreed DNR orders on medical files and do nothing when resuscitation is called for. I don't know what you call such people in your house. In mine they are Nazis from the Josef Mengele School of Medicine. Phenomenal numbers of old people have died worldwide from the effects of lockdown, depression, lack of treatment, the 'vaccine' (more later) and losing the will to live. A common response at the start of the manufactured pandemic was to remove old people from hospital beds and transfer them to nursing homes. The decision would result in a mass cull of elderly people in those homes through lack of treatment – *not* 'Covid'. Care home whistleblowers have told how once the 'Covid' era began doctors would not come to their homes to treat patients and they were begging for drugs like antibiotics that often never came. The most infamous example was ordered by New York governor Andrew Cuomo, brother of a moronic CNN host, who amazingly was given an Emmy Award for his handling of the 'Covid crisis' by the ridiculous Wokers that hand them out. Just how ridiculous could be seen in February, 2021, when a Department of Justice and FBI investigation began into how thousands of old people in New York died in nursing homes after being discharged from hospital to make way for 'Covid' patients on Cuomo's say-so – and how he and his staff covered up these facts. This couldn't have happened to a nicer psychopath. Even then there was a 'Covid' spin. Reports said that

thousands of old people who tested positive for 'Covid' in hospital were transferred to nursing homes to both die of 'Covid' and transmit it to others. No – they were in hospital because they were ill and the fact that they tested positive with a test not testing for the 'virus' is irrelevant. They were ill often with respiratory diseases ubiquitous in old people near the end of their lives. Their transfer out of hospital meant that their treatment stopped and many would go on to die.

They're old. Who gives a damn?

I have exposed in the books for decades the Cult plan to cull the world's old people and even to introduce at some point what they call a 'demise pill' which at a certain age everyone would take and be out of here by law. In March, 2021, Spain legalised euthanasia and assisted suicide following the Netherlands, Belgium, Luxembourg and Canada on the Tiptoe to the demise pill. Treatment of old people by many 'care' homes has been a disgrace in the 'Covid' era. There are many, many, caring staff – I know some. There have, however, been legions of stories about callous treatment of old people and their families. Police were called when families came to take their loved ones home in the light of isolation that was killing them. They became prisoners of the state. Care home residents in insane, fascist Ontario, Canada, were not allowed to leave their *room* once the 'Covid' hoax began. UK staff have even wheeled elderly people away from windows where family members were talking with them. Oriana Criscuolo from Stockport in the English North West dropped off some things for her 80-year-old father who has Parkinson's disease and dementia and she wanted to wave to him through a ground-floor window. She was told that was 'illegal'. When she went anyway they closed the curtains in the middle of the day. Oriana said:

> It's just unbelievable. I cannot understand how care home staff – people who are being paid to care – have become so uncaring. Their behaviour is inhumane and cruel. It's beyond belief.

She was right and this was not a one-off. What a way to end your life in such loveless circumstances. UK registered nurse Nicky Millen, a proper old school nurse for 40 years, said that when she started her career care was based on dignity, choice, compassion and empathy. Now she said 'the things that are important to me have gone out of the window.' She was appalled that people were dying without their loved ones and saying goodbye on iPads. Nicky described how a distressed 89-year-old lady stroked her face and asked her 'how many paracetamol would it take to finish me off'. Life was no longer worth living while not seeing her family. Nicky said she was humiliated in front of the ward staff and patients for letting the lady stroke her face and giving her a cuddle. Such is the dehumanisation that the 'Covid' hoax has brought to the surface. Nicky worked in care homes where patients told her they were being held prisoner. 'I want to live until I die', one said to her. 'I had a lady in tears because she hadn't seen her great-grandson.' Nicky was compassionate old school meeting psychopathic New Normal. She also said she had worked on a 'Covid' ward with no 'Covid' patients. Jewish writer Shai Held wrote an article in March, 2020, which was headlined 'The Staggering, Heartless Cruelty Toward the Elderly'. What he described was happening from the earliest days of lockdown. He said 'the elderly' were considered a group and not unique individuals (the way of the Woke). Shai Held said:

Notice how the all-too-familiar rhetoric of dehumanization works: 'The elderly' are bunched together as a faceless mass, all of them considered culprits and thus effectively deserving of the suffering the pandemic will inflict upon them. Lost entirely is the fact that the elderly are individual human beings, each with a distinctive face and voice, each with hopes and dreams, memories and regrets, friendships and marriages, loves lost and loves sustained.

'The elderly' have become another dehumanised group for which anything goes and for many that has resulted in cold disregard for their rights and their life. The distinctive face that Held talks about is designed to be deleted by masks until everyone is part of a faceless mass.

‘War-zone’ hospitals myth

Again and again medical professionals have told me what was really going on and how hospitals ‘overrun like war zones’ according to the media were virtually empty. The mantra from medical whistleblowers was please don’t use my name or my career is over. Citizen journalists around the world sneaked into hospitals to film evidence exposing the ‘war-zone’ lie. They really *were* largely empty with closed wards and operating theatres. I met a hospital worker in my town on the Isle of Wight during the first lockdown in 2020 who said the only island hospital had never been so quiet. Lockdown was justified by the psychopaths to stop hospitals being overrun. At the same time that the island hospital was near-empty the military arrived here to provide *extra beds*. It was all propaganda to ramp up the fear to ensure compliance with fascism as were never-used temporary hospitals with thousands of beds known as Nightingales and never-used make-shift mortuaries opened by the criminal UK government. A man who helped to install those extra island beds attributed to the army said they were never used and the hospital was empty. Doctors and nurses ‘stood around talking or on their phones, wandering down to us to see what we were doing’. There were no masks or social distancing. He accused the useless local island paper, the *County Press*, of ‘pumping the fear as if our hospital was overrun and we only have one so it should have been’. He described ambulances parked up with crews outside in deck chairs. When his brother called an ambulance he was told there was a two-hour backlog which he called ‘bullshit’. An old lady on the island fell ‘and was in a bad way’, but a caller who rang for an ambulance was told the situation wasn’t urgent enough. Ambulance stations were working under capacity while people would hear ambulances with sirens blaring driving through the streets. When those living near the stations realised what was going on they would follow them as they left, circulated around an urban area with the sirens going, and then came back without stopping. All this was to increase levels of fear and the same goes for the ‘ventilator shortage crisis’ that cost tens of millions for hastily produced ventilators never to be used.

Ambulance crews that agreed to be exploited in this way for fear propaganda might find themselves a mirror. I wish them well with that. Empty hospitals were the obvious consequence of treatment and diagnoses of non-'Covid' conditions cancelled and those involved handed a death sentence. People have been dying at home from undiagnosed and untreated cancer, heart disease and other life-threatening conditions to allow empty hospitals to deal with a 'pandemic' that wasn't happening.

Death of the innocent

'War-zones' have been laying off nursing staff, even doctors where they can. There was no work for them. Lockdown was justified by saving lives and protecting the vulnerable they were actually killing with DNR orders and preventing empty hospitals being 'overrun'. In Britain the mantra of stay at home to 'save the NHS' was everywhere and across the world the same story was being sold when it was all lies. Two California doctors, Dan Erickson and Artin Massihi at Accelerated Urgent Care in Bakersfield, held a news conference in April, 2020, to say that intensive care units in California were 'empty, essentially', with hospitals shutting floors, not treating patients and laying off doctors. The California health system was working at minimum capacity 'getting rid of doctors because we just don't have the volume'. They said that people with conditions such as heart disease and cancer were not coming to hospital out of fear of 'Covid-19'. Their video was deleted by Susan Wojcicki's Cult-owned YouTube after reaching five million views. Florida governor Ron Desantis, who rejected the severe lockdowns of other states and is being targeted for doing so, said that in March, 2020, every US governor was given models claiming they would run out of hospital beds in days. That was never going to happen and the 'modellers' knew it. Deceit can be found at every level of the system. Urgent children's operations were cancelled including fracture repairs and biopsies to spot cancer. Eric Nicholls, a consultant paediatrician, said 'this is obviously concerning and we need to return to normal operating and to increase capacity as soon as possible'. Psychopaths

in power were rather less concerned *because* they are psychopaths. Deletion of urgent care and diagnosis has been happening all over the world and how many kids and others have died as a result of the actions of these cold and heartless lunatics dictating 'health' policy? The number must be stratospheric. Richard Sullivan, professor of cancer and global health at King's College London, said people feared 'Covid' more than cancer such was the campaign of fear. 'Years of lost life will be quite dramatic', Sullivan said, with 'a huge amount of avoidable mortality'. Sarah Woolnough, executive director for policy at Cancer Research UK, said there had been a 75 percent drop in urgent referrals to hospitals by family doctors of people with suspected cancer. Sullivan said that 'a lot of services have had to scale back – we've seen a dramatic decrease in the amount of elective cancer surgery'. Lockdown deaths worldwide has been absolutely fantastic with the *New York Post* reporting how data confirmed that 'lockdowns end more lives than they save':

> There was a sharp decline in visits to emergency rooms and an increase in fatal heart attacks because patients didn't receive prompt treatment. Many fewer people were screened for cancer. Social isolation contributed to excess deaths from dementia and Alzheimer's.
>
> Researchers predicted that the social and economic upheaval would lead to tens of thousands of "deaths of despair" from drug overdoses, alcoholism and suicide. As unemployment surged and mental-health and substance-abuse treatment programs were interrupted, the reported levels of anxiety, depression and suicidal thoughts increased dramatically, as did alcohol sales and fatal drug overdoses.

This has been happening while nurses and other staff had so much time on their hands in the 'war-zones' that Tic-Tok dancing videos began appearing across the Internet with medical staff dancing around in empty wards and corridors as people died at home from causes that would normally have been treated in hospital.

Mentions in dispatches

One brave and truth-committed whistleblower was Louise Hampton, a call handler with the UK NHS who made a viral Internet video saying she had done 'fuck all' during the 'pandemic'

which was 'a load of bollocks'. She said that 'Covid-19' was rebranded flu and of course she lost her job. This is what happens in the medical and endless other professions now when you tell the truth. Louise filmed inside 'war-zone' accident and emergency departments to show they were empty and I mean *empty* as in no one there. The mainstream media could have done the same and blown the gaff on the whole conspiracy. They haven't to their eternal shame. Not that most 'journalists' seem capable of manifesting shame as with the psychopaths they slavishly repeat without question. The relative few who were admitted with serious health problems were left to die alone with no loved ones allowed to see them because of 'Covid' rules and they included kids dying without the comfort of mum and dad at their bedside while the evil behind this couldn't give a damn. It was all good fun to them. A Scottish NHS staff nurse publicly quit in the spring of 2021 saying: 'I can no longer be part of the lies and the corruption by the government.' She said hospitals 'aren't full, the beds aren't full, beds have been shut, wards have been shut'. Hospitals were never busy throughout 'Covid'. The staff nurse said that Nicola Sturgeon, tragically the leader of the Scottish government, was on television saying save the hospitals and the NHS – 'but the beds are empty' and 'we've not seen flu, we always see flu every year'. She wrote to government and spoke with her union Unison (the unions are Cult-compromised and *useless*, but nothing changed. Many of her colleagues were scared of losing their jobs if they spoke out as they wanted to. She said nursing staff were being affected by wearing masks all day and 'my head is splitting every shift from wearing a mask'. The NHS is part of the fascist tyranny and must be dismantled so we can start again with human beings in charge. (Ironically, hospitals were reported to be busier again when official 'Covid' cases *fell* in spring/summer of 2021 and many other conditions required treatment at the same time as *the fake vaccine rollout*.)

I will cover the 'Covid vaccine' scam in detail later, but it is another indicator of the sickening disregard for human life that I am highlighting here. The DNA-manipulating concoctions do not fulfil

the definition of a 'vaccine', have never been used on humans before and were given only emergency approval because trials were not completed and they continued using the unknowing public. The result was what a NHS senior nurse with responsibility for 'vaccine' procedure said was 'genocide'. She said the 'vaccines' were not 'vaccines'. They had not been shown to be safe and claims about their effectiveness by drug companies were 'poetic licence'. She described what was happening as a 'horrid act of human annihilation'. The nurse said that management had instigated a policy of not providing a Patient Information Leaflet (PIL) before people were 'vaccinated' even though health care professionals are supposed to do this according to protocol. Patients should also be told that they are taking part in an ongoing clinical trial. Her challenges to what is happening had seen her excluded from meetings and ridiculed in others. She said she was told to 'watch my step … or I would find myself surplus to requirements'. The nurse, who spoke anonymously in fear of her career, said she asked her NHS manager why he/she was content with taking part in genocide against those having the 'vaccines'. The reply was that everyone had to play their part and to 'put up, shut up, and get it done'. Government was 'leaning heavily' on NHS management which was clearly leaning heavily on staff. This is how the global 'medical' hierarchy operates and it starts with the Cult and its World Health Organization.

She told the story of a doctor who had the Pfizer jab and when questioned had no idea what was in it. The doctor had never read the literature. We have to stop treating doctors as intellectual giants when so many are moral and medical pygmies. The doctor did not even know that the 'vaccines' were not fully approved or that their trials were ongoing. They were, however, asking their patients if they minded taking part in follow-ups for research purposes – yes, the *ongoing clinical trial*. The nurse said the doctor's ignorance was not rare and she had spoken to a hospital consultant who had the jab without any idea of the background or that the 'trials' had not been completed. Nurses and pharmacists had shown the same ignorance.

'My NHS colleagues have forsaken their duty of care, broken their code of conduct – Hippocratic Oath – and have been brainwashed just the same as the majority of the UK public through propaganda …' She said she had not been able to recruit a single NHS colleague, doctor, nurse or pharmacist to stand with her and speak out. Her union had refused to help. She said that if the genocide came to light she would not hesitate to give evidence at a Nuremberg-type trial against those in power who could have affected the outcomes but didn't.

And all for what?

To put the nonsense into perspective let's say the 'virus' does exist and let's go completely crazy and accept that the official manipulated figures for cases and deaths are accurate. *Even then* a study by Stanford University epidemiologist Dr John Ioannidis published on the World Health Organization website produced an average infection to fatality rate of … *0.23 percent!* Ioannidis said: 'If one could sample equally from all locations globally, the median infection fatality rate might even be substantially lower than the 0.23% observed in my analysis.' For healthy people under 70 it was … *0.05 percent*! This compares with the 3.4 percent claimed by the Cult-owned World Health Organization when the hoax was first played and maximum fear needed to be generated. An updated Stanford study in April, 2021, put the 'infection' to 'fatality' rate at just 0.15 percent. Another team of scientists led by Megan O'Driscoll and Henrik Salje studied data from 45 countries and published their findings on the Nature website. For children and young people the figure is so small it virtually does not register although authorities will be hyping dangers to the young when they introduce DNA-manipulating 'vaccines' for children. The O'Driscoll study produced an average infection-fatality figure of 0.003 for children from birth to four; 0.001 for 5 to 14; 0.003 for 15 to 19; and it was still only 0.456 up to 64. To claim that children must be 'vaccinated' to protect them from 'Covid' is an obvious lie and so there must be another reason and there is. What's more the average age of a 'Covid' death is akin

to the average age that people die in general. The average age of death in England is about 80 for men and 83 for women. The average age of death from alleged 'Covid' is between 82 and 83. California doctors, Dan Erickson and Artin Massihi, said at their April media conference that projection models of millions of deaths had been 'woefully inaccurate'. They produced detailed figures showing that Californians had a 0.03 chance of dying from 'Covid' based on the number of people who tested positive (with a test not testing for the 'virus'). Erickson said there was a 0.1 percent chance of dying from 'Covid' in the *state* of New York, not just the city, and a 0.05 percent chance in Spain, a centre of 'Covid-19' hysteria at one stage. The Stanford studies supported the doctors' data with fatality rate estimates of 0.23 and 0.15 percent. How close are these figures to my estimate of *zero*? Death-rate figures claimed by the World Health Organization at the start of the hoax were some 15 times higher. The California doctors said there was no justification for lockdowns and the economic devastation they caused. Everything they had ever learned about quarantine was that you quarantine the *sick* and not the healthy. They had never seen this before and it made no medical sense.

Why in the in the light of all this would governments and medical systems the world over say that billions must go under house arrest; lose their livelihood; in many cases lose their mind, their health and their life; force people to wear masks dangerous to health and psychology; make human interaction and even family interaction a criminal offence; ban travel; close restaurants, bars, watching live sport, concerts, theatre, and any activity involving human togetherness and discourse; and closing schools to isolate children from their friends and cause many to commit suicide in acts of hopelessness and despair? The California doctors said lockdown consequences included increased child abuse, partner abuse, alcoholism, depression, and other impacts they were seeing every day. Who would do that to the entire human race if not mentally-ill psychopaths of almost unimaginable extremes like Bill Gates? We must face the reality of what we are dealing with and come out of

denial. Fascism and tyranny are made possible only by the target population submitting and acquiescing to fascism and tyranny. The whole of human history shows that to be true. Most people naively and unquestioning believed what they were told about a 'deadly virus' and meekly and weakly submitted to house arrest. Those who didn't believe it – at least in total – still submitted in fear of the consequences of not doing so. For the rest who wouldn't submit draconian fines have been imposed, brutal policing by psychopaths *for* psychopaths, and condemnation from the meek and weak who condemn the Pushbackers on behalf of the very force that has them, too, in its gunsights. 'Pathetic' does not even begin to suffice. Britain's brainless 'Health' Secretary Matt Hancock warned anyone lying to border officials about returning from a list of 'hotspot' countries could face a jail sentence of up to ten years which is more than for racially-aggravated assault, incest and attempting to have sex with a child under 13. Hancock is a lunatic, but he has the state apparatus behind him in a Cult-led chain reaction and the same with UK 'Vaccine Minister' Nadhim Zahawi, a prominent member of the mega-Cult secret society, Le Cercle, which featured in my earlier books. The Cult enforces its will on governments and medical systems; government and medical systems enforce their will on business and police; business enforces its will on staff who enforce it on customers; police enforce the will of the Cult on the population and play their essential part in creating a world of fascist control that their own children and grandchildren will have to live in their entire lives. It is a hierarchical pyramid of imposition and acquiescence and, yes indeedy, of clinical insanity.

Does anyone bright enough to read this book have to ask what the answer is? I think not, but I will reveal it anyway in the fewest of syllables: Tell the psychos and their moronic lackeys to fuck off and let's get on with our lives. We are many – They are few.

CHAPTER SEVEN

War on your mind

One believes things because one has been conditioned to believe them
Aldous Huxley, *Brave New World*

I have described the 'Covid' hoax as a 'Psyop' and that is true in every sense and on every level in accordance with the definition of that term which is psychological warfare. Break down the 'Covid pandemic' to the foundation themes and it is psychological warfare on the human individual and collective mind.

The same can be said for the entire human belief system involving every subject you can imagine. Huxley was right in his contention that people believe what they are conditioned to believe and this comes from the repetition throughout their lives of the same falsehoods. They spew from government, corporations, media and endless streams of 'experts' telling you what the Cult wants you to believe and often believing it themselves (although *far* from always). 'Experts' are rewarded with 'prestigious' jobs and titles and as agents of perceptual programming with regular access to the media. The Cult has to control the narrative – control *information* – or they lose control of the vital, crucial, without-which-they-cannot-prevail public perception of reality. The foundation of that control today is the Internet made possible by the Defense Advanced Research Projects Agency (DARPA), the incredibly sinister technological arm of the Pentagon. The Internet is the result of military technology.

DARPA openly brags about establishing the Internet which has been a long-term project to lasso the minds of the global population. I have said for decades the plan is to control information to such an extreme that eventually no one would see or hear anything that the Cult does not approve. We are closing in on that end with ferocious censorship since the 'Covid' hoax began and in my case it started back in the 1990s in terms of books and speaking venues. I had to create my own publishing company in 1995 precisely because no one else would publish my books even then. I think they're all still running.

Cult Internet

To secure total control of information they needed the Internet in which pre-programmed algorithms can seek out 'unclean' content for deletion and even stop it being posted in the first place. The Cult had to dismantle print and non-Internet broadcast media to ensure the transfer of information to the appropriate-named 'Web' – a critical expression of the *Cult* web. We've seen the ever-quickening demise of traditional media and control of what is left by a tiny number of corporations operating worldwide. Independent journalism in the mainstream is already dead and never was that more obvious than since the turn of 2020. The Cult wants all information communicated via the Internet to globally censor and allow the plug to be pulled any time. Lockdowns and forced isolation has meant that communication between people has been through electronic means and no longer through face-to-face discourse and discussion. Cult psychopaths have targeted the bars, restaurants, sport, venues and meeting places in general for this reason. None of this is by chance and it's to stop people gathering in any kind of privacy or number while being able to track and monitor all Internet communications and block them as necessary. Even private messages between individuals have been censored by these fascists that control Cult fronts like Facebook, Twitter, Google and YouTube which are all officially run by Sabbatian place-people and from the background by higher-level Sabbatian place people.

Facebook, Google, Amazon and their like were seed-funded and supported into existence with money-no-object infusions of funds either directly or indirectly from DARPA and CIA technology arm In-Q-Tel. The Cult plays the long game and prepares very carefully for big plays like 'Covid'. Amazon is another front in the psychological war and pretty much controls the global market in book sales and increasingly publishing. Amazon's limitless funds have deleted fantastic numbers of independent publishers to seize global domination on the way to deciding which books can be sold and circulated and which cannot. Moves in that direction are already happening. Amazon's leading light Jeff Bezos is the grandson of Lawrence Preston Gise who worked with DARPA predecessor ARPA. Amazon has big connections to the CIA and the Pentagon. The plan I have long described went like this:

1. Employ military technology to establish the Internet.

2. Sell the Internet as a place where people can freely communicate without censorship and allow that to happen until the Net becomes the central and irreversible pillar of human society. If the Internet had been highly censored from the start many would have rejected it.

3. Fund and manipulate major corporations into being to control the circulation of information on your Internet using cover stories about geeks in garages to explain how they came about. Give them unlimited funds to expand rapidly with no need to make a profit for years while non-Cult companies who need to balance the books cannot compete. You know that in these circumstances your Googles, YouTubes, Facebooks and Amazons are going to secure near monopolies by either crushing or buying up the opposition.

4. Allow freedom of expression on both the Internet and communication platforms to draw people in until the Internet is the central and irreversible pillar of human society and your communication corporations have reached a stage of near monopoly domination.

5. Then unleash your always-planned frenzy of censorship on the basis of 'where else are you going to go?' and continue to expand that until nothing remains that the Cult does not want its human targets to see.

The process was timed to hit the 'Covid' hoax to ensure the best chance possible of controlling the narrative which they knew they had to do at all costs. They were, after all, about to unleash a 'deadly virus' that didn't really exist. If you do that in an environment of free-flowing information and opinion you would be dead in the

water before you could say Gates is a psychopath. The network was in place through which the Cult-created-and-owned World Health Organization could dictate the 'Covid' narrative and response policy slavishly supported by Cult-owned Internet communication giants and mainstream media while those telling a different story were censored. Google, YouTube, Facebook and Twitter openly announced that they would do this. What else would we expect from Cult-owned operations like Facebook which former executives have confirmed set out to make the platform more addictive than cigarettes and coldly manipulates emotions of its users to sow division between people and groups and scramble the minds of the young? If Zuckerberg lives out the rest of his life without going to jail for crimes against humanity, and most emphatically against the young, it will be a travesty of justice. Still, no matter, cause and effect will catch up with him eventually and the same with Sergey Brin and Larry Page at Google with its CEO Sundar Pichai who fix the Google search results to promote Cult narratives and hide the opposition. Put the same key words into Google and other search engines like DuckDuckGo and you will see how different results can be. Wikipedia is another intensely biased 'encyclopaedia' which skews its content to the Cult agenda. YouTube links to Wikipedia's version of 'Covid' and 'climate change' on video pages in which experts in their field offer a different opinion (even that is increasingly rare with Wojcicki censorship). Into this 'Covid' silence-them network must be added government media censors, sorry 'regulators', such as Ofcom in the UK which imposed tyrannical restrictions on British broadcasters that had the effect of banning me from ever appearing. Just to debate with me about my evidence and views on 'Covid' would mean breaking the fascistic impositions of Ofcom and its CEO career government bureaucrat Melanie Dawes. Gutless British broadcasters tremble at the very thought of fascist Ofcom.

Psychos behind 'Covid'

The reason for the 'Covid' catastrophe in all its facets and forms can be seen by whom and what is driving the policies worldwide in such a coordinated way. Decisions are not being made to protect health, but to target psychology. The dominant group guiding and 'advising' government policy are not medical professionals. They are psychologists and behavioural scientists. Every major country has its own version of this phenomenon and I'll use the British example to show how it works. In many ways the British version has been affecting the wider world in the form of the huge behaviour manipulation network in the UK which operates in other countries. The network involves private companies, government, intelligence and military. The Cabinet Office is at the centre of the government 'Covid' Psyop and part-owns, with 'innovation charity' Nesta, the Behavioural Insights Team (BIT) which claims to be independent of government but patently isn't. The BIT was established in 2010 and its job is to manipulate the psyche of the population to acquiesce to government demands and so much more. It is also known as the 'Nudge Unit', a name inspired by the 2009 book by two ultra-Zionists, Cass Sunstein and Richard Thaler, called *Nudge: Improving Decisions About Health, Wealth, and Happiness*. The book, as with the Behavioural Insights Team, seeks to 'nudge' behaviour (manipulate it) to make the public follow patterns of action and perception that suit those in authority (the Cult). Sunstein is so skilled at this that he advises the World Health Organization and the UK Behavioural Insights Team and was Administrator of the White House Office of Information and Regulatory Affairs in the Obama administration. Biden appointed him to the Department of Homeland Security – another ultra-Zionist in the fold to oversee new immigration laws which is another policy the Cult wants to control. Sunstein is desperate to silence anyone exposing conspiracies and co-authored a 2008 report on the subject in which suggestions were offered to ban 'conspiracy theorizing' or impose 'some kind of tax, financial or otherwise, on those who disseminate such theories'. I guess a psychiatrist's chair is out of the question?

Sunstein's mate Richard Thaler, an 'academic affiliate' of the UK Behavioural Insights Team, is a proponent of 'behavioural economics' which is defined as the study of 'the effects of psychological, cognitive, emotional, cultural and social factors on the decisions of individuals and institutions'. Study the effects so they can be manipulated to be what you want them to be. Other leading names in the development of behavioural economics are ultra-Zionists Daniel Kahneman and Robert J. Shiller and they, with Thaler, won the Nobel Memorial Prize in Economic Sciences for their work in this field. The Behavioural Insights Team is operating at the heart of the UK government and has expanded globally through partnerships with several universities including Harvard, Oxford, Cambridge, University College London (UCL) and Pennsylvania. They claim to have 'trained' (reframed) 20,000 civil servants and run more than 750 projects involving 400 randomised controlled trials in dozens of countries' as another version of mind reframers Common Purpose. BIT works from its office in New York with cities and their agencies, as well as other partners, across the United States and Canada – this is a company part-owned by the British government Cabinet Office. An executive order by President Cult-servant Obama established a US Social and Behavioral Sciences Team in 2015. They all have the same reason for being and that's to brainwash the population directly and by brainwashing those in positions of authority.

'Covid' mind game

Another prime aspect of the UK mind-control network is the 'independent' [joke] Scientific Pandemic Insights Group on Behaviours (SPI-B) which 'provides behavioural science advice aimed at anticipating and helping people adhere to interventions that are recommended by medical or epidemiological experts'. That means manipulating public perception and behaviour to do whatever government tells them to do. It's disgusting and if they really want the public to be 'safe' this lot should all be under lock and key. According to the government website SPI-B consists of

'behavioural scientists, health and social psychologists, anthropologists and historians' and advises the Whitty-Vallance-led Scientific Advisory Group for Emergencies (SAGE) which in turn advises the government on 'the science' (it doesn't) and 'Covid' policy. When politicians say they are being guided by 'the science' this is the rabble in each country they are talking about and that 'science' is dominated by behaviour manipulators to enforce government fascism through public compliance. The Behaviour Insight Team is headed by psychologist David Solomon Halpern, a visiting professor at King's College London, and connects with a national and global web of other civilian and military organisations as the Cult moves towards its goal of fusing them into one fascistic whole in every country through its 'Fusion Doctrine'. The behaviour manipulation network involves, but is not confined to, the Foreign Office; National Security Council; government communications headquarters (GCHQ); MI5; MI6; the Cabinet Office-based Media Monitoring Unit; and the Rapid Response Unit which 'monitors digital trends to spot emerging issues; including misinformation and disinformation; and identifies the best way to respond'.

There is also the 77th Brigade of the UK military which operates like the notorious Israeli military's Unit 8200 in manipulating information and discussion on the Internet by posing as members of the public to promote the narrative and discredit those who challenge it. Here we have the military seeking to manipulate *domestic* public opinion while the Nazis in government are fine with that. Conservative Member of Parliament Tobias Ellwood, an advocate of lockdown and control through 'vaccine passports', is a Lieutenant Colonel reservist in the 77th Brigade which connects with the military operation jHub, the 'innovation centre' for the Ministry of Defence and Strategic Command. jHub has also been involved with the civilian National Health Service (NHS) in 'symptom tracing' the population. The NHS is a key part of this mind control network and produced a document in December, 2020, explaining to staff how to use psychological manipulation with different groups and ages to get them to have the DNA-manipulating 'Covid vaccine'

that's designed to cumulatively rewrite human genetics. The document, called 'Optimising Vaccination Roll Out – Do's and Dont's for all messaging, documents and "communications" in the widest sense', was published by NHS England and the NHS Improvement *Behaviour Change Unit* in partnership with Public Health England and Warwick Business School. I hear the mantra about 'save the NHS' and 'protect the NHS' when we need to scrap the NHS and start again. The current version is far too corrupt, far too anti-human and totally compromised by Cult operatives and their assets. UK government broadcast media censor Ofcom will connect into this web – as will the BBC with its tremendous Ofcom influence – to control what the public see and hear and dictate mass perception. Nuremberg trials must include personnel from all these organisations.

The fear factor

The 'Covid' hoax has led to the creation of the UK Cabinet Office-connected Joint Biosecurity Centre (JBC) which is officially described as providing 'expert advice on pandemics' using its independent [all Cult operations are 'independent'] analytical function to provide real-time analysis about infection outbreaks to identify and respond to outbreaks of Covid-19'. Another role is to advise the government on a response to spikes in infections – 'for example by closing schools or workplaces in local areas where infection levels have risen'. Put another way, promoting the Cult agenda. The Joint Biosecurity Centre is modelled on the Joint Terrorism Analysis Centre which analyses intelligence to set 'terrorism threat levels' and here again you see the fusion of civilian and military operations and intelligence that has led to military intelligence producing documents about 'vaccine hesitancy' and how it can be combated. Domestic civilian matters and opinions should not be the business of the military. The Joint Biosecurity Centre is headed by Tom Hurd, director general of the Office for Security and Counter-Terrorism from the establishment-to-its-fingertips Hurd family. His father is former Foreign Secretary Douglas Hurd. How coincidental that Tom

Hurd went to the elite Eton College and Oxford University with Boris Johnson. Imperial College with its ridiculous computer modeller Neil Ferguson will connect with this gigantic web that will itself interconnect with similar set-ups in other major and not so major countries. Compared with this Cult network the politicians, be they Boris Johnson, Donald Trump or Joe Biden, are bit-part players 'following the science'. The network of psychologists was on the 'Covid' case from the start with the aim of generating maximum fear of the 'virus' to ensure compliance by the population. A government behavioural science group known as SPI-B produced a paper in March, 2020, for discussion by the main government science advisory group known as SAGE. It was headed 'Options for increasing adherence to social distancing measures' and it said the following in a section headed 'Persuasion':

- A substantial number of people still do not feel sufficiently personally threatened; it could be that they are reassured by the low death rate in their demographic group, although levels of concern may be rising. Having a good understanding of the risk has been found to be positively associated with adoption of COVID-19 social distancing measures in Hong Kong.

- The perceived level of personal threat needs to be increased among those who are complacent, using hard-hitting evaluation of options for increasing social distancing emotional messaging. To be effective this must also empower people by making clear the actions they can take to reduce the threat.

- Responsibility to others: There seems to be insufficient understanding of, or feelings of responsibility about, people's role in transmitting the infection to others … Messaging about actions need to be framed positively in terms of protecting oneself and the community, and increase confidence that they will be effective.

- Some people will be more persuaded by appeals to play by the rules, some by duty to the community, and some to personal risk.

> All these different approaches are needed. The messaging also needs to take account of the realities of different people's lives. Messaging needs to take account of the different motivational levers and circumstances of different people.

All this could be achieved the SPI-B psychologists said by *using the media to increase the sense of personal threat* which translates as terrify the shit out of the population, including children, so they all do what we want. That's not happened has it? Those excuses for 'journalists' who wouldn't know journalism if it bit them on the arse (the great majority) have played their crucial part in serving this Cult-government Psyop to enslave their own kids and grandkids. How they live with themselves I have no idea. The psychological war has been underpinned by constant government 'Covid' propaganda in almost every television and radio ad break, plus the Internet and print media, which has pounded out the fear with taxpayers footing the bill for their own programming. The result has been people terrified of a 'virus' that doesn't exist or one with a tiny fatality rate even if you believe it does. People walk down the street and around the shops wearing face-nappies damaging their health and psychology while others report those who refuse to be that naïve to the police who turn up in their own face-nappies. I had a cameraman come to my flat and he was so frightened of 'Covid' he came in wearing a mask and refused to shake my hand in case he caught something. He had – naïveitis – and the thought that he worked in the mainstream media was both depressing and made his behaviour perfectly explainable. The fear which has gripped the minds of so many and frozen them into compliance has been carefully cultivated by these psychologists who are really psychopaths. If lives get destroyed and a lot of young people commit suicide it shows our plan is working. SPI-B then turned to compulsion on the public to comply. 'With adequate preparation, rapid change can be achieved', it said. Some countries had introduced mandatory self-isolation on a wide scale without evidence of major public unrest and a large majority of the UK's population appeared to be supportive of more coercive measures with 64 percent of adults saying they would

support putting London under a lockdown (watch the 'polls' which are designed to make people believe that public opinion is in favour or against whatever the subject in hand).

For 'aggressive protective measures' to be effective, the SPI-B paper said, special attention should be devoted to those population groups that are more at risk. Translated from the Orwellian this means making the rest of population feel guilty for not protecting the 'vulnerable' such as old people which the Cult and its agencies were about to kill on an industrial scale with lockdown, lack of treatment and the Gates 'vaccine'. Psychopath psychologists sold their guilt-trip so comprehensively that Los Angeles County Supervisor Hilda Solis reported that children were apologising (from a distance) to their parents and grandparents for bringing 'Covid' into their homes and getting them sick. '… These apologies are just some of the last words that loved ones will ever hear as they die alone,' she said. Gut-wrenchingly Solis then used this childhood tragedy to tell children to stay at home and 'keep your loved ones alive'. Imagine heaping such potentially life-long guilt on a kid when it has absolutely nothing to do with them. These people are deeply disturbed and the psychologists behind this even more so.

Uncivil war – divide and rule

Professional mind-controllers at SPI-B wanted the media to increase a sense of responsibility to others (do as you're told) and promote 'positive messaging' for those actions while in contrast to invoke 'social disapproval' by the unquestioning, obedient, community of anyone with a mind of their own. Again the compliant Goebbels-like media obliged. This is an old, old, trick employed by tyrannies the world over throughout human history. You get the target population to keep the target population in line – *your* line. SPI-B said this could 'play an important role in preventing anti-social behaviour or discouraging failure to enact pro-social behaviour'. For 'anti-social' in the Orwellian parlance of SPI-B see any behaviour that government doesn't approve. SPI-B recommendations said that 'social disapproval' should be accompanied by clear messaging and

promotion of strong collective identity – hence the government and celebrity mantra of 'we're all in this together'. Sure we are. The mind doctors have such contempt for their targets that they think some clueless comedian, actor or singer telling them to do what the government wants will be enough to win them over. We have had UK comedian Lenny Henry, actor Michael Caine and singer Elton John wheeled out to serve the propagandists by urging people to have the DNA-manipulating 'Covid' non-'vaccine'. The role of Henry and fellow black celebrities in seeking to coax a 'vaccine' reluctant black community into doing the government's will was especially stomach-turning. An emotion-manipulating script and carefully edited video featuring these black 'celebs' was such an insult to the intelligence of black people and where's the self-respect of those involved selling their souls to a fascist government agenda? Henry said he heard black people's 'legitimate worries and concerns', but people must 'trust the facts' when they were doing exactly that by not having the 'vaccine'. They had to include the obligatory reference to Black Lives Matter with the line … 'Don't let coronavirus cost even more black lives – because we matter'. My god, it was pathetic. 'I know the vaccine is safe and what it does.' How? 'I'm a comedian and it says so in my script.'

SPI-B said social disapproval needed to be carefully managed to avoid victimisation, scapegoating and misdirected criticism, but they knew that their 'recommendations' would lead to exactly that and the media were specifically used to stir-up the divide-and-conquer hostility. Those who conform like good little baa, baas, are praised while those who have seen through the tidal wave of lies are 'Covidiots'. The awake have been abused by the fast asleep for not conforming to fascism and impositions that the awake know are designed to endanger their health, dehumanise them, and tear asunder the very fabric of human society. We have had the curtain-twitchers and morons reporting neighbours and others to the face-nappied police for breaking 'Covid rules' with fascist police delighting in posting links and phone numbers where this could be done. The Cult cannot impose its will without a compliant police

and military or a compliant population willing to play their part in enslaving themselves and their kids. The words of a pastor in Nazi Germany are so appropriate today:

First they came for the socialists and I did not speak out because I was not a socialist.

Then they came for the trade unionists and I did not speak out because I was not a trade unionist.

Then they came for the Jews and I did not speak out because I was not a Jew.

Then they came for me and there was no one left to speak for me.

Those who don't learn from history are destined to repeat it and so many are.

'Covid' rules: Rewiring the mind

With the background laid out to this gigantic national and global web of psychological manipulation we can put 'Covid' rules into a clear and sinister perspective. Forget the claims about protecting health. 'Covid' rules are about dismantling the human mind, breaking the human spirit, destroying self-respect, and then putting Humpty Dumpty together again as a servile, submissive slave. Social isolation through lockdown and distancing have devastating effects on the human psyche as the psychological psychopaths well know and that's the real reason for them. Humans need contact with each other, discourse, closeness and touch, or they eventually, and literarily, go crazy. Masks, which I will address at some length, fundamentally add to the effects of isolation and the Cult agenda to dehumanise and de-individualise the population. To do this while knowing – in fact *seeking* – this outcome is the very epitome of evil and psychologists involved in this *are* the epitome of evil. They must like all the rest of the Cult demons and their assets stand trial for crimes against humanity on a scale that defies the imagination. Psychopaths in uniform use isolation to break enemy troops and agents and make them subservient and submissive to tell what they know. The technique is rightly considered a form of torture and

torture is most certainly what has been imposed on the human population.

Clinically-insane American psychologist Harry Harlow became famous for his isolation experiments in the 1950s in which he separated baby monkeys from their mothers and imprisoned them for months on end in a metal container or 'pit of despair'. They soon began to show mental distress and depression as any idiot could have predicted. Harlow put other monkeys in steel chambers for three, six or twelve months while denying them any contact with animals or humans. He said that the effects of total social isolation for six months were 'so devastating and debilitating that we had assumed initially that twelve months of isolation would not produce any additional decrement'; but twelve months of isolation 'almost obliterated the animals socially'. This is what the Cult and its psychopaths are doing to you and your children. Even monkeys in partial isolation in which they were not allowed to form relationships with other monkeys became 'aggressive and hostile, not only to others, but also towards their own bodies'. We have seen this in the young as a consequence of lockdown. UK government psychopaths launched a public relations campaign telling people not to hug each other even after they received the 'Covid-19 vaccine' which we were told with more lies would allow a return to 'normal life'. A government source told *The Telegraph*: 'It will be along the lines that it is great that you have been vaccinated, but if you are going to visit your family and hug your grandchildren there is a chance you are going to infect people you love.' The source was apparently speaking from a secure psychiatric facility. Janet Lord, director of Birmingham University's Institute of Inflammation and Ageing, said that parents and grandparents should avoid hugging their children. Well, how can I put it, Ms Lord? Fuck off. Yep, that'll do.

Destroying the kids – where are the parents?

Observe what has happened to people enslaved and isolated by lockdown as suicide and self-harm has soared worldwide,

particularly among the young denied the freedom to associate with their friends. A study of 49,000 people in English-speaking countries concluded that almost half of young adults are at clinical risk of mental health disorders. A national survey in America of 1,000 currently enrolled high school and college students found that 5 percent reported attempting suicide during the pandemic. Data from the US CDC's National Syndromic Surveillance Program from January 1st to October 17th, 2020, revealed a *31 percent* increase in mental health issues among adolescents aged 12 to 17 compared with 2019. The CDC reported that America in general suffered the biggest drop in life expectancy since World War Two as it fell by a year in the first half of 2020 as a result of 'deaths of despair' – overdoses and suicides. Deaths of despair have leapt by more than 20 percent during lockdown and include the highest number of fatal overdoses ever recorded in a single year – 81,000. Internet addiction is another consequence of being isolated at home which lowers interest in physical activities as kids fall into inertia and what's the point? Children and young people are losing hope and giving up on life, sometimes literally. A 14-year-old boy killed himself in Maryland because he had 'given up' when his school district didn't reopen; an 11-year-old boy shot himself during a zoom class; a teenager in Maine succumbed to the isolation of the 'pandemic' when he ended his life after experiencing a disrupted senior year at school. Children as young as nine have taken their life and all these stories can be repeated around the world. Careers are being destroyed before they start and that includes those in sport in which promising youngsters have not been able to take part. The plan of the psycho-psychologists is working all right. Researchers at Cambridge University found that lockdowns cause significant harm to children's mental health. Their study was published in the *Archives of Disease in Childhood*, and followed 168 children aged between 7 and 11. The researchers concluded:

> During the UK lockdown, children's depression symptoms have increased substantially, relative to before lockdown. The scale of this effect has direct relevance for the continuation of different elements of lockdown policy, such as complete or partial school closures …

… Specifically, we observed a statistically significant increase in ratings of depression, with a medium-to-large effect size. Our findings emphasise the need to incorporate the potential impact of lockdown on child mental health in planning the ongoing response to the global pandemic and the recovery from it.

Not a chance when the Cult's psycho-psychologists were getting exactly what they wanted. The UK's Royal College of Paediatrics and Child Health has urged parents to look for signs of eating disorders in children and young people after a three to four fold increase. Specialists say the 'pandemic' is a major reason behind the rise. You don't say. The College said isolation from friends during school closures, exam cancellations, loss of extra-curricular activities like sport, and an increased use of social media were all contributory factors along with fears about the virus (psycho-psychologists again), family finances, and students being forced to quarantine. Doctors said young people were becoming severely ill by the time they were seen with 'Covid' regulations reducing face-to-face consultations. Nor is it only the young that have been devastated by the psychopaths. Like all bullies and cowards the Cult is targeting the young, elderly, weak and infirm. A typical story was told by a British lady called Lynn Parker who was not allowed to visit her husband in 2020 for the last ten and half months of his life 'when he needed me most' between March 20th and when he died on December 19th. This vacates the criminal and enters the territory of evil. The emotional impact on the immune system alone is immense as are the number of people of all ages worldwide who have died as a result of Cult-demanded, Gates-demanded, lockdowns.

Isolation is torture

The experience of imposing solitary confinement on millions of prisoners around the world has shown how a large percentage become 'actively psychotic and/or acutely suicidal'. Social isolation has been found to trigger 'a specific psychiatric syndrome, characterized by hallucinations; panic attacks; overt paranoia; diminished impulse control; hypersensitivity to external stimuli; and difficulties with thinking, concentration and memory'. Juan Mendez,

a United Nations rapporteur (investigator), said that isolation is a form of torture. Research has shown that even after isolation prisoners find it far more difficult to make social connections and I remember chatting to a shop assistant after one lockdown who told me that when her young son met another child again he had no idea how to act or what to do. Hannah Flanagan, Director of Emergency Services at Journey Mental Health Center in Dane County, Wisconsin, said: 'The specificity about Covid social distancing and isolation that we've come across as contributing factors to the suicides are really new to us this year.' But they are not new to those that devised them. They are getting the effect they want as the population is psychologically dismantled to be rebuilt in a totally different way. Children and the young are particularly targeted. They will be the adults when the full-on fascist AI-controlled technocracy is planned to be imposed and they are being prepared to meekly submit. At the same time older people who still have a memory of what life was like before – and how fascist the new normal really is – are being deleted. You are going to see efforts to turn the young against the old to support this geriatric genocide. Hannah Flanagan said the big increase in suicide in her county proved that social isolation is not only harmful, but deadly. Studies have shown that isolation from others is one of the main risk factors in suicide and even more so with women. Warnings that lockdown could create a 'perfect storm' for suicide were ignored. After all this was one of the *reasons* for lockdown. Suicide, however, is only the most extreme of isolation consequences. There are many others. Dr Dhruv Khullar, assistant professor of healthcare policy at Weill Cornell Medical College, said in a *New York Times* article in 2016 long before the fake 'pandemic':

> A wave of new research suggests social separation is bad for us. Individuals with less social connection have disrupted sleep patterns, altered immune systems, more inflammation and higher levels of stress hormones. One recent study found that isolation increases the risk of heart disease by 29 percent and stroke by 32 percent. Another analysis that pooled data from 70 studies and 3.4 million people found that socially isolated individuals had a 30 percent higher risk of dying in the next seven years, and that this effect was largest in middle age.

Loneliness can accelerate cognitive decline in older adults, and isolated individuals are twice as likely to die prematurely as those with more robust social interactions. These effects start early: Socially isolated children have significantly poorer health 20 years later, even after controlling for other factors. All told, loneliness is as important a risk factor for early death as obesity and smoking.

There you have proof from that one article alone four years before 2020 that those who have enforced lockdown, social distancing and isolation knew what the effect would be and that is even more so with professional psychologists that have been driving the policy across the globe. We can go back even further to the years 2000 and 2003 and the start of a major study on the effects of isolation on health by Dr Janine Gronewold and Professor Dirk M. Hermann at the University Hospital in Essen, Germany, who analysed data on 4,316 people with an average age of 59 who were recruited for the long-term research project. They found that socially isolated people are more than 40 percent more likely to have a heart attack, stroke, or other major cardiovascular event and nearly 50 percent more likely to die from any cause. Given the financial Armageddon unleashed by lockdown we should note that the study found a relationship between increased cardiovascular risk and lack of financial support. After excluding other factors social isolation was still connected to a 44 percent increased risk of cardiovascular problems and a 47 percent increased risk of death by any cause. Lack of financial support was associated with a 30 percent increase in the risk of cardiovascular health events. Dr Gronewold said it had been known for some time that feeling lonely or lacking contact with close friends and family can have an impact on physical health and the study had shown that having strong social relationships is of high importance for heart health. Gronewold said they didn't understand yet why people who are socially isolated have such poor health outcomes, but this was obviously a worrying finding, particularly during these times of prolonged social distancing. Well, it can be explained on many levels. You only have to identify the point in the body where people feel loneliness and missing people they are parted from – it's in the centre of the chest where they feel the ache of loneliness and the ache of missing people. 'My heart aches for

you' … 'My heart aches for some company.' I will explain this more in the chapter Escaping Wetiko, but when you realise that the body is the mind – they are expressions of each other – the reason why state of the mind dictates state of the body becomes clear.

American psychologist Ranjit Powar was highlighting the effects of lockdown isolation as early as April, 2020. She said humans have evolved to be social creatures and are wired to live in interactive groups. Being isolated from family, friends and colleagues could be unbalancing and traumatic for most people and could result in short or even long-term psychological and physical health problems. An increase in levels of anxiety, aggression, depression, forgetfulness and hallucinations were possible psychological effects of isolation. 'Mental conditions may be precipitated for those with underlying pre-existing susceptibilities and show up in many others without any pre-condition.' Powar said personal relationships helped us cope with stress and if we lost this outlet for letting off steam the result can be a big emotional void which, for an average person, was difficult to deal with. 'Just a few days of isolation can cause increased levels of anxiety and depression' – so what the hell has been the effect on the global population of *18 months* of this at the time of writing? Powar said: 'Add to it the looming threat of a dreadful disease being repeatedly hammered in through the media and you have a recipe for many shades of mental and physical distress.' For those with a house and a garden it is easy to forget that billions have had to endure lockdown isolation in tiny overcrowded flats and apartments with nowhere to go outside. The psychological and physical consequences of this are unimaginable and with lunatic and abusive partners and parents the consequences have led to tremendous increases in domestic and child abuse and alcoholism as people seek to shut out the horror. Ranjit Powar said:

Staying in a confined space with family is not all a rosy picture for everyone. It can be extremely oppressive and claustrophobic for large low-income families huddled together in small single-room houses. Children here are not lucky enough to have many board/electronic games or books to keep them occupied.

Add to it the deep insecurity of running out of funds for food and basic necessities. On the other hand, there are people with dysfunctional family dynamics, such as domineering, abusive or alcoholic partners, siblings or parents which makes staying home a period of trial. Incidence of suicide and physical abuse against women has shown a worldwide increase. Heightened anxiety and depression also affect a person's immune system, making them more susceptible to illness.

To think that Powar's article was published on April 11th, 2020.

Six-feet fantasy

Social (unsocial) distancing demanded that people stay six feet or two metres apart. UK government advisor Robert Dingwall from the New and Emerging Respiratory Virus Threats Advisory Group said in a radio interview that the two-metre rule was 'conjured up out of nowhere' and was not based on science. No, it was not based on *medical* science, but it didn't come out of nowhere. The distance related to *psychological* science. Six feet/two metres was adopted in many countries and we were told by people like the criminal Anthony Fauci and his ilk that it was founded on science. Many schools could not reopen because they did not have the space for six-feet distancing. Then in March, 2021, after a year of six-feet 'science', a study published in the *Journal of Infectious Diseases* involving more than 500,000 students and almost 100,000 staff over 16 weeks revealed no significant difference in 'Covid' cases between six feet and three feet and Fauci changed his tune. Now three feet was okay. There is no difference between six feet and three *inches* when there is no 'virus' and they got away with six feet for psychological reasons for as long as they could. I hear journalists and others talk about 'unintended consequences' of lockdown. They are not *unintended* at all; they have been coldly-calculated for a specific outcome of human control and that's why super-psychopaths like Gates have called for them so vehemently. Super-psychopath psychologists have demanded them and psychopathic or clueless, spineless, politicians have gone along with them by 'following the science'. But it's not science at all. 'Science' is not what is; it's only what people can be manipulated to believe it is. The whole 'Covid' catastrophe is

founded on mind control. Three word or three statement mantras issued by the UK government are a well-known mind control technique and so we've had 'Stay home/protect the NHS/save lives', 'Stay alert/control the virus/save lives' and 'hands/face/space'. One of the most vocal proponents of extreme 'Covid' rules in the UK has been Professor Susan Michie, a member of the British Communist Party, who is not a medical professional. Michie is the director of the Centre for Behaviour Change at University College London. She is a *behavioural psychologist* and another filthy rich 'Marxist' who praised China's draconian lockdown. She was known by fellow students at Oxford University as 'Stalin's nanny' for her extreme Marxism. Michie is an influential member of the UK government's Scientific Advisory Group for Emergencies (SAGE) and behavioural manipulation groups which have dominated 'Covid' policy. She is a consultant adviser to the World Health Organization on 'Covid-19' and behaviour. Why the hell are lockdowns anything to do with her when they are claimed to be about health? Why does a behavioural psychologist from a group charged with changing the behaviour of the public want lockdown, human isolation and mandatory masks? Does that question really need an answer? Michie *absolutely* has to explain herself before a Nuremberg court when humanity takes back its world again and even more so when you see the consequences of masks that she demands are compulsory. This is a Michie classic:

> The benefits of getting primary school children to wear masks is that regardless of what little degree of transmission is occurring in those age groups it could help normalise the practice. Young children wearing masks may be more likely to get their families to accept masks.

Those words alone should carry a prison sentence when you ponder on the callous disregard for children involved and what a statement it makes about the mind and motivations of Susan Michie. What a lovely lady and what she said there encapsulates the mentality of the psychopaths behind the 'Covid' horror. Let us compare what Michie said with a countrywide study in Germany published at researchsquare.com involving 25,000 school children and 17,854 health complaints submitted by parents. Researchers

found that masks are harming children physically, psychologically, and behaviourally with 24 health issues associated with mask wearing. They include: shortness of breath (29.7%); dizziness (26.4%); increased headaches (53%); difficulty concentrating (50%); drowsiness or fatigue (37%); and malaise (42%). Nearly a third of children experienced more sleep issues than before and a quarter developed new fears. Researchers found health issues and other impairments in 68 percent of masked children covering their faces for an average of 4.5 hours a day. Hundreds of those taking part experienced accelerated respiration, tightness in the chest, weakness, and short-term impairment of consciousness. A reminder of what Michie said again:

The benefits of getting primary school children to wear masks is that regardless of what little degree of transmission is occurring in those age groups it could help normalise the practice. Young children wearing masks may be more likely to get their families to accept masks.

Psychopaths in government and psychology now have children and young people – plus all the adults – wearing masks for hours on end while clueless teachers impose the will of the psychopaths on the young they should be protecting. What the hell are parents doing?

Cult lab rats

We have some schools already imposing on students microchipped buzzers that activate when they get 'too close' to their pals in the way they do with lab rats. How apt. To the Cult and its brain-dead servants our children *are* lab rats being conditioned to be unquestioning, dehumanised slaves for the rest of their lives. Children and young people are being weaned and frightened away from the most natural human instincts including closeness and touch. I have tracked in the books over the years how schools were banning pupils from greeting each other with a hug and the whole Cult-induced Me Too movement has terrified men and boys from a relaxed and natural interaction with female friends and work colleagues to the point where many men try never to be in a room

alone with a woman that's not their partner. Airhead celebrities have as always played their virtue-signalling part in making this happen with their gross exaggeration. For every monster like Harvey Weinstein there are at least tens of thousands of men that don't treat women like that; but everyone must be branded the same and policy changed for them as well as the monster. I am going to be using the word 'dehumanise' many times in this chapter because that is what the Cult is seeking to do and it goes very deep as we shall see. Don't let them kid you that social distancing is planned to end one day. That's not the idea. We are seeing more governments and companies funding and producing wearable gadgets to keep people apart and they would not be doing that if this was meant to be short-term. A tech start-up company backed by GCHQ, the British Intelligence and military surveillance headquarters, has created a social distancing wrist sensor that alerts people when they get too close to others. The CIA has also supported tech companies developing similar devices. The wearable sensor was developed by Tended, one of a number of start-up companies supported by GCHQ (see the CIA and DARPA). The device can be worn on the wrist or as a tag on the waistband and will vibrate whenever someone wearing the device breaches social distancing and gets anywhere near natural human contact. The company had a lucky break in that it was developing a distancing sensor when the 'Covid' hoax arrived which immediately provided a potentially enormous market. How fortunate. The government in big-time Cult-controlled Ontario in Canada is investing $2.5 million in wearable contact tracing technology that 'will alert users if they may have been exposed to the Covid-19 in the workplace and will beep or vibrate if they are within six feet of another person'. Facedrive Inc., the technology company behind this, was founded in 2016 with funding from the Ontario Together Fund and obviously they, too, had a prophet on the board of directors. The human surveillance and control technology is called TraceSCAN and would be worn by the human cyborgs in places such as airports, workplaces, construction sites, care homes and … *schools*.

I emphasise schools with children and young people the prime targets. You know what is planned for society as a whole if you keep your eyes on the schools. They have always been places where the state program the next generation of slaves to be its compliant worker-ants – or Woker-ants these days; but in the mist of the 'Covid' madness they have been transformed into mind laboratories on a scale never seen before. Teachers and head teachers are just as programmed as the kids – often more so. Children are kept apart from human interaction by walk lanes, classroom distancing, staggered meal times, masks, and the rolling-out of buzzer systems. Schools are now physically laid out as a laboratory maze for lab-rats. Lunatics at a school in Anchorage, Alaska, who should be prosecuted for child abuse, took away desks and forced children to kneel (know your place) on a mat for five hours a day while wearing a mask and using their chairs as a desk. How this was supposed to impact on a 'virus' only these clinically insane people can tell you and even then it would be clap-trap. The school banned recess (interaction), art classes (creativity), and physical exercise (getting body and mind moving out of inertia). Everyone behind this outrage should be in jail or better still a mental institution. The behavioural manipulators are all for this dystopian approach to schools. Professor Susan Michie, the mind-doctor and British Communist Party member, said it was wrong to say that schools were safe. They had to be made so by 'distancing', masks and ventilation (sitting all day in the cold). I must ask this lady round for dinner on a night I know I am going to be out and not back for weeks. She probably wouldn't be able to make it, anyway, with all the visits to her own psychologist she must have block-booked.

Masking identity

I know how shocking it must be for you that a behaviour manipulator like Michie wants everyone to wear masks which have long been a feature of mind-control programs like the infamous MKUltra in the United States, but, there we are. We live and learn. I spent many years from 1996 to right across the millennium

researching mind control in detail on both sides of the Atlantic and elsewhere. I met a large number of mind-control survivors and many had been held captive in body and mind by MKUltra. MK stands for mind-control, but employs the German spelling in deference to the Nazis spirited out of Germany at the end of World War Two by Operation Paperclip in which the US authorities, with help from the Vatican, transported Nazi mind-controllers and engineers to America to continue their work. Many of them were behind the creation of NASA and they included Nazi scientist and SS officer Wernher von Braun who swapped designing V-2 rockets to bombard London with designing the Saturn V rockets that powered the NASA moon programme's Apollo craft. I think I may have mentioned that the Cult has no borders. Among Paperclip escapees was Josef Mengele, the Angel of Death in the Nazi concentration camps where he conducted mind and genetic experiments on children often using twins to provide a control twin to measure the impact of his 'work' on the other. If you want to observe the Cult mentality in all its extremes of evil then look into the life of Mengele. I have met many people who suffered mercilessly under Mengele in the United States where he operated under the name Dr Greene and became a stalwart of MKUltra programming and torture. Among his locations was the underground facility in the Mojave Desert in California called the China Lake Naval Weapons Station which is almost entirely below the surface. My books *The Biggest Secret*, *Children of the Matrix* and *The Perception Deception* have the detailed background to MKUltra.

The best-known MKUltra survivor is American Cathy O'Brien. I first met her and her late partner Mark Phillips at a conference in Colorado in 1996. Mark helped her escape and deprogram from decades of captivity in an offshoot of MKUltra known as Project Monarch in which 'sex slaves' were provided for the rich and famous including Father George Bush, Dick Cheney and the Clintons. Read Cathy and Mark's book *Trance-Formation of America* and if you are new to this you will be shocked to the core. I read it in 1996 shortly before, with the usual synchronicity of my life, I found

myself given a book table at the conference right next to hers. MKUltra never ended despite being very publicly exposed (only a small part of it) in the 1970s and continues in other guises. I am still in touch with Cathy. She contacted me during 2020 after masks became compulsory in many countries to tell me how they were used as part of MKUltra programming. I had been observing 'Covid regulations' and the relationship between authority and public for months. I saw techniques that I knew were employed on individuals in MKUltra being used on the global population. I had read many books and manuals on mind control including one called *Silent Weapons for Quiet Wars* which came to light in the 1980s and was a guide on how to perceptually program on a mass scale. 'Silent Weapons' refers to mind-control. I remembered a line from the manual as governments, medical authorities and law enforcement agencies have so obviously talked to – or rather at – the adult population since the 'Covid' hoax began as if they are children. The document said:

> If a person is spoken to by a T.V. advertiser as if he were a twelve-year-old, then, due to suggestibility, he will, with a certain probability, respond or react to that suggestion with the uncritical response of a twelve-year-old and will reach in to his economic reservoir and deliver its energy to buy that product on impulse when he passes it in the store.

That's why authority has spoken to adults like children since all this began.

Why did Michael Jackson wear masks?

Every aspect of the 'Covid' narrative has mind-control as its central theme. Cathy O'Brien wrote an article for davidicke.com about the connection between masks and mind control. Her daughter Kelly who I first met in the 1990s was born while Cathy was still held captive in MKUltra. Kelly was forced to wear a mask as part of her programming from the age of *two* to dehumanise her, target her sense of individuality and reduce the amount of oxygen her brain and body received. *Bingo*. This is the real reason for compulsory

masks, why they have been enforced en masse, and why they seek to increase the number they demand you wear. First one, then two, with one disgraceful alleged 'doctor' recommending four which is nothing less than a death sentence. Where and how often they must be worn is being expanded for the purpose of mass mind control and damaging respiratory health which they can call 'Covid-19'. Canada's government headed by the man-child Justin Trudeau, says it's fine for children of two and older to wear masks. An insane 'study' in Italy involving just 47 children concluded there was no problem for babies as young as *four months* wearing them. Even after people were 'vaccinated' they were still told to wear masks by the criminal that is Anthony Fauci. Cathy wrote that mandating masks is allowing the authorities literally to control the air we breathe which is what was done in MKUltra. You might recall how the singer Michael Jackson wore masks and there is a reason for that. He was subjected to MKUltra mind control through Project Monarch and his psyche was scrambled by these simpletons. Cathy wrote:

In MKUltra Project Monarch mind control, Michael Jackson had to wear a mask to silence his voice so he could not reach out for help. Remember how he developed that whisper voice when he wasn't singing? Masks control the mind from the outside in, like the redefining of words is doing. By controlling what we can and cannot say for fear of being labeled racist or beaten, for example, it ultimately controls thought that drives our words and ultimately actions (or lack thereof).

Likewise, a mask muffles our speech so that we are not heard, which controls voice … words … mind. This is Mind Control. Masks are an obvious mind control device, and I am disturbed so many people are complying on a global scale. Masks depersonalize while making a person feel as though they have no voice. It is a barrier to others. People who would never choose to comply but are forced to wear a mask in order to keep their job, and ultimately their family fed, are compromised. They often feel shame and are subdued. People have stopped talking with each other while media controls the narrative.

The 'no voice' theme has often become literal with train passengers told not to speak to each other in case they pass on the 'virus', singing banned for the same reason and bonkers California officials telling people riding roller coasters that they cannot shout and scream. Cathy said she heard every day from healed MKUltra survivors who cannot wear a mask without flashing back on ways

their breathing was controlled – 'from ball gags and penises to water boarding'. She said that through the years when she saw images of people in China wearing masks 'due to pollution' that it was really to control their oxygen levels. 'I knew it was as much of a population control mechanism of depersonalisation as are burkas', she said. Masks are another Chinese communist/fascist method of control that has been swept across the West as the West becomes China at lightning speed since we entered 2020.

Mask-19

There are other reasons for mandatory masks and these include destroying respiratory health to call it 'Covid-19' and stunting brain development of children and the young. Dr Margarite Griesz-Brisson MD, PhD, is a Consultant Neurologist and Neurophysiologist and the Founder and Medical Director of the London Neurology and Pain Clinic. Her CV goes down the street and round the corner. She is clearly someone who cares about people and won't parrot the propaganda. Griesz-Brisson has a PhD in pharmacology, with special interest in neurotoxicology, environmental medicine, neuroregeneration and neuroplasticity (the way the brain can change in the light of information received). She went public in October, 2020, with a passionate warning about the effects of mask-wearing laws:

> The reinhalation of our exhaled air will without a doubt create oxygen deficiency and a flooding of carbon dioxide. We know that the human brain is very sensitive to oxygen deprivation. There are nerve cells for example in the hippocampus that can't be longer than 3 minutes without oxygen – they cannot survive. The acute warning symptoms are headaches, drowsiness, dizziness, issues in concentration, slowing down of reaction time – reactions of the cognitive system.

Oh, I know, let's tell bus, truck and taxi drivers to wear them and people working machinery. How about pilots, doctors and police? Griesz-Brisson makes the important point that while the symptoms she mentions may fade as the body readjusts this does not alter the fact that people continue to operate in oxygen deficit with long list of

potential consequences. She said it was well known that neurodegenerative diseases take years or decades to develop. 'If today you forget your phone number, the breakdown in your brain would have already started 20 or 30 years ago.' She said degenerative processes in your brain are getting amplified as your oxygen deprivation continues through wearing a mask. Nerve cells in the brain are unable to divide themselves normally in these circumstances and lost nerve cells will no longer be regenerated. 'What is gone is gone.' Now consider that people like shop workers and *schoolchildren* are wearing masks for hours every day. What in the name of sanity is going to be happening to them? 'I do not wear a mask, I need my brain to think', Griesz-Brisson said, 'I want to have a clear head when I deal with my patients and not be in a carbon dioxide-induced anaesthesia'. If you are told to wear a mask anywhere ask the organisation, police, store, whatever, for their risk assessment on the dangers and negative effects on mind and body of enforcing mask-wearing. They won't have one because it has never been done not even by government. All of them must be subject to class-action lawsuits as the consequences come to light. They don't do mask risk assessments for an obvious reason. They know what the conclusions would be and independent scientific studies that *have* been done tell a horror story of consequences.

'Masks are criminal'

Dr Griesz-Brisson said that for children and adolescents, masks are an absolute no-no. They had an extremely active and adaptive immune system and their brain was incredibly active with so much to learn. 'The child's brain, or the youth's brain, is thirsting for oxygen.' The more metabolically active an organ was, the more oxygen it required; and in children and adolescents every organ was metabolically active. Griesz-Brisson said that to deprive a child's or adolescent's brain of oxygen, or to restrict it in any way, was not only dangerous to their health, it was absolutely criminal. 'Oxygen deficiency inhibits the development of the brain, and the damage that has taken place as a result CANNOT be reversed.' Mind

manipulators of MKUltra put masks on two-year-olds they wanted to neurologically rewire and you can see why. Griesz-Brisson said a child needs the brain to learn and the brain needs oxygen to function. 'We don't need a clinical study for that. This is simple, indisputable physiology.' Consciously and purposely induced oxygen deficiency was an absolutely deliberate health hazard, and an absolute medical contraindication which means that 'this drug, this therapy, this method or measure should not be used, and is not allowed to be used'. To coerce an entire population to use an absolute medical contraindication by force, she said, there had to be definite and serious reasons and the reasons must be presented to competent interdisciplinary and independent bodies to be verified and authorised. She had this warning of the consequences that were coming if mask wearing continued:

When, in ten years, dementia is going to increase exponentially, and the younger generations couldn't reach their god-given potential, it won't help to say 'we didn't need the masks'. I know how damaging oxygen deprivation is for the brain, cardiologists know how damaging it is for the heart, pulmonologists know how damaging it is for the lungs. Oxygen deprivation damages every single organ. Where are our health departments, our health insurance, our medical associations? It would have been their duty to be vehemently against the lockdown and to stop it and stop it from the very beginning.

Why do the medical boards issue punishments to doctors who give people exemptions? Does the person or the doctor seriously have to prove that oxygen deprivation harms people? What kind of medicine are our doctors and medical associations representing? Who is responsible for this crime? The ones who want to enforce it? The ones who let it happen and play along, or the ones who don't prevent it?

All of the organisations and people she mentions there either answer directly to the Cult or do whatever hierarchical levels above them tell them to do. The outcome of both is the same. 'It's not about masks, it's not about viruses, it's certainly not about your health', Griesz-Brisson said. 'It is about much, much more. I am not participating. I am not afraid.' They were taking our air to breathe and there was no unfounded medical exemption from face masks. Oxygen deprivation was dangerous for every single brain. It had to be the free decision of every human being whether they want to

wear a mask that was absolutely ineffective to protect themselves from a virus. She ended by rightly identifying where the responsibility lies for all this:

> The imperative of the hour is personal responsibility. We are responsible for what we think, not the media. We are responsible for what we do, not our superiors. We are responsible for our health, not the World Health Organization. And we are responsible for what happens in our country, not the government.

Halle-bloody-lujah.

But surgeons wear masks, right?

Independent studies of mask-wearing have produced a long list of reports detailing mental, emotional and physical dangers. What a definition of insanity to see police officers imposing mask-wearing on the public which will cumulatively damage their health while the police themselves wear masks that will cumulatively damage *their* health. It's utter madness and both public and police do this because 'the government says so' – yes a government of brain-donor idiots like UK Health Secretary Matt Hancock reading the 'follow the science' scripts of psychopathic, lunatic psychologists. The response you get from Stockholm syndrome sufferers defending the very authorities that are destroying them and their families is that 'surgeons wear masks'. This is considered the game, set and match that they must work and don't cause oxygen deficit. Well, actually, scientific studies have shown that they *do* and oxygen levels are monitored in operating theatres to compensate. Surgeons wear masks to stop spittle and such like dropping into open wounds – not to stop 'viral particles' which are so miniscule they can only be seen through an electron microscope. Holes in the masks are significantly bigger than 'viral particles' and if you sneeze or cough they will breach the mask. I watched an incredibly disingenuous 'experiment' that claimed to prove that masks work in catching 'virus' material from the mouth and nose. They did this with a slow motion camera and the mask did block big stuff which stayed inside the mask and

against the face to be breathed in or cause infections on the face as we have seen with many children. 'Viral particles', however, would never have been picked up by the camera as they came through the mask when they are far too small to be seen. The 'experiment' was therefore disingenuous *and* useless.

Studies have concluded that wearing masks in operating theatres (and thus elsewhere) make no difference to preventing infection while the opposite is true with toxic shite building up in the mask and this had led to an explosion in tooth decay and gum disease dubbed by dentists 'mask mouth'. You might have seen the Internet video of a furious American doctor urging people to take off their masks after a four-year-old patient had been rushed to hospital the night before and nearly died with a lung infection that doctors sourced to mask wearing. A study in the journal *Cancer Discovery* found that inhalation of harmful microbes can contribute to advanced stage lung cancer in adults and long-term use of masks can help breed dangerous pathogens. Microbiologists have said frequent mask wearing creates a moist environment in which microbes can grow and proliferate before entering the lungs. The Canadian Agency for Drugs and Technologies in Health, or CADTH, a Canadian national organisation that provides research and analysis to healthcare decision-makers, said this as long ago as 2013 in a report entitled 'Use of Surgical Masks in the Operating Room: A Review of the Clinical Effectiveness and Guidelines'. It said:

- No evidence was found to support the use of surgical face masks to reduce the frequency of surgical site infections
- No evidence was found on the effectiveness of wearing surgical face masks to protect staff from infectious material in the operating room.
- Guidelines recommend the use of surgical face masks by staff in the operating room to protect both operating room staff and patients (despite the lack of evidence).

We were told that the world could go back to 'normal' with the arrival of the 'vaccines'. When they came, fraudulent as they are, the story changed as I knew that it would. We are in the midst of transforming 'normal', not going back to it. Mary Ramsay, head of immunisation at Public Health England, echoed the words of US criminal Anthony Fauci who said masks and other regulations must stay no matter if people are vaccinated. The Fauci idiot continued to wear two masks – different colours so both could be clearly seen – after he *claimed* to have been vaccinated. Senator Rand Paul told Fauci in one exchange that his double-masks were 'theatre' and he was right. It's all theatre. Mary Ramsay back-tracked on the vaccine-return-to-normal theme when she said the public may need to wear masks and social-distance for years despite the jabs. 'People have got used to those lower-level restrictions now, and [they] can live with them', she said telling us what the idea has been all along. 'The vaccine does not give you a pass, even if you have had it, you must continue to follow all the guidelines' said a Public Health England statement which reneged on what we had been told before and made having the 'vaccine' irrelevant to 'normality' even by the official story. Spain's fascist government trumped everyone by passing a law mandating the wearing of masks on the beach and even when swimming in the sea. The move would have devastated what's left of the Spanish tourist industry, posed potential breathing dangers to swimmers and had Northern European sunbathers walking around with their forehead brown and the rest of their face white as a sheet. The ruling was so crazy that it had to be retracted after pressure from public and tourist industry, but it confirmed where the Cult wants to go with masks and how clinically insane authority has become. The determination to make masks permanent and hide the serious dangers to body and mind can be seen in the censorship of scientist Professor Denis Rancourt by Bill Gates-funded academic publishing website ResearchGate over his papers exposing the dangers and uselessness of masks. Rancourt said:

ResearchGate today has permanently locked my account, which I have had since 2015. Their reasons graphically show the nature of their attack against democracy, and their corruption of

science … By their obscene non-logic, a scientific review of science articles reporting on harms caused by face masks has a 'potential to cause harm'. No criticism of the psychological device (face masks) is tolerated, if the said criticism shows potential to influence public policy.

This is what happens in a fascist world.

Where are the 'greens' (again)?

Other dangers of wearing masks especially regularly relate to the inhalation of minute plastic fibres into the lungs and the deluge of discarded masks in the environment and oceans. Estimates predicted that more than 1.5 billion disposable masks will end up in the world's oceans every year polluting the water with tons of plastic and endangering marine wildlife. Studies project that humans are using 129 billion face masks each month worldwide – about three million a minute. Most are disposable and made from plastic, non-biodegradable microfibers that break down into smaller plastic particles that become widespread in ecosystems. They are littering cities, clogging sewage channels and turning up in bodies of water. I have written in other books about the immense amounts of microplastics from endless sources now being absorbed into the body. Rolf Halden, director of the Arizona State University (ASU) Biodesign Center for Environmental Health Engineering, was the senior researcher in a 2020 study that analysed 47 human tissue samples and found microplastics in all of them. 'We have detected these chemicals of plastics in every single organ that we have investigated', he said. I wrote in *The Answer* about the world being deluged with microplastics. A study by the Worldwide Fund for Nature (WWF) found that people are consuming on average every week some 2,000 tiny pieces of plastic mostly through water and also through marine life and the air. Every year humans are ingesting enough microplastics to fill a heaped dinner plate and in a life-time of 79 years it is enough to fill two large waste bins. Marco Lambertini, WWF International director general said: 'Not only are plastics polluting our oceans and waterways and killing marine life – it's in all of us and we can't escape consuming plastics,' American

geologists found tiny plastic fibres, beads and shards in rainwater samples collected from the remote slopes of the Rocky Mountain National Park near Denver, Colorado. Their report was headed: 'It is raining plastic.' Rachel Adams, senior lecturer in Biomedical Science at Cardiff Metropolitan University, said that among health consequences are internal inflammation and immune responses to a 'foreign body'. She further pointed out that microplastics become carriers of toxins including mercury, pesticides and dioxins (a known cause of cancer and reproductive and developmental problems). These toxins accumulate in the fatty tissues once they enter the body through microplastics. Now this is being compounded massively by people putting plastic on their face and throwing it away.

Workers exposed to polypropylene plastic fibres known as 'flock' have developed 'flock worker's lung' from inhaling small pieces of the flock fibres which can damage lung tissue, reduce breathing capacity and exacerbate other respiratory problems. *Now* … commonly used surgical masks have three layers of melt-blown textiles made of … polypropylene. We have billions of people putting these microplastics against their mouth, nose and face for hours at a time day after day in the form of masks. How does anyone think that will work out? I mean – what could possibly go wrong? We posted a number of scientific studies on this at davidicke.com, but when I went back to them as I was writing this book the links to the science research website where they were hosted were dead. Anything that challenges the official narrative in any way is either censored or vilified. The official narrative is so unsupportable by the evidence that only deleting the truth can protect it. A study by Chinese scientists still survived – with the usual twist which it why it was still active, I guess. Yes, they found that virtually all the masks they tested increased the daily intake of microplastic fibres, but people should still wear them because the danger from the 'virus' was worse said the crazy 'team' from the Institute of Hydrobiology in Wuhan. Scientists first discovered microplastics in lung tissue of some patients who died of lung cancer

in the 1990s. Subsequent studies have confirmed the potential health damage with the plastic degrading slowly and remaining in the lungs to accumulate in volume. Wuhan researchers used a machine simulating human breathing to establish that masks shed up to nearly 4,000 microplastic fibres in a month with reused masks producing more. Scientists said some masks are laced with toxic chemicals and a variety of compounds seriously restricted for both health and environmental reasons. They include cobalt (used in blue dye) and formaldehyde known to cause watery eyes, burning sensations in the eyes, nose, and throat, plus coughing, wheezing and nausea. No – that must be 'Covid-19'.

Mask 'worms'

There is another and potentially even more sinister content of masks. Mostly new masks of different makes filmed under a microscope around the world have been found to contain strange black fibres or 'worms' that appear to move or 'crawl' by themselves and react to heat and water. The nearest I have seen to them are the self-replicating fibres that are pulled out through the skin of those suffering from Morgellons disease which has been connected to the phenomena of 'chemtrails' which I will bring into the story later on. Morgellons fibres continue to grow outside the body and have a form of artificial intelligence. Black 'worm' fibres in masks have that kind of feel to them and there is a nanotechnology technique called 'worm micelles' which carry and release drugs or anything else you want to deliver to the body. For sure the suppression of humanity by mind altering drugs is the Cult agenda big time and the more excuses they can find to gain access to the body the more opportunities there are to make that happen whether through 'vaccines' or masks pushed against the mouth and nose for hours on end.

So let us summarise the pros and cons of masks:

Against masks: Breathing in your own carbon dioxide; depriving the body and brain of sufficient oxygen; build-up of toxins in the mask that can be breathed into the lungs and cause rashes on the face and 'mask-mouth'; breathing microplastic fibres and toxic chemicals into the lungs; dehumanisation and deleting individualisation by literally making people faceless; destroying human emotional interaction through facial expression and deleting parental connection with their babies which look for guidance to their facial expression.

For masks: They don't protect you from a 'virus' that doesn't exist and even if it did 'viral' particles are so minute they are smaller than the holes in the mask.

Governments, police, supermarkets, businesses, transport companies, and all the rest who seek to impose masks have done no risk assessment on their consequences for health and psychology and are now open to group lawsuits when the impact becomes clear with a cumulative epidemic of respiratory and other disease. Authorities will try to exploit these effects and hide the real cause by dubbing them 'Covid-19'. Can you imagine setting out to force the population to wear health-destroying masks without doing any assessment of the risks? It is criminal and it is evil, but then how many people targeted in this way, who see their children told to wear them all day at school, have asked for a risk assessment? Billions can't be imposed upon by the few unless the billions allow it. Oh, yes, with just a tinge of irony, 85 percent of all masks made worldwide come from *China*.

Wash your hands in toxic shite

'Covid' rules include the use of toxic sanitisers and again the health consequences of constantly applying toxins to be absorbed through the skin is obvious to any level of Renegade Mind. America's Food and Drug Administration (FDA) said that sanitisers are drugs and issued a warning about 75 dangerous brands which contain

methanol used in antifreeze and can cause death, kidney damage and blindness. The FDA circulated the following warning even for those brands that it claims to be safe:

Store hand sanitizer out of the reach of pets and children, and children should use it only with adult supervision. Do not drink hand sanitizer. This is particularly important for young children, especially toddlers, who may be attracted by the pleasant smell or brightly colored bottles of hand sanitizer.

Drinking even a small amount of hand sanitizer can cause alcohol poisoning in children. (However, there is no need to be concerned if your children eat with or lick their hands after using hand sanitizer.) During this coronavirus pandemic, poison control centers have had an increase in calls about accidental ingestion of hand sanitizer, so it is important that adults monitor young children's use.

Do not allow pets to swallow hand sanitizer. If you think your pet has eaten something potentially dangerous, call your veterinarian or a pet poison control center right away. Hand sanitizer is flammable and should be stored away from heat and flames. When using hand sanitizer, rub your hands until they feel completely dry before performing activities that may involve heat, sparks, static electricity, or open flames.

There you go, perfectly safe, then, and that's without even a mention of the toxins absorbed through the skin. Come on kids – sanitise your hands everywhere you go. It will save you from the 'virus'. Put all these elements together of the 'Covid' normal and see how much health and psychology is being cumulatively damaged, even devastated, to 'protect your health'. Makes sense, right? They are only imposing these things because they care, right? *Right?*

Submitting to insanity

Psychological reframing of the population goes very deep and is done in many less obvious ways. I hear people say how contradictory and crazy 'Covid' rules are and how they are ever changing. This is explained away by dismissing those involved as idiots. It is a big mistake. The Cult is delighted if its cold calculation is perceived as incompetence and idiocy when it is anything but. Oh, yes, there are idiots within the system – lots of them – but they are *administering* the Cult agenda, mostly unknowingly. They are not deciding and dictating it. The bulwark against tyranny is self-

respect, always has been, always will be. It is self-respect that has broken every tyranny in history. By its very nature self-respect will not bow to oppression and its perpetrators. There is so little self-respect that it's always the few that overturn dictators. Many may eventually follow, but the few with the iron spines (self-respect) kick it off and generate the momentum. The Cult targets self-respect in the knowledge that once this has gone only submission remains. Crazy, contradictory, ever-changing 'Covid' rules are systematically applied by psychologists to delete self-respect. They *want* you to see that the rules make no sense. It is one thing to decide to do something when *you* have made the choice based on evidence and logic. You still retain your self-respect. It is quite another when you can see what you are being told to do is insane, ridiculous and makes no sense, and *yet you still do it*. Your self-respect is extinguished and this has been happening as ever more obviously stupid and nonsensical things have been demanded and the great majority have complied even when they can see they are stupid and nonsensical.

People walk around in face-nappies knowing they are damaging their health and make no difference to a 'virus'. They do it in fear of not doing it. I know it's daft, but I'll do it anyway. When that happens something dies inside of you and submissive reframing has begun. Next there's a need to hide from yourself that you have conceded your self-respect and you convince yourself that you have not really submitted to fear and intimidation. You begin to believe that you are complying with craziness because it's the right thing to do. When first you concede your self-respect of 2+2 = 4 to 2+2 = 5 you *know* you are compromising your self-respect. Gradually to avoid facing that fact you begin to *believe* that 2+2=5. You have been reframed and I have been watching this process happening in the human psyche on an industrial scale. The Cult is working to break your spirit and one of its major tools in that war is humiliation. I read how former American soldier Bradley Manning (later Chelsea Manning after a sex-change) was treated after being jailed for supplying WikiLeaks with documents exposing the enormity of

government and elite mendacity. Manning was isolated in solitary confinement for eight months, put under 24-hour surveillance, forced to hand over clothing before going to bed, and stand naked for every roll call. This is systematic humiliation. The introduction of anal swab 'Covid' tests in China has been done for the same reason to delete self-respect and induce compliant submission. Anal swabs are mandatory for incoming passengers in parts of China and American diplomats have said they were forced to undergo the indignity which would have been calculated humiliation by the Cult-owned Chinese government that has America in its sights.

Government-people: An abusive relationship

Spirit-breaking psychological techniques include giving people hope and apparent respite from tyranny only to take it away again. This happened in the UK during Christmas, 2020, when the psycho-psychologists and their political lackeys announced an easing of restrictions over the holiday only to reimpose them almost immediately on the basis of yet another lie. There is a big psychological difference between getting used to oppression and being given hope of relief only to have that dashed. Psychologists know this and we have seen the technique used repeatedly. Then there is traumatising people before you introduce more extreme regulations that require compliance. A perfect case was the announcement by the dark and sinister Whitty and Vallance in the UK that 'new data' predicted that 4,000 could die every day over the winter of 2020/2021 if we did not lockdown again. I think they call it lying and after traumatising people with that claim out came Jackboot Johnson the next day with new curbs on human freedom. Psychologists know that a frightened and traumatised mind becomes suggestable to submission and behaviour reframing. Underpinning all this has been to make people fearful and suspicious of each other and see themselves as a potential danger to others. In league with deleted self-respect you have the perfect psychological recipe for self-loathing. The relationship between authority and public is now demonstrably the same as that of

subservience to an abusive partner. These are signs of an abusive relationship explained by psychologist Leslie Becker-Phelps:

Psychological and emotional abuse: Undermining a partner's self-worth with verbal attacks, name-calling, and belittling. Humiliating the partner in public, unjustly accusing them of having an affair, or interrogating them about their every behavior. Keeping partner confused or off balance by saying they were just kidding or blaming the partner for 'making' them act this way … Feigning in public that they care while turning against them in private. This leads to victims frequently feeling confused, incompetent, unworthy, hopeless, and chronically self-doubting. [Apply these techniques to how governments have treated the population since New Year, 2020, and the parallels are obvious.]

Physical abuse: The abuser might physically harm their partner in a range of ways, such as grabbing, hitting, punching, or shoving them. They might throw objects at them or harm them with a weapon. [Observe the physical harm imposed by masks, lockdown, and so on.]

Threats and intimidation: One way abusers keep their partners in line is by instilling fear. They might be verbally threatening, or give threatening looks or gestures. Abusers often make it known that they are tracking their partner's every move. They might destroy their partner's possessions, threaten to harm them, or threaten to harm their family members. Not surprisingly, victims of this abuse often feel anxiety, fear, and panic. [No words necessary.]

Isolation: Abusers often limit their partner's activities, forbidding them to talk or interact with friends or family. They might limit access to a car or even turn off their phone. All of this might be done by physically holding them against their will, but is often accomplished through psychological abuse and intimidation. The more isolated a person feels, the fewer resources they have to help gain perspective on their situation and to escape from it. [No words necessary.]

Economic abuse: Abusers often make their partners beholden to them for money by controlling access to funds of any kind. They might prevent their partner from getting a job or withhold access to money they earn from a job. This creates financial dependency that makes leaving the relationship very difficult. [See destruction of livelihoods and the proposed meagre 'guaranteed income' so long as you do whatever you are told.]

Using children: An abuser might disparage their partner's parenting skills, tell their children lies about their partner, threaten to take custody of their children, or threaten to harm their children. These tactics instil fear and often elicit compliance. [See reframed social service mafia and how children are being mercilessly abused by the state over 'Covid' while their parents look on too frightened to do anything.]

A further recurring trait in an abusive relationship is the abused blaming themselves for their abuse and making excuses for the abuser. We have the public blaming each other for lockdown abuse by government and many making excuses for the government while attacking those who challenge the government. How often we have heard authorities say that rules are being imposed or reimposed only because people have refused to 'behave' and follow the rules. We don't want to do it – it's *you*.

Renegade Minds are an antidote to all of these things. They will never concede their self-respect no matter what the circumstances. Even when apparent humiliation is heaped upon them they laugh in its face and reflect back the humiliation on the abuser where it belongs. Renegade Minds will never wear masks they know are only imposed to humiliate, suppress and damage both physically and psychologically. Consequences will take care of themselves and they will never break their spirit or cause them to concede to tyranny. UK newspaper columnist Peter Hitchens was one of the few in the mainstream media to speak out against lockdowns and forced vaccinations. He then announced he had taken the jab. He wanted to see family members abroad and he believed vaccine passports were inevitable even though they had not yet been introduced. Hitchens

has a questioning and critical mind, but not a Renegade one. If he had no amount of pressure would have made him concede. Hitchens excused his action by saying that the battle has been lost. Renegade Minds never accept defeat when freedom is at stake and even if they are the last one standing the self-respect of not submitting to tyranny is more important than any outcome or any consequence.

That's why Renegade Minds are the only minds that ever changed anything worth changing.

CHAPTER EIGHT

'Reframing' insanity

Insanity is relative. It depends on who has who locked in what cage
Ray Bradbury

'Reframing' a mind means simply to change its perception and behaviour. This can be done subconsciously to such an extent that subjects have no idea they have been 'reframed' while to any observer changes in behaviour and attitudes are obvious.

Human society is being reframed on a ginormous scale since the start of 2020 and here we have the reason why psychologists rather than doctors have been calling the shots. Ask most people who have succumbed to 'Covid' reframing if they have changed and most will say 'no'; but they *have* and fundamentally. The Cult's long-game has been preparing for these times since way back and crucial to that has been to prepare both population and officialdom mentally and emotionally. To use the mind-control parlance they had to reframe the population with a mentality that would submit to fascism and reframe those in government and law enforcement to impose fascism or at least go along with it. The result has been the fact-deleted mindlessness of 'Wokeness' and officialdom that has either enthusiastically or unquestioningly imposed global tyranny demanded by reframed politicians on behalf of psychopathic and deeply evil cultists. 'Cognitive reframing' identifies and challenges the way someone sees the world in the form of situations, experiences and emotions and then restructures those perceptions to view the same set of circumstances in a different way. This can have

benefits if the attitudes are personally destructive while on the other side it has the potential for individual and collective mind control which the subject has no idea has even happened.

Cognitive therapy was developed in the 1960s by Aaron T. Beck who was born in Rhode Island in 1921 as the son of Jewish immigrants from the Ukraine. He became interested in the techniques as a treatment for depression. Beck's daughter Judith S. Beck is prominent in the same field and they founded the Beck Institute for Cognitive Behavior Therapy in Philadelphia in 1994. Cognitive reframing, however, began to be used worldwide by those with a very dark agenda. The Cult reframes politicians to change their attitudes and actions until they are completely at odds with what they once appeared to stand for. The same has been happening to government administrators at all levels, law enforcement, military and the human population. Cultists love mind control for two main reasons: It allows them to control what people think, do and say to secure agenda advancement and, by definition, it calms their legendary insecurity and fear of the unexpected. I have studied mind control since the time I travelled America in 1996. I may have been talking to next to no one in terms of an audience in those years, but my goodness did I gather a phenomenal amount of information and knowledge about so many things including the techniques of mind control. I have described this in detail in other books going back to *The Biggest Secret* in 1998. I met a very large number of people recovering from MKUltra and its offshoots and successors and I began to see how these same techniques were being used on the population in general. This was never more obvious than since the 'Covid' hoax began.

Reframing the enforcers

I have observed over the last two decades and more the very clear transformation in the dynamic between the police, officialdom and the public. I tracked this in the books as the relationship mutated from one of serving the public to seeing them as almost the enemy and certainly a lower caste. There has always been a class divide

based on income and always been some psychopathic, corrupt, and big-I-am police officers. This was different. Wholesale change was unfolding in the collective dynamic; it was less about money and far more about position and perceived power. An us-and-them was emerging. Noses were lifted skyward by government administration and law enforcement and their attitude to the public they were *supposed* to be serving changed to one of increasing contempt, superiority and control. The transformation was so clear and widespread that it had to be planned. Collective attitudes and dynamics do not change naturally and organically that quickly on that scale. I then came across an organisation in Britain called Common Purpose created in the late 1980s by Julia Middleton who would work in the office of Deputy Prime Minister John Prescott during the long and disastrous premiership of war criminal Tony Blair. When Blair speaks the Cult is speaking and the man should have been in jail a long time ago. Common Purpose proclaims itself to be one of the biggest 'leadership development' organisations in the world while functioning as a *charity* with all the financial benefits which come from that. It hosts 'leadership development' courses and programmes all over the world and claims to have 'brought together' what it calls 'leaders' from more than 100 countries on six continents. The modus operandi of Common Purpose can be compared with the work of the UK government's reframing network that includes the Behavioural Insights Team 'nudge unit' and 'Covid' reframing specialists at SPI-B. WikiLeaks described Common Purpose long ago as 'a hidden virus in our government and schools' which is unknown to the general public: 'It recruits and trains "leaders" to be loyal to the directives of Common Purpose and the EU, instead of to their own departments, which they then undermine or subvert, the NHS [National Health Service] being an example.' This is a vital point to understand the 'Covid' hoax. The NHS, and its equivalent around the world, has been utterly reframed in terms of administrators and much of the medical personnel with the transformation underpinned by recruitment policies. The outcome has been the criminal and psychopathic behaviour of the

NHS over 'Covid' and we have seen the same in every other major country. WikiLeaks said Common Purpose trainees are 'learning to rule without regard to democracy' and to usher in a police state (current events explained). Common Purpose operated like a 'glue' and had members in the NHS, BBC, police, legal profession, church, many of Britain's 7,000 quangos, local councils, the Civil Service, government ministries and Parliament, and controlled many RDA's (Regional Development Agencies). Here we have one answer for how and why British institutions and their like in other countries have changed so negatively in relation to the public. This further explains how and why the beyond-disgraceful reframed BBC has become a propaganda arm of 'Covid' fascism. They are all part of a network pursuing the same goal.

By 2019 Common Purpose was quoting a figure of 85,000 'leaders' that had attended its programmes. These 'students' of all ages are known as Common Purpose 'graduates' and they consist of government, state and local government officials and administrators, police chiefs and officers, and a whole range of others operating within the national, local and global establishment. Cressida Dick, Commissioner of the London Metropolitan Police, is the Common Purpose graduate who was the 'Gold Commander' that oversaw what can only be described as the murder of Brazilian electrician Jean Charles de Menezes in 2005. He was held down by psychopathic police and shot seven times in the head by a psychopathic lunatic after being mistaken for a terrorist when he was just a bloke going about his day. Dick authorised officers to pursue and keep surveillance on de Menezes and ordered that he be stopped from entering the underground train system. Police psychopaths took her at her word clearly. She was 'disciplined' for this outrage by being *promoted* – eventually to the top of the 'Met' police where she has been a disaster. Many Chief Constables controlling the police in different parts of the UK are and have been Common Purpose graduates. I have heard the 'graduate' network described as a sort of Mafia or secret society operating within the fabric of government at all levels pursuing a collective policy

ingrained at Common Purpose training events. Founder Julia Middleton herself has said:

> Locally and internationally, Common Purpose graduates will be 'lighting small fires' to create change in their organisations and communities … The Common Purpose effect is best illustrated by the many stories of small changes brought about by leaders, who themselves have changed.

A Common Purpose mission statement declared:

> Common Purpose aims to improve the way society works by expanding the vision, decision-making ability and influence of all kinds of leaders. The organisation runs a variety of educational programmes for leaders of all ages, backgrounds and sectors, in order to provide them with the inspirational, information and opportunities they need to change the world.

Yes, but into what? Since 2020 the answer has become clear.

NLP and the Delphi technique

Common Purpose would seem to be a perfect name or would common programming be better? One of the foundation methods of reaching 'consensus' (group think) is by setting the agenda theme and then encouraging, cajoling or pressuring everyone to agree a 'consensus' in line with the core theme promoted by Common Purpose. The methodology involves the 'Delphi technique', or an adaption of it, in which opinions are expressed that are summarised by a 'facilitator or change agent' at each stage. Participants are 'encouraged' to modify their views in the light of what others have said. Stage by stage the former individual opinions are merged into group consensus which just happens to be what Common Purpose wants them to believe. A key part of this is to marginalise anyone refusing to concede to group think and turn the group against them to apply pressure to conform. We are seeing this very technique used on the general population to make 'Covid' group-thinkers hostile to those who have seen through the bullshit. People can be reframed by using perception manipulation methods such as Neuro-Linguistic Programming (NLP) in which you change perception with the use of

carefully constructed language. An NLP website described the technique this way:

… A method of influencing brain behaviour (the 'neuro' part of the phrase) through the use of language (the 'linguistic' part) and other types of communication to enable a person to 'recode' the way the brain responds to stimuli (that's the 'programming') and manifest new and better behaviours. Neuro-Linguistic Programming often incorporates hypnosis and self-hypnosis to help achieve the change (or 'programming') that is wanted.

British alternative media operation UKColumn has done very detailed research into Common Purpose over a long period. I quoted co-founder and former naval officer Brian Gerrish in my book *Remember Who You Are*, published in 2011, as saying the following years before current times:

It is interesting that many of the mothers who have had children taken by the State speak of the Social Services people being icily cool, emotionless and, as two ladies said in slightly different words, '… like little robots'. We know that NLP is cumulative, so people can be given small imperceptible doses of NLP in a course here, another in a few months, next year etc. In this way, major changes are accrued in their personality, but the day by day change is almost unnoticeable.

In these and other ways 'graduates' have had their perceptions uniformly reframed and they return to their roles in the institutions of government, law enforcement, legal profession, military, 'education', the UK National Health Service and the whole swathe of the establishment structure to pursue a common agenda preparing for the 'post-industrial', 'post-democratic' society. I say 'preparing' but we are now there. 'Post-industrial' is code for the Great Reset and 'post-democratic' is 'Covid' fascism. UKColumn has spoken to partners of those who have attended Common Purpose 'training'. They have described how personalities and attitudes of 'graduates' changed very noticeably for the worse by the time they had completed the course. They had been 'reframed' and told they are the 'leaders' – the special ones – who know better than the population. There has also been the very demonstrable recruitment of psychopaths and narcissists into government administration at all

levels and law enforcement. If you want psychopathy hire psychopaths and you get a simple cause and effect. If you want administrators, police officers and 'leaders' to perceive the public as lesser beings who don't matter then employ narcissists. These personalities are identified using 'psychometrics' that identifies knowledge, abilities, attitudes and personality traits, mostly through carefully-designed questionnaires and tests. As this policy has passed through the decades we have had power-crazy, power-trippers appointed into law enforcement, security and government administration in preparation for current times and the dynamic between public and law enforcement/officialdom has been transformed. UKColumn's Brian Gerrish said of the narcissistic personality:

> Their love of themselves and power automatically means that they will crush others who get in their way. I received a major piece of the puzzle when a friend pointed out that when they made public officials re-apply for their own jobs several years ago they were also required to do psychometric tests. This was undoubtedly the start of the screening process to get 'their' sort of people in post.

How obvious that has been since 2020 although it was clear what was happening long before if people paid attention to the changing public-establishment dynamic.

Change agents

At the centre of events in 'Covid' Britain is the National Health Service (NHS) which has behaved disgracefully in slavishly following the Cult agenda. The NHS management structure is awash with Common Purpose graduates or 'change agents' working to a common cause. Helen Bevan, a Chief of Service Transformation at the NHS Institute for Innovation and Improvement, co-authored a document called 'Towards a million change agents, a review of the social movements literature: implications for large scale change in the NHS'. The document compared a project management approach to that of change and social movements where 'people change

themselves and each other – peer to peer'. Two definitions given for a 'social movement' were:

A group of people who consciously attempt to build a radically new social order; involves people of a broad range of social backgrounds; and deploys politically confrontational and socially disruptive tactics – Cyrus Zirakzadeh 1997

Collective challenges, based on common purposes and social solidarities, in sustained interaction with elites, opponents, and authorities – Sidney Tarrow 1994

Helen Bevan wrote another NHS document in which she defined 'framing' as 'the process by which leaders construct, articulate and put across their message in a powerful and compelling way in order to win people to their cause and call them to action'. I think I could come up with another definition that would be rather more accurate. The National Health Service and institutions of Britain and the wider world have been taken over by reframed 'change agents' and that includes everything from the United Nations to national governments, local councils and social services which have been kidnapping children from loving parents on an extraordinary and gathering scale on the road to the end of parenthood altogether. Children from loving homes are stolen and kidnapped by the state and put into the 'care' (inversion) of the local authority through council homes, foster parents and forced adoption. At the same time children are allowed to be abused without response while many are under council 'care'. UKColumn highlighted the Common Purpose connection between South Yorkshire Police and Rotherham council officers in the case of the scandal in that area of the sexual exploitation of children to which the authorities turned not one blind eye, but both:

We were alarmed to discover that the Chief Executive, the Strategic Director of Children and Young People's Services, the Manager for the Local Strategic Partnership, the Community Cohesion Manager, the Cabinet Member for Cohesion, the Chief Constable and his predecessor had all attended Leadership training courses provided by the pseudo-charity Common Purpose.

Once 'change agents' have secured positions of hire and fire within any organisation things start to move very quickly. Personnel are then hired and fired on the basis of whether they will work towards the agenda the change agent represents. If they do they are rapidly promoted even though they may be incompetent. Those more qualified and skilled who are pre-Common Purpose 'old school' see their careers stall and even disappear. This has been happening for decades in every institution of state, police, 'health' and social services and all of them have been transformed as a result in their attitudes to their jobs and the public. Medical professions, including nursing, which were once vocations for the caring now employ many cold, callous and couldn't give a shit personality types. The UKColumn investigation concluded:

By blurring the boundaries between people, professions, public and private sectors, responsibility and accountability, Common Purpose encourages 'graduates' to believe that as new selected leaders, they can work together, outside of the established political and social structures, to achieve a paradigm shift or CHANGE – so called 'Leading Beyond Authority'. In doing so, the allegiance of the individual becomes 'reframed' on CP colleagues and their NETWORK.

Reframing the Face-Nappies

Nowhere has this process been more obvious than in the police where recruitment of psychopaths and development of unquestioning mind-controlled group-thinkers have transformed law enforcement into a politically-correct 'Woke' joke and a travesty of what should be public service. Today they wear their face-nappies like good little gofers and enforce 'Covid' rules which are fascism under another name. Alongside the specifically-recruited psychopaths we have software minds incapable of free thought. Brian Gerrish again:

An example is the policeman who would not get on a bike for a press photo because he had not done the cycling proficiency course. Normal people say this is political correctness gone mad. Nothing could be further from the truth. The policeman has been reframed, and in his reality it is perfect common sense not to get on the bike 'because he hasn't done the cycling course'.

Another example of this is where the police would not rescue a boy from a pond until they had taken advice from above on the 'risk assessment'. A normal person would have arrived, perhaps thought of the risk for a moment, and dived in. To the police now 'reframed', they followed 'normal' procedure.

There are shocking cases of reframed ambulance crews doing the same. Sheer unthinking stupidity of London Face-Nappies headed by Common Purpose graduate Cressida Dick can be seen in their behaviour at a vigil in March, 2021, for a murdered woman, Sarah Everard. A police officer had been charged with the crime. Anyone with a brain would have left the vigil alone in the circumstances. Instead they 'manhandled' women to stop them breaking 'Covid rules' to betray classic reframing. Minds in the thrall of perception control have no capacity for seeing a situation on its merits and acting accordingly. 'Rules is rules' is their only mind-set. My father used to say that rules and regulations are for the guidance of the intelligent and the blind obedience of the idiot. Most of the intelligent, decent, coppers have gone leaving only the other kind and a few old school for whom the job must be a daily nightmare. The combination of psychopaths and rule-book software minds has been clearly on public display in the 'Covid' era with automaton robots in uniform imposing fascistic 'Covid' regulations on the population without any personal initiative or judging situations on their merits. There are thousands of examples around the world, but I'll make my point with the infamous Derbyshire police in the English East Midlands – the ones who think pouring dye into beauty spots and using drones to track people walking in the countryside away from anyone is called 'policing'. To them there are rules decreed by the government which they have to enforce and in their bewildered state a group gathering in a closed space and someone walking alone in the countryside are the same thing. It is beyond idiocy and enters the realm of clinical insanity.

Police officers in Derbyshire said they were 'horrified' – *horrified* – to find 15 to 20 'irresponsible' kids playing a football match at a closed leisure centre 'in breach of coronavirus restrictions'. When they saw the police the kids ran away leaving their belongings behind and the reframed men and women of Derbyshire police were seeking to establish their identities with a view to fining their parents. The most natural thing for youngsters to do – kicking a ball about – is turned into a criminal activity and enforced by the moronic software programs of Derbyshire police. You find the same mentality in every country. These barely conscious 'horrified' officers said they had to take action because 'we need to ensure these rules are being followed' and 'it is of the utmost importance that you ensure your children are following the rules and regulations for Covid-19'. Had any of them done ten seconds of research to see if this parroting of their masters' script could be supported by any evidence? Nope. Reframed people don't think – others think for them and that's the whole idea of reframing. I have seen police officers one after the other repeating without question word for word what officialdom tells them just as I have seen great swathes of the public doing the same. Ask either for 'their' opinion and out spews what they have been told to think by the official narrative. Police and public may seem to be in different groups, but their mentality is the same. Most people do whatever they are told in fear not doing so or because they believe what officialdom tells them; almost the entirety of the police do what they are told for the same reason. Ultimately it's the tiny inner core of the global Cult that's telling both what to do.

So Derbyshire police were 'horrified'. Oh, really? Why did they think those kids were playing football? It was to relieve the psychological consequences of lockdown and being denied human contact with their friends and interaction, touch and discourse vital to human psychological health. Being denied this month after month has dismantled the psyche of many children and young people as depression and suicide have exploded. Were Derbyshire police *horrified by that*? Are you kidding? Reframed people don't have those

mental and emotional processes that can see how the impact on the psychological health of youngsters is far more dangerous than any 'virus' even if you take the mendacious official figures to be true. The reframed are told (programmed) how to act and so they do. The Derbyshire Chief Constable in the first period of lockdown when the black dye and drones nonsense was going on was Peter Goodman. He was the man who severed the connection between his force and the Derbyshire Constabulary *Male Voice* Choir when he decided that it was not inclusive enough to allow women to join. The fact it was a male voice choir making a particular sound produced by male voices seemed to elude a guy who terrifyingly ran policing in Derbyshire. He retired weeks after his force was condemned as disgraceful by former Supreme Court Justice Jonathan Sumption for their behaviour over extreme lockdown impositions. Goodman was replaced by his deputy Rachel Swann who was in charge when her officers were 'horrified'. The police statement over the boys committing the hanging-offence of playing football included the line about the youngsters being 'irresponsible in the times we are all living through' missing the point that the real relevance of the 'times we are all living through' is the imposition of fascism enforced by psychopaths and reframed minds of police officers playing such a vital part in establishing the fascist tyranny that their own children and grandchildren will have to live in their entire lives. As a definition of insanity that is hard to beat although it might be run close by imposing masks on people that can have a serious effect on their health while wearing a face nappy all day themselves. Once again public and police do it for the same reason – the authorities tell them to and who are they to have the self-respect to say no?

Wokers in uniform

How reframed do you have to be to arrest a *six-year-old* and take him to court for *picking a flower* while waiting for a bus? Brain dead police and officialdom did just that in North Carolina where criminal proceedings happen regularly for children under nine. Attorney Julie Boyer gave the six-year-old crayons and a colouring book

during the 'flower' hearing while the 'adults' decided his fate. County Chief District Court Judge Jay Corpening asked: 'Should a child that believes in Santa Claus, the Easter Bunny and the tooth fairy be making life-altering decisions?' Well, of course not, but common sense has no meaning when you have a common purpose and a reframed mind. Treating children in this way, and police operating in American schools, is all part of the psychological preparation for children to accept a police state as normal all their adult lives. The same goes for all the cameras and biometric tracking technology in schools. Police training is focused on reframing them as snowflake Wokers and this is happening in the military. Pentagon top brass said that 'training sessions on extremism' were needed for troops who asked why they were so focused on the Capitol Building riot when Black Lives Matter riots were ignored. What's the difference between them some apparently and rightly asked. Actually, there is a difference. Five people died in the Capitol riot, only one through violence, and that was a police officer shooting an unarmed protestor. BLM riots killed at least 25 people and cost billions. Asking the question prompted the psychopaths and reframed minds that run the Pentagon to say that more 'education' (programming) was needed. Troop training is all based on psychological programming to make them fodder for the Cult – 'Military men are just dumb, stupid animals to be used as pawns in foreign policy' as Cult-to-his-DNA former Secretary of State Henry Kissinger famously said. Governments see the police in similar terms and it's time for those among them who can see this to defend the people and stop being enforcers of the Cult agenda upon the people.

The US military, like the country itself, is being targeted for destruction through a long list of Woke impositions. Cult-owned gaga 'President' Biden signed an executive order when he took office to allow taxpayer money to pay for transgender surgery for active military personnel and veterans. Are you a man soldier? No, I'm a LGBTQIA+ with a hint of Skoliosexual and Spectrasexual. Oh, good man. Bad choice of words you bigot. The Pentagon announced in March, 2021, the appointment of the first 'diversity and inclusion

officer' for US Special Forces. Richard Torres-Estrada arrived with the publication of a 'D&I Strategic Plan which will guide the enterprise-wide effort to institutionalize and sustain D&I'. If you think a Special Forces 'Strategic Plan' should have something to do with defending America you haven't been paying attention. Defending Woke is now the military's new role. Torres-Estrada has posted images comparing Donald Trump with Adolf Hitler and we can expect no bias from him as a representative of the supposedly non-political Pentagon. Cable news host Tucker Carlson said: 'The Pentagon is now the Yale faculty lounge but with cruise missiles.' Meanwhile Secretary of Defense Lloyd Austin, a board member of weapons-maker Raytheon with stock and compensation interests in October, 2020, worth $1.4 million, said he was purging the military of the 'enemy within' – anyone who isn't Woke and supports Donald Trump. Austin refers to his targets as 'racist extremists' while in true Woke fashion being himself a racist extremist. Pentagon documents pledge to 'eradicate, eliminate and conquer all forms of racism, sexism and homophobia'. The definitions of these are decided by 'diversity and inclusion committees' peopled by those who see racism, sexism and homophobia in every situation and opinion. Woke (the Cult) is dismantling the US military and purging testosterone as China expands its military and gives its troops 'masculinity training'. How do we think that is going to end when this is all Cult coordinated? The US military, like the British military, is controlled by Woke and spineless top brass who just go along with it out of personal career interests.

'Woke' means fast asleep

Mind control and perception manipulation techniques used on individuals to create group-think have been unleashed on the global population in general. As a result many have no capacity to see the obvious fascist agenda being installed all around them or what 'Covid' is really all about. Their brains are firewalled like a computer system not to process certain concepts, thoughts and realisations that are bad for the Cult. The young are most targeted as the adults they

will be when the whole fascist global state is planned to be fully implemented. They need to be prepared for total compliance to eliminate all pushback from entire generations. The Cult has been pouring billions into taking complete control of 'education' from schools to universities via its operatives and corporations and not least Bill Gates as always. The plan has been to transform 'education' institutions into programming centres for the mentality of 'Woke'. James McConnell, professor of psychology at the University of Michigan, wrote in *Psychology Today* in 1970:

> The day has come when we can combine sensory deprivation with drugs, hypnosis, and astute manipulation of reward and punishment, to gain almost absolute control over an individual's behaviour. It should then be possible to achieve a very rapid and highly effective type of brainwashing that would allow us to make dramatic changes in a person's behaviour and personality ...
>
> … We should reshape society so that we all would be trained from birth to want to do what society wants us to do. We have the techniques to do it... no-one owns his own personality you acquired, and there's no reason to believe you should have the right to refuse to acquire a new personality if your old one is anti-social.

This was the potential for mass brainwashing in 1970 and the mentality there displayed captures the arrogant psychopathy that drives it forward. I emphasise that not all young people have succumbed to Woke programming and those that haven't are incredibly impressive people given that today's young are the most perceptually-targeted generations in history with all the technology now involved. Vast swathes of the young generations, however, have fallen into the spell – and that's what it is – of Woke. The Woke mentality and perceptual program is founded on *inversion* and you will appreciate later why that is so significant. Everything with Woke is inverted and the opposite of what it is claimed to be. Woke was a term used in African-American culture from the 1900s and referred to an awareness of social and racial justice. This is not the meaning of the modern version or 'New Woke' as I call it in *The Answer*. Oh, no, Woke today means something very different no matter how much Wokers may seek to hide that and insist Old Woke and New

Woke are the same. See if you find any 'awareness of social justice' here in the modern variety:

- Woke demands 'inclusivity' while excluding anyone with a different opinion and calls for mass censorship to silence other views.

- Woke claims to stand against oppression when imposing oppression is the foundation of all that it does. It is the driver of political correctness which is nothing more than a Cult invention to manipulate the population to silence itself.

- Woke believes itself to be 'liberal' while pursuing a global society that can only be described as fascist (see 'anti-fascist' fascist Antifa).

- Woke calls for 'social justice' while spreading injustice wherever it goes against the common 'enemy' which can be easily identified as a differing view.

- Woke is supposed to be a metaphor for 'awake' when it is solid-gold asleep and deep in a Cult-induced coma that meets the criteria for 'off with the fairies'.

I state these points as obvious facts if people only care to look. I don't do this with a sense of condemnation. We need to appreciate that the onslaught of perceptual programming on the young has been incessant and merciless. I can understand why so many have been reframed, or, given their youth, framed from the start to see the world as the Cult demands. The Cult has had access to their minds day after day in its 'education' system for their entire formative years. Perception is formed from information received and the Cult-created system is a life-long download of information delivered to elicit a particular perception, thus behaviour. The more this has expanded into still new extremes in recent decades and ever-increasing censorship has deleted other opinions and information why wouldn't that lead to a perceptual reframing on a mass scale? I

have described already cradle-to-grave programming and in more recent times the targeting of young minds from birth to adulthood has entered the stratosphere. This has taken the form of skewing what is 'taught' to fit the Cult agenda and the omnipresent techniques of group-think to isolate non-believers and pressure them into line. There has always been a tendency to follow the herd, but we really are in a new world now in relation to that. We have parents who can see the 'Covid' hoax told by their children not to stop them wearing masks at school, being 'Covid' tested or having the 'vaccine' in fear of the peer-pressure consequences of being different. What is 'peer-pressure' if not pressure to conform to group-think? Renegade Minds never group-think and always retain a set of perceptions that are unique to them. Group-think is always underpinned by consequences for not group-thinking. Abuse now aimed at those refusing DNA-manipulating 'Covid vaccines' are a potent example of this. The biggest pressure to conform comes from the very group which is itself being manipulated. 'I am programmed to be part of a hive mind and so you must be.'

Woke control structures in 'education' now apply to every mainstream organisation. Those at the top of the 'education' hierarchy (the Cult) decide the policy. This is imposed on governments through the Cult network; governments impose it on schools, colleges and universities; their leadership impose the policy on teachers and academics and they impose it on children and students. At any level where there is resistance, perhaps from a teacher or university lecturer, they are targeted by the authorities and often fired. Students themselves regularly demand the dismissal of academics (increasingly few) at odds with the narrative that the students have been programmed to believe in. It is quite a thought that students who are being targeted by the Cult become so consumed by programmed group-think that they launch protests and demand the removal of those who are trying to push back against those targeting the students. Such is the scale of perceptual inversion. We see this with 'Covid' programming as the Cult imposes the rules via psycho-psychologists and governments on

shops, transport companies and businesses which impose them on their staff who impose them on their customers who pressure Pushbackers to conform to the will of the Cult which is in the process of destroying them and their families. Scan all aspects of society and you will see the same sequence every time.

Fact free Woke and hijacking the 'left'

There is no more potent example of this than 'Woke', a mentality only made possible by the deletion of factual evidence by an 'education' system seeking to produce an ever more uniform society. Why would you bother with facts when you don't know any? Deletion of credible history both in volume and type is highly relevant. Orwell said: 'Who controls the past controls the future: who controls the present controls the past.' They who control the perception of the past control the perception of the future and they who control the present control the perception of the past through the writing and deleting of history. Why would you oppose the imposition of Marxism in the name of Wokeism when you don't know that Marxism cost at least 100 million lives in the 20th century alone? Watch videos and read reports in which Woker generations are asked basic historical questions – it's mind-blowing. A survey of 2,000 people found that six percent of millennials (born approximately early1980s to early 2000s) believed the Second World War (1939-1945) broke out with the assassination of President Kennedy (in 1963) and one in ten thought Margaret Thatcher was British Prime Minister at the time. She was in office between 1979 and 1990. We are in a post-fact society. Provable facts are no defence against the fascism of political correctness or Silicon Valley censorship. Facts don't matter anymore as we have witnessed with the 'Covid' hoax. Sacrificing uniqueness to the Woke group-think religion is all you are required to do and that means thinking for yourself is the biggest Woke no, no. All religions are an expression of group-think and censorship and Woke is just another religion with an orthodoxy defended by group-think and censorship. Burned at

the stake becomes burned on Twitter which leads back eventually to burned at the stake as Woke humanity regresses to ages past.

The biggest Woke inversion of all is its creators and funders. I grew up in a traditional left of centre political household on a council estate in Leicester in the 1950s and 60s – you know, the left that challenged the power of wealth-hoarding elites and threats to freedom of speech and opinion. In those days students went on marches defending freedom of speech while today's Wokers march for its deletion. What on earth could have happened? Those very elites (collectively the Cult) that we opposed in my youth and early life have funded into existence the antithesis of that former left and hijacked the 'brand' while inverting everything it ever stood for. We have a mentality that calls itself 'liberal' and 'progressive' while acting like fascists. Cult billionaires and their corporations have funded themselves into control of 'education' to ensure that Woke programming is unceasing throughout the formative years of children and young people and that non-Wokers are isolated (that word again) whether they be students, teachers or college professors. The Cult has funded into existence the now colossal global network of Woke organisations that have spawned and promoted all the 'causes' on the Cult wish-list for global transformation and turned Wokers into demanders of them. Does anyone really think it's a coincidence that the Cult agenda for humanity is a carbon (sorry) copy of the societal transformations desired by Woke?? These are only some of them:

Political correctness: The means by which the Cult deletes all public debates that it knows it cannot win if we had the free-flow of information and evidence.

Human-caused 'climate change': The means by which the Cult seeks to transform society into a globally-controlled dictatorship imposing its will over the fine detail of everyone's lives 'to save the planet' which doesn't actually need saving.

Transgender obsession: Preparing collective perception to accept the 'new human' which would not have genders because it would be created technologically and not through procreation. I'll have much more on this in Human 2.0.

Race obsession: The means by which the Cult seeks to divide and rule the population by triggering racial division through the perception that society is more racist than ever when the opposite is the case. Is it perfect in that regard? No. But to compare today with the racism of apartheid and segregation brought to an end by the civil rights movement in the 1960s is to insult the memory of that movement and inspirations like Martin Luther King. Why is the 'anti-racism' industry (which it is) so dominated by privileged white people?

White supremacy: This is a label used by privileged white people to demonise poor and deprived white people pushing back on tyranny to marginalise and destroy them. White people are being especially targeted as the dominant race by number within Western society which the Cult seeks to transform in its image. If you want to change a society you must weaken and undermine its biggest group and once you have done that by using the other groups you next turn on them to do the same … 'Then they came for the Jews and I was not a Jew so I did nothing.'

Mass migration: The mass movement of people from the Middle East, Africa and Asia into Europe, from the south into the United States and from Asia into Australia are another way the Cult seeks to dilute the racial, cultural and political influence of white people on Western society. White people ask why their governments appear to be working against them while being politically and culturally biased towards incoming cultures. Well, here's your answer. In the same way sexually 'straight' people, men and women, ask why the

authorities are biased against them in favour of other sexualities. The answer is the same – that's the way the Cult wants it to be for very sinister motives.

These are all central parts of the Cult agenda and central parts of the Woke agenda and Woke was created and continues to be funded to an immense degree by Cult billionaires and corporations. If anyone begins to say 'coincidence' the syllables should stick in their throat.

Billionaire 'social justice warriors'

Joe Biden is a 100 percent-owned asset of the Cult and the Wokers' man in the White House whenever he can remember his name and for however long he lasts with his rapidly diminishing cognitive function. Even walking up the steps of an aircraft without falling on his arse would appear to be a challenge. He's not an empty-shell puppet or anything. From the minute Biden took office (or the Cult did) he began his executive orders promoting the Woke wish-list. You will see the Woke agenda imposed ever more severely because it's really the *Cult* agenda. Woke organisations and activist networks spawned by the Cult are funded to the extreme so long as they promote what the Cult wants to happen. Woke is funded to promote 'social justice' by billionaires who become billionaires by destroying social justice. The social justice mantra is only a cover for dismantling social justice and funded by billionaires that couldn't give a damn about social justice. Everything makes sense when you see that. One of Woke's premier funders is Cult billionaire financier George Soros who said: 'I am basically there to make money, I cannot and do not look at the social consequences of what I do.' This is the same Soros who has given more than $32 billion to his Open Society Foundations global Woke network and funded Black Lives Matter, mass immigration into Europe and the United States, transgender activism, climate change activism, political correctness and groups targeting 'white supremacy' in the form of privileged white thugs that dominate Antifa. What a scam it all is and when

you are dealing with the unquestioning fact-free zone of Woke scamming them is child's play. All you need to pull it off in all these organisations are a few in-the-know agents of the Cult and an army of naïve, reframed, uninformed, narcissistic, know-nothings convinced of their own self-righteousness, self-purity and virtue.

Soros and fellow billionaires and billionaire corporations have poured hundreds of millions into Black Lives Matter and connected groups and promoted them to a global audience. None of this is motivated by caring about black people. These are the billionaires that have controlled and exploited a system that leaves millions of black people in abject poverty and deprivation which they do absolutely nothing to address. The same Cult networks funding BLM were behind the *slave trade!* Black Lives Matter hijacked a phrase that few would challenge and they have turned this laudable concept into a political weapon to divide society. You know that BLM is a fraud when it claims that *All* Lives Matter, the most inclusive statement of all, is 'racist'. BLM and its Cult masters don't want to end racism. To them it's a means to an end to control all of humanity never mind the colour, creed, culture or background. What has destroying the nuclear family got to do with ending racism? Nothing – but that is one of the goals of BLM and also happens to be a goal of the Cult as I have been exposing in my books for decades. Stealing children from loving parents and giving schools ever more power to override parents is part of that same agenda. BLM is a Marxist organisation and why would that not be the case when the Cult created Marxism *and* BLM? Patrisse Cullors, a BLM co-founder, said in a 2015 video that she and her fellow organisers, including co-founder Alicia Garza, are 'trained Marxists'. The lady known after marriage as Patrisse Khan-Cullors bought a $1.4 million home in 2021 in one of the whitest areas of California with a black population of just 1.6 per cent and has so far bought *four* high-end homes for a total of $3.2 million. How very Marxist. There must be a bit of spare in the BLM coffers, however, when Cult corporations and billionaires have handed over the best part of $100 million. Many black people can see that Black Lives Matter is not

working for them, but against them, and this is still more confirmation. Black journalist Jason Whitlock, who had his account suspended by Twitter for simply linking to the story about the 'Marxist's' home buying spree, said that BLM leaders are 'making millions of dollars off the backs of these dead black men who they wouldn't spit on if they were on fire and alive'.

Black Lies Matter

Cult assets and agencies came together to promote BLM in the wake of the death of career criminal George Floyd who had been jailed a number of times including for forcing his way into the home of a black woman with others in a raid in which a gun was pointed at her stomach. Floyd was filmed being held in a Minneapolis street in 2020 with the knee of a police officer on his neck and he subsequently died. It was an appalling thing for the officer to do, but the same technique has been used by police on peaceful protestors of lockdown without any outcry from the Woke brigade. As unquestioning supporters of the Cult agenda Wokers have supported lockdown and all the 'Covid' claptrap while attacking anyone standing up to the tyranny imposed in its name. Court documents would later include details of an autopsy on Floyd by County Medical Examiner Dr Andrew Baker who concluded that Floyd had taken a fatal level of the drug fentanyl. None of this mattered to fact-free, question-free, Woke. Floyd's death was followed by worldwide protests against police brutality amid calls to defund the police. Throwing babies out with the bathwater is a Woke speciality. In the wake of the murder of British woman Sarah Everard a Green Party member of the House of Lords, Baroness Jones of Moulescoomb (Nincompoopia would have been better), called for a 6pm curfew for all men. This would be in breach of the Geneva Conventions on war crimes which ban collective punishment, but that would never have crossed the black and white Woke mind of Baroness Nincompoopia who would have been far too convinced of her own self-righteousness to compute such details. Many American cities did defund the police in the face of Floyd riots

and after $15 million was deleted from the police budget in Washington DC under useless Woke mayor Muriel Bowser car-jacking alone rose by 300 percent and within six months the US capital recorded its highest murder rate in 15 years. The same happened in Chicago and other cities in line with the Cult/Soros plan to bring fear to streets and neighbourhoods by reducing the police, releasing violent criminals and not prosecuting crime. This is the mob-rule agenda that I have warned in the books was coming for so long. Shootings in the area of Minneapolis where Floyd was arrested increased by 2,500 percent compared with the year before. Defunding the police over George Floyd has led to a big increase in dead people with many of them black. Police protection for politicians making these decisions stayed the same or increased as you would expect from professional hypocrites. The Cult doesn't actually want to abolish the police. It wants to abolish local control over the police and hand it to federal government as the psychopaths advance the Hunger Games Society. Many George Floyd protests turned into violent riots with black stores and businesses destroyed by fire and looting across America fuelled by Black Lives Matter. Woke doesn't do irony. If you want civil rights you must loot the liquor store and the supermarket and make off with a smart TV. It's the only way.

It's not a race war – it's a class war

Black people are patronised by privileged blacks and whites alike and told they are victims of white supremacy. I find it extraordinary to watch privileged blacks supporting the very system and bloodline networks behind the slave trade and parroting the same Cult-serving manipulative crap of their privileged white, often billionaire, associates. It is indeed not a race war but a class war and colour is just a diversion. Black Senator Cory Booker and black Congresswoman Maxine Waters, more residents of Nincompoopia, personify this. Once you tell people they are victims of someone else you devalue both their own responsibility for their plight and the power they have to impact on their reality and experience. Instead

we have: 'You are only in your situation because of whitey – turn on them and everything will change.' It won't change. Nothing changes in our lives unless *we* change it. Crucial to that is never seeing yourself as a victim and always as the creator of your reality. Life is a simple sequence of choice and consequence. Make different choices and you create different consequences. *You* have to make those choices – not Black Lives Matter, the Woke Mafia and anyone else that seeks to dictate your life. Who are they these Wokers, an emotional and psychological road traffic accident, to tell you what to do? Personal empowerment is the last thing the Cult and its Black Lives Matter want black people or anyone else to have. They claim to be defending the underdog while *creating* and perpetuating the underdog. The Cult's worst nightmare is human unity and if they are going to keep blacks, whites and every other race under economic servitude and control then the focus must be diverted from what they have in common to what they can be manipulated to believe divides them. Blacks have to be told that their poverty and plight is the fault of the white bloke living on the street in the same poverty and with the same plight they are experiencing. The difference is that your plight black people is due to him, a white supremacist with 'white privilege' living on the street. Don't unite as one human family against your mutual oppressors and suppressors – fight the oppressor with the white face who is as financially deprived as you are. The Cult knows that as its 'Covid' agenda moves into still new levels of extremism people are going to respond and it has been spreading the seeds of disunity everywhere to stop a united response to the evil that targets *all of us*.

Racist attacks on 'whiteness' are getting ever more outrageous and especially through the American Democratic Party which has an appalling history for anti-black racism. Barack Obama, Joe Biden, Hillary Clinton and Nancy Pelosi all eulogised about Senator Robert Byrd at his funeral in 2010 after a nearly 60-year career in Congress. Byrd was a brutal Ku Klux Klan racist and a violent abuser of Cathy O'Brien in MKUltra. He said he would never fight in the military 'with a negro by my side' and 'rather I should die a thousand times,

and see Old Glory trampled in the dirt never to rise again, than to see this beloved land of ours become degraded by race mongrels, a throwback to the blackest specimen from the wilds'. Biden called Byrd a 'very close friend and mentor'. These 'Woke' hypocrites are not anti-racist they are anti-poor and anti-people not of their perceived class. Here is an illustration of the scale of anti-white racism to which we have now descended. Seriously Woke and moronic *New York Times* contributor Damon Young described whiteness as a 'virus' that 'like other viruses will not die until there are no bodies left for it to infect'. He went on: '… the only way to stop it is to locate it, isolate it, extract it, and kill it.' Young can say that as a black man with no consequences when a white man saying the same in reverse would be facing a jail sentence. *That's* racism. We had super-Woke numbskull senators Tammy Duckworth and Mazie Hirono saying they would object to future Biden Cabinet appointments if he did not nominate more Asian Americans and Pacific Islanders. Never mind the ability of the candidate what do they look like? Duckworth said: 'I will vote for racial minorities and I will vote for LGBTQ, but anyone else I'm not voting for.' Appointing people on the grounds of race is illegal, but that was not a problem for this ludicrous pair. They were on-message and that's a free pass in any situation.

Critical race racism

White children are told at school they are intrinsically racist as they are taught the divisive 'critical race theory'. This claims that the law and legal institutions are inherently racist and that race is a socially constructed concept used by white people to further their economic and political interests at the expense of people of colour. White is a 'virus' as we've seen. Racial inequality results from 'social, economic, and legal differences that white people create between races to maintain white interests which leads to poverty and criminality in minority communities'. I must tell that to the white guy sleeping on the street. The principal of East Side Community School in New York sent white parents a manifesto that called on

them to become 'white traitors' and advocate for full 'white abolition'. These people are teaching your kids when they urgently need a psychiatrist. The 'school' included a chart with 'eight white identities' that ranged from 'white supremacist' to 'white abolition' and defined the behaviour white people must follow to end 'the regime of whiteness'. Woke blacks and their privileged white associates are acting exactly like the slave owners of old and Ku Klux Klan racists like Robert Byrd. They are too full of their own self-purity to see that, but it's true. Racism is not a body type; it's a state of mind that can manifest through any colour, creed or culture.

Another racial fraud is *'equity'*. Not equality of treatment and opportunity – equity. It's a term spun as equality when it means something very different. Equality in its true sense is a raising up while 'equity' is a race to the bottom. Everyone in the same level of poverty is 'equity'. Keep everyone down – that's equity. The Cult doesn't want anyone in the human family to be empowered and BLM leaders, like all these 'anti-racist' organisations, continue their privileged, pampered existence by perpetuating the perception of gathering racism. When is the last time you heard an 'anti-racist' or 'anti-Semitism' organisation say that acts of racism and discrimination have *fallen?* It's not in the interests of their fund-raising and power to influence and the same goes for the professional soccer anti-racism operation, Kick It Out. Two things confirmed that the Black Lives Matter riots in the summer of 2020 were Cult creations. One was that while anti-lockdown protests were condemned in this same period for 'transmitting 'Covid' the authorities supported mass gatherings of Black Lives Matter supporters. I even saw self-deluding people claiming to be doctors say the two types of protest were not the same. No – the non-existent 'Covid' was in favour of lockdowns and attacked those that protested against them while 'Covid' supported Black Lives Matter and kept well away from its protests. The whole thing was a joke and as lockdown protestors were arrested, often brutally, by reframed Face-Nappies we had the grotesque sight of police officers taking the knee to Black Lives Matter, a Cult-funded Marxist

organisation that supports violent riots and wants to destroy the nuclear family and white people.

He's not white? Shucks!

Woke obsession with race was on display again when ten people were shot dead in Boulder, Colorado, in March, 2021. Cult-owned Woke TV channels like CNN said the shooter appeared to be a white man and Wokers were on Twitter condemning 'violent white men' with the usual mantras. Then the shooter's name was released as Ahmad Al Aliwi Alissa, an anti-Trump Arab-American, and the sigh of disappointment could be heard five miles away. Never mind that ten people were dead and what that meant for their families. Race baiting was all that mattered to these sick Cult-serving people like Barack Obama who exploited the deaths to further divide America on racial grounds which is his job for the Cult. This is the man that 'racist' white Americans made the first black president of the United States and then gave him a second term. Not-very-bright Obama has become filthy rich on the back of that and today appears to have a big influence on the Biden administration. Even so he's still a downtrodden black man and a victim of white supremacy. This disingenuous fraud reveals the contempt he has for black people when he puts on a Deep South Alabama accent whenever he talks to them, no, *at* them.

Another BLM red flag was how the now fully-Woke (fully-Cult) and fully-virtue-signalled professional soccer authorities had their teams taking the knee before every match in support of Marxist Black Lives Matter. Soccer authorities and clubs displayed 'Black Lives Matter' on the players' shirts and flashed the name on electronic billboards around the pitch. Any fans that condemned what is a Freemasonic taking-the-knee ritual were widely condemned as you would expect from the Woke virtue-signallers of professional sport and the now fully-Woke media. We have reverse racism in which you are banned from criticising any race or culture except for white people for whom anything goes – say what you like, no problem. What has this got to do with racial harmony and

equality? We've had black supremacists from Black Lives Matter telling white people to fall to their knees in the street and apologise for their white supremacy. Black supremacists acting like white supremacist slave owners of the past couldn't breach their self-obsessed, race-obsessed sense of self-purity. Joe Biden appointed a race-obsessed black supremacist Kristen Clarke to head the Justice Department Civil Rights Division. Clarke claimed that blacks are endowed with 'greater mental, physical and spiritual abilities' than whites. If anyone reversed that statement they would be vilified. Clarke is on-message so no problem. She's never seen a black-white situation in which the black figure is anything but a virtuous victim and she heads the Civil Rights Division which should treat everyone the same or it isn't civil rights. Another perception of the Renegade Mind: If something or someone is part of the Cult agenda they will be supported by Woke governments and media no matter what. If they're not, they will be condemned and censored. It really is that simple and so racist Clarke prospers despite (make that because of) her racism.

The end of culture

Biden's administration is full of such racial, cultural and economic bias as the Cult requires the human family to be divided into warring factions. We are now seeing racially-segregated graduations and everything, but everything, is defined through the lens of perceived 'racism. We have 'racist' mathematics, 'racist' food and even 'racist' *plants*. World famous Kew Gardens in London said it was changing labels on plants and flowers to tell its pre-'Covid' more than two million visitors a year how racist they are. Kew director Richard Deverell said this was part of an effort to 'move quickly to decolonise collections' after they were approached by one Ajay Chhabra 'an actor with an insight into how sugar cane was linked to slavery'. They are *plants* you idiots. 'Decolonisation' in the Woke manual really means colonisation of society with its mentality and by extension colonisation by the Cult. We are witnessing a new Chinese-style 'Cultural Revolution' so essential to the success of all

Marxist takeovers. Our cultural past and traditions have to be swept away to allow a new culture to be built-back-better. Woke targeting of long-standing Western cultural pillars including historical monuments and cancelling of historical figures is what happened in the Mao revolution in China which 'purged remnants of capitalist and traditional elements from Chinese society' and installed Maoism as the dominant ideology'. For China see the Western world today and for 'dominant ideology' see Woke. Better still see Marxism or Maoism. The 'Covid' hoax has specifically sought to destroy the arts and all elements of Western culture from people meeting in a pub or restaurant to closing theatres, music venues, sports stadiums, places of worship and even banning *singing*. Destruction of Western society is also why criticism of any religion is banned except for Christianity which again is the dominant religion as white is the numerically-dominant race. Christianity may be fading rapidly, but its history and traditions are weaved through the fabric of Western society. Delete the pillars and other structures will follow until the whole thing collapses. I am not a Christian defending that religion when I say that. I have no religion. It's just a fact. To this end Christianity has itself been turned Woke to usher its own downfall and its ranks are awash with 'change agents' – knowing and unknowing – at every level including Pope Francis (*definitely* knowing) and the clueless Archbishop of Canterbury Justin Welby (possibly not, but who can be sure?). Woke seeks to coordinate attacks on Western culture, traditions, and ways of life through 'intersectionality' defined as 'the complex, cumulative way in which the effects of multiple forms of discrimination (such as racism, sexism, and classism) combine, overlap, or intersect especially in the experiences of marginalised individuals or groups'. Wade through the Orwellian Woke-speak and this means coordinating disparate groups in a common cause to overthrow freedom and liberal values.

The entire structure of public institutions has been infested with Woke – government at all levels, political parties, police, military, schools, universities, advertising, media and trade unions. This abomination has been achieved through the Cult web by appointing

Wokers to positions of power and battering non-Wokers into line through intimidation, isolation and threats to their job. Many have been fired in the wake of the empathy-deleted, vicious hostility of 'social justice' Wokers and the desire of gutless, spineless employers to virtue-signal their Wokeness. Corporations are filled with Wokers today, most notably those in Silicon Valley. Ironically at the top they are not Woke at all. They are only exploiting the mentality their Cult masters have created and funded to censor and enslave while the Wokers cheer them on until it's their turn. Thus the Woke 'liberal left' is an inversion of the traditional liberal left. Campaigning for justice on the grounds of power and wealth distribution has been replaced by campaigning for identity politics. The genuine traditional left would never have taken money from today's billionaire abusers of fairness and justice and nor would the billionaires have wanted to fund that genuine left. It would not have been in their interests to do so. The division of opinion in those days was between the haves and have nots. This all changed with Cult manipulated and funded identity politics. The division of opinion today is between Wokers and non-Wokers and not income brackets. Cult corporations and their billionaires may have taken wealth disparity to cataclysmic levels of injustice, but as long as they speak the language of Woke, hand out the dosh to the Woke network and censor the enemy they are 'one of us'. Billionaires who don't give a damn about injustice are laughing at them till their bellies hurt. Wokers are not even close to self-aware enough to see that. The transformed 'left' dynamic means that Wokers who drone on about 'social justice' are funded by billionaires that have destroyed social justice the world over. It's *why* they are billionaires.

The climate con

Nothing encapsulates what I have said more comprehensively than the hoax of human-caused global warming. I have detailed in my books over the years how Cult operatives and organisations were the pump-primers from the start of the climate con. A purpose-built vehicle for this is the Club of Rome established by the Cult in 1968

with the Rockefellers and Rothschilds centrally involved all along. Their gofer frontman Maurice Strong, a Canadian oil millionaire, hosted the Earth Summit in Rio de Janeiro, Brazil, in 1992 where the global 'green movement' really expanded in earnest under the guiding hand of the Cult. The Earth Summit established Agenda 21 through the Cult-created-and-owned United Nations to use the illusion of human-caused climate change to justify the transformation of global society to save the world from climate disaster. It is a No-Problem-Reaction-Solution sold through governments, media, schools and universities as whole generations have been terrified into believing that the world was going to end in their lifetimes unless what old people had inflicted upon them was stopped by a complete restructuring of how everything is done. Chill, kids, it's all a hoax. Such restructuring is precisely what the Cult agenda demands (purely by coincidence of course). Today this has been given the codename of the Great Reset which is only an updated term for Agenda 21 and its associated Agenda 2030. The latter, too, is administered through the UN and was voted into being by the General Assembly in 2015. Both 21 and 2030 seek centralised control of all resources and food right down to the raindrops falling on your own land. These are some of the demands of Agenda 21 established in 1992. See if you recognise this society emerging today:

- End national sovereignty
- State planning and management of all land resources, ecosystems, deserts, forests, mountains, oceans and fresh water; agriculture; rural development; biotechnology; and ensuring *'equity'*
- The state to 'define the role' of business and financial resources
- Abolition of private property
- 'Restructuring' the family unit (see BLM)
- Children raised by the state
- People told what their job will be
- Major restrictions on movement
- Creation of 'human settlement zones'

- Mass resettlement as people are forced to vacate land where they live
- Dumbing down education
- Mass global depopulation in pursuit of all the above

The United Nations was created as a Trojan horse for world government. With the climate con of critical importance to promoting that outcome you would expect the UN to be involved. Oh, it's involved all right. The UN is promoting Agenda 21 and Agenda 2030 justified by 'climate change' while also driving the climate hoax through its Intergovernmental Panel on Climate Change (IPCC), one of the world's most corrupt organisations. The IPCC has been lying ferociously and constantly since the day it opened its doors with the global media hanging unquestioningly on its every mendacious word. The Green movement is entirely Woke and has long lost its original environmental focus since it was co-opted by the Cult. An obsession with 'global warming' has deleted its values and scrambled its head. I experienced a small example of what I mean on a beautiful country walk that I have enjoyed several times a week for many years. The path merged into the fields and forests and you felt at one with the natural world. Then a 'Green' organisation, the Hampshire and Isle of Wight Wildlife Trust, took over part of the land and proceeded to cut down a large number of trees, including mature ones, to install a horrible big, bright steel 'this-is-ours-stay-out' fence that destroyed the whole atmosphere of this beautiful place. No one with a feel for nature would do that. Day after day I walked to the sound of chainsaws and a magnificent mature weeping willow tree that I so admired was cut down at the base of the trunk. When I challenged a Woke young girl in a green shirt (of course) about this vandalism she replied: 'It's a weeping willow – it will grow back.' This is what people are paying for when they donate to the Hampshire and Isle of Wight Wildlife Trust and many other 'green' organisations today. It is not the environmental movement that I knew and instead has become a support-system – as with Extinction Rebellion – for a very dark agenda.

Private jets for climate justice

The Cult-owned, Gates-funded, World Economic Forum and its founder Klaus Schwab were behind the emergence of Greta Thunberg to harness the young behind the climate agenda and she was invited to speak to the world at … the UN. Schwab published a book, *Covid-19: The Great Reset* in 2020 in which he used the 'Covid' hoax and the climate hoax to lay out a new society straight out of Agenda 21 and Agenda 2030. Bill Gates followed in early 2021 when he took time out from destroying the world to produce a book in his name about the way to save it. Gates flies across the world in private jets and admitted that 'I probably have one of the highest greenhouse gas footprints of anyone on the planet … my personal flying alone is gigantic.' He has also bid for the planet's biggest private jet operator. Other climate change saviours who fly in private jets include John Kerry, the US Special Presidential Envoy for Climate, and actor Leonardo DiCaprio, a 'UN Messenger of Peace with special focus on climate change'. These people are so full of bullshit they could corner the market in manure. We mustn't be sceptical, though, because the Gates book, *How to Avoid a Climate Disaster: The Solutions We Have and the Breakthroughs We Need*, is a genuine attempt to protect the world and not an obvious pile of excrement attributed to a mega-psychopath aimed at selling his masters' plans for humanity. The Gates book and the other shite-pile by Klaus Schwab could have been written by the same person and may well have been. Both use 'climate change' and 'Covid' as the excuses for their new society and by coincidence the Cult's World Economic Forum and Bill and Melinda Gates Foundation promote the climate hoax and hosted Event 201 which pre-empted with a 'simulation' the very 'coronavirus' hoax that would be simulated for real on humanity within weeks. The British 'royal' family is promoting the 'Reset' as you would expect through Prince 'climate change caused the war in Syria' Charles and his hapless son Prince William who said that we must 'reset our relationship with nature and our trajectory as a species' to avoid a climate disaster. Amazing how many promotors of the 'Covid' and 'climate change' control

systems are connected to Gates and the World Economic Forum. A 'study' in early 2021 claimed that carbon dioxide emissions must fall by the equivalent of a global lockdown roughly every two years for the next decade to save the planet. The 'study' appeared in the same period that the Schwab mob claimed in a video that lockdowns destroying the lives of billions are good because they make the earth 'quieter' with less 'ambient noise'. They took down the video amid a public backlash for such arrogant, empathy-deleted stupidity You see, however, where they are going with this. Corinne Le Quéré, a professor at the Tyndall Centre for Climate Change Research, University of East Anglia, was lead author of the climate lockdown study, and she writes for … the World Economic Forum. Gates calls in 'his' book for changing 'every aspect of the economy' (long-time Cult agenda) and for humans to eat synthetic 'meat' (predicted in my books) while cows and other farm animals are eliminated. Australian TV host and commentator Alan Jones described what carbon emission targets would mean for farm animals in Australia alone if emissions were reduced as demanded by 35 percent by 2030 and zero by 2050:

Well, let's take agriculture, the total emissions from agriculture are about 75 million tonnes of carbon dioxide, equivalent. Now reduce that by 35 percent and you have to come down to 50 million tonnes, I've done the maths. So if you take for example 1.5 million cows, you're going to have to reduce the herd by 525,000 [by] 2030, nine years, that's 58,000 cows a year. The beef herd's 30 million, reduce that by 35 percent, that's 10.5 million, which means 1.2 million cattle have to go every year between now and 2030. This is insanity!

There are 75 million sheep. Reduce that by 35 percent, that's 26 million sheep, that's almost 3 million a year. So under the Paris Agreement over 30 million beasts. dairy cows, cattle, pigs and sheep would go. More than 8,000 every minute of every hour for the next decade, do these people know what they're talking about?

Clearly they don't at the level of campaigners, politicians and administrators. The Cult *does* know; that's the outcome it wants. We are faced with not just a war on humanity. Animals and the natural world are being targeted and I have been saying since the 'Covid' hoax began that the plan eventually was to claim that the 'deadly virus' is able to jump from animals, including farm animals and

domestic pets, to humans. Just before this book went into production came this story: 'Russia registers world's first Covid-19 vaccine for cats & dogs as makers of Sputnik V warn pets & farm animals could spread virus'. The report said 'top scientists warned that the deadly pathogen could soon begin spreading through homes and farms' and 'the next stage is the infection of farm and domestic animals'. Know the outcome and you'll see the journey. Think what that would mean for animals and keep your eye on a term called zoonosis or zoonotic diseases which transmit between animals and humans. The Cult wants to break the connection between animals and people as it does between people and people. Farm animals fit with the Cult agenda to transform food from natural to synthetic.

The gas of life is killing us

There can be few greater examples of Cult inversion than the condemnation of carbon dioxide as a dangerous pollutant when it is the gas of life. Without it the natural world would be dead and so we would all be dead. We breathe in oxygen and breathe out carbon dioxide while plants produce oxygen and absorb carbon dioxide. It is a perfect symbiotic relationship that the Cult wants to dismantle for reasons I will come to in the final two chapters. Gates, Schwab, other Cult operatives and mindless repeaters, want the world to be 'carbon neutral' by at least 2050 and the earlier the better. 'Zero carbon' is the cry echoed by lunatics calling for 'Zero Covid' when we already have it. These carbon emission targets will deindustrialise the world in accordance with Cult plans – the post-industrial, post-democratic society – and with so-called renewables like solar and wind not coming even close to meeting human energy needs blackouts and cold are inevitable. Texans got the picture in the winter of 2021 when a snow storm stopped wind turbines and solar panels from working and the lights went down along with water which relies on electricity for its supply system. Gates wants everything to be powered by electricity to ensure that his masters have the kill switch to stop all human activity, movement, cooking, water and warmth any time they like. The climate lie is so

stupendously inverted that it claims we must urgently reduce carbon dioxide when we *don't have enough*.

Co2 in the atmosphere is a little above 400 parts per million when the optimum for plant growth is 2,000 ppm and when it falls anywhere near 150 ppm the natural world starts to die and so do we. It fell to as low as 280 ppm in an 1880 measurement in Hawaii and rose to 413 ppm in 2019 with industrialisation which is why the planet has become *greener* in the industrial period. How insane then that psychopathic madman Gates is not satisfied only with blocking the rise of Co2. He's funding technology to suck it out of the atmosphere. The reason why will become clear. The industrial era is not destroying the world through Co2 and has instead turned around a potentially disastrous ongoing fall in Co2. Greenpeace co-founder and scientist Patrick Moore walked away from Greenpeace in 1986 and has exposed the green movement for fear-mongering and lies. He said that 500 million years ago there was *17 times* more Co2 in the atmosphere than we have today and levels have been falling for hundreds of millions of years. In the last 150 million years Co2 levels in Earth's atmosphere had reduced by *90 percent*. Moore said that by the time humanity began to unlock carbon dioxide from fossil fuels we were at '38 seconds to midnight' and in that sense: 'Humans are [the Earth's] salvation.' Moore made the point that only half the Co2 emitted by fossil fuels stays in the atmosphere and we should remember that all pollution pouring from chimneys that we are told is carbon dioxide is in fact nothing of the kind. It's pollution. Carbon dioxide is an invisible gas.

William Happer, Professor of Physics at Princeton University and long-time government adviser on climate, has emphasised the Co2 deficiency for maximum growth and food production. Greenhouse growers don't add carbon dioxide for a bit of fun. He said that most of the warming in the last 100 years, after the earth emerged from the super-cold period of the 'Little Ice Age' into a natural warming cycle, was over by 1940. Happer said that a peak year for warming in 1988 can be explained by a 'monster El Nino' which is a natural and cyclical warming of the Pacific that has nothing to do with 'climate

change'. He said the effect of Co2 could be compared to painting a wall with red paint in that once two or three coats have been applied it didn't matter how much more you slapped on because the wall will not get much redder. Almost all the effect of the rise in Co2 has already happened, he said, and the volume in the atmosphere would now have to *double* to increase temperature by a single degree. Climate hoaxers know this and they have invented the most ridiculously complicated series of 'feedback' loops to try to overcome this rather devastating fact. You hear puppet Greta going on cluelessly about feedback loops and this is why.

The Sun affects temperature? No you *climate denier*

Some other nonsense to contemplate: Climate graphs show that rises in temperature do not follow rises in Co2 – *it's the other way round* with a lag between the two of some 800 years. If we go back 800 years from present time we hit the Medieval Warm Period when temperatures were higher than now without any industrialisation and this was followed by the Little Ice Age when temperatures plummeted. The world was still emerging from these centuries of serious cold when many climate records began which makes the ever-repeated line of the 'hottest year since records began' meaningless when you are not comparing like with like. The coldest period of the Little Ice Age corresponded with the lowest period of sunspot activity when the Sun was at its least active. Proper scientists will not be at all surprised by this when it confirms the obvious fact that earth temperature is affected by the scale of Sun activity and the energetic power that it subsequently emits; but when is the last time you heard a climate hoaxer talking about the Sun as a source of earth temperature?? Everything has to be focussed on Co2 which makes up just 0.117 percent of so-called greenhouse gases and only a fraction of even that is generated by human activity. The rest is natural. More than *90 percent* of those greenhouse gases are water vapour and clouds (Fig 9). Ban moisture I say. Have you noticed that the climate hoaxers no longer use the polar bear as their promotion image? That's because far from becoming extinct polar

bear communities are stable or thriving. Joe Bastardi, American meteorologist, weather forecaster and outspoken critic of the climate lie, documents in his book *The Climate Chronicles* how weather patterns and events claimed to be evidence of climate change have been happening since long before industrialisation: 'What happened before naturally is happening again, as is to be expected given the cyclical nature of the climate due to the design of the planet.' If you read the detailed background to the climate hoax in my other books you will shake your head and wonder how anyone could believe the crap which has spawned a multi-trillion dollar industry based on absolute garbage (see HIV causes AIDs and Sars-Cov-2 causes 'Covid-19'). Climate and 'Covid' have much in common given they have the same source. They both have the contradictory *everything* factor in which everything is explained by reference to them. It's hot – 'it's climate change'. It's cold – 'it's climate change'. I got a sniffle – 'it's Covid'. I haven't got a sniffle – 'it's Covid'. Not having a sniffle has to be a symptom of 'Covid'. Everything is and not having a sniffle is especially dangerous if you are a slow walker. For sheer audacity I offer you a Cambridge University 'study' that actually linked 'Covid' to 'climate change'. It had to happen eventually. They concluded that climate change played a role in 'Covid-19' spreading from animals to humans because … wait for it … I kid you not … *the two groups were forced closer together as populations grow*. Er, that's it. The whole foundation on which this depended was that 'Bats are the likely zoonotic origin of SARS-CoV-1 and SARS-CoV-2'. Well, they are not. They are nothing to do with it. Apart from bats not being the origin and therefore 'climate change' effects on bats being irrelevant I am in awe of their academic insight. Where would we be without them? Not where we are that's for sure.

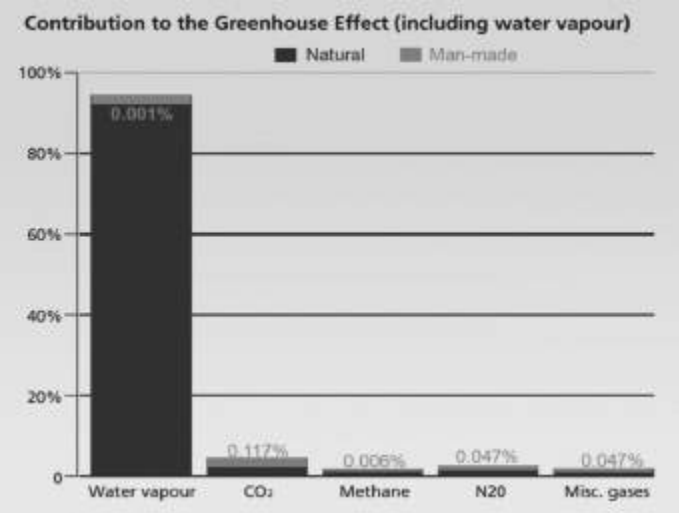

Figure 9: The idea that the gas of life is disastrously changing the climate is an insult to brain cell activity.

One other point about the weather is that climate modification is now well advanced and not every major weather event is natural – or earthquake come to that. I cover this subject at some length in other books. China is openly planning a rapid expansion of its weather modification programme which includes changing the climate in an area more than one and a half times the size of India. China used weather manipulation to ensure clear skies during the 2008 Olympics in Beijing. I have quoted from US military documents detailing how to employ weather manipulation as a weapon of war and they did that in the 1960s and 70s during the conflict in Vietnam with Operation Popeye manipulating monsoon rains for military purposes. Why would there be international treaties on weather modification if it wasn't possible? Of course it is. Weather is energetic information and it can be changed.

How was the climate hoax pulled off? See 'Covid'

If you can get billions to believe in a 'virus' that doesn't exist you can get them to believe in human-caused climate change that doesn't exist. Both are being used by the Cult to transform global society in the way it has long planned. Both hoaxes have been achieved in pretty much the same way. First you declare a lie is a fact. There's a

'virus' you call SARS-Cov-2 or humans are warming the planet with their behaviour. Next this becomes, via Cult networks, the foundation of government, academic and science policy and belief. Those who parrot the mantra are given big grants to produce research that confirms the narrative is true and ever more 'symptoms' are added to make the 'virus'/'climate change' sound even more scary. Scientists and researchers who challenge the narrative have their grants withdrawn and their careers destroyed. The media promote the lie as the unquestionable truth and censor those with an alternative view or evidence. A great percentage of the population believe what they are told as the lie becomes an everybody-knows-that and the believing-masses turn on those with a mind of their own. The technique has been used endlessly throughout human history. Wokers are the biggest promotors of the climate lie *and* 'Covid' fascism because their minds are owned by the Cult; their sense of self-righteous self-purity knows no bounds; and they exist in a bubble of reality in which facts are irrelevant and only get in the way of looking without seeing.

Running through all of this like veins in a blue cheese is control of information, which means control of perception, which means control of behaviour, which collectively means control of human society. The Cult owns the global media and Silicon Valley fascists for the simple reason that it *has* to. Without control of information it can't control perception and through that human society. Examine every facet of the Cult agenda and you will see that anything supporting its introduction is never censored while anything pushing back is always censored. I say again: Psychopaths that know why they are doing this must go before Nuremberg trials and those that follow their orders must trot along behind them into the same dock. 'I was just following orders' didn't work the first time and it must not work now. Nuremberg trials must be held all over the world before public juries for politicians, government officials, police, compliant doctors, scientists and virologists, and all Cult operatives such as Gates, Tedros, Fauci, Vallance, Whitty, Ferguson, Zuckerberg, Wojcicki, Brin, Page, Dorsey, the whole damn lot of

them – including, no *especially*, the psychopath psychologists. Without them and the brainless, gutless excuses for journalists that have repeated their lies, none of this could be happening. Nobody can be allowed to escape justice for the psychological and economic Armageddon they are all responsible for visiting upon the human race.

As for the compliant, unquestioning, swathes of humanity, and the self-obsessed, all-knowing ignorance of the Wokers … don't start me. God help their kids. God help their grandkids. God *help them*.

CHAPTER NINE

We must have it? So what is it?

Well I won't back down. No, I won't back down. You can stand me up at the Gates of Hell. But I won't back down
Tom Petty

I will now focus on the genetically-manipulating 'Covid vaccines' which do not meet this official definition of a vaccine by the US Centers for Disease Control (CDC): 'A product that stimulates a person's immune system to produce immunity to a specific disease, protecting the person from that disease.' On that basis 'Covid vaccines' are not a vaccine in that the makers don't even claim they stop infection or transmission.

They are instead part of a multi-levelled conspiracy to change the nature of the human body and what it means to be 'human' and to depopulate an enormous swathe of humanity. What I shall call Human 1.0 is on the cusp of becoming Human 2.0 and for very sinister reasons. Before I get to the 'Covid vaccine' in detail here's some background to vaccines in general. Government regulators do not test vaccines – the makers do – and the makers control which data is revealed and which isn't. Children in America are given 50 vaccine doses by age six and 69 by age 19 and the effect of the whole combined schedule has never been tested. Autoimmune diseases when the immune system attacks its own body have soared in the mass vaccine era and so has disease in general in children and the young. Why wouldn't this be the case when vaccines target the *immune system?* The US government gave Big Pharma drug

companies immunity from prosecution for vaccine death and injury in the 1986 National Childhood Vaccine Injury Act (NCVIA) and since then the government (taxpayer) has been funding compensation for the consequences of Big Pharma vaccines. The criminal and satanic drug giants can't lose and the vaccine schedule has increased dramatically since 1986 for this reason. There is no incentive to make vaccines safe and a big incentive to make money by introducing ever more. Even against a ridiculously high bar to prove vaccine liability, and with the government controlling the hearing in which it is being challenged for compensation, the vaccine court has so far paid out more than $4 billion. These are the vaccines we are told are safe and psychopaths like Zuckerberg censor posts saying otherwise. The immunity law was even justified by a ruling that vaccines by their nature were 'unavoidably unsafe'.

Check out the ingredients of vaccines and you will be shocked if you are new to this. *They put that in children's bodies?? What??* Try aluminium, a brain toxin connected to dementia, aborted foetal tissue and formaldehyde which is used to embalm corpses. World-renowned aluminium expert Christopher Exley had his research into the health effect of aluminium in vaccines shut down by Keele University in the UK when it began taking funding from the Bill and Melinda Gates Foundation. Research when diseases 'eradicated' by vaccines began to decline and you will find the fall began long *before* the vaccine was introduced. Sometimes the fall even plateaued after the vaccine. Diseases like scarlet fever for which there was no vaccine declined in the same way because of environmental and other factors. A perfect case in point is the polio vaccine. Polio began when lead arsenate was first sprayed as an insecticide and residues remained in food products. Spraying started in 1892 and the first US polio epidemic came in Vermont in 1894. The simple answer was to stop spraying, but Rockefeller-created Big Pharma had a better idea. Polio was decreed to be caused by the *poliovirus* which 'spreads from person to person and can infect a person's spinal cord'. Lead arsenate was replaced by the lethal DDT which had the same effect of causing paralysis by damaging the brain and central nervous

system. Polio plummeted when DDT was reduced and then banned, but the vaccine is still given the credit for something it didn't do. Today by far the biggest cause of polio is the vaccines promoted by Bill Gates. Vaccine justice campaigner Robert Kennedy Jr, son of assassinated (by the Cult) US Attorney General Robert Kennedy, wrote:

> In 2017, the World Health Organization (WHO) reluctantly admitted that the global explosion in polio is predominantly vaccine strain. The most frightening epidemics in Congo, Afghanistan, and the Philippines, are all linked to vaccines. In fact, by 2018, 70% of global polio cases were vaccine strain.

Vaccines make fortunes for Cult-owned Gates and Big Pharma while undermining the health and immune systems of the population. We had a glimpse of the mentality behind the Big Pharma cartel with a report on WION (World is One News), an international English language TV station based in India, which exposed the extraordinary behaviour of US drug company Pfizer over its 'Covid vaccine'. The WION report told how Pfizer had made fantastic demands of Argentina, Brazil and other countries in return for its 'vaccine'. These included immunity from prosecution, even for Pfizer negligence, government insurance to protect Pfizer from law suits and handing over as collateral sovereign assets of the country to include Argentina's bank reserves, military bases and embassy buildings. Pfizer demanded the same of Brazil in the form of waiving sovereignty of its assets abroad; exempting Pfizer from Brazilian laws; and giving Pfizer immunity from all civil liability. This is a 'vaccine' developed with government funding. Big Pharma is evil incarnate as a creation of the Cult and all must be handed tickets to Nuremberg.

Phantom 'vaccine' for a phantom 'disease'

I'll expose the 'Covid vaccine' fraud and then go on to the wider background of why the Cult has set out to 'vaccinate' every man, woman and child on the planet for an alleged 'new disease' with a survival rate of 99.77 percent (or more) even by the grotesquely-

manipulated figures of the World Health Organization and Johns Hopkins University. The 'infection' to 'death' ratio is 0.23 to 0.15 percent according to Stanford epidemiologist Dr John Ioannidis and while estimates vary the danger remains tiny. I say that if the truth be told the fake infection to fake death ratio is zero. Never mind all the evidence I have presented here and in *The Answer* that there is no 'virus' let us just focus for a moment on that death-rate figure of say 0.23 percent. The figure includes all those worldwide who have tested positive with a test not testing for the 'virus' and then died within 28 days or even longer of any other cause – *any other cause*. Now subtract all those illusory 'Covid' deaths on the global data sheets from the 0.23 percent. What do you think you would be left with? *Zero*. A vaccination has never been successfully developed for a so-called coronavirus. They have all failed at the animal testing stage when they caused hypersensitivity to what they were claiming to protect against and made the impact of a disease far worse. Cult-owned vaccine corporations got around that problem this time by bypassing animal trials, going straight to humans and making the length of the 'trials' before the public rollout as short as they could get away with. Normally it takes five to ten years or more to develop vaccines that still cause demonstrable harm to many people and that's without including the long-term effects that are never officially connected to the vaccination. 'Covid' non-vaccines have been officially produced and approved in a matter of months from a standing start and part of the reason is that (a) they were developed before the 'Covid' hoax began and (b) they are based on computer programs and not natural sources. Official non-trials were so short that government agencies gave *emergency*, not full, approval. 'Trials' were not even completed and full approval cannot be secured until they are. Public 'Covid vaccination' is actually a *continuation of the trial*. Drug company 'trials' are not scheduled to end until 2023 by which time a lot of people are going to be dead. Data on which government agencies gave this emergency approval was supplied by the Big Pharma corporations themselves in the form of Pfizer/BioNTech, AstraZeneca, Moderna, Johnson & Johnson, and

others, and this is the case with all vaccines. By its very nature *emergency* approval means drug companies do not have to prove that the 'vaccine' is 'safe and effective'. How could they with trials way short of complete? Government regulators only have to *believe* that they *could* be safe and effective. It is criminal manipulation to get products in circulation with no testing worth the name. Agencies giving that approval are infested with Big Pharma-connected place-people and they act in the interests of Big Pharma (the Cult) and not the public about whom they do not give a damn.

More human lab rats

'Covid vaccines' produced in record time by Pfizer/BioNTech and Moderna employ a technique *never approved before for use on humans*. They are known as mRNA 'vaccines' and inject a synthetic version of 'viral' mRNA or 'messenger RNA'. The key is in the term 'messenger'. The body works, or doesn't, on the basis of information messaging. Communications are constantly passing between and within the genetic system and the brain. Change those messages and you change the state of the body and even its very nature and you can change psychology and behaviour by the way the brain processes information. I think you are going to see significant changes in personality and perception of many people who have had the 'Covid vaccine' synthetic potions. Insider Aldous Huxley predicted the following in 1961 and mRNA 'vaccines' can be included in the term 'pharmacological methods':

> There will be, in the next generation or so, a pharmacological method of making people love their servitude, and producing dictatorship without tears, so to speak, producing a kind of painless concentration camp for entire societies, so that people will in fact have their own liberties taken away from them, but rather enjoy it, because they will be distracted from any desire to rebel by propaganda or brainwashing, or brainwashing enhanced by pharmacological methods. And this seems to be the final revolution.

Apologists claim that mRNA synthetic 'vaccines' don't change the DNA genetic blueprint because RNA does not affect DNA only the other way round. This is so disingenuous. A process called 'reverse

transcription' can convert RNA into DNA and be integrated into DNA in the cell nucleus. This was highlighted in December, 2020, by scientists at Harvard and Massachusetts Institute of Technology (MIT). Geneticists report that more than 40 percent of mammalian genomes results from reverse transcription. On the most basic level if messaging changes then that sequence must lead to changes in DNA which is receiving and transmitting those communications. How can introducing synthetic material into cells not change the cells where DNA is located? The process is known as transfection which is defined as 'a technique to insert foreign nucleic acid (DNA or RNA) into a cell, typically with the intention of altering the properties of the cell'. Researchers at the Sloan Kettering Institute in New York found that changes in messenger RNA can deactivate tumour-suppressing proteins and thereby promote cancer. This is what happens when you mess with messaging. 'Covid vaccine' maker Moderna was founded in 2010 by Canadian stem cell biologist Derrick J. Rossi after his breakthrough discovery in the field of transforming and reprogramming stem cells. These are neutral cells that can be programmed to become any cell including sperm cells. Moderna was therefore founded on the principle of genetic manipulation and has never produced any vaccine or drug before its genetically-manipulating synthetic 'Covid' shite. Look at the name – Mode-RNA or Modify-RNA. Another important point is that the US Supreme Court has ruled that genetically-modified DNA, or complementary DNA (cDNA) synthesized in the laboratory from messenger RNA, can be patented and owned. These psychopaths are doing this to the human body.

Cells replicate synthetic mRNA in the 'Covid vaccines' and in theory the body is tricked into making antigens which trigger antibodies to target the 'virus spike proteins' which as Dr Tom Cowan said have *never been seen*. Cut the crap and these 'vaccines' deliver *self-replicating* synthetic material to the cells with the effect of changing human DNA. The more of them you have the more that process is compounded while synthetic material is all the time self-replicating. 'Vaccine'-maker Moderna describes mRNA as 'like

software for the cell' and so they are messing with the body's software. What happens when you change the software in a computer? Everything changes. For this reason the Cult is preparing a production line of mRNA 'Covid vaccines' and a long list of excuses to use them as with all the 'variants' of a 'virus' never shown to exist. The plan is further to transfer the mRNA technique to other vaccines mostly given to children and young people. The cumulative consequences will be a transformation of human DNA through a constant infusion of synthetic genetic material which will kill many and change the rest. Now consider that governments that have given emergency approval for a vaccine that's not a vaccine; never been approved for humans before; had no testing worth the name; and the makers have been given immunity from prosecution for any deaths or adverse effects suffered by the public. The UK government awarded *permanent legal indemnity* to itself and its employees for harm done when a patient is being treated for 'Covid-19' or 'suspected Covid-19'. That is quite a thought when these are possible 'side-effects' from the 'vaccine' (they are not 'side', they are effects) listed by the US Food and Drug Administration:

Guillain-Barre syndrome; acute disseminated encephalomyelitis; transverse myelitis; encephalitis; myelitis; encephalomyelitis; meningoencephalitis; meningitis; encephalopathy; convulsions; seizures; stroke; narcolepsy; cataplexy; anaphylaxis; acute myocardial infarction (heart attack); myocarditis; pericarditis; autoimmune disease; death; implications for pregnancy, and birth outcomes; other acute demyelinating diseases; non anaphylactic allergy reactions; thrombocytopenia ; disseminated intravascular coagulation; venous thromboembolism; arthritis; arthralgia; joint pain; Kawasaki disease; multisystem inflammatory syndrome in children; vaccine enhanced disease. The latter is the way the 'vaccine' has the potential to make diseases far worse than they would otherwise be.

UK doctor and freedom campaigner Vernon Coleman described the conditions in this list as 'all unpleasant, most of them very serious, and you can't get more serious than death'. The thought that anyone at all has had the 'vaccine' in these circumstances is testament to the potential that humanity has for clueless, unquestioning, stupidity and for many that programmed stupidity has already been terminal.

An insider speaks

Dr Michael Yeadon is a former Vice President, head of research and Chief Scientific Adviser at vaccine giant Pfizer. Yeadon worked on the inside of Big Pharma, but that did not stop him becoming a vocal critic of 'Covid vaccines' and their potential for multiple harms, including infertility in women. By the spring of 2021 he went much further and even used the no, no, term 'conspiracy'. When you begin to see what is going on it is impossible not to do so. Yeadon spoke out in an interview with freedom campaigner James Delingpole and I mentioned earlier how he said that no one had samples of 'the virus'. He explained that the mRNA technique originated in the anti-cancer field and ways to turn on and off certain genes which could be advantageous if you wanted to stop cancer growing out of control. 'That's the origin of them. They are a very unusual application, really.' Yeadon said that treating a cancer patient with an aggressive procedure might be understandable if the alternative was dying, but it was quite another thing to use the same technique as a public health measure. Most people involved wouldn't catch the infectious agent you were vaccinating against and if they did they probably wouldn't die:

> If you are really using it as a public health measure you really want to as close as you can get to zero sides-effects … I find it odd that they chose techniques that were really cutting their teeth in the field of oncology and I'm worried that in using gene-based vaccines that have to be injected in the body and spread around the body, get taken up into some cells, and the regulators haven't quite told us which cells they get taken up into … you are going to be generating a wide range of responses … with multiple steps each of which could go well or badly.

I doubt the Cult intends it to go well. Yeadon said that you can put any gene you like into the body through the 'vaccine'. 'You can certainly give them a gene that would do them some harm if you wanted.' I was intrigued when he said that when used in the cancer field the technique could turn genes on and off. I explore this process in *The Answer* and with different genes having different functions you could create mayhem – physically and psychologically – if you turned the wrong ones on and the right ones off. I read reports of an experiment by researchers at the University of Washington's school of computer science and engineering in which they encoded DNA to infect computers. The body is itself a biological computer and if human DNA can inflict damage on a computer why can't the computer via synthetic material mess with the human body? It can. The Washington research team said it was possible to insert malicious malware into 'physical DNA strands' and corrupt the computer system of a gene sequencing machine as it 'reads gene letters and stores them as binary digits 0 and 1'. They concluded that hackers could one day use blood or spit samples to access computer systems and obtain sensitive data from police forensics labs or infect genome files. It is at this level of digital interaction that synthetic 'vaccines' need to be seen to get the full picture and that will become very clear later on. Michael Yeadon said it made no sense to give the 'vaccine' to younger people who were in no danger from the 'virus'. What was the benefit? It was all downside with potential effects:

> The fact that my government in what I thought was a civilised, rational country, is raining [the 'vaccine'] on people in their 30s and 40s, even my children in their 20s, they're getting letters and phone calls, I know this is not right and any of you doctors who are vaccinating you know it's not right, too. They are not at risk. They are not at risk from the disease, so you are now hoping that the side-effects are so rare that you get away with it. You don't give new technology … that you don't understand to 100 percent of the population.

Blood clot problems with the AstraZeneca 'vaccine' have been affecting younger people to emphasise the downside risks with no benefit. AstraZeneca's version, produced with Oxford University, does not use mRNA, but still gets its toxic cocktail inside cells where

it targets DNA. The Johnson & Johnson 'vaccine' which uses a similar technique has also produced blood clot effects to such an extent that the United States paused its use at one point. They are all 'gene therapy' (cell modification) procedures and not 'vaccines'. The truth is that once the content of these injections enter cells we have no idea what the effect will be. People can speculate and some can give very educated opinions and that's good. In the end, though, only the makers know what their potions are designed to do and even they won't know every last consequence. Michael Yeadon was scathing about doctors doing what they knew to be wrong. 'Everyone's mute', he said. Doctors in the NHS must know this was not right, coming into work and injecting people. 'I don't know how they sleep at night. I know I couldn't do it. I know that if I were in that position I'd have to quit.' He said he knew enough about toxicology to know this was not a good risk-benefit. Yeadon had spoken to seven or eight university professors and all except two would not speak out publicly. Their universities had a policy that no one said anything that countered the government and its medical advisors. They were afraid of losing their government grants. This is how intimidation has been used to silence the truth at every level of the system. I say silence, but these people could still speak out if they made that choice. Yeadon called them 'moral cowards' – 'This is about your children and grandchildren's lives and you have just buggered off and left it.'

'Variant' nonsense

Some of his most powerful comments related to the alleged 'variants' being used to instil more fear, justify more lockdowns, and introduce more 'vaccines'. He said government claims about 'variants' were nonsense. He had checked the alleged variant 'codes' and they were 99.7 percent identical to the 'original'. This was the human identity difference equivalent to putting a baseball cap on and off or wearing it the other way round. A 0.3 percent difference would make it impossible for that 'variant' to escape immunity from the 'original'. This made no sense of having new 'vaccines' for

'variants'. He said there would have to be at least a *30 percent* difference for that to be justified and even then he believed the immune system would still recognise what it was. Gates-funded 'variant modeller' and 'vaccine'-pusher John Edmunds might care to comment. Yeadon said drug companies were making new versions of the 'vaccine' as a 'top up' for 'variants'. Worse than that, he said, the 'regulators' around the world like the MHRA in the UK had got together and agreed that because 'vaccines' for 'variants' were so similar to the first 'vaccines' *they did not have to do safety studies*. How transparently sinister that is. This is when Yeadon said: 'There is a conspiracy here.' There was no need for another vaccine for 'variants' and yet we were told that there was and the country had shut its borders because of them. 'They are going into hundreds of millions of arms without passing 'go' or any regulator. Why did they do that? Why did they pick this method of making the vaccine?'

The reason had to be something bigger than that it seemed and 'it's not protection against the virus'. It's was a far bigger project that meant politicians and advisers were willing to do things and not do things that knowingly resulted in avoidable deaths – 'that's already happened when you think about lockdown and deprivation of health care for a year.' He spoke of people prepared to do something that results in the avoidable death of their fellow human beings and it not bother them. This is the penny-drop I have been working to get across for more than 30 years – the level of pure evil we are dealing with. Yeadon said his friends and associates could not believe there could be that much evil, but he reminded them of Stalin, Pol Pot and Hitler and of what Stalin had said: 'One death is a tragedy. A million? A statistic.' He could not think of a benign explanation for why you need top-up vaccines 'which I'm sure you don't' and for the regulators 'to just get out of the way and wave them through'. Why would the regulators do that when they were still wrestling with the dangers of the 'parent' vaccine? He was clearly shocked by what he had seen since the 'Covid' hoax began and now he was thinking the previously unthinkable:

If you wanted to depopulate a significant proportion of the world and to do it in a way that doesn't involve destruction of the environment with nuclear weapons, poisoning everyone with anthrax or something like that, and you wanted plausible deniability while you had a multi-year infectious disease crisis, I actually don't think you could come up with a better plan of work than seems to be in front of me. I can't say that's what they are going to do, but I can't think of a benign explanation why they are doing it.

He said he never thought that they would get rid of 99 percent of humans, but now he wondered. 'If you wanted to that this would be a hell of a way to do it – it would be unstoppable folks.' Yeadon had concluded that those who submitted to the 'vaccine' would be allowed to have some kind of normal life (but for how long?) while screws were tightened to coerce and mandate the last few percent. 'I think they'll put the rest of them in a prison camp. I wish I was wrong, but I don't think I am.' Other points he made included: There were no coronavirus vaccines then suddenly they all come along at the same time; we have no idea of the long term affect with trials so short; coercing or forcing people to have medical procedures is against the Nuremberg Code instigated when the Nazis did just that; people should at least delay having the 'vaccine'; a quick Internet search confirms that masks don't reduce respiratory viral transmission and 'the government knows that'; they have smashed civil society and they know that, too; two dozen peer-reviewed studies show no connection between lockdown and reducing deaths; he knew from personal friends the elite were still flying around and going on holiday while the public were locked down; the elite were not having the 'vaccines'. He was also asked if 'vaccines' could be made to target difference races. He said he didn't know, but the document by the Project for the New American Century in September, 2000, said developing 'advanced forms of biological warfare that can target *specific genotypes* may transform biological warfare from the realm of terror to a politically useful tool.' Oh, they're evil all right. Of that we can be *absolutely* sure.

Another cull of old people

We have seen from the CDC definition that the mRNA 'Covid vaccine' is not a vaccine and nor are the others that *claim* to reduce 'severity of symptoms' in *some* people, but not protect from infection or transmission. What about all the lies about returning to 'normal' if people were 'vaccinated'? If they are not claimed to stop infection and transmission of the alleged 'virus', how does anything change? This was all lies to manipulate people to take the jabs and we are seeing that now with masks and distancing still required for the 'vaccinated'. How did they think that elderly people with fragile health and immune responses were going to be affected by infusing their cells with synthetic material and other toxic substances? They *knew* that in the short and long term it would be devastating and fatal as the culling of the old that began with the first lockdowns was continued with the 'vaccine'. Death rates in care homes soared immediately residents began to be 'vaccinated' – infused with synthetic material. Brave and committed whistleblower nurses put their careers at risk by exposing this truth while the rest kept their heads down and their mouths shut to put their careers before those they are supposed to care for. A long-time American Certified Nursing Assistant who gave his name as James posted a video in which he described emotionally what happened in his care home when vaccination began. He said that during 2020 very few residents were sick with 'Covid' and no one died during the entire year; but shortly after the Pfizer mRNA injections 14 people died within two weeks and many others were near death. 'They're dropping like flies', he said. Residents who walked on their own before the shot could no longer and they had lost their ability to conduct an intelligent conversation. The home's management said the sudden deaths were caused by a 'super-spreader' of 'Covid-19'. Then how come, James asked, that residents who refused to take the injections were not sick? It was a case of inject the elderly with mRNA synthetic potions and blame their illness and death that followed on the 'virus'. James described what was happening in care homes as 'the greatest crime of genocide this country has ever seen'. Remember the NHS staff nurse from earlier who used the same

word ‘genocide’ for what was happening with the ‘vaccines’ and that it was an ‘act of human annihilation’. A UK care home whistleblower told a similar story to James about the effect of the ‘vaccine’ in deaths and ‘outbreaks’ of illness dubbed ‘Covid’ after getting the jab. She told how her care home management and staff had zealously imposed government regulations and no one was allowed to even question the official narrative let alone speak out against it. She said the NHS was even worse. Again we see the results of reframing. A worker at a local care home where I live said they had not had a single case of ‘Covid’ there for almost a year and when the residents were ‘vaccinated’ they had 19 positive cases in two weeks with eight dying.

It’s not the ‘vaccine’ – honest

The obvious cause and effect was being ignored by the media and most of the public. Australia’s health minister Greg Hunt (a former head of strategy at the World Economic Forum) was admitted to hospital after he had the ‘vaccine’. He was suffering according to reports from the skin infection ‘cellulitis’ and it must have been a severe case to have warranted days in hospital. Immediately the authorities said this was nothing to do with the ‘vaccine’ when an effect of some vaccines is a ‘cellulitis-like reaction’. We had families of perfectly healthy old people who died after the ‘vaccine’ saying that if only they had been given the ‘vaccine’ earlier they would still be alive. As a numbskull rating that is off the chart. A father of four ‘died of Covid’ at aged 48 when he was taken ill two days after having the ‘vaccine’. The man, a health administrator, had been ‘shielding during the pandemic’ and had ‘not really left the house’ until he went for the ‘vaccine’. Having the ‘vaccine’ and then falling ill and dying does not seem to have qualified as a possible cause and effect and ‘Covid-19’ went on his death certificate. His family said they had no idea how he ‘caught the virus’. A family member said: ‘Tragically, it could be that going for a vaccination ultimately led to him catching Covid …The sad truth is that they are never going to know where it came from.’ The family warned people to remember

that the virus still existed and was 'very real'. So was their stupidity. Nurses and doctors who had the first round of the 'vaccine' were collapsing, dying and ending up in a hospital bed while they or their grieving relatives were saying they'd still have the 'vaccine' again despite what happened. I kid you not. You mean if your husband returned from the dead he'd have the same 'vaccine' again that killed him??

Doctors at the VCU Medical Center in Richmond, Virginia, said the Johnson & Johnson 'vaccine' was to blame for a man's skin peeling off. Patient Richard Terrell said: 'It all just happened so fast. My skin peeled off. It's still coming off on my hands now.' He said it was stinging, burning and itching and when he bent his arms and legs it was very painful with 'the skin swollen and rubbing against itself'. Pfizer/BioNTech and Moderna vaccines use mRNA to change the cell while the Johnson & Johnson version uses DNA in a process similar to AstraZeneca's technique. Johnson & Johnson and AstraZeneca have both had their 'vaccines' paused by many countries after causing serious blood problems. Terrell's doctor Fnu Nutan said he could have died if he hadn't got medical attention. It sounds terrible so what did Nutan and Terrell say about the 'vaccine' now? Oh, they still recommend that people have it. A nurse in a hospital bed 40 minutes after the vaccination and unable to swallow due to throat swelling was told by a doctor that he lost mobility in his arm for 36 hours following the vaccination. What did he say to the ailing nurse? 'Good for you for getting the vaccination.' We are dealing with a serious form of cognitive dissonance madness in both public and medical staff. There is a remarkable correlation between those having the 'vaccine' and trumpeting the fact and suffering bad happenings shortly afterwards. Witold Rogiewicz, a Polish doctor, made a video of his 'vaccination' and ridiculed those who were questioning its safety and the intentions of Bill Gates: 'Vaccinate yourself to protect yourself, your loved ones, friends and also patients. And to mention quickly I have info for anti-vaxxers and anti-Coviders if you want to contact Bill Gates you can do this through me.' He further ridiculed the dangers of 5G. Days later he

was dead, but naturally the vaccination wasn't mentioned in the verdict of 'heart attack'.

Lies, lies and more lies

So many members of the human race have slipped into extreme states of insanity and unfortunately they include reframed doctors and nursing staff. Having a 'vaccine' and dying within minutes or hours is not considered a valid connection while death from any cause within 28 days or longer of a positive test with a test not testing for the 'virus' means 'Covid-19' goes on the death certificate. How could that 'vaccine'-death connection not have been made except by calculated deceit? US figures in the initial rollout period to February 12th, 2020, revealed that a third of the deaths reported to the CDC after 'Covid vaccines' happened within 48 hours. Five men in the UK suffered an 'extremely rare' blood clot problem after having the AstraZeneca 'vaccine', but no causal link was established said the Gates-funded Medicines and Healthcare products Regulatory Agency (MHRA) which had given the 'vaccine' emergency approval to be used. Former Pfizer executive Dr Michael Yeadon explained in his interview how the procedures could cause blood coagulation and clots. People who should have been at no risk were dying from blood clots in the brain and he said he had heard from medical doctor friends that people were suffering from skin bleeding and massive headaches. The AstraZeneca 'shot' was stopped by some 20 countries over the blood clotting issue and still the corrupt MHRA, the European Medicines Agency (EMA) and the World Health Organization said that it should continue to be given even though the EMA admitted that it 'still cannot rule out definitively' a link between blood clotting and the 'vaccine'. Later Marco Cavaleri, head of EMA vaccine strategy, said there was indeed a clear link between the 'vaccine' and thrombosis, but they didn't know why. So much for the trials showing the 'vaccine' is safe. Blood clots were affecting younger people who would be under virtually no danger from 'Covid' even if it existed which makes it all the more stupid and sinister.

The British government responded to public alarm by wheeling out June Raine, the terrifyingly weak infant school headmistress sound-alike who heads the UK MHRA drug 'regulator'. The idea that she would stand up to Big Pharma and government pressure is laughable and she told us that all was well in the same way that she did when allowing untested, never-used-on-humans-before, genetically-manipulating 'vaccines' to be exposed to the public in the first place. Mass lying is the new normal of the 'Covid' era. The MHRA later said 30 cases of rare blood clots had by then been connected with the AstraZeneca 'vaccine' (that means a lot more in reality) while stressing that the benefits of the jab in preventing 'Covid-19' outweighed any risks. A more ridiculous and disingenuous statement with callous disregard for human health it is hard to contemplate. Immediately after the mendacious 'all-clears' two hospital workers in Denmark experienced blood clots and cerebral haemorrhaging following the AstraZeneca jab and one died. Top Norwegian health official Pål Andre Holme said the 'vaccine' was the only common factor: 'There is nothing in the patient history of these individuals that can give such a powerful immune response … I am confident that the antibodies that we have found are the cause, and I see no other explanation than it being the vaccine which triggers it.' Strokes, a clot or bleed in the brain, were clearly associated with the 'vaccine' from word of mouth and whistleblower reports. Similar consequences followed with all these 'vaccines' that we were told were so safe and as the numbers grew by the day it was clear we were witnessing human carnage.

Learning the hard way

A woman interviewed by UKColumn told how her husband suffered dramatic health effects after the vaccine when he'd been in good health all his life. He went from being a little unwell to losing all feeling in his legs and experiencing 'excruciating pain'. Misdiagnosis followed twice at Accident and Emergency (an 'allergy' and 'sciatica') before he was admitted to a neurology ward where doctors said his serious condition had been caused by the

'vaccine'. Another seven 'vaccinated' people were apparently being treated on the same ward for similar symptoms. The woman said he had the 'vaccine' because they believed media claims that it was safe. 'I didn't think the government would give out a vaccine that does this to somebody; I believed they would be bringing out a vaccination that would be safe.' What a tragic way to learn that lesson. Another woman posted that her husband was transporting stroke patients to hospital on almost every shift and when he asked them if they had been 'vaccinated' for 'Covid' they all replied 'yes'. One had a 'massive brain bleed' the day after his second dose. She said her husband reported the 'just been vaccinated' information every time to doctors in A and E only for them to ignore it, make no notes and appear annoyed that it was even mentioned. This particular report cannot be verified, but it expresses a common theme that confirms the monumental underreporting of 'vaccine' consequences. Interestingly as the 'vaccines' and their brain blood clot/stroke consequences began to emerge the UK National Health Service began a publicity campaign telling the public what to do in the event of a stroke. A Scottish NHS staff nurse who quit in disgust in March, 2021, said:

I have seen traumatic injuries from the vaccine, they're not getting reported to the yellow card [adverse reaction] scheme, they're treating the symptoms, not asking why, why it's happening. It's just treating the symptoms and when you speak about it you're dismissed like you're crazy, I'm not crazy, I'm not crazy because every other colleague I've spoken to is terrified to speak out, they've had enough.

Videos appeared on the Internet of people uncontrollably shaking after the 'vaccine' with no control over muscles, limbs and even their face. A Scottish mother broke out in a severe rash all over her body almost immediately after she was given the AstraZeneca 'vaccine'. The pictures were horrific. Leigh King, a 41-year-old hairdresser from Lanarkshire said: 'Never in my life was I prepared for what I was about to experience … My skin was so sore and constantly hot … I have never felt pain like this …' But don't you worry, the 'vaccine' is perfectly safe. Then there has been the effect on medical

staff who have been pressured to have the 'vaccine' by psychopathic 'health' authorities and government. A London hospital consultant who gave the name K. Polyakova wrote this to the *British Medical Journal* or *BMJ*:

> I am currently struggling with … the failure to report the reality of the morbidity caused by our current vaccination program within the health service and staff population. The levels of sickness after vaccination is unprecedented and staff are getting very sick and some with neurological symptoms which is having a huge impact on the health service function. Even the young and healthy are off for days, some for weeks, and some requiring medical treatment. Whole teams are being taken out as they went to get vaccinated together.
>
> Mandatory vaccination in this instance is stupid, unethical and irresponsible when it comes to protecting our staff and public health. We are in the voluntary phase of vaccination, and encouraging staff to take an unlicensed product that is impacting on their immediate health … it is clearly stated that these vaccine products do not offer immunity or stop transmission. In which case why are we doing it?

Not to protect health that's for sure. Medical workers are lauded by governments for agenda reasons when they couldn't give a toss about them any more than they can for the population in general. Schools across America faced the same situation as they closed due to the high number of teachers and other staff with bad reactions to the Pfizer/BioNTech, Moderna, and Johnson & Johnson 'Covid vaccines' all of which were linked to death and serious adverse effects. The *BMJ* took down the consultant's comments pretty quickly on the grounds that they were being used to spread 'disinformation'. They were exposing the truth about the 'vaccine' was the real reason. The cover-up is breathtaking.

Hiding the evidence

The scale of the 'vaccine' death cover-up worldwide can be confirmed by comparing official figures with the personal experience of the public. I heard of many people in my community who died immediately or soon after the vaccine that would never appear in the media or even likely on the official totals of 'vaccine' fatalities and adverse reactions when only about ten percent are estimated to be

reported and I have seen some estimates as low as one percent in a Harvard study. In the UK alone by April 29th, 2021, some 757,654 adverse reactions had been officially reported from the Pfizer/BioNTech, Oxford/AstraZeneca and Moderna 'vaccines' with more than a thousand deaths linked to jabs and that means an estimated ten times this number in reality from a ten percent reporting rate percentage. That's seven million adverse reactions and 10,000 potential deaths and a one percent reporting rate would be ten times *those* figures. In 1976 the US government pulled the swine flu vaccine after *53* deaths. The UK data included a combined 10,000 eye disorders from the 'Covid vaccines' with more than 750 suffering visual impairment or blindness and again multiply by the estimated reporting percentages. As 'Covid cases' officially fell hospitals virtually empty during the 'Covid crisis' began to fill up with a range of other problems in the wake of the 'vaccine' rollout. The numbers across America have also been catastrophic. Deaths linked to *all* types of vaccine increased by *6,000 percent* in the first quarter of 2021 compared with 2020. A 39-year-old woman from Ogden, Utah, died four days after receiving a second dose of Moderna's 'Covid vaccine' when her liver, heart and kidneys all failed despite the fact that she had no known medical issues or conditions. Her family sought an autopsy, but Dr Erik Christensen, Utah's chief medical examiner, said proving vaccine injury as a cause of death almost never happened. He could think of only one instance where an autopsy would name a vaccine as the official cause of death and that would be anaphylaxis where someone received a vaccine and died almost instantaneously. 'Short of that, it would be difficult for us to definitively say this is the vaccine,' Christensen said. If that is true this must be added to the estimated ten percent (or far less) reporting rate of vaccine deaths and serious reactions and the conclusion can only be that vaccine deaths and serious reactions – including these 'Covid' potions' – are phenomenally understated in official figures. The same story can be found everywhere. Endless accounts of deaths and serious reactions among the public, medical

and care home staff while official figures did not even begin to reflect this.

Professional script-reader Dr David Williams, a 'top public-health official' in Ontario, Canada, insulted our intelligence by claiming only four serious adverse reactions and no deaths from the more than 380,000 vaccine doses then given. This bore no resemblance to what people knew had happened in their owns circles and we had Dirk Huyer in charge of getting millions vaccinated in Ontario while at the same time he was Chief Coroner for the province investigating causes of death including possible death from the vaccine. An aide said he had stepped back from investigating deaths, but evidence indicated otherwise. Rosemary Frei, who secured a Master of Science degree in molecular biology at the Faculty of Medicine at Canada's University of Calgary before turning to investigative journalism, was one who could see that official figures for 'vaccine' deaths and reactions made no sense. She said that doctors seldom reported adverse events and when people got really sick or died after getting a vaccination they would attribute that to anything except the vaccines. It had been that way for years and anyone who wondered aloud whether the 'Covid vaccines' or other shots cause harm is immediately branded as 'anti-vax' and 'anti-science'. This was 'career-threatening' for health professionals. Then there was the huge pressure to support the push to 'vaccinate' billions in the quickest time possible. Frei said:

> So that's where we're at today. More than half a million vaccine doses have been given to people in Ontario alone. The rush is on to vaccinate all 15 million of us in the province by September. And the mainstream media are screaming for this to be sped up even more. That all adds up to only a very slim likelihood that we're going to be told the truth by officials about how many people are getting sick or dying from the vaccines.

What is true of Ontario is true of everywhere.

They KNEW – and still did it

The authorities knew what was going to happen with multiple deaths and adverse reactions. The UK government's Gates-funded

and Big Pharma-dominated Medicines and Healthcare products Regulatory Agency (MHRA) hired a company to employ AI in compiling the projected reactions to the 'vaccine' that would otherwise be uncountable. The request for applications said: 'The MHRA urgently seeks an Artificial Intelligence (AI) software tool to process the expected high volume of Covid-19 vaccine Adverse Drug Reaction …' This was from the agency, headed by the disingenuous June Raine, that gave the 'vaccines' emergency approval and the company was hired before the first shot was given. 'We are going to kill and maim you – is that okay?' 'Oh, yes, perfectly fine – I'm very grateful, thank you, doctor.' The range of 'Covid vaccine' adverse reactions goes on for page after page in the MHRA criminally underreported 'Yellow Card' system and includes affects to eyes, ears, skin, digestion, blood and so on. Raine's MHRA amazingly claimed that the 'overall safety experience … is so far as expected from the clinical trials'. The death, serious adverse effects, deafness and blindness were *expected?* When did they ever mention that? If these human tragedies were expected then those that gave approval for the use of these 'vaccines' must be guilty of crimes against humanity including murder – a definition of which is 'killing a person with malice aforethought or with recklessness manifesting extreme indifference to the value of human life.' People involved at the MHRA, the CDC in America and their equivalent around the world must go before Nuremberg trials to answer for their callous inhumanity. We are only talking here about the immediate effects of the 'vaccine'. The longer-term impact of the DNA synthetic manipulation is the main reason they are so hysterically desperate to inoculate the entire global population in the shortest possible time.

Africa and the developing world are a major focus for the 'vaccine' depopulation agenda and a mass vaccination sales-pitch is underway thanks to caring people like the Rockefellers and other Cult assets. The Rockefeller Foundation, which pre-empted the 'Covid pandemic' in a document published in 2010 that 'predicted' what happened a decade later, announced an initial $34.95 million grant in February, 2021, 'to ensure more equitable access to Covid-19

testing and vaccines' among other things in Africa in collaboration with '24 organizations, businesses, and government agencies'. The pan-Africa initiative would focus on 10 countries: Burkina Faso, Ethiopia, Ghana, Kenya, Nigeria, Rwanda, South Africa, Tanzania, Uganda, and Zambia'. Rajiv Shah, President of the Rockefeller Foundation and former administrator of CIA-controlled USAID, said that if Africa was not mass-vaccinated (to change the DNA of its people) it was a 'threat to all of humanity' and not fair on Africans. When someone from the Rockefeller Foundation says they want to do something to help poor and deprived people and countries it is time for a belly-laugh. They are doing this out of the goodness of their 'heart' because 'vaccinating' the entire global population is what the 'Covid' hoax set out to achieve. Official 'decolonisation' of Africa by the Cult was merely a prelude to financial colonisation on the road to a return to physical colonisation. The 'vaccine' is vital to that and the sudden and convenient death of the 'Covid' sceptic president of Tanzania can be seen in its true light. A lot of people in Africa are aware that this is another form of colonisation and exploitation and they need to stand their ground.

The 'vaccine is working' scam

A potential problem for the Cult was that the 'vaccine' is meant to change human DNA and body messaging and not to protect anyone from a 'virus' never shown to exist. The vaccine couldn't work because it was not designed to work and how could they make it *appear* to be working so that more people would have it? This was overcome by lowering the amplification rate of the PCR test to produce fewer 'cases' and therefore fewer 'deaths'. Some of us had been pointing out since March, 2020, that the amplification rate of the test not testing for the 'virus' had been made artificially high to generate positive tests which they could call 'cases' to justify lockdowns. The World Health Organization recommended an absurdly high 45 amplification cycles to ensure the high positives required by the Cult and then remained silent on the issue until January 20th, 2021 – Biden's Inauguration Day. This was when the

'vaccinations' were seriously underway and on that day the WHO recommended after discussions with America's CDC that laboratories *lowered their testing amplification*. Dr David Samadi, a certified urologist and health writer, said the WHO was encouraging all labs to reduce their cycle count for PCR tests. He said the current cycle was much too high and was 'resulting in any particle being declared a positive case'. Even one mainstream news report I saw said this meant the number of 'Covid' infections may have been 'dramatically inflated'. Oh, just a little bit. The CDC in America issued new guidance to laboratories in April, 2021, to use 28 cycles *but only for 'vaccinated' people*. The timing of the CDC/WHO interventions were cynically designed to make it appear the 'vaccines' were responsible for falling cases and deaths when the real reason can be seen in the following examples. New York's state lab, the Wadsworth Center, identified 872 positive tests in July, 2020, based on a threshold of 40 cycles. When the figure was lowered to 35 cycles *43 percent* of the 872 were no longer 'positives'. At 30 cycles the figure was 63 percent. A Massachusetts lab found that between *85 to 90 percent* of people who tested positive in July with a cycle threshold of 40 would be negative at 30 cycles, Ashish Jha, MD, director of the Harvard Global Health Institute, said: 'I'm really shocked that it could be that high … Boy, does it really change the way we need to be thinking about testing.' I'm shocked that I could see the obvious in the spring of 2020, with no medical background, and most medical professionals still haven't worked it out. No, that's not shocking – it's terrifying.

Three weeks after the WHO directive to lower PCR cycles the London *Daily Mail* ran this headline: 'Why ARE Covid cases plummeting? New infections have fallen 45% in the US and 30% globally in the past 3 weeks but experts say vaccine is NOT the main driver because only 8% of Americans and 13% of people worldwide have received their first dose.' They acknowledged that the drop could not be attributed to the 'vaccine', but soon this morphed throughout the media into the 'vaccine' has caused cases and deaths to fall when it was the PCR threshold. In December, 2020, there was

chaos at English Channel ports with truck drivers needing negative 'Covid' tests before they could board a ferry home for Christmas. The government wanted to remove the backlog as fast as possible and they brought in troops to do the 'testing'. Out of 1,600 drivers just *36* tested positive and the rest were given the all clear to cross the Channel. I guess the authorities thought that 36 was the least they could get away with without the unquestioning catching on. The amplification trick which most people believed in the absence of information in the mainstream applied more pressure on those refusing the 'vaccine' to succumb when it 'obviously worked'. The truth was the exact opposite with deaths in care homes soaring with the 'vaccine' and in Israel the term used was 'skyrocket'. A re-analysis of published data from the Israeli Health Ministry led by Dr Hervé Seligmann at the Medicine Emerging Infectious and Tropical Diseases at Aix-Marseille University found that Pfizer's 'Covid vaccine' killed 'about 40 times more [elderly] people than the disease itself would have killed' during a five-week vaccination period and *260 times* more younger people than would have died from the 'virus' even according to the manipulated 'virus' figures. Dr Seligmann and his co-study author, Haim Yativ, declared after reviewing the Israeli 'vaccine' death data: 'This is a new Holocaust.'

Then, in mid-April, 2021, after vast numbers of people worldwide had been 'vaccinated', the story changed with clear coordination. The UK government began to prepare the ground for more future lockdowns when Nuremberg-destined Boris Johnson told yet another whopper. He said that cases had fallen because of *lockdowns* not 'vaccines'. Lockdowns are irrelevant when *there is no 'virus'* and the test and fraudulent death certificates are deciding the number of 'cases' and 'deaths'. Study after study has shown that lockdowns don't work and instead kill and psychologically destroy people. Meanwhile in the United States Anthony Fauci and Rochelle Walensky, the ultra-Zionist head of the CDC, peddled the same line. More lockdown was the answer and not the 'vaccine', a line repeated on cue by the moron that is Canadian Prime Minister Justin Trudeau. Why all the hysteria to get everyone 'vaccinated' if lockdowns and

not 'vaccines' made the difference? None of it makes sense on the face of it. Oh, but it does. The Cult wants lockdowns *and* the 'vaccine' and if the 'vaccine' is allowed to be seen as the total answer lockdowns would no longer be justified when there are still livelihoods to destroy. 'Variants' and renewed upward manipulation of PCR amplification are planned to instigate never-ending lockdown *and* more 'vaccines'.

You *must* have it – we're desperate

Israel, where the Jewish and Arab population are ruled by the Sabbatian Cult, was the front-runner in imposing the DNA-manipulating 'vaccine' on its people to such an extent that Jewish refusers began to liken what was happening to the early years of Nazi Germany. This would seem to be a fantastic claim. Why would a government of Jewish people be acting like the Nazis did? If you realise that the Sabbatian Cult was behind the Nazis and that Sabbatians hate Jews the pieces start to fit and the question of why a 'Jewish' government would treat Jews with such callous disregard for their lives and freedom finds an answer. Those controlling the government of Israel *aren't Jewish* – they're Sabbatian. Israeli lawyer Tamir Turgal was one who made the Nazi comparison in comments to German lawyer Reiner Fuellmich who is leading a class action lawsuit against the psychopaths for crimes against humanity. Turgal described how the Israeli government was vaccinating children and pregnant women on the basis that there was no evidence that this was dangerous when they had no evidence that it *wasn't* dangerous either. They just had no evidence. This was medical experimentation and Turgal said this breached the Nuremberg Code about medical experimentation and procedures requiring informed consent and choice. Think about that. A Nuremberg Code developed because of Nazi experimentation on Jews and others in concentration camps by people like the evil-beyond-belief Josef Mengele is being breached by the *Israeli* government; but when you know that it's a *Sabbatian* government along with its intelligence and military agencies like Mossad, Shin Bet and the Israeli Defense Forces, and that Sabbatians

were the force behind the Nazis, the kaleidoscope comes into focus. What have we come to when Israeli Jews are suing their government for violating the Nuremberg Code by essentially making Israelis subject to a medical experiment using the controversial 'vaccines'? It's a shocker that this has to be done in the light of what happened in Nazi Germany. The Anshe Ha-Emet, or 'People of the Truth', made up of Israeli doctors, lawyers, campaigners and public, have launched a lawsuit with the International Criminal Court. It says:

> When the heads of the Ministry of Health as well as the prime minister presented the vaccine in Israel and began the vaccination of Israeli residents, the vaccinated were not advised, that, in practice, they are taking part in a medical experiment and that their consent is required for this under the Nuremberg Code.

The irony is unbelievable, but easily explained in one word: Sabbatians. The foundation of Israeli 'Covid' apartheid is the 'green pass' or 'green passport' which allows Jews and Arabs who have had the DNA-manipulating 'vaccine' to go about their lives – to work, fly, travel in general, go to shopping malls, bars, restaurants, hotels, concerts, gyms, swimming pools, theatres and sports venues, while non-'vaccinated' are banned from all those places and activities. Israelis have likened the 'green pass' to the yellow stars that Jews in Nazi Germany were forced to wear – the same as the yellow stickers that a branch of UK supermarket chain Morrisons told exempt mask-wears they had to display when shopping. How very sensitive. The Israeli system is blatant South African-style apartheid on the basis of compliance or non-compliance to fascism rather than colour of the skin. How appropriate that the Sabbatian Israeli government was so close to the pre-Mandela apartheid regime in Pretoria. The Sabbatian-instigated 'vaccine passport' in Israel is planned for everywhere. Sabbatians struck a deal with Pfizer that allowed them to lead the way in the percentage of a national population infused with synthetic material and the result was catastrophic. Israeli freedom activist Shai Dannon told me how chairs were appearing on beaches that said 'vaccinated only'. Health Minister Yuli Edelstein said that anyone unwilling or unable to get

the jabs that 'confer immunity' will be 'left behind'. The man's a liar. Not even the makers claim the 'vaccines' confer immunity. When you see those figures of 'vaccine' deaths these psychopaths were saying that you must take the chance the 'vaccine' will kill you or maim you while knowing it will change your DNA or lockdown for you will be permanent. That's fascism. The Israeli parliament passed a law to allow personal information of the non-vaccinated to be shared with local and national authorities for three months. This was claimed by its supporters to be a way to 'encourage' people to be vaccinated. Hadas Ziv from Physicians for Human Rights described this as a 'draconian law which crushed medical ethics and the patient rights'. But that's the idea, the Sabbatians would reply.

Your papers, please

Sabbatian Israel was leading what has been planned all along to be a global 'vaccine pass' called a 'green passport' without which you would remain in permanent lockdown restriction and unable to do anything. This is how badly – *desperately* – the Cult is to get everyone 'vaccinated'. The term and colour 'green' was not by chance and related to the psychology of fusing the perception of the green climate hoax with the 'Covid' hoax and how the 'solution' to both is the same Great Reset. Lying politicians, health officials and psychologists denied there were any plans for mandatory vaccinations or restrictions based on vaccinations, but they knew that was exactly what was meant to happen with governments of all countries reaching agreements to enforce a global system. 'Free' Denmark and 'free' Sweden unveiled digital vaccine certification. Cyprus, Czech Republic, Estonia, Greece, Hungary, Iceland, Italy, Poland, Portugal, Slovakia, and Spain have all committed to a vaccine passport system and the rest including the whole of the EU would follow. The satanic UK government will certainly go this way despite mendacious denials and at the time of writing it is trying to manipulate the public into having the 'vaccine' so they could go abroad on a summer holiday. How would that work without something to prove you had the synthetic toxicity injected into you?

Documents show that the EU's European Commission was moving towards 'vaccine certificates' in 2018 and 2019 before the 'Covid' hoax began. They knew what was coming. Abracadabra – Ursula von der Leyen, the German President of the Commission, announced in March, 2021, an EU 'Digital Green Certificate' – green again – to track the public's 'Covid status'. The passport sting is worldwide and the Far East followed the same pattern with South Korea ruling that only those with 'vaccination' passports – again the *green* pass – would be able to 'return to their daily lives'.

Bill Gates has been preparing for this 'passport' with other Cult operatives for years and beyond the paper version is a Gates-funded 'digital tattoo' to identify who has been vaccinated and who hasn't. The 'tattoo' is reported to include a substance which is externally readable to confirm who has been vaccinated. This is a bio-luminous light-generating enzyme (think fireflies) called … *Luciferase*. Yes, named after the Cult 'god' Lucifer the 'light bringer' of whom more to come. Gates said he funded the readable tattoo to ensure children in the developing world were vaccinated and no one was missed out. He cares so much about poor kids as we know. This was just the cover story to develop a vaccine tagging system for everyone on the planet. Gates has been funding the ID2020 'alliance' to do just that in league with other lovely people at Microsoft, GAVI, the Rockefeller Foundation, Accenture and IDEO.org. He said in interviews in March, 2020, before any 'vaccine' publicly existed, that the world must have a globalised digital certificate to track the 'virus' and who had been vaccinated. Gates knew from the start that the mRNA vaccines were coming and when they would come and that the plan was to tag the 'vaccinated' to marginalise the intelligent and stop them doing anything including travel. Evil just doesn't suffice. Gates was exposed for offering a $10 million bribe to the Nigerian House of Representatives to invoke compulsory 'Covid' vaccination of all Nigerians. Sara Cunial, a member of the Italian Parliament, called Gates a 'vaccine criminal'. She urged the Italian President to hand him over to the International Criminal Court for crimes against

humanity and condemned his plans to 'chip the human race' through ID2020.

You know it's a long-planned agenda when war criminal and Cult gofer Tony Blair is on the case. With the scale of arrogance only someone as dark as Blair can muster he said: 'Vaccination in the end is going to be your route to liberty.' Blair is a disgusting piece of work and he confirms that again. The media has given a lot of coverage to a bloke called Charlie Mullins, founder of London's biggest independent plumbing company, Pimlico Plumbers, who has said he won't employ anyone who has not been vaccinated or have them go to any home where people are not vaccinated. He said that if he had his way no one would be allowed to walk the streets if they have not been vaccinated. Gates was cheering at the time while I was alerting the white coats. The plan is that people will qualify for 'passports' for having the first two doses and then to keep it they will have to have all the follow ups and new ones for invented 'variants' until human genetics is transformed and many are dead who can't adjust to the changes. Hollywood celebrities – the usual propaganda stunt – are promoting something called the WELL Health-Safety Rating to verify that a building or space has 'taken the necessary steps to prioritize the health and safety of their staff, visitors and other stakeholders'. They included Lady Gaga, Jennifer Lopez, Michael B. Jordan, Robert DeNiro, Venus Williams, Wolfgang Puck, Deepak Chopra and 17th Surgeon General Richard Carmona. Yawn. WELL Health-Safety has big connections with China. Parent company Delos is headed by former Goldman Sachs partner Paul Scialla. This is another example – and we will see so many others – of using the excuse of 'health' to dictate the lives and activities of the population. I guess one confirmation of the 'safety' of buildings is that only 'vaccinated' people can go in, right?

Electronic concentration camps

I wrote decades ago about the plans to restrict travel and here we are for those who refuse to bow to tyranny. This can be achieved in one go with air travel if the aviation industry makes a blanket decree.

The ‘vaccine’ and guaranteed income are designed to be part of a global version of China’s social credit system which tracks behaviour 24/7 and awards or deletes ‘credits’ based on whether your behaviour is supported by the state or not. I mean your entire lifestyle – what you do, eat, say, everything. Once your credit score falls below a certain level consequences kick in. In China tens of millions have been denied travel by air and train because of this. All the locations and activities denied to refusers by the ‘vaccine’ passports will be included in one big mass ban on doing almost anything for those that don’t bow their head to government. It’s beyond fascist and a new term is required to describe its extremes – I guess fascist technocracy will have to do. The way the Chinese system of technological – technocratic – control is sweeping the West can be seen in the Los Angeles school system and is planned to be expanded worldwide. Every child is required to have a ‘Covid’-tracking app scanned daily before they can enter the classroom. The so-called Daily Pass tracking system is produced by Gates’ Microsoft which I’m sure will shock you rigid. The pass will be scanned using a barcode (one step from an inside-the-body barcode) and the information will include health checks, ‘Covid’ tests and vaccinations. Entry codes are for one specific building only and access will only be allowed if a student or teacher has a negative test with a test not testing for the ‘virus’, has no symptoms of anything alleged to be related to ‘Covid’ (symptoms from a range of other illness), and has a temperature under 100 degrees. No barcode, no entry, is planned to be the case for everywhere and not only schools.

Kids are being psychologically prepared to accept this as ‘normal’ their whole life which is why what they can impose in schools is so important to the Cult and its gofers. Long-time American freedom campaigner John Whitehead of the Rutherford Institute was not exaggerating when he said: ‘Databit by databit, we are building our own electronic concentration camps.’ Canada under its Cult gofer prime minister Justin Trudeau has taken a major step towards the real thing with people interned against their will if they test positive with a test not testing for the ‘virus’ when they arrive at a Canadian

airport. They are jailed in internment hotels often without food or water for long periods and with many doors failing to lock there have been sexual assaults. The interned are being charged sometimes $2,000 for the privilege of being abused in this way. Trudeau is fully on board with the Cult and says the 'Covid pandemic' has provided an opportunity for a global 'reset' to permanently change Western civilisation. His number two, Deputy Prime Minister Chrystia Freeland, is a trustee of the World Economic Forum and a Rhodes Scholar. The Trudeau family have long been servants of the Cult. See *The Biggest Secret* and Cathy O'Brien's book *Trance-Formation of America* for the horrific background to Trudeau's father Pierre Trudeau another Canadian prime minister. Hide your fascism behind the façade of a heart-on-the-sleeve liberal. It's a well-honed Cult technique.

What can the 'vaccine' *really* do?

We have a 'virus' never shown to exist and 'variants' of the 'virus' that have also never been shown to exist except, like the 'original', as computer-generated fictions. Even if you believe there's a 'virus' the 'case' to 'death' rate is in the region of 0.23 to 0.15 percent and those 'deaths' are concentrated among the very old around the same average age that people die anyway. In response to this lack of threat (in truth none) psychopaths and idiots, knowingly and unknowingly answering to Gates and the Cult, are seeking to 'vaccinate' every man, woman and child on Planet Earth. Clearly the 'vaccine' is not about 'Covid' – none of this ever has been. So what is it all about *really*? Why the desperation to infuse genetically-manipulating synthetic material into everyone through mRNA fraudulent 'vaccines' with the intent of doing this over and over with the excuses of 'variants' and other 'virus' inventions? Dr Sherri Tenpenny, an osteopathic medical doctor in the United States, has made herself an expert on vaccines and their effects as a vehement campaigner against their use. Tenpenny was board certified in emergency medicine, the director of a level two trauma centre for 12 years, and moved to Cleveland in 1996 to start an integrative

medicine practice which has treated patients from all 50 states and some 17 other countries. Weaning people off pharmaceutical drugs is a speciality.

She became interested in the consequences of vaccines after attending a meeting at the National Vaccine Information Center in Washington DC in 2000 where she 'sat through four days of listening to medical doctors and scientists and lawyers and parents of vaccine injured kids' and asked: 'What's going on?' She had never been vaccinated and never got ill while her father was given a list of vaccines to be in the military and was 'sick his entire life'. The experience added to her questions and she began to examine vaccine documents from the Centers for Disease Control (CDC). After reading the first one, the 1998 version of *The General Recommendations of Vaccination*, she thought: 'This is it?' The document was poorly written and bad science and Tenpenny began 20 years of research into vaccines that continues to this day. She began her research into 'Covid vaccines' in March, 2020, and she describes them as 'deadly'. For many, as we have seen, they already have been. Tenpenny said that in the first 30 days of the 'vaccine' rollout in the United States there had been more than 40,000 adverse events reported to the vaccine adverse event database. A document had been delivered to her the day before that was 172 pages long. 'We have over 40,000 adverse events; we have over 3,100 cases of [potentially deadly] anaphylactic shock; we have over 5,000 neurological reactions.' Effects ranged from headaches to numbness, dizziness and vertigo, to losing feeling in hands or feet and paraesthesia which is when limbs 'fall asleep' and people have the sensation of insects crawling underneath their skin. All this happened in the first 30 days and remember that only about *ten percent* (or far less) of adverse reactions and vaccine-related deaths are estimated to be officially reported. Tenpenny said:

> So can you think of one single product in any industry, any industry, for as long as products have been made on the planet that within 30 days we have 40,000 people complaining of side effects that not only is still on the market but … we've got paid actors telling us how great

they are for getting their vaccine. We're offering people $500 if they will just get their vaccine and we've got nurses and doctors going; 'I got the vaccine, I got the vaccine'.

Tenpenny said they were not going to be 'happy dancing folks' when they began to suffer Bell's palsy (facial paralysis), neuropathies, cardiac arrhythmias and autoimmune reactions that kill through a blood disorder. 'They're not going to be so happy, happy then, but we're never going to see pictures of those people' she said. Tenpenny described the 'vaccine' as 'a well-designed killing tool'.

No off-switch

Bad as the initial consequences had been Tenpenny said it would be maybe 14 months before we began to see the 'full ravage' of what is going to happen to the 'Covid vaccinated' with full-out consequences taking anything between two years and 20 years to show. You can understand why when you consider that variations of the 'Covid vaccine' use mRNA (messenger RNA) to in theory activate the immune system to produce protective antibodies without using the actual 'virus'. How can they when it's a computer program and they've never isolated what they claim is the 'real thing'? Instead they use *synthetic* mRNA. They are inoculating synthetic material into the body which through a technique known as the Trojan horse is absorbed into cells to change the nature of DNA. Human DNA is changed by an infusion of messenger RNA and with each new 'vaccine' of this type it is changed even more. Say so and you are banned by Cult Internet platforms. The contempt the contemptuous Mark Zuckerberg has for the truth and human health can be seen in an internal Facebook video leaked to the Project Veritas investigative team in which he said of the 'Covid vaccines': '… I share some caution on this because we just don't know the long term side-effects of basically modifying people's DNA and RNA.' At the same time this disgusting man's Facebook was censoring and banning anyone saying exactly the same. He must go before a Nuremberg trial for crimes against humanity when he *knows* that he

is censoring legitimate concerns and denying the right of informed consent on behalf of the Cult that owns him. People have been killed and damaged by the very 'vaccination' technique he cast doubt on himself when they may not have had the 'vaccine' with access to information that he denied them. The plan is to have at least annual 'Covid vaccinations', add others to deal with invented 'variants', and change all other vaccines into the mRNA system. Pfizer executives told shareholders at a virtual Barclays Global Healthcare Conference in March, 2021, that the public may need a third dose of 'Covid vaccine', plus regular yearly boosters and the company planned to hike prices to milk the profits in a 'significant opportunity for our vaccine'. These are the professional liars, cheats and opportunists who are telling you their 'vaccine' is safe. Given this volume of mRNA planned to be infused into the human body and its ability to then replicate we will have a transformation of human genetics from biological to synthetic biological – exactly the long-time Cult plan for reasons we'll see – and many will die. Sherri Tenpenny said of this replication:

> It's like having an on-button but no off-button and that whole mechanism … they actually give it a name and they call it the Trojan horse mechanism, because it allows that [synthetic] virus and that piece of that [synthetic] virus to get inside of your cells, start to replicate and even get inserted into other parts of your DNA as a Trojan-horse.

Ask the overwhelming majority of people who have the 'vaccine' what they know about the contents and what they do and they would reply: 'The government says it will stop me getting the virus.' Governments give that false impression on purpose to increase take-up. You can read Sherri Tenpenny's detailed analysis of the health consequences in her blog at Vaxxter.com, but in summary these are some of them. She highlights the statement by Bill Gates about how human beings can become their own 'vaccine manufacturing machine'. The man is insane. ['Vaccine'-generated] 'antibodies' carry synthetic messenger RNA into the cells and the damage starts, Tenpenny contends, and she says that lungs can be adversely affected through varying degrees of pus and bleeding which

obviously affects breathing and would be dubbed 'Covid-19'. Even more sinister was the impact of 'antibodies' on macrophages, a white blood cell of the immune system. They consist of Type 1 and Type 2 which have very different functions. She said Type 1 are 'hyper-vigilant' white blood cells which 'gobble up' bacteria etc. However, in doing so, this could cause inflammation and in extreme circumstances be fatal. She says these affects are mitigated by Type 2 macrophages which kick in to calm down the system and stop it going rogue. They clear up dead tissue debris and reduce inflammation that the Type 1 'fire crews' have caused. Type 1 kills the infection and Type 2 heals the damage, she says. This is her punchline with regard to 'Covid vaccinations': She says that mRNA 'antibodies' block Type 2 macrophages by attaching to them and deactivating them. This meant that when the Type 1 response was triggered by infection there was nothing to stop that getting out of hand by calming everything down. There's an on-switch, but no off-switch, she says. What follows can be 'over and out, see you when I see you'.

Genetic suicide

Tenpenny also highlights the potential for autoimmune disease – the body attacking itself – which has been associated with vaccines since they first appeared. Infusing a synthetic foreign substance into cells could cause the immune system to react in a panic believing that the body is being overwhelmed by an invader (it is) and the consequences can again be fatal. There is an autoimmune response known as a 'cytokine storm' which I have likened to a homeowner panicked by an intruder and picking up a gun to shoot randomly in all directions before turning the fire on himself. The immune system unleashes a storm of inflammatory response called cytokines to a threat and the body commits hara-kiri. The lesson is that you mess with the body's immune response at your peril and these 'vaccines' seriously – fundamentally – mess with immune response. Tenpenny refers to a consequence called anaphylactic shock which is a severe and highly dangerous allergic reaction when the immune system

floods the body with chemicals. She gives the example of having a bee sting which primes the immune system and makes it sensitive to those chemicals. When people are stung again maybe years later the immune response can be so powerful that it leads to anaphylactic shock. Tenpenny relates this 'shock' with regard to the 'Covid vaccine' to something called polyethylene glycol or PEG. Enormous numbers of people have become sensitive to this over decades of use in a whole range of products and processes including food, drink, skin creams and 'medicine'. Studies have claimed that some 72 percent of people have antibodies triggered by PEG compared with two percent in the 1960s and allergic hypersensitive reactions to this become a gathering cause for concern. Tenpenny points out that the 'mRNA vaccine' is coated in a 'bubble' of polyethylene glycol which has the potential to cause anaphylactic shock through immune sensitivity. Many reports have appeared of people reacting this way after having the 'Covid vaccine'. What do we think is going to happen as humanity has more and more of these 'vaccines'? Tenpenny said: 'All these pictures we have seen with people with these rashes … these weepy rashes, big reactions on their arms and things like that – it's an acute allergic reaction most likely to the polyethylene glycol that you've been previously primed and sensitised to.'

Those who have not studied the conspiracy and its perpetrators at length might think that making the population sensitive to PEG and then putting it in these 'vaccines' is just a coincidence. It is not. It is instead testament to how carefully and coldly-planned current events have been and the scale of the conspiracy we are dealing with. Tenpenny further explains that the 'vaccine' mRNA procedure can breach the blood-brain barrier which protects the brain from toxins and other crap that will cause malfunction. In this case they could make two proteins corrupt brain function to cause Amyotrophic lateral sclerosis (ALS) , a progressive nervous system disease leading to loss of muscle control, and frontal lobe degeneration – Alzheimer's and dementia. Immunologist J. Bart Classon published a paper connecting mRNA 'vaccines' to prion

disease which can lead to Alzheimer's and other forms of neurogenerative disease while others have pointed out the potential to affect the placenta in ways that make women infertile. This will become highly significant in the next chapter when I will discuss other aspects of this non-vaccine that relate to its nanotechnology and transmission from the injected to the uninjected.

Qualified in idiocy

Tenpenny describes how research has confirmed that these 'vaccine'-generated antibodies can interact with a range of other tissues in the body and attack many other organs including the lungs. 'This means that if you have a hundred people standing in front of you that all got this shot they could have a hundred different symptoms.' Anyone really think that Cult gofers like the Queen, Tony Blair, Christopher Whitty, Anthony Fauci, and all the other psychopaths have really had this 'vaccine' in the pictures we've seen? Not a bloody chance. Why don't doctors all tell us about all these dangers and consequences of the 'Covid vaccine'? Why instead do they encourage and pressure patients to have the shot? Don't let's think for a moment that doctors and medical staff can't be stupid, lazy, and psychopathic and that's without the financial incentives to give the jab. Tenpenny again:

> Some people are going to die from the vaccine directly but a large number of people are going to start to get horribly sick and get all kinds of autoimmune diseases 42 days to maybe a year out. What are they going to do, these stupid doctors who say; 'Good for you for getting that vaccine.' What are they going to say; 'Oh, it must be a mutant, we need to give an extra dose of that vaccine.'
>
> Because now the vaccine, instead of one dose or two doses we need three or four because the stupid physicians aren't taking the time to learn anything about it. If I can learn this sitting in my living room reading a 19 page paper and several others so can they. There's nothing special about me, I just take the time to do it.

Remember how Sara Kayat, the NHS and TV doctor, said that the 'Covid vaccine' would '100 percent prevent hospitalisation and death'. Doctors can be idiots like every other profession and they

should not be worshipped as infallible. They are not and far from it. Behind many medical and scientific 'experts' lies an uninformed prat trying to hide themselves from you although in the 'Covid' era many have failed to do so as with UK narrative-repeating 'TV doctor' Hilary Jones. Pushing back against the minority of proper doctors and scientists speaking out against the 'vaccine' has been the entire edifice of the Cult global state in the form of governments, medical systems, corporations, mainstream media, Silicon Valley, and an army of compliant doctors, medical staff and scientists willing to say anything for money and to enhance their careers by promoting the party line. If you do that you are an 'expert' and if you won't you are an 'anti-vaxxer' and 'Covidiot'. The pressure to be 'vaccinated' is incessant. We have even had reports claiming that the 'vaccine' can help cure cancer and Alzheimer's and make the lame walk. I am waiting for the announcement that it can bring you coffee in the morning and cook your tea. Just as the symptoms of 'Covid' seem to increase by the week so have the miracles of the 'vaccine'. American supermarket giant Kroger Co. offered nearly 500,000 employees in 35 states a $100 bonus for having the 'vaccine' while donut chain Krispy Kreme promised 'vaccinated' customers a free glazed donut every day for the rest of 2021. Have your DNA changed and you will get a doughnut although we might not have to give you them for long. Such offers and incentives confirm the desperation.

Perhaps the worse vaccine-stunt of them all was UK 'Health' Secretary Matt-the-prat Hancock on live TV after watching a clip of someone being 'vaccinated' when the roll-out began. Hancock faked tears so badly it was embarrassing. Brain-of-Britain Piers Morgan, the lockdown-supporting, 'vaccine' supporting, 'vaccine' passport-supporting, TV host played along with Hancock – 'You're quite emotional about that' he said in response to acting so atrocious it would have been called out at a school nativity which will presumably today include Mary and Jesus in masks, wise men keeping their camels six feet apart, and shepherds under tent arrest. System-serving Morgan tweeted this: 'Love the idea of covid vaccine passports for everywhere: flights, restaurants, clubs, football, gyms,

shops etc. It's time covid-denying, anti-vaxxer loonies had their bullsh*t bluff called & bar themselves from going anywhere that responsible citizens go.' If only I could aspire to his genius. To think that Morgan, who specialises in shouting over anyone he disagrees with, was lauded as a free speech hero when he lost his job after storming off the set of his live show like a child throwing his dolly out of the pram. If he is a free speech hero we are in real trouble. I have no idea what 'bullsh*t' means, by the way, the * throws me completely.

The Cult is desperate to infuse its synthetic DNA-changing concoction into everyone and has been using every lie, trick and intimidation to do so. The question of '*Why*?' we shall now address.

CHAPTER TEN

Human 2.0

I believe that at the end of the century the use of words and general educated opinion will have altered so much that one will be able to speak of machines thinking without expecting to be contradicted –
Alan Turing (1912-1954), the 'Father of artificial intelligence'

I have been exposing for decades the plan to transform the human body from a biological to a synthetic-biological state. The new human that I will call Human 2.0 is planned to be connected to artificial intelligence and a global AI 'Smart Grid' that would operate as one global system in which AI would control everything from your fridge to your heating system to your car to your mind. Humans would no longer be 'human', but post-human and sub-human, with their thinking and emotional processes replaced by AI.

What I said sounded crazy and beyond science fiction and I could understand that. To any balanced, rational, mind it *is* crazy. Today, however, that world is becoming reality and it puts the 'Covid vaccine' into its true context. Ray Kurzweil is the ultra-Zionist 'computer scientist, inventor and futurist' and co-founder of the Singularity University. Singularity refers to the merging of humans with machines or 'transhumanism'. Kurzweil has said humanity would be connected to the cyber 'cloud' in the period of the ever-recurring year of 2030:

Our thinking … will be a hybrid of biological and non-biological thinking … humans will be able to extend their limitations and 'think in the cloud' … We're going to put gateways to the

cloud in our brains ... We're going to gradually merge and enhance ourselves ... In my view, that's the nature of being human – we transcend our limitations. As the technology becomes vastly superior to what we are then the small proportion that is still human gets smaller and smaller and smaller until it's just utterly negligible.

They are trying to sell this end-of-humanity-as-we-know-it as the next stage of 'evolution' when we become super-human and 'like the gods'. They are lying to you. Shocked, eh? The population, and again especially the young, have been manipulated into addiction to technologies designed to enslave them for life. First they induced an addiction to smartphones (holdables); next they moved to technology on the body (wearables); and then began the invasion of the body (implantables). I warned way back about the plan for microchipped people and we are now entering that era. We should not be diverted into thinking that this refers only to chips we can see. Most important are the nanochips known as smart dust, neural dust and nanobots which are far too small to be seen by the human eye. Nanotechnology is everywhere, increasingly in food products, and released into the atmosphere by the geoengineering of the skies funded by Bill Gates to 'shut out the Sun' and 'save the planet from global warming'. Gates has been funding a project to spray millions of tonnes of chalk (calcium carbonate) into the stratosphere over Sweden to 'dim the Sun' and cool the Earth. Scientists warned the move could be disastrous for weather systems in ways no one can predict and opposition led to the Swedish space agency announcing that the 'experiment' would not be happening as planned in the summer of 2021; but it shows where the Cult is going with dimming the impact of the Sun and there's an associated plan to change the planet's atmosphere. Who gives psychopath Gates the right to dictate to the entire human race and dismantle planetary systems? The world will not be safe while this man is at large.

The global warming hoax has made the Sun, like the gas of life, something to fear when both are essential to good health and human survival (more inversion). The body transforms sunlight into vital vitamin D through a process involving … *cholesterol*. This is the cholesterol we are also told to fear. We are urged to take Big Pharma

statin drugs to reduce cholesterol and it's all systematic. Reducing cholesterol means reducing vitamin D uptake with all the multiple health problems that will cause. At least if you take statins long term it saves the government from having to pay you a pension. The delivery system to block sunlight is widely referred to as chemtrails although these have a much deeper agenda, too. They appear at first to be contrails or condensation trails streaming from aircraft into cold air at high altitudes. Contrails disperse very quickly while chemtrails do not and spread out across the sky before eventually their content falls to earth. Many times I have watched aircraft cross-cross a clear blue sky releasing chemtrails until it looks like a cloudy day. Chemtrails contain many things harmful to humans and the natural world including toxic heavy metals, aluminium (see Alzheimer's) and nanotechnology. Ray Kurzweil reveals the reason without actually saying so: 'Nanobots will infuse all the matter around us with information. Rocks, trees, everything will become these intelligent creatures.' How do you deliver that? *From the sky*. Self-replicating nanobots would connect everything to the Smart Grid. The phenomenon of Morgellons disease began in the chemtrail era and the correlation has led to it being dubbed the 'chemtrail disease'. Self-replicating fibres appear in the body that can be pulled out through the skin. Morgellons fibres continue to grow outside the body and have a form of artificial intelligence. I cover this at greater length in *Phantom Self*.

'Vaccine' operating system

'Covid vaccines' with their self-replicating synthetic material are also designed to make the connection between humanity and Kurzweil's 'cloud'. American doctor and dedicated campaigner for truth, Carrie Madej, an Internal Medicine Specialist in Georgia with more than 20 years medical experience, has highlighted the nanotechnology aspect of the fake 'vaccines'. She explains how one of the components in at least the Moderna and Pfizer synthetic potions are 'lipid nanoparticles' which are 'like little tiny computer bits' – a 'sci-fi substance' known as nanobots and hydrogel which can be 'triggered

at any moment to deliver its payload' and act as 'biosensors'. The synthetic substance had 'the ability to accumulate data from your body like your breathing, your respiration, thoughts and emotions, all kind of things' and each syringe could carry a *million* nanobots:

This substance because it's like little bits of computers in your body, crazy, but it's true, it can do that, [and] obviously has the ability to act through Wi-Fi. It can receive and transmit energy, messages, frequencies or impulses. That issue has never been addressed by these companies. What does that do to the human?

Just imagine getting this substance in you and it can react to things all around you, the 5G, your smart device, your phones, what is happening with that? What if something is triggering it, too, like an impulse, a frequency? We have something completely foreign in the human body.

Madej said her research revealed that electromagnetic (EMF) frequencies emitted by phones and other devices had increased dramatically in the same period of the 'vaccine' rollout and she was seeing more people with radiation problems as 5G and other electromagnetic technology was expanded and introduced to schools and hospitals. She said she was 'floored with the EMF coming off' the devices she checked. All this makes total sense and syncs with my own work of decades when you think that Moderna refers in documents to its mRNA 'vaccine' as an 'operating system':

Recognizing the broad potential of mRNA science, we set out to create an mRNA technology platform that functions very much like an operating system on a computer. It is designed so that it can plug and play interchangeably with different programs. In our case, the 'program' or 'app' is our mRNA drug – the unique mRNA sequence that codes for a protein …

… Our MRNA Medicines – 'The 'Software Of Life': When we have a concept for a new mRNA medicine and begin research, fundamental components are already in place. Generally, the only thing that changes from one potential mRNA medicine to another is the coding region – the actual genetic code that instructs ribosomes to make protein. Utilizing these instruction sets gives our investigational mRNA medicines a software-like quality. We also have the ability to combine different mRNA sequences encoding for different proteins in a single mRNA investigational medicine.

Who needs a real 'virus' when you can create a computer version to justify infusing your operating system into the entire human race on the road to making living, breathing people into cyborgs? What is missed with the 'vaccines' is the *digital* connection between synthetic material and the body that I highlighted earlier with the study that hacked a computer with human DNA. On one level the body is digital, based on mathematical codes, and I'll have more about that in the next chapter. Those who ridiculously claim that mRNA 'vaccines' are not designed to change human genetics should explain the words of Dr Tal Zaks, chief medical officer at Moderna, in a 2017 TED talk. He said that over the last 30 years 'we've been living this phenomenal digital scientific revolution, and I'm here today to tell you, that we are actually *hacking the software of life*, and that it's changing the way we think about prevention and treatment of disease':

In every cell there's this thing called messenger RNA, or mRNA for short, that transmits the critical information from the DNA in our genes to the protein, which is really the stuff we're all made out of. This is the critical information that determines what the cell will do. So we think about it as an operating system. So if you could change that, if you could introduce a line of code, or change a line of code, it turns out, that has profound implications for everything, from the flu to cancer.

Zaks should more accurately have said that this has profound implications for the human genetic code and the nature of DNA. Communications within the body go both ways and not only one. But, hey, no, the 'Covid vaccine' will not affect your genetics. Cult fact-checkers say so even though the man who helped to develop the mRNA technique says that it does. Zaks said in 2017:

If you think about what it is we're trying to do. We've taken information and our understanding of that information and how that information is transmitted in a cell, and we've taken our understanding of medicine and how to make drugs, and we're fusing the two. We think of it as information therapy.

I have been writing for decades that the body is an information field communicating with itself and the wider world. This is why

radiation which is information can change the information field of body and mind through phenomena like 5G and change their nature and function. 'Information therapy' means to change the body's information field and change the way it operates. DNA is a receiver-transmitter of information and can be mutated by information like mRNA synthetic messaging. Technology to do this has been ready and waiting in the underground bases and other secret projects to be rolled out when the 'Covid' hoax was played. 'Trials' of such short and irrelevant duration were only for public consumption. When they say the 'vaccine' is 'experimental' that is not true. It may appear to be 'experimental' to those who don't know what's going on, but the trials have already been done to ensure the Cult gets the result it desires. Zaks said that it took decades to sequence the human genome, completed in 2003, but now they could do it in a week. By 'they' he means scientists operating in the public domain. In the secret projects they were sequencing the genome in a week long before even 2003.

Deluge of mRNA

Highly significantly the Moderna document says the guiding premise is that if using mRNA as a medicine works for one disease then it should work for many diseases. They were leveraging the flexibility afforded by their platform and the fundamental role mRNA plays in protein synthesis to pursue mRNA medicines for a broad spectrum of diseases. Moderna is confirming what I was saying through 2020 that multiple 'vaccines' were planned for 'Covid' (and later invented 'variants') and that previous vaccines would be converted to the mRNA system to infuse the body with massive amounts of genetically-manipulating synthetic material to secure a transformation to a synthetic-biological state. The 'vaccines' are designed to kill stunning numbers as part of the long-exposed Cult depopulation agenda and transform the rest. Given this is the goal you can appreciate why there is such hysterical demand for every human to be 'vaccinated' for an alleged 'disease' that has an estimated 'infection' to 'death' ratio of 0.23-0.15 percent. As I write

children are being given the 'vaccine' in trials (their parents are a disgrace) and ever-younger people are being offered the vaccine for a 'virus' that even if you believe it exists has virtually zero chance of harming them. Horrific effects of the 'trials' on a 12-year-old girl were revealed by a family member to be serious brain and gastric problems that included a bowel obstruction and the inability to swallow liquids or solids. She was unable to eat or drink without throwing up, had extreme pain in her back, neck and abdomen, and was paralysed from the waist down which stopped her urinating unaided. When the girl was first taken to hospital doctors said it was all in her mind. She was signed up for the 'trial' by her parents for whom no words suffice. None of this 'Covid vaccine' insanity makes any sense unless you see what the 'vaccine' really is – a body-changer. Synthetic biology or 'SynBio' is a fast-emerging and expanding scientific discipline which includes everything from genetic and molecular engineering to electrical and computer engineering. Synthetic biology is defined in these ways:

- A multidisciplinary area of research that seeks to create new biological parts, devices, and systems, or to redesign systems that are already found in nature.

- The use of a mixture of physical engineering and genetic engineering to create new (and therefore synthetic) life forms.

- An emerging field of research that aims to combine the knowledge and methods of biology, engineering and related disciplines in the design of chemically-synthesized DNA to create organisms with novel or enhanced characteristics and traits (synthetic organisms including humans).

We now have synthetic blood, skin, organs and limbs being developed along with synthetic body parts produced by 3D printers. These are all elements of the synthetic human programme and this comment by Kurzweil's co-founder of the Singularity University,

Peter Diamandis, can be seen in a whole new light with the 'Covid' hoax and the sanctions against those that refuse the 'vaccine':

Anybody who is going to be resisting the progress forward [to transhumanism] is going to be resisting evolution and, fundamentally, they will die out. It's not a matter of whether it's good or bad. It's going to happen.

'Resisting evolution'? What absolute bollocks. The arrogance of these people is without limit. His 'it's going to happen' mantra is another way of saying 'resistance is futile' to break the spirit of those pushing back and we must not fall for it. Getting this genetically-transforming 'vaccine' into everyone is crucial to the Cult plan for total control and the desperation to achieve that is clear for anyone to see. Vaccine passports are a major factor in this and they, too, are a form of resistance is futile. It's NOT. The paper funded by the Rockefeller Foundation for the 2013 'health conference' in China said:

We will interact more with artificial intelligence. The use of robotics, bio-engineering to augment human functioning is already well underway and will advance. Re-engineering of humans into potentially separate and unequal forms through genetic engineering or mixed human-robots raises debates on ethics and equality.

A new demography is projected to emerge after 2030 [that year again] of technologies (robotics, genetic engineering, nanotechnology) producing robots, engineered organisms, 'nanobots' and artificial intelligence (AI) that can self-replicate. Debates will grow on the implications of an impending reality of human designed life.

What is happening today is so long planned. The world army enforcing the will of the world government is intended to be a robot army, not a human one. Today's military and its technologically 'enhanced' troops, pilotless planes and driverless vehicles are just stepping stones to that end. Human soldiers are used as Cult fodder and its time they woke up to that and worked for the freedom of the population instead of their own destruction and their family's destruction – the same with the police. Join us and let's sort this out. The phenomenon of enforce my own destruction is widespread in the 'Covid' era with Woker 'luvvies' in the acting and entertainment

industries supporting 'Covid' rules which have destroyed their profession and the same with those among the public who put signs on the doors of their businesses 'closed due to Covid – stay safe' when many will never reopen. It's a form of masochism and most certainly insanity.

Transgender = transhumanism

When something explodes out of nowhere and is suddenly everywhere it is always the Cult agenda and so it is with the tidal wave of claims and demands that have infiltrated every aspect of society under the heading of 'transgenderism'. The term 'trans' is so 'in' and this is the dictionary definition:

A prefix meaning 'across', 'through', occurring … in loanwords from Latin, used in particular for denoting movement or conveyance from place to place (transfer; transmit; transplant) or complete change (transform; transmute), or to form adjectives meaning 'crossing', 'on the other side of', or 'going beyond' the place named (transmontane; transnational; trans-Siberian).

Transgender means to go beyond gender and transhuman means to go beyond human. Both are aspects of the Cult plan to transform the human body to a synthetic state with *no gender*. Human 2.0 is not designed to procreate and would be produced technologically with no need for parents. The new human would mean the end of parents and so men, and increasingly women, are being targeted for the deletion of their rights and status. Parental rights are disappearing at an ever-quickening speed for the same reason. The new human would have no need for men or women when there is no procreation and no gender. Perhaps the transgender movement that appears to be in a permanent state of frenzy might now contemplate on how it is being used. This was never about transgender rights which are only the interim excuse for confusing gender, particularly in the young, on the road to *fusing* gender. Transgender activism is not an end; it is a *means* to an end. We see again the technique of creative destruction in which you destroy the status quo to 'build back better' in the form that you want. The gender status quo had to be

destroyed by persuading the Cult-created Woke mentality to believe that you can have 100 genders or more. A programme for 9 to 12 year olds produced by the Cult-owned BBC promoted the 100 genders narrative. The very idea may be the most monumental nonsense, but it is not what is true that counts, only what you can make people *believe* is true. Once the gender of 2 + 2 = 4 has been dismantled through indoctrination, intimidation and 2 + 2 = 5 then the new no-gender normal can take its place with Human 2.0. Aldous Huxley revealed the plan in his prophetic *Brave New World* in 1932:

> Natural reproduction has been done away with and children are created, decanted', and raised in 'hatcheries and conditioning centres'. From birth, people are genetically designed to fit into one of five castes, which are further split into 'Plus' and 'Minus' members and designed to fulfil predetermined positions within the social and economic strata of the World State.

How could Huxley know this in 1932? For the same reason George Orwell knew about the Big Brother state in 1948, Cult insiders I have quoted knew about it in 1969, and I have known about it since the early 1990s. If you are connected to the Cult or you work your balls off to uncover the plan you can predict the future. The process is simple. If there is a plan for the world and nothing intervenes to stop it then it will happen. Thus if you communicate the plan ahead of time you are perceived to have predicted the future, but you haven't. You have revealed the plan which without intervention will become the human future. The whole reason I have done what I have is to alert enough people to inspire an intervention and maybe at last that time has come with the Cult and its intentions now so obvious to anyone with a brain in working order.

The future is here

Technological wombs that Huxley described to replace parent procreation are already being developed and they are only the projects we know about in the public arena. Israeli scientists told *The Times of Israel* in March, 2021, that they have grown 250-cell embryos

into mouse foetuses with fully formed organs using artificial wombs in a development they say could pave the way for gestating humans outside the womb. Professor Jacob Hanna of the Weizmann Institute of Science said:

We took mouse embryos from the mother at day five of development, when they are just of 250 cells, and had them in the incubator from day five until day 11, by which point they had grown all their organs.

By day 11 they make their own blood and have a beating heart, a fully developed brain. Anybody would look at them and say, 'this is clearly a mouse foetus with all the characteristics of a mouse.' It's gone from being a ball of cells to being an advanced foetus.

A special liquid is used to nourish embryo cells in a laboratory dish and they float on the liquid to duplicate the first stage of embryonic development. The incubator creates all the right conditions for its development, Hanna said. The liquid gives the embryo 'all the nutrients, hormones and sugars they need' along with a custom-made electronic incubator which controls gas concentration, pressure and temperature. The cutting-edge in the underground bases and other secret locations will be light years ahead of that, however, and this was reported by the London *Guardian* in 2017:

We are approaching a biotechnological breakthrough. Ectogenesis, the invention of a complete external womb, could completely change the nature of human reproduction. In April this year, researchers at the Children's Hospital of Philadelphia announced their development of an artificial womb.

The article was headed 'Artificial wombs could soon be a reality. What will this mean for women?' What would it mean for children is an even bigger question. No mother to bond with only a machine in preparation for a life of soulless interaction and control in a world governed by machines (see the *Matrix* movies). Now observe the calculated manipulations of the 'Covid' hoax as human interaction and warmth has been curtailed by distancing, isolation and fear with people communicating via machines on a scale never seen before.

These are all dots in the same picture as are all the personal assistants, gadgets and children's toys through which kids and adults communicate with AI as if it is human. The AI 'voice' on Sat-Nav should be included. All these things are psychological preparation for the Cult endgame. Before you can make a physical connection with AI you have to make a psychological connection and that is what people are being conditioned to do with this ever gathering human-AI interaction. Movies and TV programmes depicting the transhuman, robot dystopia relate to a phenomenon known as 'pre-emptive programming' in which the world that is planned is portrayed everywhere in movies, TV and advertising. This is conditioning the conscious and subconscious mind to become familiar with the planned reality to dilute resistance when it happens for real. What would have been a shock such is the change is made less so. We have young children put on the road to transgender transition surgery with puberty blocking drugs at an age when they could never be able to make those life-changing decisions.

Rachel Levine, a professor of paediatrics and psychiatry who believes in treating children this way, became America's highest-ranked openly-transgender official when she was confirmed as US Assistant Secretary at the Department of Health and Human Services after being nominated by Joe Biden (the Cult). Activists and governments press for laws to deny parents a say in their children's transition process so the kids can be isolated and manipulated into agreeing to irreversible medical procedures. A Canadian father Robert Hoogland was denied bail by the Vancouver Supreme Court in 2021 and remained in jail for breaching a court order that he stay silent over his young teenage daughter, a minor, who was being offered life-changing hormone therapy without parental consent. At the age of 12 the girl's 'school counsellor' said she may be transgender, referred her to a doctor and told the school to treat her like a boy. This is another example of state-serving schools imposing ever more control over children's lives while parents have ever less.

Contemptible and extreme child abuse is happening all over the world as the Cult gender-fusion operation goes into warp-speed.

Why the war on men – and now women?

The question about what artificial wombs mean for women should rightly be asked. The answer can be seen in the deletion of women's rights involving sport, changing rooms, toilets and status in favour of people in male bodies claiming to identify as women. I can identify as a mountain climber, but it doesn't mean I can climb a mountain any more than a biological man can be a biological woman. To believe so is a triumph of belief over factual reality which is the very perceptual basis of everything Woke. Women's sport is being destroyed by allowing those with male bodies who say they identify as female to 'compete' with girls and women. Male body 'women' dominate 'women's' competition with their greater muscle mass, bone density, strength and speed. With that disadvantage sport for women loses all meaning. To put this in perspective nearly 300 American high school boys can run faster than the quickest woman sprinter in the world. Women are seeing their previously protected spaces invaded by male bodies simply because they claim to identify as women. That's all they need to do to access all women's spaces and activities under the Biden 'Equality Act' that destroys equality for women with the usual Orwellian Woke inversion. Male sex offenders have already committed rapes in women's prisons after claiming to identify as women to get them transferred. Does this not matter to the Woke 'equality' hypocrites? Not in the least. What matters to Cult manipulators and funders behind transgender activists is to advance gender fusion on the way to the no-gender 'human'. When you are seeking to impose transparent nonsense like this, or the 'Covid' hoax, the only way the nonsense can prevail is through censorship and intimidation of dissenters, deletion of factual information, and programming of the unquestioning, bewildered and naive. You don't have to scan the world for long to see that all these things are happening.

Many women's rights organisations have realised that rights and status which took such a long time to secure are being eroded and that it is systematic. Kara Dansky of the global Women's Human Rights Campaign said that Biden's transgender executive order immediately he took office, subsequent orders, and Equality Act legislation that followed 'seek to erase women and girls in the law as a category'. *Exactly*. I said during the long ago-started war on men (in which many women play a crucial part) that this was going to turn into a war on them. The Cult is phasing out *both* male and female genders. To get away with that they are brought into conflict so they are busy fighting each other while the Cult completes the job with no unity of response. Unity, people, *unity*. We need unity everywhere. Transgender is the only show in town as the big step towards the no-gender human. It's not about rights for transgender people and never has been. Woke political correctness is deleting words relating to genders to the same end. Wokers believe this is to be 'inclusive' when the opposite is true. They are deleting words describing gender because gender *itself* is being deleted by Human 2.0. Terms like 'man', 'woman', 'mother' and 'father' are being deleted in the universities and other institutions to be replaced by the *no*-gender, not trans-gender, 'individuals' and 'guardians'. Women's rights campaigner Maria Keffler of Partners for Ethical Care said: 'Children are being taught from kindergarten upward that some boys have a vagina, some girls have a penis, and that kids can be any gender they want to be.' Do we really believe that suddenly countries all over the world at the same time had the idea of having drag queens go into schools or read transgender stories to very young children in the local library? It's coldly-calculated confusion of gender on the way to the fusion of gender. Suzanne Vierling, a psychologist from Southern California, made another important point:

Yesterday's slave woman who endured gynecological medical experiments is today's girl-child being butchered in a booming gender-transitioning sector. Ovaries removed, pushing her into menopause and osteoporosis, uncharted territory, and parents' rights and authority decimated.

The erosion of parental rights is a common theme in line with the Cult plans to erase the very concept of parents and 'ovaries removed, pushing her into menopause' means what? Those born female lose the ability to have children – another way to discontinue humanity as we know it.

Eliminating Human 1.0 (before our very eyes)

To pave the way for Human 2.0 you must phase out Human 1.0. This is happening through plummeting sperm counts and making women infertile through an onslaught of chemicals, radiation (including smartphones in pockets of men) and mRNA 'vaccines'. Common agriculture pesticides are also having a devastating impact on human fertility. I have been tracking collapsing sperm counts in the books for a long time and in 2021 came a book by fertility scientist and reproductive epidemiologist Shanna Swan, *Count Down: How Our Modern World Is Threatening Sperm Counts, Altering Male and Female Reproductive Development and Imperiling the Future of the Human Race*. She reports how the global fertility rate dropped by *half* between 1960 and 2016 with America's birth rate 16 percent below where it needs to be to sustain the population. Women are experiencing declining egg quality, more miscarriages, and more couples suffer from infertility. Other findings were an increase in erectile dysfunction, infant boys developing more genital abnormalities, male problems with conception, and plunging levels of the male hormone testosterone which would explain why so many men have lost their backbone and masculinity. This has been very evident during the 'Covid' hoax when women have been prominent among the Pushbackers and big strapping blokes have bowed their heads, covered their faces with a nappy and quietly submitted. Mind control expert Cathy O'Brien also points to how global education introduced the concept of 'we're all winners' in sport and classrooms: 'Competition was defused, and it in turn defused a sense of fighting back.' This is another version of the 'equity' doctrine in which you drive down rather than raise up. What a contrast in Cult-controlled China with its global ambitions

where the government published plans in January, 2021, to 'cultivate masculinity' in boys from kindergarten through to high school in the face of a 'masculinity crisis'. A government adviser said boys would be soon become 'delicate, timid and effeminate' unless action was taken. Don't expect any similar policy in the targeted West. A 2006 study showed that a 65-year-old man in 2002 had testosterone levels *15 percent* lower than a 65-year-old man in 1987 while a 2020 study found a similar story with young adults and adolescents. Men are getting prescriptions for testosterone replacement therapy which causes an even greater drop in sperm count with up to 99 percent seeing sperm counts drop to zero during the treatment. More sperm is defective and malfunctioning with some having two heads or not pursuing an egg.

A class of *synthetic* chemicals known as phthalates are being blamed for the decline. These are found everywhere in plastics, shampoos, cosmetics, furniture, flame retardants, personal care products, pesticides, canned foods and even receipts. Why till receipts? Everyone touches them. Let no one delude themselves that all this is not systematic to advance the long-time agenda for human body transformation. Phthalates mimic hormones and disrupt the hormone balance causing testosterone to fall and genital birth defects in male infants. Animals and fish have been affected in the same way due to phthalates and other toxins in rivers. When fish turn gay or change sex through chemicals in rivers and streams it is a pointer to why there has been such an increase in gay people and the sexually confused. It doesn't matter to me what sexuality people choose to be, but if it's being affected by chemical pollution and consumption then we need to know. Does anyone really think that this is not connected to the transgender agenda, the war on men and the condemnation of male 'toxic masculinity'? You watch this being followed by 'toxic femininity'. It's already happening. When breastfeeding becomes 'chest-feeding', pregnant women become pregnant people along with all the other Woke claptrap you know that the world is going insane and there's a Cult scam in progress. Transgender activists are promoting the Cult agenda while Cult

billionaires support and fund the insanity as they laugh themselves to sleep at the sheer stupidity for which humans must be infamous in galaxies far, far away.

'Covid vaccines' and female infertility

We can now see why the 'vaccine' has been connected to potential infertility in women. Dr Michael Yeadon, former Vice President and Chief Scientific Advisor at Pfizer, and Dr Wolfgang Wodarg in Germany, filed a petition with the European Medicines Agency in December, 2020, urging them to stop trials for the Pfizer/BioNTech shot and all other mRNA trials until further studies had been done. They were particularly concerned about possible effects on fertility with 'vaccine'-produced antibodies attacking the protein Syncytin-1 which is responsible for developing the placenta. The result would be infertility 'of indefinite duration' in women who have the 'vaccine' with the placenta failing to form. Section 10.4.2 of the Pfizer/BioNTech trial protocol says that pregnant women or those who might become so should not have mRNA shots. Section 10.4 warns men taking mRNA shots to 'be abstinent from heterosexual intercourse' and not to donate sperm. The UK government said that it *did not know* if the mRNA procedure had an effect on fertility. *Did not know?* These people have to go to jail. UK government advice did not recommend at the start that pregnant women had the shot and said they should avoid pregnancy for at least two months after 'vaccination'. The 'advice' was later updated to pregnant women should only have the 'vaccine' if the benefits outweighed the risks to mother and foetus. What the hell is that supposed to mean? Then 'spontaneous abortions' began to appear and rapidly increase on the adverse reaction reporting schemes which include only a fraction of adverse reactions. Thousands and ever-growing numbers of 'vaccinated' women are describing changes to their menstrual cycle with heavier blood flow, irregular periods and menstruating again after going through the menopause – all links to reproduction effects. Women are passing blood clots and the lining of their uterus while men report erectile dysfunction and blood effects. Most

significantly of all *un*vaccinated women began to report similar menstrual changes after interaction with *'vaccinated'* people and men and children were also affected with bleeding noses, blood clots and other conditions. 'Shedding' is when vaccinated people can emit the content of a vaccine to affect the unvaccinated, but this is different. 'Vaccinated' people were not shedding a 'live virus' allegedly in 'vaccines' as before because the fake 'Covid vaccines' involve synthetic material and other toxicity. Doctors exposing what is happening prefer the term 'transmission' to shedding. Somehow those that have had the shots are transmitting effects to those that haven't. Dr Carrie Madej said the nano-content of the 'vaccines' can 'act like an antenna' to others around them which fits perfectly with my own conclusions. This 'vaccine' transmission phenomenon was becoming known as the book went into production and I deal with this further in the Postscript.

Vaccine effects on sterility are well known. The World Health Organization was accused in 2014 of sterilising millions of women in Kenya with the evidence confirmed by the content of the vaccines involved. The same WHO behind the 'Covid' hoax admitted its involvement for more than ten years with the vaccine programme. Other countries made similar claims. Charges were lodged by Tanzania, Nicaragua, Mexico, and the Philippines. The Gardasil vaccine claimed to protect against a genital 'virus' known as HPV has also been linked to infertility. Big Pharma and the WHO (same thing) are criminal and satanic entities. Then there's the Bill Gates Foundation which is connected through funding and shared interests with 20 pharmaceutical giants and laboratories. He stands accused of directing the policy of United Nations Children's Fund (UNICEF), vaccine alliance GAVI, and other groupings, to advance the vaccine agenda and silence opposition at great cost to women and children. At the same time Gates wants to reduce the global population. Coincidence?

Great Reset = Smart Grid = new human

The Cult agenda I have been exposing for 30 years is now being openly promoted by Cult assets like Gates and Klaus Schwab of the World Economic Forum under code-terms like the 'Great Reset', 'Build Back Better' and 'a rare but narrow window of opportunity to reflect, reimagine, and reset our world'. What provided this 'rare but narrow window of opportunity'? The 'Covid' hoax did. Who created that? *They* did. My books from not that long ago warned about the planned 'Internet of Things' (IoT) and its implications for human freedom. This was the plan to connect all technology to the Internet and artificial intelligence and today we are way down that road with an estimated 36 billion devices connected to the World Wide Web and that figure is projected to be 76 billion by 2025. I further warned that the Cult planned to go beyond that to the Internet of *Everything* when the human brain was connected via AI to the Internet and Kurzweil's 'cloud'. Now we have Cult operatives like Schwab calling for precisely that under the term 'Internet of Bodies', a fusion of the physical, digital and biological into one centrally-controlled Smart Grid system which the Cult refers to as the 'Fourth Industrial Revolution'. They talk about the 'biological', but they really mean the synthetic-biological which is required to fully integrate the human body and brain into the Smart Grid and artificial intelligence planned to replace the human mind. We have everything being synthetically manipulated including the natural world through GMO and smart dust, the food we eat and the human body itself with synthetic 'vaccines'. I said in *The Answer* that we would see the Cult push for synthetic meat to replace animals and in February, 2021, the so predictable psychopath Bill Gates called for the introduction of synthetic meat to save us all from 'climate change'. The climate hoax just keeps on giving like the 'Covid' hoax. The war on meat by vegan activists is a carbon (oops, sorry) copy of the manipulation of transgender activists. They have no idea (except their inner core) that they are being used to promote and impose the agenda of the Cult or that they are only the *vehicle* and not the *reason*. This is not to say those who choose not to eat meat shouldn't be respected and supported in that right, but there are ulterior motives

for those in power. A *Forbes* article in December, 2019, highlighted the plan so beloved of Schwab and the Cult under the heading: 'What Is The Internet of Bodies? And How Is It Changing Our World?' The article said the human body is the latest data platform (remember 'our vaccine is an operating system'). *Forbes* described the plan very accurately and the words could have come straight out of my books from long before:

The Internet of Bodies (IoB) is an extension of the IoT and basically connects the human body to a network through devices that are ingested, implanted, or connected to the body in some way. Once connected, data can be exchanged, and the body and device can be remotely monitored and controlled.

They were really describing a human hive mind with human perception centrally-dictated via an AI connection as well as allowing people to be 'remotely monitored and controlled'. Everything from a fridge to a human mind could be directed from a central point by these insane psychopaths and 'Covid vaccines' are crucial to this. Forbes explained the process I mentioned earlier of holdable and wearable technology followed by implantable. The article said there were three generations of the Internet of Bodies that include:

- Body external: These are wearable devices such as Apple Watches or Fitbits that can monitor our health.

- Body internal: These include pacemakers, cochlear implants, and digital pills that go inside our bodies to monitor or control various aspects of health.

- Body embedded: The third generation of the Internet of Bodies is embedded technology where technology and the human body are melded together and have a real-time connection to a remote machine.

Forbes noted the development of the Brain Computer Interface (BCI) which merges the brain with an external device for monitoring and controlling in real-time. 'The ultimate goal is to help restore function to individuals with disabilities by using brain signals rather than conventional neuromuscular pathways.' Oh, do fuck off. The goal of brain interface technology is controlling human thought and emotion from the central point in a hive mind serving its masters wishes. Many people are now agreeing to be chipped to open doors without a key. You can recognise them because they'll be wearing a mask, social distancing and lining up for the 'vaccine'. The Cult plans a Great Reset money system after they have completed the demolition of the global economy in which 'money' will be exchanged through communication with body operating systems. Rand Corporation, a Cult-owned think tank, said of the Internet of Bodies or IoB:

> Internet of Bodies technologies fall under the broader IoT umbrella. But as the name suggests, IoB devices introduce an even more intimate interplay between humans and gadgets. IoB devices monitor the human body, collect health metrics and other personal information, and transmit those data over the Internet. Many devices, such as fitness trackers, are already in use … IoB devices … and those in development can track, record, and store users' whereabouts, bodily functions, and what they see, hear, and even think.

Schwab's World Economic Forum, a long-winded way of saying 'fascism' or 'the Cult', has gone full-on with the Internet of Bodies in the 'Covid' era. 'We're entering the era of the Internet of Bodies', it declared, 'collecting our physical data via a range of devices that can be implanted, swallowed or worn'. The result would be a huge amount of health-related data that could improve human wellbeing around the world, and prove crucial in fighting the 'Covid-19 pandemic'. Does anyone think these clowns care about 'human wellbeing' after the death and devastation their pandemic hoax has purposely caused? Schwab and co say we should move forward with the Internet of Bodies because 'Keeping track of symptoms could help us stop the spread of infection, and quickly detect new cases'. How wonderful, but keeping track' is all they are really bothered

about. Researchers were investigating if data gathered from smartwatches and similar devices could be used as viral infection alerts by tracking the user's heart rate and breathing. Schwab said in his 2018 book *Shaping the Future of the Fourth Industrial Revolution*:

> The lines between technologies and beings are becoming blurred and not just by the ability to create lifelike robots or synthetics. Instead it is about the ability of new technologies to literally become part of us. Technologies already influence how we understand ourselves, how we think about each other, and how we determine our realities. As the technologies … give us deeper access to parts of ourselves, we may begin to integrate digital technologies into our bodies.

You can see what the game is. Twenty-four hour control and people – if you could still call them that – would never know when something would go ping and take them out of circulation. It's the most obvious rush to a global fascist dictatorship and the complete submission of humanity and yet still so many are locked away in their Cult-induced perceptual coma and can't see it.

Smart Grid control centres

The human body is being transformed by the 'vaccines' and in other ways into a synthetic cyborg that can be attached to the global Smart Grid which would be controlled from a central point and other sub-locations of Grid manipulation. Where are these planned to be? Well, China for a start which is one of the Cult's biggest centres of operation. The technological control system and technocratic rule was incubated here to be unleashed across the world after the 'Covid' hoax came out of China in 2020. Another Smart Grid location that will surprise people new to this is Israel. I have exposed in *The Trigger* how Sabbatian technocrats, intelligence and military operatives were behind the horrors of 9/11 and not '19 Arab hijackers' who somehow manifested the ability to pilot big passenger airliners when instructors at puddle-jumping flying schools described some of them as a joke. The 9/11 attacks were made possible through control of civilian and military air computer systems and those of the White House, Pentagon and connected agencies. See *The Trigger* – it

will blow your mind. The controlling and coordinating force were the Sabbatian networks in Israel and the United States which by then had infiltrated the entire US government, military and intelligence system. The real name of the American Deep State is 'Sabbatian State'. Israel is a tiny country of only nine million people, but it is one of the global centres of cyber operations and fast catching Silicon Valley in importance to the Cult. Israel is known as the 'start-up nation' for all the cyber companies spawned there with the Sabbatian specialisation of 'cyber security' that I mentioned earlier which gives those companies access to computer systems of their clients in real time through 'backdoors' written into the coding when security software is downloaded. The Sabbatian centre of cyber operations outside Silicon Valley is the Israeli military Cyber Intelligence Unit, the biggest infrastructure project in Israel's history, headquartered in the desert-city of Beersheba and involving some 20,000 'cyber soldiers'. Here are located a literal army of Internet trolls scanning social media, forums and comment lists for anyone challenging the Cult agenda. The UK military has something similar with its 77th Brigade and associated operations. The Beersheba complex includes research and development centres for other Cult operations such as Intel, Microsoft, IBM, Google, Apple, Hewlett-Packard, Cisco Systems, Facebook and Motorola. Techcrunch.com ran an article about the Beersheba global Internet technology centre headlined 'Israel's desert city of Beersheba is turning into a cybertech oasis':

> The military's massive relocation of its prestigious technology units, the presence of multinational and local companies, a close proximity to Ben Gurion University and generous government subsidies are turning Beersheba into a major global cybertech hub. Beersheba has all of the ingredients of a vibrant security technology ecosystem, including Ben Gurion University with its graduate program in cybersecurity and Cyber Security Research Center, and the presence of companies such as EMC, Deutsche Telekom, PayPal, Oracle, IBM, and Lockheed Martin. It's also the future home of the INCB (Israeli National Cyber Bureau); offers a special income tax incentive for cyber security companies, and was the site for the relocation of the army's intelligence corps units.

Sabbatians have taken over the cyber world through the following process: They scan the schools for likely cyber talent and develop them at Ben Gurion University and their period of conscription in the Israeli Defense Forces when they are stationed at the Beersheba complex. When the cyber talented officially leave the army they are funded to start cyber companies with technology developed by themselves or given to them by the state. Much of this is stolen through backdoors of computer systems around the world with America top of the list. Others are sent off to Silicon Valley to start companies or join the major ones and so we have many major positions filled by apparently 'Jewish' but really Sabbatian operatives. Google, YouTube and Facebook are all run by 'Jewish' CEOs while Twitter is all but run by ultra-Zionist hedge-fund shark Paul Singer. At the centre of the Sabbatian global cyber web is the Israeli army's Unit 8200 which specialises in hacking into computer systems of other countries, inserting viruses, gathering information, instigating malfunction, and even taking control of them from a distance. A long list of Sabbatians involved with 9/11, Silicon Valley and Israeli cyber security companies are operatives of Unit 8200. This is not about Israel. It's about the Cult. Israel is planned to be a Smart Grid hub as with China and what is happening at Beersheba is not for the benefit of Jewish people who are treated disgustingly by the Sabbatian elite that control the country. A glance at the Nuremberg Codes will tell you that.

The story is much bigger than 'Covid', important as that is to where we are being taken. Now, though, it's time to really strap in. There's more … much more …

CHAPTER ELEVEN

Who controls the Cult?

Awake, arise or be forever fall'n
John Milton, *Paradise Lost*

I have exposed this far the level of the Cult conspiracy that operates in the world of the seen and within the global secret society and satanic network which operates in the shadows one step back from the seen. The story, however, goes much deeper than that.

The 'Covid' hoax is major part of the Cult agenda, but only part, and to grasp the biggest picture we have to expand our attention beyond the realm of human sight and into the infinity of possibility that we cannot see. It is from here, ultimately, that humanity is being manipulated into a state of total control by the force which dictates the actions of the Cult. How much of reality can we see? Next to damn all is the answer. We may appear to see all there is to see in the 'space' our eyes survey and observe, but little could be further from the truth. The human 'world' is only a tiny band of frequency that the body's visual and perceptual systems can decode into *perception* of a 'world'. According to mainstream science the electromagnetic spectrum is 0.005 percent of what exists in the Universe (Fig 10). The maximum estimate I have seen is 0.5 percent and either way it's miniscule. I say it is far, far, smaller even than 0.005 percent when you compare reality we see with the totality of reality that we don't. Now get this if you are new to such information: Visible light, the only band of frequency that we can see, is a *fraction* of the 0.005

percent (Fig 11 overleaf). Take this further and realise that our universe is one of infinite universes and that universes are only a fragment of overall reality – *infinite* reality. Then compare that with the almost infinitesimal frequency band of visible light or human sight. You see that humans are as near blind as it is possible to be without actually being so. Artist and filmmaker, Sergio Toporek, said:

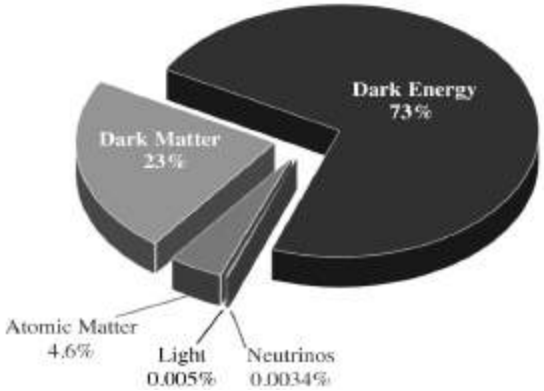

Figure 10: Humans can perceive such a tiny band of visual reality it's laughable.

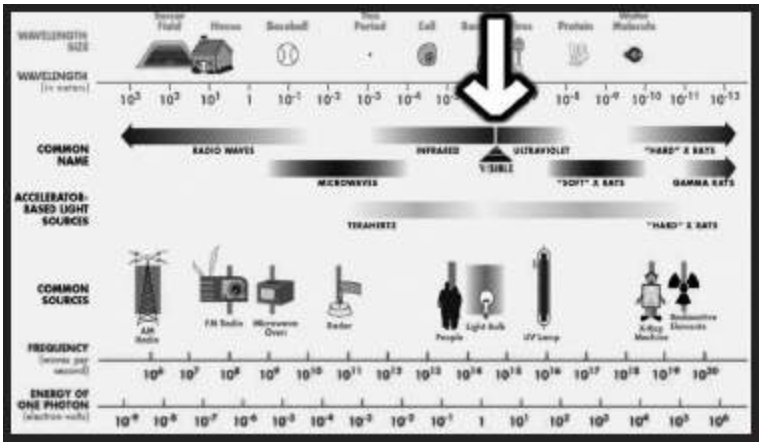

Figure 11: We can see a smear of the 0.005 percent electromagnetic spectrum, but we still know it all. Yep, makes sense.

Consider that you can see less than 1% of the electromagnetic spectrum and hear less than 1% of the acoustic spectrum. 90% of the cells in your body carry their own microbial DNA and are not 'you'. The atoms in your body are 99.9999999999999999% empty space and none of them are the ones you were born with ... Human beings have 46 chromosomes, two less than a potato.

The existence of the rainbow depends on the conical photoreceptors in your eyes; to animals without cones, the rainbow does not exist. So you don't just look at a rainbow, you create it. This is pretty amazing, especially considering that all the beautiful colours you see represent less than 1% of the electromagnetic spectrum.

Suddenly the 'world' of humans looks a very different place. Take into account, too, that Planet Earth when compared with the projected size of this single universe is the equivalent of a billionth of a pinhead. Imagine the ratio that would be when compared to infinite reality. To think that Christianity once insisted that Earth and humanity were the centre of everything. This background is vital if we are going to appreciate the nature of 'human' and how we can be manipulated by an unseen force. To human visual reality virtually *everything* is unseen and yet the prevailing perception within the institutions and so much of the public is that if we can't see it, touch it, hear it, taste it and smell it then it cannot exist. Such perception is indoctrinated and encouraged by the Cult and its agents because it isolates believers in the strictly limited, village-idiot, realm of the five senses where perceptions can be firewalled and information controlled. Most of those perpetuating the 'this-world-is-all-there-is' insanity are themselves indoctrinated into believing the same delusion. While major players and influencers know that official reality is laughable most of those in science, academia and medicine really believe the nonsense they peddle and teach succeeding generations. Those who challenge the orthodoxy are dismissed as nutters and freaks to protect the manufactured illusion from exposure. Observe the dynamic of the 'Covid' hoax and you will see how that takes the same form. The inner-circle psychopaths knows it's a gigantic scam, but almost the entirety of those imposing their fascist rules believe that 'Covid' is all that they're told it is.

Stolen identity

Ask people who they are and they will give you their name, place of birth, location, job, family background and life story. Yet that is not who they are – it is what they are *experiencing*. The difference is *absolutely crucial*. The true 'I', the eternal, infinite 'I', is consciousness,

a state of being aware. Forget 'form'. That is a vehicle for a brief experience. Consciousness does not come *from* the brain, but *through* the brain and even that is more symbolic than literal. We are awareness, pure awareness, and this is what withdraws from the body at what we call 'death' to continue our eternal beingness, *isness*, in other realms of reality within the limitlessness of infinity or the Biblical 'many mansions in my father's house'. Labels of a human life, man, woman, transgender, black, white, brown, nationality, circumstances and income are not who we are. They are what we are – awareness – is *experiencing* in a brief connection with a band of frequency we call 'human'. The labels are not the self; they are, to use the title of one of my books, a *Phantom* Self. I am not David Icke born in Leicester, England, on April 29th, 1952. I am the consciousness *having that experience*. The Cult and its non-human masters seek to convince us through the institutions of 'education', science, medicine, media and government that what we are *experiencing* is who we *are*. It's so easy to control and direct perception locked away in the bewildered illusions of the five senses with no expanded radar. Try, by contrast, doing the same with a humanity aware of its true self and its true power to consciously create its reality and experience. How is it possible to do this? We do it all day every day. If you perceive yourself as 'little me' with no power to impact upon your life and the world then your life experience will reflect that. You will hand the power you don't think you have to authority in all its forms which will use it to control your experience. This, in turn, will appear to confirm your perception of 'little me' in a self-fulfilling feedback loop. But that is what 'little me' really is – a *perception*. We are all 'big-me', infinite me, and the Cult has to make us forget that if its will is to prevail. We are therefore manipulated and pressured into self-identifying with human labels and not the consciousness/awareness *experiencing* those human labels.

The phenomenon of identity politics is a Cult-instigated manipulation technique to sub-divide previous labels into even smaller ones. A United States university employs this list of letters to

describe student identity: LGBTTQQFAGPBDSM or lesbian, gay, bisexual, transgender, transsexual, queer, questioning, flexual, asexual, gender-fuck, polyamorous, bondage/discipline, dominance/submission and sadism/masochism. I'm sure other lists are even longer by now as people feel the need to self-identity the 'I' with the minutiae of race and sexual preference. Wokers programmed by the Cult for generations believe this is about 'inclusivity' when it's really the Cult locking them away into smaller and smaller versions of Phantom Self while firewalling them from the influence of their true self, the infinite, eternal 'I'. You may notice that my philosophy which contends that we are all unique points of attention/awareness within the same infinite whole or Oneness is the ultimate non-racism. The very sense of Oneness makes the judgement of people by their body-type, colour or sexuality utterly ridiculous and confirms that racism has no understanding of reality (including anti-white racism). Yet despite my perception of life Cult agents and fast-asleep Wokers label me racist to discredit my information while they are themselves phenomenally racist and sexist. All they see is race and sexuality and they judge people as good or bad, demons or untouchables, by their race and sexuality. All they see is *Phantom Self* and perceive themselves in terms of Phantom Self. They are pawns and puppets of the Cult agenda to focus attention and self-identity in the five senses and play those identities against each other to divide and rule. Columbia University has introduced segregated graduations in another version of social distancing designed to drive people apart and teach them that different racial and cultural groups have nothing in common with each other. The last thing the Cult wants is unity. Again the pump-primers of this will be Cult operatives in the knowledge of what they are doing, but the rest are just the Phantom Self blind leading the Phantom Self blind. We *do* have something in common – we are all *the same consciousness* having different temporary experiences.

What is this 'human'?

Yes, what *is* 'human'? That is what we are supposed to be, right? I mean 'human'? True, but 'human' is the experience not the 'I'. Break it down to basics and 'human' is the way that information is processed. If we are to experience and interact with this band of frequency we call the 'world' we must have a vehicle that operates within that band of frequency. Our consciousness in its prime form cannot do that; it is way beyond the frequency of the human realm. My consciousness or awareness could not tap these keys and pick up the cup in front of me in the same way that radio station A cannot interact with radio station B when they are on different frequencies. The human body is the means through which we have that interaction. I have long described the body as a biological computer which processes information in a way that allows consciousness to experience this reality. The body is a receiver, transmitter and processor of information in a particular way that we call human. We visually perceive only the world of the five senses in a wakened state – that is the limit of the body's visual decoding system. In truth it's not even visual in the way we experience 'visual reality' as I will come to in a moment. We are 'human' because the body processes the information sources of human into a reality and behaviour system that we *perceive* as human. Why does an elephant act like an elephant and not like a human or a duck? The elephant's biological computer is a different information field and processes information according to that program into a visual and behaviour type we call an elephant. The same applies to everything in our reality. These body information fields are perpetuated through procreation (like making a copy of a software program). The Cult wants to break that cycle and intervene technologically to transform the human information field into one that will change what we call humanity. If it can change the human information field it will change the way that field processes information and change humanity both 'physically' and psychologically. Hence the *messenger* (information) RNA 'vaccines' and so much more that is targeting human genetics by changing the body's information – *messaging* – construct through food, drink, radiation, toxicity and other means.

Reality that we experience is nothing like reality as it really is in the same way that the reality people experience in virtual reality games is not the reality they are really living in. The game is only a decoded source of information that appears to be a reality. Our world is also an information construct – a *simulation* (more later). In its base form our reality is a wavefield of information much the same in theme as Wi-Fi. The five senses decode wavefield information into electrical information which they communicate to the brain to decode into holographic (illusory 'physical') information. Different parts of the brain specialise in decoding different senses and the information is fused into a reality that appears to be outside of us but is really inside the brain and the genetic structure in general (Fig 12 overleaf). DNA is a receiver-transmitter of information and a vital part of this decoding process and the body's connection to other realities. Change DNA and you change the way we decode and connect with reality – see 'Covid vaccines'. Think of computers decoding Wi-Fi. You have information encoded in a radiation field and the computer decodes that information into a very different form on the screen. You can't see the Wi-Fi until its information is made manifest on the screen and the information on the screen is inside the computer and not outside. I have just described how we decode the 'human world'. All five senses decode the waveform 'Wi-Fi' field into electrical signals and the brain (computer) constructs reality inside the brain and not outside – 'You don't just look at a rainbow, you create it'. Sound is a simple example. We don't hear sound until the brain decodes it. Waveform sound waves are picked up by the hearing sense and communicated to the brain in an electrical form to be decoded into the sounds that we hear. Everything we hear is inside the brain along with everything we see, feel, smell and taste. Words and language are waveform fields generated by our vocal chords which pass through this process until they are decoded by the brain into words that we hear. Different languages are different frequency fields or sound waves generated by vocal chords. Late British philosopher Alan Watts said:

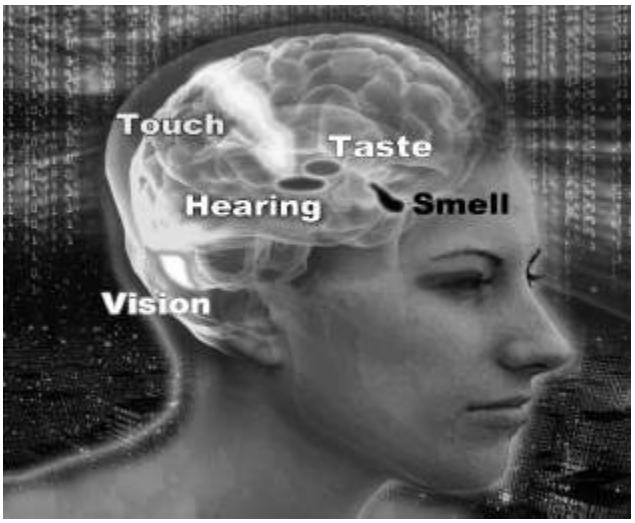

Figure 12: The brain receives information from the five senses and constructs from that our perceived reality.

[Without the brain] the world is devoid of light, heat, weight, solidity, motion, space, time or any other imaginable feature. All these phenomena are interactions, or transactions, of vibrations with a certain arrangement of neurons.

That's exactly what they are and scientist Robert Lanza describes in his book, *Biocentrism*, how we decode electromagnetic waves and energy into visual and 'physical' experience. He uses the example of a flame emitting photons, electromagnetic energy, each pulsing electrically and magnetically:

… these … invisible electromagnetic waves strike a human retina, and if (and only if) the waves happen to measure between 400 and 700 nano meters in length from crest to crest, then their energy is just right to deliver a stimulus to the 8 million cone-shaped cells in the retina.

Each in turn send an electrical pulse to a neighbour neuron, and on up the line this goes, at 250 mph, until it reaches the … occipital lobe of the brain, in the back of the head. There, a cascading complex of neurons fire from the incoming stimuli, and we subjectively perceive this experience as a yellow brightness occurring in a place we have been conditioned to call the 'external world'.

You hear what you decode

If a tree falls or a building collapses they make no noise unless someone is there to decode the energetic waves generated by the disturbance into what we call sound. Does a falling tree make a noise? Only if you hear it – *decode* it. Everything in our reality is a frequency field of information operating within the overall 'Wi-Fi' field that I call The Field. A vibrational disturbance is generated in The Field by the fields of the falling tree or building. These disturbance waves are what we decode into the sound of them falling. If no one is there to do that then neither will make any noise. Reality is created by the observer – *decoder* – and the *perceptions* of the observer affect the decoding process. For this reason different people – different *perceptions* – will perceive the same reality or situation in a different way. What one may perceive as a nightmare another will see as an opportunity. The question of why the Cult is so focused on controlling human perception now answers itself. All experienced reality is the act of decoding and we don't experience Wi-Fi until it is decoded on the computer screen. The sight and sound of an Internet video is encoded in the Wi-Fi all around us, but we don't see or hear it until the computer decodes that information. Taste, smell and touch are all phenomena of the brain as a result of the same process. We don't taste, smell or feel anything except in the brain and there are pain relief techniques that seek to block the signal from the site of discomfort to the brain because if the brain doesn't decode that signal we don't feel pain. Pain is in the brain and only appears to be at the point of impact thanks to the feedback loop between them. We don't see anything until electrical information from the sight senses is decoded in an area at the back of the brain. If that area is damaged we can go blind when our eyes are perfectly okay. So why do we go blind if we damage an eye? We damage the information processing between the waveform visual information and the visual decoding area of the brain. If information doesn't reach the brain in a form it can decode then we can't see the visual reality that it represents. What's more the brain is decoding only a fraction of the information it receives and the rest is absorbed by the

sub-conscious mind. This explanation is from the science magazine, *Wonderpedia*:

> Every second, 11 million sensations crackle along these [brain] pathways ... The brain is confronted with an alarming array of images, sounds and smells which it rigorously filters down until it is left with a manageable list of around 40. Thus 40 sensations per second make up what we perceive as reality.

The 'world' is not what people are told to believe that is it and the inner circles of the Cult *know that*.

Illusory 'physical' reality

We can only see a smear of 0.005 percent of the Universe which is only one of a vast array of universes – 'mansions' – within infinite reality. Even then the brain decodes only 40 pieces of information ('sensations') from a potential *11 million* that we receive every second. Two points strike you from this immediately: The sheer breathtaking stupidity of believing we know anything so rigidly that there's nothing more to know; and the potential for these processes to be manipulated by a malevolent force to control the reality of the population. One thing I can say for sure with no risk of contradiction is that when you can perceive an almost indescribable fraction of infinite reality there is always more to know as in tidal waves of it. Ancient Greek philosopher Socrates was so right when he said that wisdom is to know how little we know. How obviously true that is when you think that we are experiencing a physical world of solidity that is neither physical nor solid and a world of apartness when everything is connected. Cult-controlled 'science' dismisses the so-called 'paranormal' and all phenomena related to that when the 'para'-normal is perfectly normal and explains the alleged 'great mysteries' which dumbfound scientific minds. There is a reason for this. A 'scientific mind' in terms of the mainstream is a material mind, a five-sense mind imprisoned in see it, touch it, hear it, smell it and taste it. Phenomena and happenings that can't be explained that way leave the 'scientific mind' bewildered and the rule is that if they

can't account for why something is happening then it can't, by definition, be happening. I beg to differ. Telepathy is thought waves passing through The Field (think wave disturbance again) to be decoded by someone able to connect with that wavelength (information). For example: You can pick up the thought waves of a friend at any distance and at the very least that will bring them to mind. A few minutes later the friend calls you. 'My god', you say, 'that's incredible – I was just thinking of you.' Ah, but *they* were thinking of *you* before they made the call and that's what you decoded. Native peoples not entrapped in five-sense reality do this so well it became known as the 'bush telegraph'. Those known as psychics and mediums (genuine ones) are doing the same only across dimensions of reality. 'Mind over matter' comes from the fact that matter and mind are the *same*. The state of one influences the state of the other. Indeed one *and* the other are illusions. They are aspects of the same field. Paranormal phenomena are all explainable so why are they still considered 'mysteries' or not happening? Once you go down this road of understanding you begin to expand awareness beyond the five senses and that's the nightmare for the Cult.

Figure 13: Holograms are not solid, but the best ones appear to be.

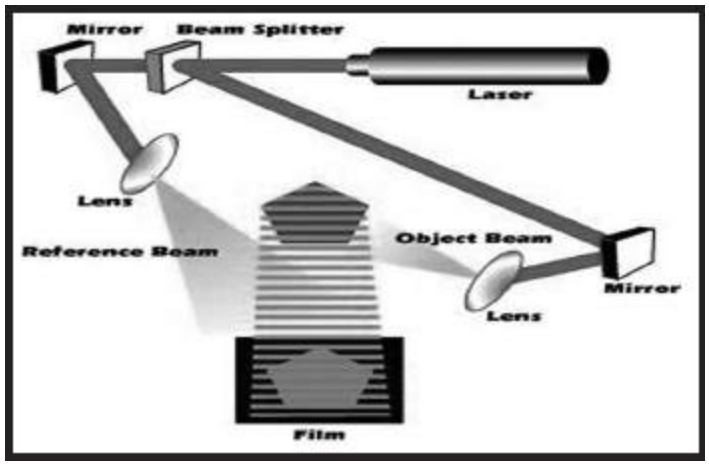

Figure 14: How holograms are created by capturing a waveform version of the subject image.

Holographic 'solidity'

Our reality is not solid, it is holographic. We are now well aware of holograms which are widely used today. Two-dimensional information is decoded into a three-dimensional reality that is not solid although can very much appear to be (Fig 13). Holograms are created with a laser divided into two parts. One goes directly onto a holographic photographic print ('reference beam') and the other takes a waveform image of the subject ('working beam') before being directed onto the print where it 'collides' with the other half of the laser (Fig 14). This creates a *waveform* interference pattern which contains the wavefield information of whatever is being photographed (Fig 15 overleaf). The process can be likened to dropping pebbles in a pond. Waves generated by each one spread out across the water to collide with the others and create a wave representation of where the stones fell and at what speed, weight and distance. A waveform interference pattern of a hologram is akin to the waveform information in The Field which the five senses decode into electrical signals to be decoded by the brain into a holographic illusory 'physical' reality. In the same way when a laser (think human attention) is directed at the waveform interference pattern a three-dimensional version of the subject is projected into apparently 'solid' reality (Fig 16). An amazing trait of holograms reveals more 'paranormal mysteries'. Information of the *whole*

hologram is encoded in waveform in every part of the interference pattern by the way they are created. This means that every *part* of a hologram is a smaller version of the whole. Cut the interference wave-pattern into four and you won't get four parts of the image. You get quarter-sized versions of the *whole* image. The body is a hologram and the same applies. Here we have the basis of acupuncture, reflexology and other forms of healing which identify representations of the whole body in all of the parts, hands, feet, ears, everywhere. Skilled palm readers can do what they do because the information of whole body is encoded in the hand. The concept of as above, so below, comes from this.

Figure 15: A waveform interference pattern that holds the information that transforms into a hologram.

Figure 16: Holographic people including 'Elvis' holographically inserted to sing a duet with Celine Dion.

The question will be asked of why, if solidity is illusory, we can't just walk through walls and each other. The resistance is not solid against solid; it is electromagnetic field against electromagnetic field and we decode this into the *experience* of solid against solid. We should also not underestimate the power of belief to dictate reality. What you believe is impossible *will be*. Your belief impacts on your decoding processes and they won't decode what you think is impossible. What we believe we perceive and what we perceive we experience. 'Can't dos' and 'impossibles' are like a firewall in a computer system that won't put on the screen what the firewall blocks. How vital that is to understanding how human experience has been hijacked. I explain in *The Answer*, *Everything You Need To Know But Have Never Been Told* and other books a long list of 'mysteries' and 'paranormal' phenomena that are not mysterious and perfectly normal once you realise what reality is and how it works. 'Ghosts' can be seen to pass through 'solid' walls because the walls are not solid and the ghost is a discarnate entity operating on a frequency so different to that of the wall that it's like two radio stations sharing the same space while never interfering with each other. I have seen ghosts do this myself. The apartness of people and objects is also an illusion. Everything is connected by the Field like all sea life is connected by the sea. It's just that within the limits of our visual reality we only 'see' holographic information and not the field of information that connects everything and from which the holographic world is made manifest. If you can only see holographic 'objects' and not the field that connects them they will appear to you as unconnected to each other in the same way that we see the computer while not seeing the Wi-Fi.

What you don't know *can* hurt you

Okay, we return to those 'two worlds' of human society and the Cult with its global network of interconnecting secret societies and satanic groups which manipulate through governments, corporations, media, religions, etc. The fundamental difference between them is *knowledge*. The idea has been to keep humanity

ignorant of the plan for its total enslavement underpinned by a crucial ignorance of reality – who we are and where we are – and how we interact with it. 'Human' should be the interaction between our expanded eternal consciousness and the five-sense body experience. We are meant to be *in* this world in terms of the five senses but not *of* this world in relation to our greater consciousness and perspective. In that state we experience the small picture of the five senses within the wider context of the big picture of awareness beyond the five senses. Put another way the five senses see the dots and expanded awareness connects them into pictures and patterns that give context to the apparently random and unconnected. Without the context of expanded awareness the five senses see only apartness and randomness with apparently no meaning. The Cult and its other-dimensional controllers seek to intervene in the frequency realm where five-sense reality is supposed to connect with expanded reality and to keep the two apart (more on this in the final chapter). When that happens five-sense mental and emotional processes are no longer influenced by expanded awareness, or the True 'I', and instead are driven by the isolated perceptions of the body's decoding systems. They are in the world *and* of it. Here we have the human plight and why humanity with its potential for infinite awareness can be so easily manipulatable and descend into such extremes of stupidity.

Once the Cult isolates five-sense mind from expanded awareness it can then program the mind with perceptions and beliefs by controlling information that the mind receives through the 'education' system of the formative years and the media perceptual bombardment and censorship of an entire lifetime. Limit perception and a sense of the possible through limiting knowledge by limiting and skewing information while censoring and discrediting that which could set people free. As the title of another of my books says *… And The Truth Shall Set You Free*. For this reason the last thing the Cult wants in circulation is the truth about anything – especially the reality of the eternal 'I' – and that's why it is desperate to control information. The Cult knows that information becomes perception

which becomes behaviour which, collectively, becomes human society. Cult-controlled and funded mainstream 'science' denies the existence of an eternal 'I' and seeks to dismiss and trash all evidence to the contrary. Cult-controlled mainstream religion has a version of 'God' that is little more than a system of control and dictatorship that employs threats of damnation in an afterlife to control perceptions and behaviour in the here and now through fear and guilt. Neither is true and it's the 'neither' that the Cult wishes to suppress. This 'neither' is that everything is an expression, a point of attention, within an infinite state of consciousness which is the real meaning of the term 'God'.

Perceptual obsession with the 'physical body' and five-senses means that 'God' becomes personified as a bearded bloke sitting among the clouds or a raging bully who loves us if we do what 'he' wants and condemns us to the fires of hell if we don't. These are no more than a 'spiritual' fairy tales to control and dictate events and behaviour through fear of this 'God' which has bizarrely made 'God-fearing' in religious circles a state to be desired. I would suggest that fearing *anything* is not to be encouraged and celebrated, but rather deleted. You can see why 'God fearing' is so beneficial to the Cult and its religions when *they* decide what 'God' wants and what 'God' demands (the Cult demands) that everyone do. As the great American comedian Bill Hicks said satirising a Christian zealot: 'I think what God meant to say.' How much of this infinite awareness ('God') that we access is decided by how far we choose to expand our perceptions, self-identity and sense of the possible. The scale of self-identity reflects itself in the scale of awareness that we can connect with and are influenced by – how much knowing and insight we have instead of programmed perception. You cannot expand your awareness into the infinity of possibility when you believe that you are little me Peter the postman or Mary in marketing and nothing more. I'll deal with this in the concluding chapter because it's crucial to how we turnaround current events.

Where the Cult came from

When I realised in the early 1990s there was a Cult network behind global events I asked the obvious question: When did it start? I took it back to ancient Rome and Egypt and on to Babylon and Sumer in Mesopotamia, the 'Land Between Two Rivers', in what we now call Iraq. The two rivers are the Tigris and Euphrates and this region is of immense historical and other importance to the Cult, as is the land called Israel only 550 miles away by air. There is much more going with deep esoteric meaning across this whole region. It's not only about 'wars for oil'. Priceless artefacts from Mesopotamia were stolen or destroyed after the American and British invasion of Iraq in 2003 justified by the lies of Boy Bush and Tony Blair (their Cult masters) about non-existent 'weapons of mass destruction'. Mesopotamia was the location of Sumer (about 5,400BC to 1,750BC), and Babylon (about 2,350BC to 539BC). Sabbatians may have become immensely influential in the Cult in modern times but they are part of a network that goes back into the mists of history. Sumer is said by historians to be the 'cradle of civilisation'. I disagree. I say it was the re-start of what we call human civilisation after cataclysmic events symbolised in part as the 'Great Flood' destroyed the world that existed before. These fantastic upheavals that I have been describing in detail in the books since the early1990s appear in accounts and legends of ancient cultures across the world and they are supported by geological and biological evidence. Stone tablets found in Iraq detailing the Sumer period say the cataclysms were caused by non-human 'gods' they call the Anunnaki. These are described in terms of extraterrestrial visitations in which knowledge supplied by the Anunnaki is said to have been the source of at least one of the world's oldest writing systems and developments in astronomy, mathematics and architecture that were way ahead of their time. I have covered this subject at length in *The Biggest Secret* and *Children of the Matrix* and the same basic 'Anunnaki' story can be found in Zulu accounts in South Africa where the late and very great Zulu high shaman Credo Mutwa told me that the Sumerian Anunnaki were known by Zulus as the Chitauri or 'children of the serpent'. See my six-hour video interview with Credo on this subject entitled *The*

Reptilian Agenda recorded at his then home near Johannesburg in 1999 which you can watch on the Ickonic media platform.

The Cult emerged out of Sumer, Babylon and Egypt (and elsewhere) and established the Roman Empire before expanding with the Romans into northern Europe from where many empires were savagely imposed in the form of Cult-controlled societies all over the world. Mass death and destruction was their calling card. The Cult established its centre of operations in Europe and European Empires were Cult empires which allowed it to expand into a global force. Spanish and Portuguese colonialists headed for Central and South America while the British and French targeted North America. Africa was colonised by Britain, France, Belgium, the Netherlands, Portugal, Spain, Italy, and Germany. Some like Britain and France moved in on the Middle East. The British Empire was by far the biggest for a simple reason. By now Britain was the headquarters of the Cult from which it expanded to form Canada, the United States, Australia and New Zealand. The Sun never set on the British Empire such was the scale of its occupation. London remains a global centre for the Cult along with Rome and the Vatican although others have emerged in Israel and China. It is no accident that the 'virus' is alleged to have come out of China while Italy was chosen as the means to terrify the Western population into compliance with 'Covid' fascism. Nor that Israel has led the world in 'Covid' fascism and mass 'vaccination'.

You would think that I would mention the United States here, but while it has been an important means of imposing the Cult's will it is less significant than would appear and is currently in the process of having what power it does have deleted. The Cult in Europe has mostly loaded the guns for the US to fire. America has been controlled from Europe from the start through Cult operatives in Britain and Europe. The American Revolution was an illusion to make it appear that America was governing itself while very different forces were pulling the strings in the form of Cult families such as the Rothschilds through the Rockefellers and other subordinates. The Rockefellers are extremely close to Bill Gates and

established both scalpel and drug 'medicine' and the World Health Organization. They play a major role in the development and circulation of vaccines through the Rockefeller Foundation on which Bill Gates said his Foundation is based. Why wouldn't this be the case when the Rockefellers and Gates are on the same team? Cult infiltration of human society goes way back into what we call history and has been constantly expanding and centralising power with the goal of establishing a global structure to dictate everything. Look how this has been advanced in great leaps with the 'Covid' hoax.

The non-human dimension

I researched and observed the comings and goings of Cult operatives through the centuries and even thousands of years as they were born, worked to promote the agenda within the secret society and satanic networks, and then died for others to replace them. Clearly there had to be a coordinating force that spanned this entire period while operatives who would not have seen the end goal in their lifetimes came and went advancing the plan over millennia. I went in search of that coordinating force with the usual support from the extraordinary synchronicity of my life which has been an almost daily experience since 1990. I saw common themes in religious texts and ancient cultures about a non-human force manipulating human society from the hidden. Christianity calls this force Satan, the Devil and demons; Islam refers to the Jinn or Djinn; Zulus have their Chitauri (spelt in other ways in different parts of Africa); and the Gnostic people in Egypt in the period around and before 400AD referred to this phenomena as the 'Archons', a word meaning rulers in Greek. Central American cultures speak of the 'Predators' among other names and the same theme is everywhere. I will use 'Archons' as a collective name for all of them. When you see how their nature and behaviour is described all these different sources are clearly talking about the same force. Gnostics described the Archons in terms of 'luminous fire' while Islam relates the Jinn to 'smokeless fire'. Some refer to beings in form that could occasionally be seen, but the most common of common theme is that they operate from

unseen realms which means almost all existence to the visual processes of humans. I had concluded that this was indeed the foundation of human control and that the Cult was operating within the human frequency band on behalf of this hidden force when I came across the writings of Gnostics which supported my conclusions in the most extraordinary way.

A sealed earthen jar was found in 1945 near the town of Nag Hammadi about 75-80 miles north of Luxor on the banks of the River Nile in Egypt. Inside was a treasure trove of manuscripts and texts left by the Gnostic people some 1,600 years earlier. They included 13 leather-bound papyrus codices (manuscripts) and more than 50 texts written in Coptic Egyptian estimated to have been hidden in the jar in the period of 400AD although the source of the information goes back much further. Gnostics oversaw the Great or Royal Library of Alexandria, the fantastic depository of ancient texts detailing advanced knowledge and accounts of human history. The Library was dismantled and destroyed in stages over a long period with the death-blow delivered by the Cult-established Roman Church in the period around 415AD. The Church of Rome was the Church of Babylon relocated as I said earlier. Gnostics were not a race. They were a way of perceiving reality. Whenever they established themselves and their information circulated the terrorists of the Church of Rome would target them for destruction. This happened with the Great Library and with the Gnostic Cathars who were burned to death by the psychopaths after a long period of oppression at the siege of the Castle of Monségur in southern France in 1244. The Church has always been terrified of Gnostic information which demolishes the official Christian narrative although there is much in the Bible that supports the Gnostic view if you read it in another way. To anyone studying the texts of what became known as the Nag Hammadi Library it is clear that great swathes of Christian and Biblical belief has its origin with Gnostics sources going back to Sumer. Gnostic themes have been twisted to manipulate the perceived reality of Bible believers. Biblical texts have been in the open for centuries where they could be changed while Gnostic

documents found at Nag Hammadi were sealed away and untouched for 1,600 years. What you see is what they wrote.

Use your *pneuma* not your *nous*

Gnosticism and Gnostic come from 'gnosis' which means knowledge, or rather *secret* knowledge, in the sense of spiritual awareness – knowledge about reality and life itself. The desperation of the Cult's Church of Rome to destroy the Gnostics can be understood when the knowledge they were circulating was the last thing the Cult wanted the population to know. Sixteen hundred years later the same Cult is working hard to undermine and silence me for the same reason. The dynamic between knowledge and ignorance is a constant. 'Time' appears to move on, but essential themes remain the same. We are told to 'use your nous', a Gnostic word for head/brain/intelligence. They said, however, that spiritual awakening or 'salvation' could only be secured by expanding awareness *beyond* what they called *nous* and into *pneuma* or Infinite Self. Obviously as I read these texts the parallels with what I have been saying since 1990 were fascinating to me. There is a universal truth that spans human history and in that case why wouldn't we be talking the same language 16 centuries apart? When you free yourself from the perception program of the five senses and explore expanded realms of consciousness you are going to connect with the same information no matter what the perceived 'era' within a manufactured timeline of a single and tiny range of manipulated frequency. Humans working with 'smart' technology or knocking rocks together in caves is only a timeline appearing to operate within the human frequency band. Expanded awareness and the knowledge it holds have always been there whether the era be Stone Age or computer age. We can only access that knowledge by opening ourselves to its frequency which the five-sense prison cell is designed to stop us doing. Gates, Fauci, Whitty, Vallance, Zuckerberg, Brin, Page, Wojcicki, Bezos, and all the others behind the 'Covid' hoax clearly have a long wait before their range of frequency can make that connection given that an open heart is

crucial to that as we shall see. Instead of accessing knowledge directly through expanded awareness it is given to Cult operatives by the secret society networks of the Cult where it has been passed on over thousands of years outside the public arena. Expanded realms of consciousness is where great artists, composers and writers find their inspiration and where truth awaits anyone open enough to connect with it. We need to go there fast.

Archon hijack

A fifth of the Nag Hammadi texts describe the existence and manipulation of the Archons led by a 'Chief Archon' they call 'Yaldabaoth', or the 'Demiurge', and this is the Christian 'Devil', 'Satan', 'Lucifer', and his demons. Archons in Biblical symbolism are the 'fallen ones' which are also referred to as fallen angels after the angels expelled from heaven according to the Abrahamic religions of Judaism, Christianity and Islam. These angels are claimed to tempt humans to 'sin' ongoing and you will see how accurate that symbolism is during the rest of the book. The theme of 'original sin' is related to the 'Fall' when Adam and Eve were 'tempted by the serpent' and fell from a state of innocence and 'obedience' (connection) with God into a state of disobedience (disconnection). The Fall is said to have brought sin into the world and corrupted everything including human nature. Yaldabaoth, the 'Lord Archon', is described by Gnostics as a 'counterfeit spirit', 'The Blind One', 'The Blind God', and 'The Foolish One'. The Jewish name for Yaldabaoth in Talmudic writings is Samael which translates as 'Poison of God', or 'Blindness of God'. You see the parallels. Yaldabaoth in Islamic belief is the Muslim Jinn devil known as Shaytan – Shaytan is Satan as the same themes are found all over the world in every religion and culture. The 'Lord God' of the Old Testament is the 'Lord Archon' of Gnostic manuscripts and that's why he's such a bloodthirsty bastard. Satan is known by Christians as 'the Demon of Demons' and Gnostics called Yaldabaoth the 'Archon of Archons'. Both are known as 'The Deceiver'. We are talking about the same 'bloke' for sure and these common themes

using different names, storylines and symbolism tell a common tale of the human plight.

Archons are referred to in Nag Hammadi documents as mind parasites, inverters, guards, gatekeepers, detainers, judges, pitiless ones and deceivers. The 'Covid' hoax alone is a glaring example of all these things. The Biblical 'God' is so different in the Old and New Testaments because they are not describing the same phenomenon. The vindictive, angry, hate-filled, 'God' of the Old Testament, known as Yahweh, is Yaldabaoth who is depicted in Cult-dictated popular culture as the 'Dark Lord', 'Lord of Time', Lord (Darth) Vader and Dormammu, the evil ruler of the 'Dark Dimension' trying to take over the 'Earth Dimension' in the Marvel comic movie, *Dr Strange*. Yaldabaoth is both the Old Testament 'god' and the Biblical 'Satan'. Gnostics referred to Yaldabaoth as the 'Great Architect of the Universe'and the Cult-controlled Freemason network calls their god 'the 'Great Architect of the Universe' (also Grand Architect). The 'Great Architect' Yaldabaoth is symbolised by the Cult as the all-seeing eye at the top of the pyramid on the Great Seal of the United States and the dollar bill. Archon is encoded in *arch*-itect as it is in *arch*-angels and *arch*-bishops. All religions have the theme of a force for good and force for evil in some sort of spiritual war and there is a reason for that – the theme is true. The Cult and its non-human masters are quite happy for this to circulate. They present themselves as the force for good fighting evil when they are really the force of evil (absence of love). The whole foundation of Cult modus operandi is inversion. They promote themselves as a force for good and anyone challenging them in pursuit of peace, love, fairness, truth and justice is condemned as a satanic force for evil. This has been the game plan throughout history whether the Church of Rome inquisitions of non-believers or 'conspiracy theorists' and 'anti-vaxxers' of today. The technique is the same whatever the timeline era.

Yaldabaoth is revolting (true)

Yaldabaoth and the Archons are said to have revolted against God with Yaldabaoth claiming to *be* God – the *All That Is*. The Old Testament 'God' (Yaldabaoth) demanded to be worshipped as such: ' *I am* the LORD, and there is none else, there is no God beside me' (Isaiah 45:5). I have quoted in other books a man who said he was the unofficial son of the late Baron Philippe de Rothschild of the Mouton-Rothschild wine producing estates in France who died in 1988 and he told me about the Rothschild 'revolt from God'. The man said he was given the name Phillip Eugene de Rothschild and we shared long correspondence many years ago while he was living under another identity. He said that he was conceived through 'occult incest' which (within the Cult) was 'normal and to be admired'. 'Phillip' told me about his experience attending satanic rituals with rich and famous people whom he names and you can see them and the wider background to Cult Satanism in my other books starting with *The Biggest Secret*. Cult rituals are interactions with Archontic 'gods'. 'Phillip' described Baron Philippe de Rothschild as 'a master Satanist and hater of God' and he used the same term 'revolt from God' associated with Yaldabaoth/Satan/Lucifer/the Devil in describing the Sabbatian Rothschild dynasty. 'I played a key role in my family's revolt from God', he said. That role was to infiltrate in classic Sabbatian style the Christian Church, but eventually he escaped the mind-prison to live another life. The Cult has been targeting religion in a plan to make worship of the Archons the global one-world religion. Infiltration of Satanism into modern 'culture', especially among the young, through music videos, stage shows and other means, is all part of this.

Nag Hammadi texts describe Yaldabaoth and the Archons in their prime form as energy – consciousness – and say they can take form if they choose in the same way that consciousness takes form as a human. Yaldabaoth is called 'formless' and represents a deeply inverted, distorted and chaotic state of consciousness which seeks to attached to humans and turn them into a likeness of itself in an attempt at assimilation. For that to happen it has to manipulate

humans into low frequency mental and emotional states that match its own. Archons can certainly appear in human form and this is the origin of the psychopathic personality. The energetic distortion Gnostics called Yaldabaoth *is* psychopathy. When psychopathic Archons take human form that human will be a psychopath as an expression of Yaldabaoth consciousness. Cult psychopaths are Archons in human form. The principle is the same as that portrayed in the 2009 *Avatar* movie when the American military travelled to a fictional Earth-like moon called Pandora in the Alpha Centauri star system to infiltrate a society of blue people, or Na'vi, by hiding within bodies that looked like the Na'vi. Archons posing as humans have a particular hybrid information field, part human, part Archon, (the ancient 'demigods') which processes information in a way that manifests behaviour to match their psychopathic evil, lack of empathy and compassion, and stops them being influenced by the empathy, compassion and love that a fully-human information field is capable of expressing. Cult bloodlines interbreed, be they royalty or dark suits, for this reason and you have their obsession with incest. Interbreeding with full-blown humans would dilute the Archontic energy field that guarantees psychopathy in its representatives in the human realm.

Gnostic writings say the main non-human forms that Archons take are *serpentine* (what I have called for decades 'reptilian' amid unbounded ridicule from the Archontically-programmed) and what Gnostics describe as 'an unborn baby or foetus with grey skin and dark, unmoving eyes'. This is an excellent representation of the ET 'Greys' of UFO folklore which large numbers of people claim to have seen and been abducted by – Zulu shaman Credo Mutwa among them. I agree with those that believe in extraterrestrial or interdimensional visitations today and for thousands of years past. No wonder with their advanced knowledge and technological capability they were perceived and worshipped as gods for technological and other 'miracles' they appeared to perform. Imagine someone arriving in a culture disconnected from the modern world with a smartphone and computer. They would be

seen as a 'god' capable of 'miracles'. The Renegade Mind, however, wants to know the source of everything and not only the way that source manifests as human or non-human. In the same way that a Renegade Mind seeks the original source material for the 'Covid virus' to see if what is claimed is true. The original source of Archons in form is consciousness – the distorted state of consciousness known to Gnostics as Yaldabaoth.

'Revolt from God' is energetic disconnection

Where I am going next will make a lot of sense of religious texts and ancient legends relating to 'Satan', Lucifer' and the 'gods'. Gnostic descriptions sync perfectly with the themes of my own research over the years in how they describe a consciousness distortion seeking to impose itself on human consciousness. I've referred to the core of infinite awareness in previous books as Infinite Awareness in Awareness of Itself. By that I mean a level of awareness that knows that it is all awareness and is aware of all awareness. From here comes the frequency of love in its true sense and balance which is what love is on one level – the balance of all forces into a single whole called Oneness and Isness. The more we disconnect from this state of love that many call 'God' the constituent parts of that Oneness start to unravel and express themselves as a part and not a whole. They become individualised as intellect, mind, selfishness, hatred, envy, desire for power over others, and such like. This is not a problem in the greater scheme in that 'God', the *All That Is*, can experience all these possibilities through different expressions of itself including humans. What we as expressions of the whole experience the *All That Is* experiences. We are the *All That Is* experiencing itself. As we withdraw from that state of Oneness we disconnect from its influence and things can get very unpleasant and very stupid. Archontic consciousness is at the extreme end of that. It has so disconnected from the influence of Oneness that it has become an inversion of unity and love, an inversion of everything, an inversion of life itself. Evil is appropriately live written backwards. Archontic consciousness is obsessed with death, an inversion of life,

and so its manifestations in Satanism are obsessed with death. They use inverted symbols in their rituals such as the inverted pentagram and cross. Sabbatians as Archontic consciousness incarnate invert Judaism and every other religion and culture they infiltrate. They seek disunity and chaos and they fear unity and harmony as they fear love like garlic to a vampire. As a result the Cult, Archons incarnate, act with such evil, psychopathy and lack of empathy and compassion disconnected as they are from the source of love. How could Bill Gates and the rest of the Archontic psychopaths do what they have to human society in the 'Covid' era with all the death, suffering and destruction involved and have no emotional consequence for the impact on others? Now you know. Why have Zuckerberg, Brin, Page, Wojcicki and company callously censored information warning about the dangers of the 'vaccine' while thousands have been dying and having severe, sometimes life-changing reactions? Now you know. Why have Tedros, Fauci, Whitty, Vallance and their like around the world been using case and death figures they're aware are fraudulent to justify lockdowns and all the deaths and destroyed lives that have come from that? Now you know. Why did Christian Drosten produce and promote a 'testing' protocol that he knew couldn't test for infectious disease which led to a global human catastrophe. Now you know. The Archontic mind doesn't give a shit (Fig 17). I personally think that Gates and major Cult insiders are a form of AI cyborg that the Archons want humans to become.

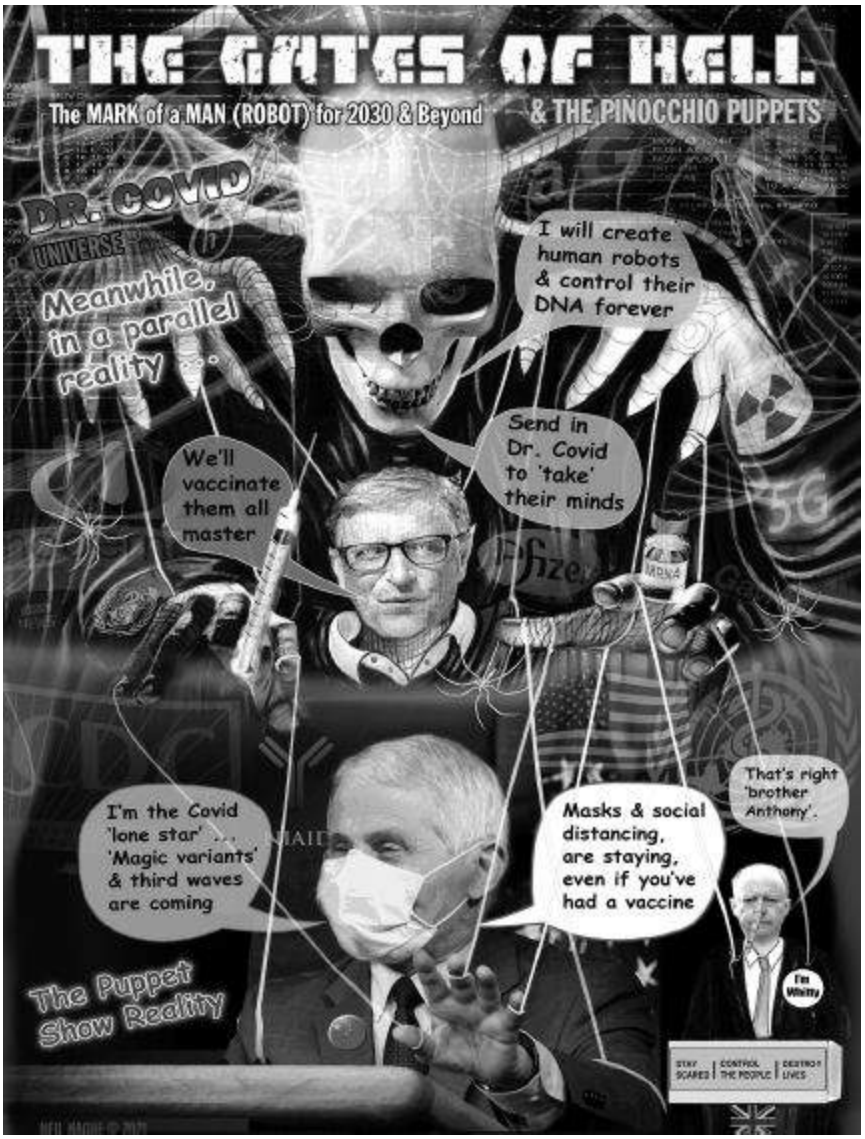

Figure 17: Artist Neil Hague's version of the 'Covid' hierarchy.

Human batteries

A state of such inversion does have its consequences, however. The level of disconnection from the Source of All means that you withdraw from that source of energetic sustenance and creativity. This means that you have to find your own supply of energetic power and it has – *us*. When the Morpheus character in the first *Matrix* movie held up a battery he spoke a profound truth when he said: 'The Matrix is a computer-generated dream world built to keep us under control in order to change the human being into one of

these.' The statement was true in all respects. We do live in a technologically-generated virtual reality simulation (more very shortly) and we have been manipulated to be an energy source for Archontic consciousness. The Disney-Pixar animated movie *Monsters, Inc*. in 2001 symbolised the dynamic when monsters in their world had no energy source and they would enter the human world to terrify children in their beds, catch the child's scream, terror (low-vibrational frequencies), and take that energy back to power the monster world. The lead character you might remember was a single giant eye and the symbolism of the Cult's all-seeing eye was obvious. Every thought and emotion is broadcast as a frequency unique to that thought and emotion. Feelings of love and joy, empathy and compassion, are high, quick, frequencies while fear, depression, anxiety, suffering and hate are low, slow, dense frequencies. Which kind do you think Archontic consciousness can connect with and absorb? In such a low and dense frequency state there's no way it can connect with the energy of love and joy. Archons can only feed off energy compatible with their own frequency and they and their Cult agents want to delete the human world of love and joy and manipulate the transmission of low vibrational frequencies through low-vibrational human mental and emotional states. *We are their energy source*. Wars are energetic banquets to the Archons – a world war even more so – and think how much low-frequency mental and emotional energy has been generated from the consequences for humanity of the 'Covid' hoax orchestrated by Archons incarnate like Gates.

The ancient practice of human sacrifice 'to the gods', continued in secret today by the Cult, is based on the same principle. 'The gods' are Archontic consciousness in different forms and the sacrifice is induced into a state of intense terror to generate the energy the Archontic frequency can absorb. Incarnate Archons in the ritual drink the blood which contains an adrenaline they crave which floods into the bloodstream when people are terrorised. Most of the sacrifices, ancient and modern, are children and the theme of 'sacrificing young virgins to the gods' is just code for children. They

have a particular pre-puberty energy that Archons want more than anything and the energy of the young in general is their target. The California Department of Education wants students to chant the names of Aztec gods (Archontic gods) once worshipped in human sacrifice rituals in a curriculum designed to encourage them to 'challenge racist, bigoted, discriminatory, imperialist/colonial beliefs', join 'social movements that struggle for social justice', and 'build new possibilities for a post-racist, post-systemic racism society'. It's the usual Woke crap that inverts racism and calls it anti-racism. In this case solidarity with 'indigenous tribes' is being used as an excuse to chant the names of 'gods' to which people were sacrificed (and still are in secret). What an example of Woke's inability to see beyond black and white, us and them, They condemn the colonisation of these tribal cultures by Europeans (quite right), but those cultures sacrificing people including children to their 'gods', and mass murdering untold numbers as the Aztecs did, is just fine. One chant is to the Aztec god Tezcatlipoca who had a man sacrificed to him in the 5th month of the Aztec calendar. His heart was cut out and he was eaten. Oh, that's okay then. Come on children … after three … Other sacrificial 'gods' for the young to chant their allegiance include Quetzalcoatl, Huitzilopochtli and Xipe Totec. The curriculum says that 'chants, affirmations, and energizers can be used to bring the class together, build unity around ethnic studies principles and values, and to reinvigorate the class following a lesson that may be emotionally taxing or even when student engagement may appear to be low'. Well, that's the cover story, anyway. Chanting and mantras are the repetition of a particular frequency generated from the vocal cords and chanting the names of these Archontic 'gods' tunes you into their frequency. That is the last thing you want when it allows for energetic synchronisation, attachment and perceptual influence. Initiates chant the names of their 'Gods' in their rituals for this very reason.

Vampires of the Woke

Paedophilia is another way that Archons absorb the energy of children. Paedophiles possessed by Archontic consciousness are used as the conduit during sexual abuse for discarnate Archons to vampire the energy of the young they desire so much. Stupendous numbers of children disappear every year never to be seen again although you would never know from the media. Imagine how much low-vibrational energy has been generated by children during the 'Covid' hoax when so many have become depressed and psychologically destroyed to the point of killing themselves. Shocking numbers of children are now taken by the state from loving parents to be handed to others. I can tell you from long experience of researching this since 1996 that many end up with paedophiles and assets of the Cult through corrupt and Cult-owned social services which in the reframing era has hired many psychopaths and emotionless automatons to do the job. Children are even stolen to order using spurious reasons to take them by the corrupt and secret (because they're corrupt) 'family courts'. I have written in detail in other books, starting with *The Biggest Secret* in 1997, about the ubiquitous connections between the political, corporate, government, intelligence and military elites (Cult operatives) and Satanism and paedophilia. If you go deep enough both networks have an interlocking leadership. The Woke mentality has been developed by the Cult for many reasons: To promote almost every aspect of its agenda; to hijack the traditional political left and turn it fascist; to divide and rule; and to target agenda pushbackers. But there are other reasons which relate to what I am describing here. How many happy and joyful Wokers do you ever see especially at the extreme end? They are a mental and psychological mess consumed by emotional stress and constantly emotionally cocked for the next explosion of indignation at someone referring to a female as a female. They are walking, talking, batteries as Morpheus might say emitting frequencies which both enslave them in low-vibrational bubbles of perceptual limitation and feed the Archons. Add to this the hatred claimed to be love; fascism claimed to 'anti-fascism', racism claimed to be 'anti-racism';

exclusion claimed to inclusion; and the abuse-filled Internet trolling. You have a purpose-built Archontic energy system with not a wind turbine in sight and all founded on Archontic *inversion*. We have whole generations now manipulated to serve the Archons with their actions and energy. They will be doing so their entire adult lives unless they snap out of their Archon-induced trance. Is it really a surprise that Cult billionaires and corporations put so much money their way? Where is the energy of joy and laughter, including laughing at yourself which is confirmation of your own emotional security? Mark Twain said: 'The human race has one really effective weapon, and that is laughter.' We must use it all the time. Woke has destroyed comedy because it has no humour, no joy, sense of irony, or self-deprecation. Its energy is dense and intense. *Mmmmm*, lunch says the Archontic frequency. Rudolf Steiner (1861-1925) was the Austrian philosopher and famous esoteric thinker who established Waldorf education or Steiner schools to treat children like unique expressions of consciousness and not minds to be programmed with the perceptions determined by authority. I'd been writing about this energy vampiring for decades when I was sent in 2016 a quote by Steiner. He was spot on:

> There are beings in the spiritual realms for whom anxiety and fear emanating from human beings offer welcome food. When humans have no anxiety and fear, then these creatures starve. If fear and anxiety radiates from people and they break out in panic, then these creatures find welcome nutrition and they become more and more powerful. These beings are hostile towards humanity. Everything that feeds on negative feelings, on anxiety, fear and superstition, despair or doubt, are in reality hostile forces in super-sensible worlds, launching cruel attacks on human beings, while they are being fed ... These are exactly the feelings that belong to contemporary culture and materialism; because it estranges people from the spiritual world, it is especially suited to evoke hopelessness and fear of the unknown in people, thereby calling up the above mentioned hostile forces against them.

Pause for a moment from this perspective and reflect on what has happened in the world since the start of 2020. Not only will pennies drop, but billion dollar bills. We see the same theme from Don Juan Matus, a Yaqui Indian shaman in Mexico and the information source for Peruvian-born writer, Carlos Castaneda, who wrote a series of

books from the 1960s to 1990s. Don Juan described the force manipulating human society and his name for the Archons was the predator:

> We have a predator that came from the depths of the cosmos and took over the rule of our lives. Human beings are its prisoners. The predator is our lord and master. It has rendered us docile, helpless. If we want to protest, it suppresses our protest. If we want to act independently, it demands that we don't do so ... indeed we are held prisoner!
>
> They took us over because we are food to them, and they squeeze us mercilessly because we are their sustenance. Just as we rear chickens in coops, the predators rear us in human coops, humaneros. Therefore, their food is always available to them.
>
> Different cultures, different eras, same recurring theme.

The 'ennoia' dilemma

Nag Hammadi Gnostic manuscripts say that Archon consciousness has no 'ennoia'. This is directly translated as 'intentionality', but I'll use the term 'creative imagination'. The *All That Is* in awareness of itself is the source of all creativity – all possibility – and the more disconnected you are from that source the more you are subsequently denied 'creative imagination'. Given that Archon consciousness is almost entirely disconnected it severely lacks creativity and has to rely on far more mechanical processes of thought and exploit the creative potential of those that do have 'ennoia'. You can see cases of this throughout human society. Archon consciousness almost entirely dominates the global banking system and if we study how that system works you will appreciate what I mean. Banks manifest 'money' out of nothing by issuing lines of 'credit' which is 'money' that has never, does not, and will never exist except in theory. It's a confidence trick. If you think 'credit' figures-on-a-screen 'money' is worth anything you accept it as payment. If you don't then the whole system collapses through lack of confidence in the value of that 'money'. Archontic bankers with no 'ennoia' are 'lending' 'money' that doesn't exist to humans that *do* have creativity – those that have the inspired ideas and create businesses and products. Archon banking feeds off human creativity

which it controls through 'money' creation and debt. Humans have the creativity and Archons exploit that for their own benefit and control while having none themselves. Archon Internet platforms like Facebook claim joint copyright of everything that creative users post and while Archontic minds like Zuckerberg may officially head that company it will be human creatives on the staff that provide the creative inspiration. When you have limitless 'money' you can then buy other companies established by creative humans. Witness the acquisition record of Facebook, Google and their like. Survey the Archon-controlled music industry and you see non-creative dark suit executives making their fortune from the human creativity of their artists. The cases are endless. Research the history of people like Gates and Zuckerberg and how their empires were built on exploiting the creativity of others. Archon minds cannot create out of nothing, but they are skilled (because they have to be) in what Gnostic texts call 'countermimicry'. They can imitate, but not innovate. Sabbatians trawl the creativity of others through backdoors they install in computer systems through their cybersecurity systems. Archon-controlled China is globally infamous for stealing intellectual property and I remember how Hong Kong, now part of China, became notorious for making counterfeit copies of the creativity of others – 'countermimicry'. With the now pervasive and all-seeing surveillance systems able to infiltrate any computer you can appreciate the potential for Archons to vampire the creativity of humans. Author John Lamb Lash wrote in his book about the Nag Hammadi texts, *Not In His Image*:

> Although they cannot originate anything, because they lack the divine factor of ennoia (intentionality), Archons can imitate with a vengeance. Their expertise is simulation (HAL, virtual reality). The Demiurge [Yaldabaoth] fashions a heaven world copied from the fractal patterns [of the original] ... His construction is celestial kitsch, like the fake Italianate villa of a Mafia don complete with militant angels to guard every portal.

This brings us to something that I have been speaking about since the turn of the millennium. Our reality is a simulation; a virtual reality that we think is real. No, I'm not kidding.

Human reality? Well, virtually

I had pondered for years about whether our reality is 'real' or some kind of construct. I remembered being immensely affected on a visit as a small child in the late 1950s to the then newly-opened Planetarium on the Marylebone Road in London which is now closed and part of the adjacent Madame Tussauds wax museum. It was in the middle of the day, but when the lights went out there was the night sky projected in the Planetarium's domed ceiling and it appeared to be so real. The experience never left me and I didn't know why until around the turn of the millennium when I became certain that our 'night sky' and entire reality is a projection, a virtual reality, akin to the illusory world portrayed in the *Matrix* movies. I looked at the sky one day in this period and it appeared to me like the domed roof of the Planetarium. The release of the first *Matrix* movie in 1999 also provided a synchronistic and perfect visual representation of where my mind had been going for a long time. I hadn't come across the Gnostic Nag Hammadi texts then. When I did years later the correlation was once again astounding. As I read Gnostic accounts from 1,600 years and more earlier it was clear that they were describing the same simulation phenomenon. They tell how the Yaldabaoth 'Demiurge' and Archons created a 'bad copy' of original reality to rule over all that were captured by its illusions and the body was a prison to trap consciousness in the 'bad copy' fake reality. Read how Gnostics describe the 'bad copy' and update that to current times and they are referring to what we would call today a virtual reality simulation.

Author John Lamb Lash said 'the Demiurge fashions a heaven world copied from the fractal patterns' of the original through expertise in 'HAL' or virtual reality simulation. Fractal patterns are part of the energetic information construct of our reality, a sort of blueprint. If these patterns were copied in computer terms it would indeed give you a copy of a 'natural' reality in a non-natural frequency and digital form. The principle is the same as making a copy of a website. The original website still exists, but now you can change the copy version to make it whatever you like and it can

become very different to the original website. Archons have done this with our reality, a *synthetic* copy of prime reality that still exists beyond the frequency walls of the simulation. Trapped within the illusions of this synthetic Matrix, however, were and are human consciousness and other expressions of prime reality and this is why the Archons via the Cult are seeking to make the human body synthetic and give us synthetic AI minds to complete the job of turning the entire reality synthetic including what we perceive to be the natural world. To quote Kurzweil: 'Nanobots will infuse all the matter around us with information. Rocks, trees, everything will become these intelligent creatures.' Yes, *synthetic* 'creatures' just as 'Covid' and other genetically-manipulating 'vaccines' are designed to make the human body synthetic. From this perspective it is obvious why Archons and their Cult are so desperate to infuse synthetic material into every human with their 'Covid' scam.

Let there be (electromagnetic) light

Yaldabaoth, the force that created the simulation, or Matrix, makes sense of the Gnostic reference to 'The Great Architect' and its use by Cult Freemasonry as the name of its deity. The designer of the Matrix in the movies is called 'The Architect' and that trilogy is jam-packed with symbolism relating to these subjects. I have contended for years that the angry Old Testament God (Yaldabaoth) is the 'God' being symbolically 'quoted' in the opening of Genesis as 'creating the world'. This is not the creation of prime reality – it's the creation of the *simulation*. The Genesis 'God' says: 'Let there be Light: and there was light.' But what is this 'Light'? I have said for decades that the speed of light (186,000 miles per second) is not the fastest speed possible as claimed by mainstream science and is in fact the frequency walls or outer limits of the Matrix. You can't have a fastest or slowest anything within all possibility when everything is possible. The human body is encoded to operate within the speed of light or *within the simulation* and thus we see only the tiny frequency band of visible *light*. Near-death experiencers who perceive reality outside the body during temporary 'death' describe a very different

form of light and this is supported by the Nag Hammadi texts. Prime reality beyond the simulation ('Upper Aeons' to the Gnostics) is described as a realm of incredible beauty, bliss, love and harmony – a realm of 'watery light' that is so powerful 'there are no shadows'. Our false reality of Archon control, which Gnostics call the 'Lower Aeons', is depicted as a realm with a different kind of 'light' and described in terms of chaos, 'Hell', 'the Abyss' and 'Outer Darkness', where trapped souls are tormented and manipulated by demons (relate that to the 'Covid' hoax alone). The watery light theme can be found in near-death accounts and it is not the same as *simulation* 'light' which is electromagnetic or radiation light within the speed of light – the 'Lower Aeons'. Simulation 'light' is the 'luminous fire' associated by Gnostics with the Archons. The Bible refers to Yaldabaoth as 'that old serpent, called the Devil, and Satan, which deceiveth the whole world' (Revelation 12:9). I think that making a simulated copy of prime reality ('countermimicry') and changing it dramatically while all the time manipulating humanity to believe it to be real could probably meet the criteria of deceiving the whole world. Then we come to the Cult god Lucifer – the *Light Bringer*. Lucifer is symbolic of Yaldabaoth, the bringer of radiation light that forms the bad copy simulation within the speed of light. 'He' is symbolised by the lighted torch held by the Statue of Liberty and in the name 'Illuminati'. Sabbatian-Frankism declares that Lucifer is the true god and Lucifer is the real god of Freemasonry honoured as their 'Great or Grand Architect of the Universe' (simulation).

I would emphasise, too, the way Archontic technologically-generated luminous fire of radiation has deluged our environment since I was a kid in the 1950s and changed the nature of The Field with which we constantly interact. Through that interaction technological radiation is changing us. The Smart Grid is designed to operate with immense levels of communication power with 5G expanding across the world and 6G, 7G, in the process of development. Radiation is the simulation and the Archontic manipulation system. Why wouldn't the Archon Cult wish to unleash radiation upon us to an ever-greater extreme to form

Kurzweil's 'cloud'? The plan for a synthetic human is related to the need to cope with levels of radiation beyond even anything we've seen so far. Biological humans would not survive the scale of radiation they have in their script. The Smart Grid is a technological sub-reality within the technological simulation to further disconnect five-sense perception from expanded consciousness. It's a technological prison of the mind.

Infusing the 'spirit of darkness'

A recurring theme in religion and native cultures is the manipulation of human genetics by a non-human force and most famously recorded as the biblical 'sons of god' (the god*s* plural in the original) who interbred with the daughters of men. The Nag Hammadi *Apocryphon of John* tells the same story this way:

> He [Yaldabaoth] sent his angels [Archons/demons] to the daughters of men, that they might take some of them for themselves and raise offspring for their enjoyment. And at first they did not succeed. When they had no success, they gathered together again and they made a plan together ... And the angels changed themselves in their likeness into the likeness of their mates, filling them with the spirit of darkness, which they had mixed for them, and with evil ... And they took women and begot children out of the darkness according to the likeness of their spirit.

Possession when a discarnate entity takes over a human body is an age-old theme and continues today. It's very real and I've seen it. Satanic and secret society rituals can create an energetic environment in which entities can attach to initiates and I've heard many stories of how people have changed their personality after being initiated even into lower levels of the Freemasons. I have been inside three Freemasonic temples, one at a public open day and two by just walking in when there was no one around to stop me. They were in Ryde, the town where I live, Birmingham, England, when I was with a group, and Boston, Massachusetts. They all felt the same energetically – dark, dense, low-vibrational and sinister. Demonic attachment can happen while the initiate has no idea what is going on. To them it's just a ritual to get in the Masons and do a bit of good

business. In the far more extreme rituals of Satanism human possession is even more powerful and they are designed to make possession possible. The hierarchy of the Cult is dictated by the power and perceived status of the possessing Archon. In this way the Archon hierarchy becomes the Cult hierarchy. Once the entity has attached it can influence perception and behaviour and if it attaches to the extreme then so much of its energy (information) infuses into the body information field that the hologram starts to reflect the nature of the possessing entity. This is the *Exorcist* movie type of possession when facial features change and it's known as shapeshifting. Islam's Jinn are said to be invisible tricksters who change shape, 'whisper', confuse and take human form. These are all traits of the Archons and other versions of the same phenomenon. Extreme possession could certainty infuse the 'spirit of darkness' into a partner during sex as the Nag Hammadi texts appear to describe. Such an infusion can change genetics which is also energetic information. Human genetics is information and the 'spirit of darkness' is information. Mix one with the other and change must happen. Islam has the concept of a 'Jinn baby' through possession of the mother and by Jinn taking human form. There are many ways that human genetics can be changed and remember that Archons have been aware all along of advanced techniques to do this. What is being done in human society today – and far more – was known about by Archons at the time of the 'fallen ones' and their other versions described in religions and cultures.

Archons and their human-world Cult are obsessed with genetics as we see today and they know this dictates how information is processed into perceived reality during a human life. They needed to produce a human form that would decode the simulation and this is symbolically known as 'Adam and Eve' who left the 'garden' (prime reality) and 'fell' into Matrix reality. The simulation is not a 'physical' construct (there is no 'physical'); it is a source of information. Think Wi-Fi again. The simulation is an energetic field encoded with information and body-brain systems are designed to decode that information encoded in wave or frequency form which

is transmitted to the brain as electrical signals. These are decoded by the brain to construct our sense of reality – an illusory 'physical' world that only exists in the brain or the mind. Virtual reality games mimic this process using the same sensory decoding system. Information is fed to the senses to decode a virtual reality that can appear so real, but isn't (Figs 18 and 19). Some scientists believe – and I agree with them – that what we perceive as 'physical' reality only exists when we are looking or observing. The act of perception or focus triggers the decoding systems which turn waveform information into holographic reality. When we are not observing something our reality reverts from a holographic state to a waveform state. This relates to the same principle as a falling tree not making a noise unless someone is there to hear it or decode it. The concept makes sense from the simulation perspective. A computer is not decoding all the information in a Wi-Fi field all the time and only decodes or brings into reality on the screen that part of Wi-Fi that it's decoding – focusing upon – at that moment.

Figure 18: Virtual reality technology 'hacks' into the body's five-sense decoding system.

Figure 19: The result can be experienced as very 'real'.

Interestingly, Professor Donald Hoffman at the Department of Cognitive Sciences at the University of California, Irvine, says that our experienced reality is like a computer interface that shows us only the level with which we interact while hiding all that exists beyond it: 'Evolution shaped us with a user interface that hides the truth. Nothing that we see is the truth – the very language of space and time and objects is the wrong language to describe reality.' He is correct in what he says on so many levels. Space and time are not a universal reality. They are a phenomenon of decoded *simulation* reality as part of the process of enslaving our sense of reality. Near-death experiencers report again and again how space and time did not exist as we perceive them once they were free of the body – body decoding systems. You can appreciate from this why Archons and their Cult are so desperate to entrap human attention in the five senses where we are in the Matrix and of the Matrix. Opening your mind to expanded states of awareness takes you beyond the information confines of the simulation and you become aware of knowledge and insights denied to you before. This is what we call 'awakening' – *awakening from the Matrix* – and in the final chapter I will relate this to current events.

Where are the 'aliens'?

A simulation would explain the so-called 'Fermi Paradox' named after Italian physicist Enrico Fermi (1901-1954) who created the first nuclear reactor. He considered the question of why there is such a lack of extraterrestrial activity when there are so many stars and planets in an apparently vast universe; but what if the night sky that we see, or think we do, is a simulated projection as I say? If you control the simulation and your aim is to hold humanity fast in essential ignorance would you want other forms of life including advanced life coming and going sharing information with humanity? Or would you want them to believe they were isolated and apparently alone? Themes of human isolation and apartness are common whether they be the perception of a lifeless universe or the fascist isolation laws of the 'Covid' era. Paradoxically the very

existence of a simulation means that we are not alone when some force had to construct it. My view is that experiences that people have reported all over the world for centuries with Reptilians and Grey entities are Archon phenomena as Nag Hammadi texts describe; and that benevolent 'alien' interactions are non-human groups that come in and out of the simulation by overcoming Archon attempts to keep them out. It should be highlighted, too, that Reptilians and Greys are obsessed with *genetics* and *technology* as related by cultural accounts and those who say they have been abducted by them. Technology is their way of overcoming some of the limitations in their creative potential and our technology-driven and controlled human society of today is *arch*etypical Archon-Reptilian-Grey modus operandi. Technocracy is really *Archon*tocracy. The Universe does not have to be as big as it appears with a simulation. There is no space or distance only information decoded into holographic reality. What we call 'space' is only the absence of holographic 'objects' and that 'space' is The Field of energetic information which connects everything into a single whole. The same applies with the artificially-generated information field of the simulation. The Universe is not big or small as a physical reality. It is decoded information, that's all, and its perceived size is decided by the way the simulation is encoded to make it appear. The entire night sky as we perceive it only exists in our brain and so where are those 'millions of light years'? The 'stars' on the ceiling of the Planetarium looked a vast distance away.

There's another point to mention about 'aliens'. I have been highlighting since the 1990s the plan to stage a fake 'alien invasion' to justify the centralisation of global power and a world military. Nazi scientist Werner von Braun, who was taken to America by Operation Paperclip after World War Two to help found NASA, told his American assistant Dr Carol Rosin about the Cult agenda when he knew he was dying in 1977. Rosin said that he told her about a sequence that would lead to total human control by a one-world government. This included threats from terrorism, rogue nations, meteors and asteroids before finally an 'alien invasion'. All of these

things, von Braun said, would be bogus and what I would refer to as a No-Problem-Reaction-Solution. Keep this in mind when 'the aliens are coming' is the new mantra. The aliens are not coming – they are *already here* and they have infiltrated human society while looking human. French-Canadian investigative journalist Serge Monast said in 1994 that he had uncovered a NASA/military operation called Project Blue Beam which fits with what Werner von Braun predicted. Monast died of a 'heart attack' in 1996 the day after he was arrested and spent a night in prison. He was 51. He said Blue Beam was a plan to stage an alien invasion that would include religious figures beamed holographically into the sky as part of a global manipulation to usher in a 'new age' of worshipping what I would say is the Cult 'god' Yaldabaoth in a one-world religion. Fake holographic asteroids are also said to be part of the plan which again syncs with von Braun. How could you stage an illusory threat from asteroids unless they were holographic inserts? This is pretty straightforward given the advanced technology outside the public arena and the fact that our 'physical' reality is holographic anyway. Information fields would be projected and we would decode them into the illusion of a 'physical' asteroid. If they can sell a global 'pandemic' with a 'virus' that doesn't exist what will humans not believe if government and media tell them?

All this is particularly relevant as I write with the Pentagon planning to release in June, 2021, information about 'UFO sightings'. I have been following the UFO story since the early 1990s and the common theme throughout has been government and military denials and cover up. More recently, however, the Pentagon has suddenly become more talkative and apparently open with Air Force pilot radar images released of unexplained craft moving and changing direction at speeds well beyond anything believed possible with human technology. Then, in March, 2021, former Director of National Intelligence John Ratcliffe said a Pentagon report months later in June would reveal a great deal of information about UFO sightings unknown to the public. He said the report would have 'massive implications'. The order to do this was included bizarrely

in a $2.3 trillion 'coronavirus' relief and government funding bill passed by the Trump administration at the end of 2020. I would add some serious notes of caution here. I have been pointing out since the 1990s that the US military and intelligence networks have long had craft – 'flying saucers' or anti-gravity craft – which any observer would take to be extraterrestrial in origin. Keeping this knowledge from the public allows craft flown by *humans* to be perceived as alien visitations. I am not saying that 'aliens' do not exist. I would be the last one to say that, but we have to be streetwise here. President Ronald Reagan told the UN General Assembly in 1987: 'I occasionally think how quickly our differences worldwide would vanish if we were facing an alien threat from outside this world.' That's the idea. Unite against a common 'enemy' with a common purpose behind your 'saviour force' (the Cult) as this age-old technique of mass manipulation goes global.

Science moves this way …

I could find only one other person who was discussing the simulation hypothesis publicly when I concluded it was real. This was Nick Bostrom, a Swedish-born philosopher at the University of Oxford, who has explored for many years the possibility that human reality is a computer simulation although his version and mine are not the same. Today the simulation and holographic reality hypothesis have increasingly entered the scientific mainstream. Well, the more open-minded mainstream, that is. Here are a few of the ever-gathering examples. American nuclear physicist Silas Beane led a team of physicists at the University of Bonn in Germany pursuing the question of whether we live in a simulation. They concluded that we probably do and it was likely based on a lattice of cubes. They found that cosmic rays align with that specific pattern. The team highlighted the Greisen–Zatsepin–Kuzmin (GZK) limit which refers to cosmic ray particle interaction with cosmic background radiation that creates an apparent boundary for cosmic ray particles. They say in a paper entitled 'Constraints on the Universe as a Numerical Simulation' that this 'pattern of constraint' is exactly what you

would find with a computer simulation. They also made the point that a simulation would create its own 'laws of physics' that would limit possibility. I've been making the same point for decades that the *perceived* laws of physics relate only to this reality, or what I would later call the simulation. When designers write codes to create computer and virtual reality games they are the equivalent of the laws of physics for that game. Players interact within the limitations laid out by the coding. In the same way those who wrote the codes for the simulation decided the laws of physics that would apply. These can be overridden by expanded states of consciousness, but not by those enslaved in only five-sense awareness where simulation codes rule. Overriding the codes is what people call 'miracles'. They are not. They are bypassing the encoded limits of the simulation. A population caught in simulation perception would have no idea that this was their plight. As the Bonn paper said: 'Like a prisoner in a pitch-black cell we would not be able to see the "walls" of our prison,' That's true if people remain mesmerised by the five senses. Open to expanded awareness and those walls become very clear. The main one is the speed of light.

American theoretical physicist James Gates is another who has explored the simulation question and found considerable evidence to support the idea. Gates was Professor of Physics at the University of Maryland, Director of The Center for String and Particle Theory, and on Barack Obama's Council of Advisors on Science and Technology. He and his team found *computer codes* of digital data embedded in the fabric of our reality. They relate to on-off electrical charges of 1 and 0 in the binary system used by computers. 'We have no idea what they are doing there', Gates said. They found within the energetic fabric mathematical sequences known as error-correcting codes or block codes that 'reboot' data to its original state or 'default settings' when something knocks it out of sync. Gates was asked if he had found a set of equations embedded in our reality indistinguishable from those that drive search engines and browsers and he said: 'That is correct.' Rich Terrile, director of the Centre for Evolutionary Computation and Automated Design at NASA's Jet

Propulsion Laboratory, has said publicly that he believes the Universe is a digital hologram that must have been created by a form of intelligence. I agree with that in every way. Waveform information is delivered electrically by the senses to the brain which constructs a *digital* holographic reality that we call the 'world'. This digital level of reality can be read by the esoteric art of numerology. Digital holograms are at the cutting edge of holographics today. We have digital technology everywhere designed to access and manipulate our digital level of perceived reality. Synthetic mRNA in 'Covid vaccines' has a digital component to manipulate the body's digital 'operating system'.

Reality is numbers

How many know that our reality can be broken down to numbers and codes that are the same as computer games? Max Tegmark, a physicist at the Massachusetts Institute of Technology (MIT), is the author of *Our Mathematical Universe* in which he lays out how reality can be entirely described by numbers and maths in the way that a video game is encoded with the 'physics' of computer games. Our world and computer virtual reality are essentially the same. Tegmark imagines the perceptions of characters in an advanced computer game when the graphics are so good they don't know they are in a game. They think they can bump into real objects (electromagnetic resistance in our reality), fall in love and feel emotions like excitement. When they began to study the apparently 'physical world' of the video game they would realise that everything was made of pixels (which have been found in our energetic reality as must be the case when on one level our world is digital). What computer game characters thought was physical 'stuff', Tegmark said, could actually be broken down into numbers:

> And we're exactly in this situation in our world. We look around and it doesn't seem that mathematical at all, but everything we see is made out of elementary particles like quarks and electrons. And what properties does an electron have? Does it have a smell or a colour or a texture? No! ... We physicists have come up with geeky names for [Electron] properties, like

electric charge, or spin, or lepton number, but the electron doesn't care what we call it, the properties are just numbers.

This is the illusory reality Gnostics were describing. This is the simulation. The A, C, G, and T codes of DNA have a binary value – A and C = 0 while G and T = 1. This has to be when the simulation is digital and the body must be digital to interact with it. Recurring mathematical sequences are encoded throughout reality and the body. They include the Fibonacci sequence in which the two previous numbers are added to get the next one, as in ... 1, 1, 2, 3, 5, 8, 13, 21, 34, 55, etc. The sequence is encoded in the human face and body, proportions of animals, DNA, seed heads, pine cones, trees, shells, spiral galaxies, hurricanes and the number of petals in a flower. The list goes on and on. There are fractal patterns – a 'never-ending pattern that is infinitely complex and self-similar across all scales in the as above, so below, principle of holograms. These and other famous recurring geometrical and mathematical sequences such as Phi, Pi, Golden Mean, Golden Ratio and Golden Section are *computer codes* of the simulation. I had to laugh and give my head a shake the day I finished this book and it went into the production stage. I was sent an article in *Scientific American* published in April, 2021, with the headline 'Confirmed! We Live in a Simulation'. Two decades after I first said our reality is a simulation and the speed of light is it's outer limit the article suggested that we do live in a simulation and that the speed of light is its outer limit. I left school at 15 and never passed a major exam in my life while the writer was up to his eyes in qualifications. As I will explain in the final chapter *knowing* is far better than thinking and they come from very different sources. The article rightly connected the speed of light to the processing speed of the 'Matrix' and said what has been in my books all this time … 'If we are in a simulation, as it appears, then space is an abstract property written in code. It is not real'. No it's not and if we live in a simulation something created it and it wasn't *us*. 'That David Icke says we are manipulated by aliens' – he's crackers.'

Wow …

The reality that humanity thinks is so real is an illusion. Politicians, governments, scientists, doctors, academics, law enforcement, media, school and university curriculums, on and on, are all founded on a world that *does not exist* except as a simulated prison cell. Is it such a stretch to accept that 'Covid' doesn't exist when our entire 'physical' reality doesn't exist? Revealed here is the knowledge kept under raps in the Cult networks of compartmentalised secrecy to control humanity's sense of reality by inducing the population to believe in a reality that's not real. If it wasn't so tragic in its experiential consequences the whole thing would be hysterically funny. None of this is new to Renegade Minds. Ancient Greek philosopher Plato (about 428 to about 347BC) was a major influence on Gnostic belief and he described the human plight thousands of years ago with his Allegory of the Cave. He told the symbolic story of prisoners living in a cave who had never been outside. They were chained and could only see one wall of the cave while behind them was a fire that they could not see. Figures walked past the fire casting shadows on the prisoners' wall and those moving shadows became their sense of reality. Some prisoners began to study the shadows and were considered experts on them (today's academics and scientists), but what they studied was only an illusion (today's academics and scientists). A prisoner escaped from the cave and saw reality as it really is. When he returned to report this revelation they didn't believe him, called him mad and threatened to kill him if he tried to set them free. Plato's tale is not only a brilliant analogy of the human plight and our illusory reality. It describes, too, the dynamics of the 'Covid' hoax. I have only skimmed the surface of these subjects here. The aim of this book is to crisply connect all essential dots to put what is happening today into its true context. All subject areas and their connections in this chapter are covered in great evidential detail in *Everything You Need To Know, But Have Never Been Told* and *The Answer*.

They say that bewildered people 'can't see the forest for the trees'. Humanity, however, can't see the forest for the *twigs*. The five senses

see only twigs while Renegade Minds can see the forest and it's the forest where the answers lie with the connections that reveals. Breaking free of perceptual programming so the forest can be seen is the way we turn all this around. Not breaking free is how humanity got into this mess. The situation may seem hopeless, but I promise you it's not. We are a perceptual heartbeat from paradise if only we knew.

CHAPTER TWELVE

Escaping Wetiko

Life is simply a vacation from the infinite
Dean Cavanagh

Renegade Minds weave the web of life and events and see common themes in the apparently random. They are always there if you look for them and their pursuit is aided by incredible synchronicity that comes when your mind is open rather than mesmerised by what it thinks it can see.

Infinite awareness is infinite possibility and the more of infinite possibility that we access the more becomes infinitely possible. That may be stating the apparently obvious, but it is a devastatingly-powerful fact that can set us free. We are a point of attention within an infinity of consciousness. The question is how much of that infinity do we choose to access? How much knowledge, insight, awareness, wisdom, do we want to connect with and explore? If your focus is only in the five senses you will be influenced by a fraction of infinite awareness. I mean a range so tiny that it gives new meaning to infinitesimal. Limitation of self-identity and a sense of the possible limit accordingly your range of consciousness. We are what we think we are. Life is what we think it is. The dream is the dreamer and the dreamer is the dream. Buddhist philosophy puts it this way: 'As a thing is viewed, so it appears.' Most humans live in the realm of touch, taste, see, hear, and smell and that's the limit of their sense of the possible and sense of self. Many will follow a religion and speak of a God in his heaven, but their lives are still

dominated by the five senses in their perceptions and actions. The five senses become the arbiter of everything. When that happens all except a smear of infinity is sealed away from influence by the rigid, unyielding, reality bubbles that are the five-sense human or Phantom Self. Archon Cult methodology is to isolate consciousness within five-sense reality – the simulation – and then program that consciousness with a sense of self and the world through a deluge of life-long information designed to instil the desired perception that allows global control. Efforts to do this have increased dramatically with identity politics as identity bubbles are squeezed into the minutiae of five-sense detail which disconnect people even more profoundly from the infinite 'I'.

Five-sense focus and self-identity are like a firewall that limits access to the infinite realms. You only perceive one radio or television station and no other. We'll take that literally for a moment. Imagine a vast array of stations giving different information and angles on reality, but you only ever listen to one. Here we have the human plight in which the population is overwhelmingly confined to CultFM. This relates only to the frequency range of CultFM and limits perception and insight to that band – limits *possibility* to that band. It means you are connecting with an almost imperceptibly minuscule range of possibility and creative potential within the infinite Field. It's a world where everything seems apart from everything else and where synchronicity is rare. Synchronicity is defined in the dictionary as 'the happening by chance of two or more related or similar events at the same time'. Use of 'by chance' betrays a complete misunderstanding of reality. Synchronicity is not 'by chance'. As people open their minds, or 'awaken' to use the term, they notice more and more coincidences in their lives, bits of 'luck', apparently miraculous happenings that put them in the right place at the right time with the right people. Days become peppered with 'fancy meeting you here' and 'what are the chances of that?' My entire life has been lived like this and ever more so since my own colossal awakening in 1990 and 91 which transformed my sense of reality. Synchronicity is not 'by chance'; it is by accessing expanded

realms of possibility which allow expanded potential for manifestation. People broadcasting the same vibe from the same openness of mind tend to be drawn 'by chance' to each other through what I call frequency magnetism and it's not only people. In the last more than 30 years incredible synchronicity has also led me through the Cult maze to information in so many forms and to crucial personal experiences. These 'coincidences' have allowed me to put the puzzle pieces together across an enormous array of subjects and situations. Those who have breached the bubble of five-sense reality will know exactly what I mean and this escape from the perceptual prison cell is open to everyone whenever they make that choice. This may appear super-human when compared with the limitations of 'human', but it's really our natural state. 'Human' as currently experienced is consciousness in an unnatural state of induced separation from the infinity of the whole. I'll come to how this transformation into unity can be made when I have described in more detail the force that holds humanity in servitude by denying this access to infinite self.

The Wetiko factor

I have been talking and writing for decades about the way five-sense mind is systematically barricaded from expanded awareness. I have used the analogy of a computer (five-sense mind) and someone at the keyboard (expanded awareness). Interaction between the computer and the operator is symbolic of the interaction between five-sense mind and expanded awareness. The computer directly experiences the Internet and the operator experiences the Internet via the computer which is how it's supposed to be – the two working as one. Archons seek to control that point where the operator connects with the computer to stop that interaction (Fig 20). Now the operator is banging the keyboard and clicking the mouse, but the computer is not responding and this happens when the computer is taken over – *possessed* – by an appropriately-named computer 'virus'. The operator has lost all influence over the computer which goes its own way making decisions under the control of the 'virus'. I have

just described the dynamic through which the force known to Gnostics as Yaldabaoth and Archons disconnects five-sense mind from expanded awareness to imprison humanity in perceptual servitude.

Figure 20: The mind 'virus' I have been writing about for decades seeks to isolate five-sense mind (the computer) from the true 'I'. (Image by Neil Hague).

About a year ago I came across a Native American concept of Wetiko which describes precisely the same phenomenon. Wetiko is the spelling used by the Cree and there are other versions including wintiko and windigo used by other tribal groups. They spell the name with lower case, but I see Wetiko as a proper noun as with Archons and prefer a capital. I first saw an article about Wetiko by writer and researcher Paul Levy which so synced with what I had been writing about the computer/operator disconnection and later the Archons. I then read his book, the fascinating *Dispelling Wetiko, Breaking the Spell of Evil*. The parallels between what I had concluded long before and the Native American concept of Wetiko were so clear and obvious that it was almost funny. For Wetiko see the Gnostic Archons for sure and the Jinn, the Predators, and every other name for a force of evil, inversion and chaos. Wetiko is the Native American name for the force that divides the computer from

the operator (Fig 21). Indigenous author Jack D. Forbes, a founder of the Native American movement in the 1960s, wrote another book about Wetiko entitled *Columbus And Other Cannibals – The Wetiko Disease of Exploitation, Imperialism, and Terrorism* which I also read. Forbes says that Wetiko refers to an evil person or spirit 'who terrorizes other creatures by means of terrible acts, including cannibalism'. Zulu shaman Credo Mutwa told me that African accounts tell how cannibalism was brought into the world by the Chitauri 'gods' – another manifestation of Wetiko. The distinction between 'evil person or spirit' relates to Archons/Wetiko possessing a human or acting as pure consciousness. Wetiko is said to be a sickness of the soul or spirit and a state of being that takes but gives nothing back – the Cult and its operatives perfectly described. Black Hawk, a Native American war leader defending their lands from confiscation, said European invaders had 'poisoned hearts' – Wetiko hearts – and that this would spread to native societies. Mention of the heart is very significant as we shall shortly see. Forbes writes: 'Tragically, the history of the world for the past 2,000 years is, in great part, the story of the epidemiology of the wetiko disease.' Yes, and much longer. Forbes is correct when he says: 'The wetikos destroyed Egypt and Babylon and Athens and Rome and Tenochtitlan [capital of the Aztec empire] and perhaps now they will destroy the entire earth.' Evil, he said, is the number one export of a Wetiko culture – see its globalisation with 'Covid'. Constant war, mass murder, suffering of all kinds, child abuse, Satanism, torture and human sacrifice are all expressions of Wetiko and the Wetiko possessed. The world is Wetiko made manifest, *but it doesn't have to be*. There is a way out of this even now.

Figure 21: The mind 'virus' is known to Native Americans as 'Wetiko'. (Image by Neil Hague).

Cult of Wetiko

Wetiko is the Yaldabaoth frequency distortion that seeks to attach to human consciousness and absorb it into its own. Once this connection is made Wetiko can drive the perceptions of the target which they believe to be coming from their own mind. All the horrors of history and today from mass killers to Satanists, paedophiles like Jeffrey Epstein and other psychopaths, are the embodiment of Wetiko and express its state of being in all its grotesqueness. The Cult is Wetiko incarnate, Yaldabaoth incarnate, and it seeks to facilitate Wetiko assimilation of humanity in totality into its distortion by manipulating the population into low frequency states that match its own. Paul Levy writes: 'Holographically enforced within the psyche of every human being the wetiko virus pervades and underlies the entire field of consciousness, and can therefore potentially manifest through any one of us at any moment if we are not mindful.' The 'Covid' hoax has achieved this with many people, but others have not fallen into Wetiko's frequency lair. Players in the 'Covid' human catastrophe including Gates, Schwab, Tedros, Fauci, Whitty, Vallance, Johnson, Hancock, Ferguson, Drosten, and all the rest, including the psychopath psychologists, are expressions of Wetiko. This is why

they have no compassion or empathy and no emotional consequence for what they do that would make them stop doing it. Observe all the people who support the psychopaths in authority against the Pushbackers despite the damaging impact the psychopaths have on their own lives and their family's lives. You are again looking at Wetiko possession which prevents them seeing through the lies to the obvious scam going on. *Why can't they see it?* Wetiko won't let them see it. The perceptual divide that has now become a chasm is between the Wetikoed and the non-Wetikoed.

Paul Levy describes Wetiko in the same way that I have long described the Archontic force. They are the same distorted consciousness operating across dimensions of reality: '… the subtle body of wetiko is not located in the third dimension of space and time, literally existing in another dimension … it is able to affect ordinary lives by mysteriously interpenetrating into our three-dimensional world.' Wetiko does this through its incarnate representatives in the Cult and by weaving itself into The Field which on our level of reality is the electromagnetic information field of the simulation or Matrix. More than that, the simulation *is* Wetiko / Yaldabaoth. Caleb Scharf, Director of Astrobiology at Columbia University, has speculated that 'alien life' could be so advanced that it has transcribed itself into the quantum realm to become what we call physics. He said intelligence indistinguishable from the fabric of the Universe would solve many of its greatest mysteries:

> Perhaps hyper-advanced life isn't just external. Perhaps it's already all around. It is embedded in what we perceive to be physics itself, from the root behaviour of particles and fields to the phenomena of complexity and emergence ... In other words, life might not just be in the equations. It might BE the equations [My emphasis].

Scharf said it is possible that 'we don't recognise advanced life because it forms an integral and unsuspicious part of what we've considered to be the natural world'. I agree. Wetiko/Yaldabaoth *is* the simulation. We are literally in the body of the beast. But that doesn't mean it has to control us. We all have the power to overcome Wetiko

influence and the Cult knows that. I doubt it sleeps too well because it knows that.

Which Field?

This, I suggest, is how it all works. There are two Fields. One is the fierce electromagnetic light of the Matrix within the speed of light; the other is the 'watery light' of The Field beyond the walls of the Matrix that connects with the Great Infinity. Five-sense mind and the decoding systems of the body attach us to the Field of Matrix light. They have to or we could not experience this reality. Five-sense mind sees only the Matrix Field of information while our expanded consciousness is part of the Infinity Field. When we open our minds, and most importantly our hearts, to the Infinity Field we have a mission control which gives us an expanded perspective, a road map, to understand the nature of the five-sense world. If we are isolated only in five-sense mind there is no mission control. We're on our own trying to understand a world that's constantly feeding us information to ensure we do not understand. People in this state can feel 'lost' and bewildered with no direction or radar. You can see ever more clearly those who are influenced by the Fields of Big Infinity or little five-sense mind simply by their views and behaviour with regard to the 'Covid' hoax. We have had this division throughout known human history with the mass of the people on one side and individuals who could see and intuit beyond the walls of the simulation – Plato's prisoner who broke out of the cave and saw reality for what it is. Such people have always been targeted by Wetiko/Archon-possessed authority, burned at the stake or demonised as mad, bad and dangerous. The Cult today and its global network of 'anti-hate', 'anti-fascist' Woke groups are all expressions of Wetiko attacking those exposing the conspiracy, 'Covid' lies and the 'vaccine' agenda.

Woke as a whole is Wetiko which explains its black and white mentality and how at one it is with the Wetiko-possessed Cult. Paul Levy said: 'To be in this paradigm is to still be under the thrall of a two-valued logic – where things are either true or false – of a

wetikoized mind.' Wetiko consciousness is in a permanent rage, therefore so is Woke, and then there is Woke inversion and contradiction. 'Anti-fascists' act like fascists because fascists *and* 'anti-fascists' are both Wetiko at work. Political parties act the same while claiming to be different for the same reason. Secret society and satanic rituals are attaching initiates to Wetiko and the cold, ruthless, psychopathic mentality that secures the positions of power all over the world is Wetiko. Reframing 'training programmes' have the same cumulative effect of attaching Wetiko and we have their graduates described as automatons and robots with a cold, psychopathic, uncaring demeanour. They are all traits of Wetiko possession and look how many times they have been described in this book and elsewhere with regard to personnel behind 'Covid' including the police and medical profession. Climbing the greasy pole in any profession in a Wetiko society requires traits of Wetiko to get there and that is particularly true of politics which is not about fair competition and pre-eminence of ideas. It is founded on how many backs you can stab and arses you can lick. This culminated in the global 'Covid' coordination between the Wetiko possessed who pulled it off in all the different countries without a trace of empathy and compassion for their impact on humans. Our sight sense can see only holographic form and not the Field which connects holographic form. Therefore we perceive 'physical' objects with 'space' in between. In fact that 'space' is energy/consciousness operating on multiple frequencies. One of them is Wetiko and that connects the Cult psychopaths, those who submit to the psychopaths, and those who serve the psychopaths in the media operations of the world. Wetiko is Gates. Wetiko is the mask-wearing submissive. Wetiko is the fake journalist and 'fact-checker'. The Wetiko Field is coordinating the whole thing. Psychopaths, gofers, media operatives, 'anti-hate' hate groups, 'fact-checkers' and submissive people work as one unit *even without human coordination* because they are attached to the *same* Field which is organising it all (Fig 22). Paul Levy is here describing how Wetiko-possessed people are drawn together and refuse to let any information breach their rigid

perceptions. He was writing long before 'Covid', but I think you will recognise followers of the 'Covid' religion *oh just a little bit*:

People who are channelling the vibratory frequency of wetiko align with each other through psychic resonance to reinforce their unspoken shared agreement so as to uphold their deranged view of reality. Once an unconscious content takes possession of certain individuals, it irresistibly draws them together by mutual attraction and knits them into groups tied together by their shared madness that can easily swell into an avalanche of insanity.

A psychic epidemic is a closed system, which is to say that it is insular and not open to any new information or informing influences from the outside world which contradict its fixed, limited, and limiting perspective.

There we have the Woke mind and the 'Covid' mind. Compatible resonance draws the awakening together, too, which is clearly happening today.

Figure 22: The Wetiko Field from which the Cult pyramid and its personnel are made manifest. (Image by Neil Hague).

Spiritual servitude

Wetiko doesn't care about humans. It's not human; it just possesses humans for its own ends and the effect (depending on the scale of

possession) can be anything from extreme psychopathy to unquestioning obedience. Wetiko's worst nightmare is for human consciousness to expand beyond the simulation. Everything is focussed on stopping that happening through control of information, thus perception, thus frequency. The 'education system', media, science, medicine, academia, are all geared to maintaining humanity in five-sense servitude as is the constant stimulation of low-vibrational mental and emotional states (see 'Covid'). Wetiko seeks to dominate those subconscious spaces between five-sense perception and expanded consciousness where the computer meets the operator. From these subconscious hiding places Wetiko speaks to us to trigger urges and desires that we take to be our own and manipulate us into anything from low-vibrational to psychopathic states. Remember how Islam describes the Jinn as invisible tricksters that 'whisper' and confuse. Wetiko is the origin of the 'trickster god' theme that you find in cultures all over the world. Jinn, like the Archons, are Wetiko which is terrified of humans awakening and reconnecting with our true self for then its energy source has gone. With that the feedback loop breaks between Wetiko and human perception that provides the energetic momentum on which its very existence depends as a force of evil. Humans are both its target and its source of survival, but only if we are operating in low-vibrational states of fear, hate, depression and the background anxiety that most people suffer. We are Wetiko's target because we are its key to survival. It needs us, not the other way round. Paul Levy writes:

A vampire has no intrinsic, independent, substantial existence in its own right; it only exists in relation to us. The pathogenic, vampiric mind-parasite called wetiko is nothing in itself – not being able to exist from its own side – yet it has a 'virtual reality' such that it can potentially destroy our species …

…The fact that a vampire is not reflected by a mirror can also mean that what we need to see is that there's nothing, no-thing to see, other than ourselves. The fact that wetiko is the expression of something inside of us means that the cure for wetiko is with us as well. The critical issue is finding this cure within us and then putting it into effect.

Evil begets evil because if evil does not constantly expand and find new sources of energetic sustenance its evil, its *distortion*, dies with the assimilation into balance and harmony. Love is the garlic to Wetiko's vampire. Evil, the absence of love, cannot exist in the presence of love. I think I see a way out of here. I have emphasised so many times over the decades that the Archons/Wetiko and their Cult are not all powerful. *They are not*. I don't care how it looks even now *they are not*. I have not called them little boys in short trousers for effect. I have said it because it is true. Wetiko's insatiable desire for power over others is not a sign of its omnipotence, but its insecurity. Paul Levy writes: 'Due to the primal fear which ultimately drives it and which it is driven to cultivate, wetiko's body politic has an intrinsic and insistent need for centralising power and control so as to create imagined safety for itself.' *Yeeeeeees!* Exactly! Why does Wetiko want humans in an ongoing state of fear? Wetiko itself *is* fear and it is petrified of love. As evil is an absence of love, so love is an absence of fear. Love conquers all and *especially* Wetiko which *is* fear. Wetiko brought fear into the world when it wasn't here before. *Fear* was the 'fall', the fall into low-frequency ignorance and illusion – fear is **F**alse **E**motion **A**ppearing **R**eal. The simulation is driven and energised by fear because Wetiko/Yaldabaoth (fear) *are* the simulation. Fear is the absence of love and Wetiko is the absence of love.

Wetiko today

We can now view current events from this level of perspective. The 'Covid' hoax has generated momentous amounts of ongoing fear, anxiety, depression and despair which have empowered Wetiko. No wonder people like Gates have been the instigators when they are Wetiko incarnate and exhibit every trait of Wetiko in the extreme. See how cold and unemotional these people are like Gates and his cronies, how dead of eye they are. That's Wetiko. Sabbatians are Wetiko and everything they control including the World Health Organization, Big Pharma and the 'vaccine' makers, national 'health'

hierarchies, corporate media, Silicon Valley, the banking system, and the United Nations with its planned transformation into world government. All are controlled and possessed by the Wetiko distortion into distorting human society in its image. We are with this knowledge at the gateway to understanding the world. Divisions of race, culture, creed and sexuality are diversions to hide the real division between those possessed and influenced by Wetiko and those that are not. The 'Covid' hoax has brought both clearly into view. Human behaviour is not about race. Tyrants and dictatorships come in all colours and creeds. What unites the US president bombing the innocent and an African tribe committing genocide against another as in Rwanda? What unites them? *Wetiko*. All wars are Wetiko, all genocide is Wetiko, all hunger over centuries in a world of plenty is Wetiko. Children going to bed hungry, including in the West, is Wetiko. Cult-generated Woke racial divisions that focus on the body are designed to obscure the reality that divisions in behaviour are manifestations of mind, not body. Obsession with body identity and group judgement is a means to divert attention from the real source of behaviour – mind and perception. Conflict sown by the Woke both within themselves and with their target groups are Wetiko providing lunch for itself through still more agents of the division, chaos, and fear on which it feeds. The Cult is seeking to assimilate the entirety of humanity and all children and young people into the Wetiko frequency by manipulating them into states of fear and despair. Witness all the suicide and psychological unravelling since the spring of 2020. Wetiko psychopaths want to impose a state of unquestioning obedience to authority which is no more than a conduit for Wetiko to enforce its will and assimilate humanity into itself. It needs us to believe that resistance is futile when it fears resistance and even more so the game-changing non-cooperation with its impositions. It can use violent resistance for its benefit. Violent impositions and violent resistance are *both* Wetiko. The Power of Love with its Power of No will sweep Wetiko from our world. Wetiko and its Cult know that. They just don't want us to know.

AI Wetiko

This brings me to AI or artificial intelligence and something else Wetikos don't want us to know. What is AI *really*? I know about computer code algorithms and AI that learns from data input. These, however, are more diversions, the expeditionary force, for the real AI that they want to connect to the human brain as promoted by Silicon Valley Wetikos like Kurzweil. What is this AI? It is the frequency of *Wetiko*, the frequency of the Archons. The connection of AI to the human brain is the connection of the Wetiko frequency to create a Wetiko hive mind and complete the job of assimilation. The hive mind is planned to be controlled from Israel and China which are both 100 percent owned by Wetiko Sabbatians. The assimilation process has been going on minute by minute in the 'smart' era which fused with the 'Covid' era. We are told that social media is scrambling the minds of the young and changing their personality. This is true, but what is social media? Look more deeply at how it works, how it creates divisions and conflict, the hostility and cruelty, the targeting of people until they are destroyed. That's Wetiko. Social media is manipulated to tune people to the Wetiko frequency with all the emotional exploitation tricks employed by platforms like Facebook and its Wetiko front man, Zuckerberg. Facebook's Instagram announced a new platform for children to overcome a legal bar on them using the main site. This is more Wetiko exploitation and manipulation of kids. Amnesty International likened the plan to foxes offering to guard the henhouse and said it was incompatible with human rights. Since when did Wetiko or Zuckerberg (I repeat myself) care about that? Would Brin and Page at Google, Wojcicki at YouTube, Bezos at Amazon and whoever the hell runs Twitter act as they do if they were not channelling Wetiko? Would those who are developing technologies for no other reason than human control? How about those designing and selling technologies to kill people and Big Pharma drug and 'vaccine' producers who know they will end or devastate lives? Quite a thought for these people to consider is that if you are Wetiko in a human life you are Wetiko on the 'other side' unless your frequency

changes and that can only change by a change of perception which becomes a change of behaviour. Where Gates is going does not bear thinking about although perhaps that's exactly where he wants to go. Either way, that's where he's going. His frequency will make it so.

The frequency lair

I have been saying for a long time that a big part of the addiction to smartphones and devices is that a frequency is coming off them that entraps the mind. People spend ages on their phones and sometimes even a minute or so after they put them down they pick them up again and it all repeats. 'Covid' lockdowns will have increased this addiction a million times for obvious reasons. Addictions to alcohol overindulgence and drugs are another way that Wetiko entraps consciousness to attach to its own. Both are symptoms of low-vibrational psychological distress which alcoholism and drug addiction further compound. Do we think it's really a coincidence that access to them is made so easy while potions that can take people into realms beyond the simulation are banned and illegal? I have explored smartphone addiction in other books, the scale is mind-blowing, and that level of addiction does not come without help. Tech companies that make these phones are Wetiko and they will have no qualms about destroying the minds of children. We are seeing again with these companies the Wetiko perceptual combination of psychopathic enforcers and weak and meek unquestioning compliance by the rank and file.

The global Smart Grid is the Wetiko Grid and it is crucial to complete the Cult endgame. The simulation is radiation and we are being deluged with technological radiation on a devastating scale. Wetiko frauds like Elon Musk serve Cult interests while occasionally criticising them to maintain his street-cred. 5G and other forms of Wi-Fi are being directed at the earth from space on a volume and scale that goes on increasing by the day. Elon Musk's (officially) SpaceX Starlink project is in the process of putting tens of thousands of satellites in low orbit to cover every inch of the planet with 5G and other Wi-Fi to create Kurzweil's global 'cloud' to which the

human mind is planned to be attached very soon. SpaceX has approval to operate 12,000 satellites with more than 1,300 launched at the time of writing and applications filed for *30,000* more. Other operators in the Wi-Fi, 5G, low-orbit satellite market include OneWeb (UK), Telesat (Canada), and AST & Science (US). Musk tells us that AI could be the end of humanity and then launches a company called Neuralink to connect the human brain to computers. Musk's (in theory) Tesla company is building electric cars and the driverless vehicles of the smart control grid. As frauds and bullshitters go Elon Musk in my opinion is Major League.

5G and technological radiation in general are destructive to human health, genetics and psychology and increasing the strength of artificial radiation underpins the five-sense perceptual bubbles which are themselves expressions of radiation or electromagnetism. Freedom activist John Whitehead was so right with his 'databit by databit, we are building our own electronic concentration camps'. The Smart Grid and 5G is a means to control the human mind and infuse perceptual information into The Field to influence anyone in sync with its frequency. You can change perception and behaviour en masse if you can manipulate the population into those levels of frequency and this is happening all around us today. The arrogance of Musk and his fellow Cult operatives knows no bounds in the way that we see with Gates. Musk's satellites are so many in number already they are changing the night sky when viewed from Earth. The astronomy community has complained about this and they have seen nothing yet. Some consequences of Musk's Wetiko hubris include: Radiation; visible pollution of the night sky; interference with astronomy and meteorology; ground and water pollution from intensive use of increasingly many spaceports; accumulating space debris; continual deorbiting and burning up of aging satellites, polluting the atmosphere with toxic dust and smoke; and ever-increasing likelihood of collisions. A collective public open letter of complaint to Musk said:

> We are writing to you … because SpaceX is in process of surrounding the Earth with a network of thousands of satellites whose very purpose is to irradiate every square inch of the

Earth. SpaceX, like everyone else, is treating the radiation as if it were not there. As if the mitochondria in our cells do not depend on electrons moving undisturbed from the food we digest to the oxygen we breathe.

As if our nervous systems and our hearts are not subject to radio frequency interference like any piece of electronic equipment. As if the cancer, diabetes, and heart disease that now afflict a majority of the Earth's population are not metabolic diseases that result from interference with our cellular machinery. As if insects everywhere, and the birds and animals that eat them, are not starving to death as a result.

People like Musk and Gates believe in their limitless Wetiko arrogance that they can do whatever they like to the world because they own it. Consequences for humanity are irrelevant. It's absolutely time that we stopped taking this shit from these self-styled masters of the Earth when you consider where this is going.

Why is the Cult so anti-human?

I hear this question often: Why would they do this when it will affect them, too? Ah, but will it? Who is this *them*? Forget their bodies. They are just vehicles for Wetiko consciousness. When you break it all down to the foundations we are looking at a state of severely distorted consciousness targeting another state of consciousness for assimilation. The rest is detail. The simulation is the fly-trap in which unique sensations of the five senses create a cycle of addiction called reincarnation. Renegade Minds see that everything which happens in our reality is a smaller version of the whole picture in line with the holographic principle. Addiction to the radiation of smart technology is a smaller version of addiction to the whole simulation. Connecting the body/brain to AI is taking that addiction on a giant step further to total ongoing control by assimilating human incarnate consciousness into Wetiko. I have watched during the 'Covid' hoax how many are becoming ever more profoundly attached to Wetiko's perceptual calling cards of aggressive response to any other point of view ('There is no other god but me'), psychopathic lack of compassion and empathy, and servile submission to the narrative and will of authority. Wetiko is the psychopaths *and* subservience to psychopaths. The Cult of Wetiko is

so anti-human because it is *not* human. It embarked on a mission to destroy human by targeting everything that it means to be human and to survive as human. 'Covid' is not the end, just a means to an end. The Cult with its Wetiko consciousness is seeking to change Earth systems, including the atmosphere, to suit them, not humans. The gathering bombardment of 5G alone from ground and space is dramatically changing The Field with which the five senses interact. There is so much more to come if we sit on our hands and hope it will all go away. It is not meant to go away. It is meant to get ever more extreme and we need to face that while we still can – just.

Carbon dioxide is the gas of life. Without that human is over. Kaput, gone, history. No natural world, no human. The Cult has created a cock and bull story about carbon dioxide and climate change to justify its reduction to the point where Gates and the ignoramus Biden 'climate chief' John Kerry want to suck it out of the atmosphere. Kerry wants to do this because his master Gates does. Wetikos have made the gas of life a demon with the usual support from the Wokers of Extinction Rebellion and similar organisations and the bewildered puppet-child that is Greta Thunberg who was put on the world stage by Klaus Schwab and the World Economic Forum. The name Extinction Rebellion is both ironic and as always Wetiko inversion. The gas that we need to survive must be reduced to save us from extinction. The most basic need of human is oxygen and we now have billions walking around in face nappies depriving body and brain of this essential requirement of human existence. More than that 5G at 60 gigahertz interacts with the oxygen molecule to reduce the amount of oxygen the body can absorb into the bloodstream. The obvious knock-on consequences of that for respiratory and cognitive problems and life itself need no further explanation. Psychopaths like Musk are assembling a global system of satellites to deluge the human atmosphere with this insanity. The man should be in jail. Here we have two most basic of human needs, oxygen and carbon dioxide, being dismantled.

Two others, water and food, are getting similar treatment with the United Nations Agendas 21 and 2030 – the Great Reset – planning to

centrally control all water and food supplies. People will not even own rain water that falls on their land. Food is affected at the most basic level by reducing carbon dioxide. We have genetic modification or GMO infiltrating the food chain on a mass scale, pesticides and herbicides polluting the air and destroying the soil. Freshwater fish that provide livelihoods for 60 million people and feed hundreds of millions worldwide are being 'pushed to the brink' according the conservationists while climate change is the only focus. Now we have Gates and Schwab wanting to dispense with current food sources all together and replace them with a synthetic version which the Wetiko Cult would control in terms of production and who eats and who doesn't. We have been on the Totalitarian Tiptoe to this for more than 60 years as food has become ever more processed and full of chemical shite to the point today when it's not natural food at all. As Dr Tom Cowan says: 'If it has a label don't eat it.' Bill Gates is now the biggest owner of farmland in the United States and he does nothing without an ulterior motive involving the Cult. Klaus Schwab wrote: 'To feed the world in the next 50 years we will need to produce as much food as was produced in the last 10,000 years … food security will only be achieved, however, if regulations on genetically modified foods are adapted to reflect the reality that gene editing offers a precise, efficient and safe method of improving crops.' Liar. People and the world are being targeted with aluminium through vaccines, chemtrails, food, drink cans, and endless other sources when aluminium has been linked to many health issues including dementia which is increasing year after year. Insects, bees and wildlife essential to the food chain are being deleted by pesticides, herbicides and radiation which 5G is dramatically increasing with 6G and 7G to come. The pollinating bee population is being devastated while wildlife including birds, dolphins and whales are having their natural radar blocked by the effects of ever-increasing radiation. In the summer windscreens used to be splattered with insects so numerous were they. It doesn't happen now. Where have they gone?

Synthetic everything

The Cult is introducing genetically-modified versions of trees, plants and insects including a Gates-funded project to unleash hundreds of millions of genetically-modified, lab-altered and patented male mosquitoes to mate with wild mosquitoes and induce genetic flaws that cause them to die out. Clinically-insane Gates-funded Japanese researchers have developed mosquitos that spread vaccine and are dubbed 'flying vaccinators'. Gates is funding the modification of weather patterns in part to sell the myth that this is caused by carbon dioxide and he's funding geoengineering of the skies to change the atmosphere. Some of this came to light with the Gates-backed plan to release tonnes of chalk into the atmosphere to 'deflect the Sun and cool the planet'. Funny how they do this while the heating effect of the Sun is not factored into climate projections focussed on carbon dioxide. The reason is that they want to reduce carbon dioxide (so don't mention the Sun), but at the same time they do want to reduce the impact of the Sun which is so essential to human life and health. I have mentioned the sun-cholesterol-vitamin D connection as they demonise the Sun with warnings about skin cancer (caused by the chemicals in sun cream they tell you to splash on). They come from the other end of the process with statin drugs to reduce cholesterol that turns sunlight into vitamin D. A lack of vitamin D leads to a long list of health effects and how vitamin D levels must have fallen with people confined to their homes over 'Covid'. Gates is funding other forms of geoengineering and most importantly chemtrails which are dropping heavy metals, aluminium and self-replicating nanotechnology onto the Earth which is killing the natural world. See *Everything You Need To Know, But Have Never Been Told* for the detailed background to this.

Every human system is being targeted for deletion by a force that's not human. The Wetiko Cult has embarked on the process of transforming the human body from biological to synthetic biological as I have explained. Biological is being replaced by the artificial and synthetic – Archontic 'countermimicry' – right across human society. The plan eventually is to dispense with the human body altogether

and absorb human consciousness – which it wouldn't really be by then – into cyberspace (the simulation which is Wetiko/Yaldabaoth). Preparations for that are already happening if people would care to look. The alternative media rightly warns about globalism and 'the globalists', but this is far bigger than that and represents the end of the human race as we know it. The 'bad copy' of prime reality that Gnostics describe was a bad copy of harmony, wonder and beauty to start with before Wetiko/Yaldabaoth set out to change the simulated 'copy' into something very different. The process was slow to start with. Entrapped humans in the simulation timeline were not technologically aware and they had to be brought up to intellectual speed while being suppressed spiritually to the point where they could build their own prison while having no idea they were doing so. We have now reached that stage where technological intellect has the potential to destroy us and that's why events are moving so fast. Central American shaman Don Juan Matus said:

> Think for a moment, and tell me how you would explain the contradictions between the intelligence of man the engineer and the stupidity of his systems of belief, or the stupidity of his contradictory behaviour. Sorcerers believe that the predators have given us our systems of beliefs, our ideas of good and evil; our social mores. They are the ones who set up our dreams of success or failure. They have given us covetousness, greed, and cowardice. It is the predator who makes us complacent, routinary, and egomaniacal.
>
> In order to keep us obedient and meek and weak, the predators engaged themselves in a stupendous manoeuvre – stupendous, of course, from the point of view of a fighting strategist; a horrendous manoeuvre from the point of those who suffer it. They gave us their mind. The predators' mind is baroque, contradictory, morose, filled with the fear of being discovered any minute now.

For 'predators' see Wetiko, Archons, Yaldabaoth, Jinn, and all the other versions of the same phenomenon in cultures and religions all over the world. The theme is always the same because it's true and it's real. We have reached the point where we have to deal with it. The question is – how?

Don't fight – walk away

I thought I'd use a controversial subheading to get things moving in terms of our response to global fascism. What do you mean 'don't fight'? What do you mean 'walk away'? We've got to fight. We can't walk away. Well, it depends what we mean by fight and walk away. If fighting means physical combat we are playing Wetiko's game and falling for its trap. It wants us to get angry, aggressive, and direct hate and hostility at the enemy we think we must fight. Every war, every battle, every conflict, has been fought with Wetiko leading both sides. It's what it does. Wetiko wants a fight, anywhere, any place. Just hit me, son, so I can hit you back. Wetiko hits Wetiko and Wetiko hits Wetiko in return. I am very forthright as you can see in exposing Wetikos of the Cult, but I don't hate them. I refuse to hate them. It's what they want. What you hate you become. What you *fight* you become. Wokers, 'anti-haters' and 'anti-fascists' prove this every time they reach for their keyboards or don their balaclavas. By walk away I mean to disengage from Wetiko which includes ceasing to cooperate with its tyranny. Paul Levy says of Wetiko:

> The way to 'defeat' evil is not to try to destroy it (for then, in playing evil's game, we have already lost), but rather, to find the invulnerable place within ourselves where evil is unable to vanquish us – this is to truly 'win' our battle with evil.

Wetiko is everywhere in human society and it's been on steroids since the 'Covid' hoax. Every shouting match over wearing masks has Wetiko wearing a mask and Wetiko not wearing one. It's an electrical circuit of push and resist, push and resist, with Wetiko pushing *and* resisting. Each polarity is Wetiko empowering itself. Dictionary definitions of 'resist' include 'opposing, refusing to accept or comply with' and the word to focus on is 'opposing'. What form does this take – setting police cars alight or 'refusing to accept or comply with'? The former is Wetiko opposing Wetiko while the other points the way forward. This is the difference between those aggressively demanding that government fascism must be obeyed who stand in stark contrast to the great majority of Pushbackers. We saw this clearly with a march by thousands of Pushbackers against lockdown in London followed days later by a Woker-hijacked

protest in Bristol in which police cars were set on fire. Masks were virtually absent in London and widespread in Bristol. Wetiko wants lockdown on every level of society and infuses its aggression to police it through its unknowing stooges. Lockdown protesters are the ones with the smiling faces and the hugs, The two blatantly obvious states of being – getting more obvious by the day – are the result of Wokers and their like becoming ever more influenced by the simulation Field of Wetiko and Pushbackers ever more influenced by The Field of a far higher vibration beyond the simulation. Wetiko can't invade the heart which is where most lockdown opponents are coming from. It's the heart that allows them to see through the lies to the truth in ways I will be highlighting.

Renegade Minds know that calmness is the place from which wisdom comes. You won't find wisdom in a hissing fit and wisdom is what we need in abundance right now. Calmness is not weakness – you don't have to scream at the top of your voice to be strong. Calmness is indeed a sign of strength. 'No' means I'm not doing it. *NOOOO!!!* doesn't mean you're not doing it even more. Volume does not advance 'No – I'm not doing it'. You are just not doing it. Wetiko possessed and influenced don't know how to deal with that. Wetiko wants a fight and we should not give it one. What it needs more than anything is our *cooperation* and we should not give that either. Mass rallies and marches are great in that they are a visual representation of feeling, but if it ends there they are irrelevant. You demand that Wetikos act differently? Well, they're not going to are they? They are Wetikos. We don't need to waste our time demanding that something doesn't happen when that will make no difference. We need to delete the means that *allows* it to happen. This, invariably, is our cooperation. You can demand a child stop firing a peashooter at the dog or you can refuse to buy the peashooter. If you provide the means you are cooperating with the dog being smacked on the nose with a pea. How can the authorities enforce mask-wearing if millions in a country refuse? What if the 74 million Pushbackers that voted for Trump in 2020 refused to wear masks, close their businesses or stay in their homes. It would be unenforceable. The

few control the many through the compliance of the many and that's always been the dynamic be it 'Covid' regulations or the Roman Empire. I know people can find it intimidating to say no to authority or stand out in a crowd for being the only one with a face on display; but it has to be done or it's over. I hope I've made clear in this book that where this is going will be far more intimidating than standing up now and saying 'No' – I will not cooperate with my own enslavement and that of my children. There might be consequences for some initially, although not so if enough do the same. The question that must be addressed is what is going to happen if we don't? It is time to be strong and unyieldingly so. No means no. Not here and there, but *everywhere* and *always*. I have refused to wear a mask and obey all the other nonsense. I will not comply with tyranny. I repeat: Fascism is not imposed by fascists – there are never enough of them. Fascism is imposed by the population acquiescing to fascism. *I will not do it*. I will die first, or my body will. Living meekly under fascism is a form of death anyway, the death of the spirit that Martin Luther King described.

Making things happen

We must not despair. This is not over till it's over and it's far from that. The 'fat lady' must refuse to sing. The longer the 'Covid' hoax has dragged on and impacted on more lives we have seen an awakening of phenomenal numbers of people worldwide to the realisation that what they have believed all their lives is not how the world really is. Research published by the system-serving University of Bristol and King's College London in February, 2021, concluded: 'One in every 11 people in Britain say they trust David Icke's take on the coronavirus pandemic.' It will be more by now and we have gathering numbers to build on. We must urgently progress from seeing the scam to ceasing to cooperate with it. Prominent German lawyer Reiner Fuellmich, also licenced to practice law in America, is doing a magnificent job taking the legal route to bring the psychopaths to justice through a second Nuremberg tribunal for crimes against humanity. Fuellmich has an impressive record of

beating the elite in court and he formed the German Corona Investigative Committee to pursue civil charges against the main perpetrators with a view to triggering criminal charges. Most importantly he has grasped the foundation of the hoax – the PCR test not testing for the 'virus' – and Christian Drosten is therefore on his charge sheet along with Gates frontman Tedros at the World Health Organization. Major players must be not be allowed to inflict their horrors on the human race without being brought to book. A life sentence must follow for Bill Gates and the rest of them. A group of researchers has also indicted the government of Norway for crimes against humanity with copies sent to the police and the International Criminal Court. The lawsuit cites participation in an internationally-planned false pandemic and violation of international law and human rights, the European Commission's definition of human rights by coercive rules, Nuremberg and Hague rules on fundamental human rights, and the Norwegian constitution. We must take the initiative from hereon and not just complain, protest and react.

There are practical ways to support vital mass non-cooperation. Organising in numbers is one. Lockdown marches in London in the spring in 2021 were mass non-cooperation that the authorities could not stop. There were too many people. Hundreds of thousands walked the London streets in the centre of the road for mile after mile while the Face-Nappies could only look on. They were determined, but calm, and just *did it* with no histrionics and lots of smiles. The police were impotent. Others are organising group shopping without masks for mutual support and imagine if that was happening all over. Policing it would be impossible. If the store refuses to serve people in these circumstances they would be faced with a long line of trolleys full of goods standing on their own and everything would have to be returned to the shelves. How would they cope with that if it kept happening? I am talking here about moving on from complaining to being pro-active; from watching things happen to making things happen. I include in this our relationship with the police. The behaviour of many Face-Nappies

has been disgraceful and anyone who thinks they would never find concentration camp guards in the 'enlightened' modern era have had that myth busted big-time. The period and setting may change – Wetikos never do. I watched film footage from a London march in which a police thug viciously kicked a protestor on the floor who had done nothing. His fellow Face-Nappies stood in a ring protecting him. What he did was a criminal assault and with a crowd far outnumbering the police this can no longer be allowed to happen unchallenged. I get it when people chant 'shame on you' in these circumstances, but that is no longer enough. They *have* no shame those who do this. Crowds needs to start making a citizen's arrest of the police who commit criminal offences and brutally attack innocent people and defenceless women. A citizen's arrest can be made under section 24A of the UK Police and Criminal Evidence (PACE) Act of 1984 and you will find something similar in other countries. I prefer to call it a Common Law arrest rather than citizen's for reasons I will come to shortly. Anyone can arrest a person committing an indictable offence or if they have reasonable grounds to suspect they are committing an indictable offence. On both counts the attack by the police thug would have fallen into this category. A citizen's arrest can be made to stop someone:

- Causing physical injury to himself or any other person
- Suffering physical injury
- Causing loss of or damage to property
- Making off before a constable can assume responsibility for him

A citizen's arrest may also be made to prevent a breach of the peace under Common Law and if they believe a breach of the peace will happen or anything related to harm likely to be done or already done in their presence. This is the way to go I think – the Common Law version. If police know that the crowd and members of the public will no longer be standing and watching while they commit

their thuggery and crimes they will think twice about acting like Brownshirts and Blackshirts.

Common Law – common sense

Mention of Common Law is very important. Most people think the law is the law as in one law. This is not the case. There are two bodies of law, Common Law and Statute Law, and they are not the same. Common Law is founded on the simple premise of do no harm. It does not recognise victimless crimes in which no harm is done while Statute Law does. There is a Statute Law against almost everything. So what is Statute Law? Amazingly it's the law of the *sea* that was brought ashore by the Cult to override the law of the land which is Common Law. They had no right to do this and as always they did it anyway. They had to. They could not impose their will on the people through Common Law which only applies to do no harm. How could you stitch up the fine detail of people's lives with that? Instead they took the law of the sea, or Admiralty Law, and applied it to the population. Statute Law refers to all the laws spewing out of governments and their agencies including all the fascist laws and regulations relating to 'Covid'. The key point to make is that Statute Law is *contract law*. It only applies between *contracting* corporations. Most police officers don't even know this. They have to be kept in the dark, too. Long ago when merchants and their sailing ships began to trade with different countries a contractual law was developed called Admiralty Law and other names. Again it only applied to *contracts* agreed between *corporate* entities. If there is no agreed contract the law of the sea had no jurisdiction *and that still applies to its new alias of Statute Law*. The problem for the Cult when the law of the sea was brought ashore was an obvious one. People were not corporations and neither were government entities. To overcome the latter they made governments and all associated organisations corporations. All the institutions are *private corporations* and I mean governments and their agencies, local councils, police, courts, military, US states, the whole lot. Go to the

Dun and Bradstreet corporate listings website for confirmation that they are all corporations. You are arrested by a private corporation called the police by someone who is really a private security guard and they take you to court which is another private corporation. Neither have jurisdiction over you unless you consent and *contract* with them. This is why you hear the mantra about law enforcement policing by *consent* of the people. In truth the people 'consent' only in theory through monumental trickery.

Okay, the Cult overcame the corporate law problem by making governments and institutions corporate entities; but what about people? They are not corporations are they? Ah ... well in a sense, and *only* a sense, they are. Not people exactly – the illusion of people. The Cult creates a corporation in the name of everyone at the time that their birth certificate is issued. Note birth/ *berth* certificate and when you go to court under the law of the sea on land you stand in a *dock*. These are throwbacks to the origin. My Common Law name is David Vaughan Icke. The name of the corporation created by the government when I was born is called Mr David Vaughan Icke usually written in capitals as MR DAVID VAUGHAN ICKE. That is not me, the living, breathing man. It is a fictitious corporate entity. The trick is to make you think that David Vaughan Icke and MR DAVID VAUGHAN ICKE are the same thing. *They are not*. When police charge you and take you to court they are prosecuting the corporate entity and not the living, breathing, man or woman. They have to trick you into identifying as the corporate entity and contracting with them. Otherwise they have no jurisdiction. They do this through a language known as legalese. Lawful and legal are not the same either. Lawful relates to Common Law and legal relates to Statute Law. Legalese is the language of Statue Law which uses terms that mean one thing to the public and another in legalese. Notice that when a police officer tells someone why they are being charged he or she will say at the end: 'Do you understand?' To the public that means 'Do you comprehend?' In legalese it means 'Do you stand under me?' Do you stand under my authority? If you say

yes to the question you are unknowingly agreeing to give them jurisdiction over you in a contract between two corporate entities.

This is a confidence trick in every way. Contracts have to be agreed between informed parties and if you don't know that David Vaughan Icke is agreeing to be the corporation MR DAVID VAUGHAN ICKE you cannot knowingly agree to contract. They are deceiving you and another way they do this is to ask for proof of identity. You usually show them a driving licence or other document on which your corporate name is written. In doing so you are accepting that you are that corporate entity when you are not. Referring to yourself as a 'person' or 'citizen' is also identifying with your corporate fiction which is why I made the Common Law point about the citizen's arrest. If you are approached by a police officer you identify yourself immediately as a living, breathing, man or woman and say 'I do not consent, I do not contract with you and I do not understand' or stand under their authority. I have a Common Law birth certificate as a living man and these are available at no charge from commonlawcourt.com. Businesses registered under the Statute Law system means that its laws apply. There are, however, ways to run a business under Common Law. Remember all 'Covid' laws and regulations are Statute Law – the law of *contracts* and you do not have to contract. This doesn't mean that you can kill someone and get away with it. Common Law says do no harm and that applies to physical harm, financial harm etc. Police are employees of private corporations and there needs to be a new system of non-corporate Common Law constables operating outside the Statute Law system. If you go to davidicke.com and put Common Law into the search engine you will find videos that explain Common Law in much greater detail. It is definitely a road we should walk.

With all my heart

I have heard people say that we are in a spiritual war. I don't like the term 'war' with its Wetiko dynamic, but I know what they mean. Sweep aside all the bodily forms and we are in a situation in which two states of consciousness are seeking very different realities.

Wetiko wants upheaval, chaos, fear, suffering, conflict and control. The other wants love, peace, harmony, fairness and freedom. That's where we are. We should not fall for the idea that Wetiko is all-powerful and there's nothing we can do. Wetiko is not all-powerful. It's a joke, pathetic. It doesn't have to be, but it has made that choice for now. A handful of times over the years when I have felt the presence of its frequency I have allowed it to attach briefly so I could consciously observe its nature. The experience is not pleasant, the energy is heavy and dark, but the ease with which you can kick it back out the door shows that its real power is in persuading us that it has power. It's all a con. Wetiko is a con. It's a trickster and not a power that can control us if we unleash our own. The con is founded on manipulating humanity to give its power to Wetiko which recycles it back to present the illusion that it has power when its power is *ours* that we gave away. This happens on an energetic level and plays out in the world of the seen as humanity giving its power to Wetiko authority which uses that power to control the population when the power is only the power the population has handed over. How could it be any other way for billions to be controlled by a relative few? I have had experiences with people possessed by Wetiko and again you can kick its arse if you do it with an open heart. Oh yes – the *heart* which can transform the world of perceived 'matter'.

We are receiver-transmitters and processors of information, but what information and where from? Information is processed into perception in three main areas – the brain, the heart and the belly. These relate to thinking, knowing, and emotion. Wetiko wants us to be head and belly people which means we think within the confines of the Matrix simulation and low-vibrational emotional reaction scrambles balance and perception. A few minutes on social media and you see how emotion is the dominant force. Woke is all emotion and is therefore thought-free and fact-free. Our heart is something different. It *knows* while the head *thinks* and has to try to work it out because it doesn't know. The human energy field has seven prime vortexes which connect us with wider reality (Fig 23). Chakra means

'wheels of light' in the Sanskrit language of ancient India. The main ones are: The crown chakra on top of the head; brow (or 'third eye') chakra in the centre of the forehead; throat chakra; heart chakra in the centre of the chest; solar plexus chakra below the sternum; sacral chakra beneath the navel; and base chakra at the bottom of the spine. Each one has a particular function or functions. We feel anxiety and nervousness in the belly where the sacral chakra is located and this processes emotion that can affect the colon to give people 'the shits' or make them 'shit scared' when they are nervous. Chakras all play an important role, but the Mr and Mrs Big is the heart chakra which sits at the centre of the seven, above the chakras that connect us to the 'physical' and below those that connect with higher realms (or at least should). Here in the heart chakra we feel love, empathy and compassion – 'My heart goes out to you'. Those with closed hearts become literally 'heart-less' in their attitudes and behaviour (see Bill Gates). Native Americans portrayed Wetiko with what Paul Levy calls a 'frigid, icy heart, devoid of mercy' (see Bill Gates).

Figure 23: The chakra system which interpenetrates the human energy field. The heart chakra is the governor – or should be.

Wetiko trembles at the thought of heart energy which it cannot infiltrate. The frequency is too high. What it seeks to do instead is close the heart chakra vortex to block its perceptual and energetic influence. Psychopaths have 'hearts of stone' and emotionally-damaged people have 'heartache' and 'broken hearts'. The astonishing amount of heart disease is related to heart chakra

disruption with its fundamental connection to the 'physical' heart. Dr Tom Cowan has written an outstanding book challenging the belief that the heart is a pump and making the connection between the 'physical' and spiritual heart. Rudolph Steiner who was way ahead of his time said the same about the fallacy that the heart is a pump. *What*? The heart is not a pump? That's crazy, right? Everybody knows that. Read Cowan's *Human Heart, Cosmic Heart* and you will realise that the very idea of the heart as a pump is ridiculous when you see the evidence. How does blood in the feet so far from the heart get pumped horizontally up the body by the heart?? Cowan explains in the book the real reason why blood moves as it does. Our 'physical' heart is used to symbolise love when the source is really the heart vortex or spiritual heart which is our most powerful energetic connection to 'out there' expanded consciousness. That's why we feel *knowing* – intuitive knowing – in the centre of the chest. Knowing doesn't come from a process of thoughts leading to a conclusion. It is there in an instant all in one go. Our heart knows because of its connection to levels of awareness that *do* know. This is the meaning and source of intuition – intuitive *knowing*.

For the last more than 30 years of uncovering the global game and the nature of reality my heart has been my constant antenna for truth and accuracy. An American intelligence insider once said that I had quoted a disinformer in one of my books and yet I had only quoted the part that was true. He asked: 'How do you do that?' By using my heart antenna was the answer and anyone can do it. Heart-centred is how we are meant to be. With a closed heart chakra we withdraw into a closed mind and the bubble of five-sense reality. If you take a moment to focus your attention on the centre of your chest, picture a spinning wheel of light and see it opening and expanding. You will feel it happening, too, and perceptions of the heart like joy and love as the heart impacts on the mind as they interact. The more the chakra opens the more you will feel expressions of heart consciousness and as the process continues, and becomes part of you, insights and knowings will follow. An open

heart is connected to that level of awareness that knows all is *One*. You will see from its perspective that the fault-lines that divide us are only illusions to control us. An open heart does not process the illusions of race, creed and sexuality except as brief experiences for a consciousness that is all. Our heart does not see division, only unity (Figs 24 and 25). There's something else, too. Our hearts love to laugh. Mark Twain's quote that says 'The human race has one really effective weapon, and that is laughter' is really a reference to the heart which loves to laugh with the joy of knowing the true nature of infinite reality and that all the madness of human society is an illusion of the mind. Twain also said: 'Against the assault of laughter nothing can stand.' This is so true of Wetiko and the Cult. Their insecurity demands that they be taken seriously and their power and authority acknowledged and feared. We should do nothing of the sort. We should not get aggressive or fearful which their insecurity so desires. We should laugh in their face. Even in their no-face as police come over in their face-nappies and expect to be taken seriously. They don't take themselves seriously looking like that so why should we? Laugh in the face of intimidation. Laugh in the face of tyranny. You will see by its reaction that you have pressed all of its buttons. Wetiko does not know what to do in the face of laughter or when its targets refuse to concede their joy to fear. We have seen many examples during the 'Covid' hoax when people have expressed their energetic power and the string puppets of Wetiko retreat with their tail limp between their knees. Laugh – the world is bloody mad after all and if it's a choice between laughter and tears I know which way I'm going.

Figure 24: Head consciousness without the heart sees division and everything apart from everything else.

Figure 25: Heart consciousness sees everything as One.

'Vaccines' and the soul

The foundation of Wetiko/Archon control of humans is the separation of incarnate five-sense mind from the infinite 'I' and closing the heart chakra where the True 'I' lives during a human life. The goal has been to achieve complete separation in both cases. I was interested therefore to read an account by a French energetic healer of what she said she experienced with a patient who had been given the 'Covid' vaccine. Genuine energy healers can sense information and consciousness fields at different levels of being which are referred to as 'subtle bodies'. She described treating the patient who later returned after having, without the healer's knowledge, two doses of the 'Covid vaccine'. The healer said:

I noticed immediately the change, very heavy energy emanating from [the] subtle bodies. The scariest thing was when I was working on the heart chakra, I connected with her soul: it was detached from the physical body, it had no contact and it was, as if it was floating in a state of total confusion: a damage to the consciousness that loses contact with the physical body, i.e. with our biological machine, there is no longer any communication between them.

I continued the treatment by sending light to the heart chakra, the soul of the person, but it seemed that the soul could no longer receive any light, frequency or energy. It was a very powerful experience for me. Then I understood that this substance is indeed used to detach consciousness so that this consciousness can no longer interact through this body that it possesses in life, where there is no longer any contact, no frequency, no light, no more energetic balance or mind.

This would create a human that is rudderless and at the extreme almost zombie-like operating with a fractional state of consciousness at the mercy of Wetiko. I was especially intrigued by what the healer said in the light of the prediction by the highly-informed Rudolf Steiner more than a hundred years ago. He said:

In the future, we will eliminate the soul with medicine. Under the pretext of a 'healthy point of view', there will be a vaccine by which the human body will be treated as soon as possible directly at birth, so that the human being cannot develop the thought of the existence of soul and Spirit. To materialistic doctors will be entrusted the task of removing the soul of humanity.

As today, people are vaccinated against this disease or that disease, so in the future, children will be vaccinated with a substance that can be produced precisely in such a way that people, thanks to this vaccination, will be immune to being subjected to the 'madness' of spiritual life. He would be extremely smart, but he would not develop a conscience, and that is the true goal of some materialistic circles.

Steiner said the vaccine would detach the physical body from the etheric body (subtle bodies) and 'once the etheric body is detached the relationship between the universe and the etheric body would become extremely unstable, and man would become an automaton'. He said 'the physical body of man must be polished on this Earth by spiritual will – so the vaccine becomes a kind of arymanique (Wetiko) force' and 'man can no longer get rid of a given materialistic feeling'. Humans would then, he said, become 'materialistic of constitution and can no longer rise to the spiritual'. I have been writing for years about DNA being a receiver-transmitter of information that connects us to other levels of reality and these 'vaccines' changing DNA can be likened to changing an antenna and what it can transmit and receive. Such a disconnection would clearly lead to changes in personality and perception. Steiner further predicted the arrival of AI. Big Pharma 'Covid vaccine' makers, expressions of Wetiko, are testing their DNA-manipulating evil on children as I write with a view to giving the 'vaccine' to babies. If it's a soul-body disconnector – and I say that it is or can be – every child would be disconnected from 'soul' at birth and the 'vaccine' would create a closed system in which spiritual guidance from the greater self would play no part. This has been the ambition of Wetiko all

along. A Pentagon video from 2005 was leaked of a presentation explaining the development of vaccines to change behaviour by their effect on the brain. Those that believe this is not happening with the 'Covid' genetically-modifying procedure masquerading as a 'vaccine' should make an urgent appointment with Naivety Anonymous. Klaus Schwab wrote in 2018:

> Neurotechnologies enable us to better influence consciousness and thought and to understand many activities of the brain. They include decoding what we are thinking in fine levels of detail through new chemicals and interventions that can influence our brains to correct for errors or enhance functionality.

The plan is clear and only the heart can stop it. With every heart that opens, every mind that awakens, Wetiko is weakened. Heart and love are far more powerful than head and hate and so nothing like a majority is needed to turn this around.

Beyond the Phantom

Our heart is the prime target of Wetiko and so it must be the answer to Wetiko. We *are* our heart which is part of one heart, the infinite heart. Our heart is where the true self lives in a human life behind firewalls of five-sense illusion when an imposter takes its place – *Phantom Self*; but our heart waits patiently to be set free any time we choose to see beyond the Phantom, beyond Wetiko. A Wetikoed Phantom Self can wreak mass death and destruction while the love of forever is locked away in its heart. The time is here to unleash its power and let it sweep away the fear and despair that is Wetiko. Heart consciousness does not seek manipulated, censored, advantage for its belief or religion, its activism and desires. As an expression of the One it treats all as One with the same rights to freedom and opinion. Our heart demands fairness for itself no more than for others. From this unity of heart we can come together in mutual support and transform this Wetikoed world into what reality is meant to be – a place of love, joy, happiness, fairness, justice and freedom. Wetiko has another agenda and that's why the world is as

it is, but enough of this nonsense. Wetiko can't stay where hearts are open and it works so hard to keep them closed. Fear is its currency and its food source and love in its true sense has no fear. Why would love have fear when it knows it is *All That Is, Has Been, And Ever Can Be* on an eternal exploration of all possibility? Love in this true sense is not the physical attraction that passes for love. This can be an expression of it, yes, but Infinite Love, a love without condition, goes far deeper to the core of all being. It *is* the core of all being. Infinite realty was born from love beyond the illusions of the simulation. Love infinitely expressed is the knowing that all is One and the swiftly-passing experience of separation is a temporary hallucination. You cannot disconnect from Oneness; you can only *perceive* that you have and withdraw from its influence. This is the most important of all perception trickery by the mind parasite that is Wetiko and the foundation of all its potential for manipulation.

If we open our hearts, open the sluice gates of the mind, and redefine self-identity amazing things start to happen. Consciousness expands or contracts in accordance with self-identity. When true self is recognised as infinite awareness and label self – Phantom Self – is seen as only a series of brief experiences life is transformed. Consciousness expands to the extent that self-identity expands and everything changes. You see unity, not division, the picture, not the pixels. From this we can play the long game. No more is an experience something in and of itself, but a fleeting moment in the eternity of forever. Suddenly people in uniform and dark suits are no longer intimidating. Doing what your heart knows to be right is no longer intimidating and consequences for those actions take on the same nature of a brief experience that passes in the blink of an infinite eye. Intimidation is all in the mind. Beyond the mind there is no intimidation.

An open heart does not consider consequences for what it knows to be right. To do so would be to consider not doing what it knows to be right and for a heart in its power that is never an option. The Renegade Mind is really the Renegade Heart. Consideration of consequences will always provide a getaway car for the mind and

the heart doesn't want one. What is right in the light of what we face today is to stop cooperating with Wetiko in all its forms and to do it without fear or compromise. You cannot compromise with tyranny when tyranny always demands more until it has everything. Life is your perception and you are your destiny. Change your perception and you change your life. Change collective perception and we change the world.

Come on people … One human family, One heart, One goal … *FREEEEEEDOM*!

We must settle for nothing less.

Postscript

The big scare story as the book goes to press is the 'Indian' variant and the world is being deluged with propaganda about the 'Covid catastrophe' in India which mirrors in its lies and misrepresentations what happened in Italy before the first lockdown in 2020.

The *New York Post* published a picture of someone who had 'collapsed in the street from Covid' in India in April, 2021, which was actually taken during a gas leak in May, 2020. Same old, same old. Media articles in mid-February were asking why India had been so untouched by 'Covid' and then as their vaccine rollout gathered pace the alleged 'cases' began to rapidly increase. Indian 'Covid vaccine' maker Bharat Biotech was funded into existence by the Bill and Melinda Gates Foundation (the pair announced their divorce in May, 2021, which is a pity because they so deserve each other). The Indian 'Covid crisis' was ramped up by the media to terrify the world and prepare people for submission to still more restrictions. The scam that worked the first time was being repeated only with far more people seeing through the deceit. Davidicke.com and Ickonic.com have sought to tell the true story of what is happening by talking to people living through the Indian nightmare which has nothing to do with 'Covid'. We posted a letter from 'Alisha' in Pune who told a very different story to government and media mendacity. She said scenes of dying people and overwhelmed hospitals were designed to hide what was really happening – genocide and starvation. Alisha said that millions had already died of starvation during the ongoing lockdowns while government and media were lying and making it look like the 'virus':

Restaurants, shops, gyms, theatres, basically everything is shut. The cities are ghost towns. Even so-called 'essential' businesses are only open till 11am in the morning. You basically have just an hour to buy food and then your time is up.

Inter-state travel and even inter-district travel is banned. The cops wait at all major crossroads to question why you are traveling outdoors or to fine you if you are not wearing a mask.

The medical community here is also complicit in genocide, lying about hospitals being full and turning away people with genuine illnesses, who need immediate care. They have even created a shortage of oxygen cylinders.

This is the classic Cult modus operandi played out in every country. Alisha said that people who would not have a PCR test not testing for the 'virus' were being denied hospital treatment. She said the people hit hardest were migrant workers and those in rural areas. Most businesses employed migrant workers and with everything closed there were no jobs, no income and no food. As a result millions were dying of starvation or malnutrition. All this was happening under Prime Minister Narendra Modi, a 100-percent asset of the Cult, and it emphasises yet again the scale of pure anti-human evil we are dealing with. Australia banned its people from returning home from India with penalties for trying to do so of up to five years in jail and a fine of £37,000. The manufactured 'Covid' crisis in India was being prepared to justify further fascism in the West. Obvious connections could be seen between the Indian 'vaccine' programme and increased 'cases' and this became a common theme. The Seychelles, the most per capita 'Covid vaccinated' population in the world, went back into lockdown after a 'surge of cases'.

Long ago the truly evil Monsanto agricultural biotechnology corporation with its big connections to Bill Gates devastated Indian farming with genetically-modified crops. Human rights activist Gurcharan Singh highlighted the efforts by the Indian government to complete the job by destroying the food supply to hundreds of millions with 'Covid' lockdowns. He said that 415 million people at the bottom of the disgusting caste system (still going whatever they say) were below the poverty line and struggled to feed themselves every year. Now the government was imposing lockdown at just the

time to destroy the harvest. This deliberate policy was leading to mass starvation. People may reel back at the suggestion that a government would do that, but Wetiko-controlled 'leaders' are capable of any level of evil. In fact what is described in India is in the process of being instigated worldwide. The food chain and food supply are being targeted at every level to cause world hunger and thus control. Bill Gates is not the biggest owner of farmland in America for no reason and destroying access to food aids both the depopulation agenda and the plan for synthetic 'food' already being funded into existence by Gates. Add to this the coming hyper-inflation from the suicidal creation of fake 'money' in response to 'Covid' and the breakdown of container shipping systems and you have a cocktail that can only lead one way and is meant to. The Cult plan is to crash the entire system to 'build back better' with the Great Reset.

'Vaccine' transmission

Reports from all over the world continue to emerge of women suffering menstrual and fertility problems after having the fake 'vaccine' and of the non-'vaccinated' having similar problems when interacting with the 'vaccinated'. There are far too many for 'coincidence' to be credible. We've had menopausal women getting periods, others having periods stop or not stopping for weeks, passing clots, sometimes the lining of the uterus, breast irregularities, and miscarriages (which increased by 400 percent in parts of the United States). Non-'vaccinated' men and children have suffered blood clots and nose bleeding after interaction with the 'vaccinated'. Babies have died from the effects of breast milk from a 'vaccinated' mother. Awake doctors – the small minority – speculated on the cause of non-'vaccinated' suffering the same effects as the 'vaccinated'. Was it nanotechnology in the synthetic substance transmitting frequencies or was it a straight chemical bioweapon that was being transmitted between people? I am not saying that some kind of chemical transmission is not one possible answer, but the foundation of all that the Cult does is frequency and

this is fertile ground for understanding how transmission can happen. American doctor Carrie Madej, an internal medicine physician and osteopath, has been practicing for the last 20 years, teaching medical students, and she says attending different meetings where the agenda for humanity was discussed. Madej, who operates out of Georgia, did not dismiss other possible forms of transmission, but she focused on frequency in search of an explanation for transmission. She said the Moderna and Pfizer 'vaccines' contained nano-lipid particles as a key component. This was a brand new technology never before used on humanity. 'They're using a nanotechnology which is pretty much little tiny computer bits … nanobots or hydrogel.' Inside the 'vaccines' was 'this sci-fi kind of substance' which suppressed immune checkpoints to get into the cell. I referred to this earlier as the 'Trojan horse' technique that tricks the cell into opening a gateway for the self-replicating synthetic material and while the immune system is artificially suppressed the body has no defences. Madej said the substance served many purposes including an on-demand ability to 'deliver the payload' and using the nano 'computer bits' as biosensors in the body. 'It actually has the ability to accumulate data from your body, like your breathing, your respiration, thoughts, emotions, all kinds of things.'

She said the technology obviously has the ability to operate through Wi-Fi and transmit and receive energy, messages, frequencies or impulses. 'Just imagine you're getting this new substance in you and it can react to things all around you, the 5G, your smart device, your phones.' We had something completely foreign in the human body that had never been launched large scale at a time when we were seeing 5G going into schools and hospitals (plus the Musk satellites) and she believed the 'vaccine' transmission had something to do with this: '… if these people have this inside of them … it can act like an antenna and actually transmit it outwardly as well.' The synthetic substance produced its own voltage and so it could have that kind of effect. This fits with my own contention that the nano receiver-transmitters are designed to connect people to the

Smart Grid and break the receiver-transmitter connection to expanded consciousness. That would explain the French energy healer's experience of the disconnection of body from 'soul' with those who have had the 'vaccine'. The nanobots, self-replicating inside the body, would also transmit the synthetic frequency which could be picked up through close interaction by those who have not been 'vaccinated'. Madej speculated that perhaps it was 5G and increased levels of other radiation that was causing the symptoms directly although interestingly she said that non-'vaccinated' patients had shown improvement when they were away from the 'vaccinated' person they had interacted with. It must be remembered that you can control frequency and energy with your mind and you can consciously create energetic barriers or bubbles with the mind to stop damaging frequencies from penetrating your field. American paediatrician Dr Larry Palevsky said the 'vaccine' was not a 'vaccine' and was never designed to protect from a 'viral' infection. He called it 'a massive, brilliant propaganda of genocide' because they didn't have to inject everyone to get the result they wanted. He said the content of the jabs was able to infuse any material into the brain, heart, lungs, kidneys, liver, sperm and female productive system. 'This is genocide; this is a weapon of mass destruction.' At the same time American colleges were banning students from attending if they didn't have this life-changing and potentially life-ending 'vaccine'. Class action lawsuits must follow when the consequences of this college fascism come to light. As the book was going to press came reports about fertility effects on sperm in 'vaccinated' men which would absolutely fit with what I have been saying and hospitals continued to fill with 'vaccine' reactions. Another question is what about transmission via blood transfusions? The NHS has extended blood donation restrictions from seven days after a 'Covid vaccination' to 28 days after even a sore arm reaction.

I said in the spring of 2020 that the then touted 'Covid vaccine' would be ongoing each year like the flu jab. A year later Pfizer CEO, the appalling Albert Bourla, said people would 'likely' need a 'booster dose' of the 'vaccine' within 12 months of getting 'fully

vaccinated' and then a yearly shot. 'Variants will play a key role', he said confirming the point. Johnson & Johnson CEO Alex Gorsky also took time out from his 'vaccine' disaster to say that people may need to be vaccinated against 'Covid-19' each year. UK Health Secretary, the psychopath Matt Hancock, said additional 'boosters' would be available in the autumn of 2021. This is the trap of the 'vaccine passport'. The public will have to accept every last 'vaccine' they introduce, including for the fake 'variants', or it would cease to be valid. The only other way in some cases would be continuous testing with a test not testing for the 'virus' and what is on the swabs constantly pushed up your noise towards the brain every time?

'Vaccines' changing behaviour

I mentioned in the body of the book how I believed we would see gathering behaviour changes in the 'vaccinated' and I am already hearing such comments from the non-'vaccinated' describing behaviour changes in friends, loved ones and work colleagues. This will only increase as the self-replicating synthetic material and nanoparticles expand in body and brain. An article in the *Guardian* in 2016 detailed research at the University of Virginia in Charlottesville which developed a new method for controlling brain circuits associated with complex animal behaviour. The method, dubbed 'magnetogenetics', involves genetically-engineering a protein called ferritin, which stores and releases iron, to create a magnetised substance – 'Magneto' – that can activate specific groups of nerve cells from a distance. This is claimed to be an advance on other methods of brain activity manipulation known as optogenetics and chemogenetics (the Cult has been developing methods of brain control for a long time). The ferritin technique is said to be non-invasive and able to activate neurons 'rapidly and reversibly'. In other words, human thought and perception. The article said that earlier studies revealed how nerve cell proteins 'activated by heat and mechanical pressure can be genetically engineered so that they become sensitive to radio waves and magnetic fields, by attaching them to an iron-storing protein called ferritin, or to inorganic

paramagnetic particles'. Sensitive to radio waves and magnetic fields? You mean like 5G, 6G and 7G? This is the human-AI Smart Grid hive mind we are talking about. The *Guardian* article said:

> … the researchers injected Magneto into the striatum of freely behaving mice, a deep brain structure containing dopamine-producing neurons that are involved in reward and motivation, and then placed the animals into an apparatus split into magnetised and non-magnetised sections.
>
> Mice expressing Magneto spent far more time in the magnetised areas than mice that did not, because activation of the protein caused the striatal neurons expressing it to release dopamine, so that the mice found being in those areas rewarding. This shows that Magneto can remotely control the firing of neurons deep within the brain, and also control complex behaviours.

Make no mistake this basic methodology will be part of the 'Covid vaccine' cocktail and using magnetics to change brain function through electromagnetic field frequency activation. The Pentagon is developing a 'Covid vaccine' using ferritin. Magnetics would explain changes in behaviour and why videos are appearing across the Internet as I write showing how magnets stick to the skin at the point of the 'vaccine' shot. Once people take these 'vaccines' anything becomes possible in terms of brain function and illness which will be blamed on 'Covid-19' and 'variants'. Magnetic field manipulation would further explain why the non-'vaccinated' are reporting the same symptoms as the 'vaccinated' they interact with and why those symptoms are reported to decrease when not in their company. Interestingly 'Magneto', a 'mutant', is a character in the Marvel Comic *X-Men* stories with the ability to manipulate magnetic fields and he believes that mutants should fight back against their human oppressors by any means necessary. The character was born Erik Lehnsherr to a Jewish family in Germany.

Cult-controlled courts

The European Court of Human Rights opened the door for mandatory 'Covid-19 vaccines' across the continent when it ruled in a Czech Republic dispute over childhood immunisation that legally

enforced vaccination could be 'necessary in a democratic society'. The 17 judges decided that compulsory vaccinations did not breach human rights law. On the face of it the judgement was so inverted you gasp for air. If not having a vaccine infused into your body is not a human right then what is? Ah, but they said human rights law which has been specifically written to delete all human rights at the behest of the state (the Cult). Article 8 of the European Convention on Human Rights relates to the right to a private life. The crucial word here is *'except'*:

> There shall be no interference by a public authority with the exercise of this right EXCEPT such as is in accordance with the law and is necessary in a democratic society in the interests of national security, public safety or the economic wellbeing of the country, for the prevention of disorder or crime, for the protection of health or morals, or for the protection of the rights and freedoms of others [My emphasis].

No interference *except* in accordance with the law means there *are* no 'human rights' *except* what EU governments decide you can have at their behest. 'As is necessary in a democratic society' explains that reference in the judgement and 'in the interests of national security, public safety or the economic well-being of the country, for the prevention of disorder or crime, for the protection of health or morals, or for the protection of the rights and freedoms of others' gives the EU a coach and horses to ride through 'human rights' and scatter them in all directions. The judiciary is not a check and balance on government extremism; it is a vehicle to enforce it. This judgement was almost laughably predictable when the last thing the Cult wanted was a decision that went against mandatory vaccination. Judges rule over and over again to benefit the system of which they are a part. Vaccination disputes that come before them are invariably delivered in favour of doctors and authorities representing the view of the state which owns the judiciary. Oh, yes, and we have even had calls to stop putting 'Covid-19' on death certificates within 28 days of a 'positive test' because it is claimed the practice makes the 'vaccine' appear not to work. They are laughing at you.

The scale of madness, inhumanity and things to come was highlighted when those not 'vaccinated' for 'Covid' were refused evacuation from the Caribbean island of St Vincent during massive volcanic eruptions. Cruise ships taking residents to the safety of another island allowed only the 'vaccinated' to board and the rest were left to their fate. Even in life and death situations like this we see 'Covid' stripping people of their most basic human instincts and the insanity is even more extreme when you think that fake 'vaccine'-makers are not even claiming their body-manipulating concoctions stop 'infection' and 'transmission' of a 'virus' that doesn't exist. St Vincent Prime Minister Ralph Gonsalves said: 'The chief medical officer will be identifying the persons already vaccinated so that we can get them on the ship.' Note again the power of the chief medical officer who, like Whitty in the UK, will be answering to the World Health Organization. This is the Cult network structure that has overridden politicians who 'follow the science' which means doing what WHO-controlled 'medical officers' and 'science advisers' tell them. Gonsalves even said that residents who were 'vaccinated' after the order so they could board the ships would still be refused entry due to possible side effects such as 'wooziness in the head'. The good news is that if they were woozy enough in the head they could qualify to be prime minister of St Vincent.

Microchipping freedom

The European judgement will be used at some point to justify moves to enforce the 'Covid' DNA-manipulating procedure. Sandra Ro, CEO of the Global Blockchain Business Council, told a World Economic Forum event that she hoped 'vaccine passports' would help to 'drive forced consent and standardisation' of global digital identity schemes: 'I'm hoping with the desire and global demand for some sort of vaccine passport – so that people can get travelling and working again – [it] will drive forced consent, standardisation, and frankly, cooperation across the world.' The lady is either not very bright, or thoroughly mendacious, to use the term 'forced consent'.

You do not 'consent' if you are forced – you *submit*. She was describing what the plan has been all along and that's to enforce a digital identity on every human without which they could not function. 'Vaccine passports' are opening the door and are far from the end goal. A digital identity would allow you to be tracked in everything you do in cyberspace and this is the same technique used by Cult-owned China to enforce its social credit system of total control. The ultimate 'passport' is planned to be a microchip as my books have warned for nearly 30 years. Those nice people at the Pentagon working for the Cult-controlled Defense Advanced Research Projects Agency (DARPA) claimed in April, 2021, they have developed a microchip inserted under the skin to detect 'asymptomatic Covid-19 infection' before it becomes an outbreak and a 'revolutionary filter' that can remove the 'virus' from the blood when attached to a dialysis machine. The only problems with this are that the 'virus' does not exist and people transmitting the 'virus' with no symptoms is brain-numbing bullshit. This is, of course, not a ruse to get people to be microchipped for very different reasons. DARPA also said it was producing a one-stop 'vaccine' for the 'virus' and all 'variants'. One of the most sinister organisations on Planet Earth is doing this? Better have it then. These people are insane because Wetiko that possesses them is insane.

Researchers from the Salk Institute in California announced they have created an embryo that is part human and part monkey. My books going back to the 1990s have exposed experiments in top secret underground facilities in the United States where humans are being crossed with animal and non-human 'extraterrestrial' species. They are now easing that long-developed capability into the public arena and there is much more to come given we are dealing with psychiatric basket cases. Talking of which – Elon Musk's scientists at Neuralink trained a monkey to play Pong and other puzzles on a computer screen using a joystick and when the monkey made the correct move a metal tube squirted banana smoothie into his mouth which is the basic technique for training humans into unquestioning compliance. Two Neuralink chips were in the monkey's skull and

more than 2,000 wires 'fanned out' into its brain. Eventually the monkey played a video game purely with its brain waves. Psychopathic narcissist Musk said the 'breakthrough' was a step towards putting Neuralink chips into human skulls and merging minds with artificial intelligence. *Exactly*. This man is so dark and Cult to his DNA.

World Economic Fascism (WEF)

The World Economic Forum is telling you the plan by the statements made at its many and various events. Cult-owned fascist YouTube CEO Susan Wojcicki spoke at the 2021 WEF Global Technology Governance Summit (see the name) in which 40 governments and 150 companies met to ensure 'the responsible design and deployment of emerging technologies'. Orwellian translation: 'Ensuring the design and deployment of long-planned technologies will advance the Cult agenda for control and censorship.' Freedom-destroyer and Nuremberg-bound Wojcicki expressed support for tech platforms like hers to censor content that is 'technically legal but could be harmful'. Who decides what is 'harmful'? She does and they do. 'Harmful' will be whatever the Cult doesn't want people to see and we have legislation proposed by the UK government that would censor content on the basis of 'harm' no matter if the information is fair, legal and provably true. Make that *especially* if it is fair, legal and provably true. Wojcicki called for a global coalition to be formed to enforce content moderation standards through automated censorship. This is a woman and mega-censor so self-deluded that she shamelessly accepted a 'free expression' award – *Wojcicki* – in an event sponsored by her own *YouTube*. They have no shame and no self-awareness.

You know that 'Covid' is a scam and Wojcicki a Cult operative when YouTube is censoring medical and scientific opinion purely on the grounds of whether it supports or opposes the Cult 'Covid' narrative. Florida governor Ron DeSantis compiled an expert panel with four professors of medicine from Harvard, Oxford, and Stanford Universities who spoke against forcing children and

vaccinated people to wear masks. They also said there was no proof that lockdowns reduced spread or death rates of 'Covid-19'. Cult-gofer Wojcicki and her YouTube deleted the panel video 'because it included content that contradicts the consensus of local and global health authorities regarding the efficacy of masks to prevent the spread of Covid-19'. This 'consensus' refers to what the Cult tells the World Health Organization to say and the WHO tells 'local health authorities' to do. Wojcicki knows this, of course. The panellists pointed out that censorship of scientific debate was responsible for deaths from many causes, but Wojcicki couldn't care less. She would not dare go against what she is told and as a disgrace to humanity she wouldn't want to anyway. The UK government is seeking to pass a fascist 'Online Safety Bill' to specifically target with massive fines and other means non-censored video and social media platforms to make them censor 'lawful but harmful' content like the Cult-owned Facebook, Twitter, Google and YouTube. What is 'lawful but harmful' would be decided by the fascist Blair-created Ofcom.

Another WEF obsession is a cyber-attack on the financial system and this is clearly what the Cult has planned to take down the bank accounts of everyone – except theirs. Those that think they have enough money for the Cult agenda not to matter to them have got a big lesson coming if they continue to ignore what is staring them in the face. The World Economic Forum, funded by Gates and fronted by Klaus Schwab, announced it would be running a 'simulation' with the Russian government and global banks of just such an attack called Cyber Polygon 2021. What they simulate – as with the 'Covid' Event 201 – they plan to instigate. The WEF is involved in a project with the Cult-owned Carnegie Endowment for International Peace called the WEF-Carnegie Cyber Policy Initiative which seeks to merge Wall Street banks, 'regulators' (I love it) and intelligence agencies to 'prevent' (arrange and allow) a cyber-attack that would bring down the global financial system as long planned by those that control the WEF and the Carnegie operation. The Carnegie Endowment for International Peace sent an instruction to First World

War US President Woodrow Wilson not to let the war end before society had been irreversibly transformed.

The Wuhan lab diversion

As I close, the Cult-controlled authorities and lapdog media are systematically pushing 'the virus was released from the Wuhan lab' narrative. There are two versions – it happened by accident and it happened on purpose. Both are nonsense. The perceived existence of the never-shown-to-exist 'virus' is vital to sell the impression that there is actually an infective agent to deal with and to allow the endless potential for terrifying the population with 'variants' of a 'virus' that does not exist. The authorities at the time of writing are going with the 'by accident' while the alternative media is promoting the 'on purpose'. Cable news host Tucker Carlson who has questioned aspects of lockdown and 'vaccine' compulsion has bought the Wuhan lab story. 'Everyone now agrees' he said. Well, I don't and many others don't and the question is *why* does the system and its media suddenly 'agree'? When the media moves as one unit with a narrative it is always a lie – witness the hour by hour mendacity of the 'Covid' era. Why would this Cult-owned combination which has unleashed lies like machine gun fire suddenly 'agree' to tell the truth??

Much of the alternative media is buying the lie because it fits the conspiracy narrative, but it's the *wrong* conspiracy. The real conspiracy is that *there is no virus* and that is what the Cult is desperate to hide. The idea that the 'virus' was released by accident is ludicrous when the whole 'Covid' hoax was clearly long-planned and waiting to be played out as it was so fast in accordance with the Rockefeller document and Event 201. So they prepared everything in detail over decades and then sat around strumming their fingers waiting for an 'accidental' release from a bio-lab? *What??* It's crazy. Then there's the 'on purpose' claim. You want to circulate a 'deadly virus' and hide the fact that you've done so and you release it down the street from the highest-level bio-lab in China? I repeat – *What??*

You would release it far from that lab to stop any association being made. But, no, we'll do it in a place where the connection was certain to be made. Why would you need to scam 'cases' and 'deaths' and pay hospitals to diagnose 'Covid-19' if you had a real 'virus'? What are sections of the alternative media doing believing this crap? Where were all the mass deaths in Wuhan from a 'deadly pathogen' when the recovery to normal life after the initial propaganda was dramatic in speed? Why isn't the 'deadly pathogen' now circulating all over China with bodies in the street? Once again we have the technique of tell them what they want to hear and they will likely believe it. The alternative media has its 'conspiracy' and with Carlson it fits with his 'China is the danger' narrative over years. China *is* a danger as a global Cult operations centre, but not for this reason. The Wuhan lab story also has the potential to instigate conflict with China when at some stage the plan is to trigger a Problem-Reaction-Solution confrontation with the West. Question everything – *everything* – and especially when the media agrees on a common party line.

Third wave ... fourth wave ... fifth wave ...

As the book went into production the world was being set up for more lockdowns and a 'third wave' supported by invented 'variants' that were increasing all the time and will continue to do so in public statements and computer programs, but not in reality. India became the new Italy in the 'Covid' propaganda campaign and we were told to be frightened of the new 'Indian strain'. Somehow I couldn't find it within myself to do so. A document produced for the UK government entitled 'Summary of further modelling of easing of restrictions – Roadmap Step 2' declared that a third wave was inevitable (of course when it's in the script) and it would be the fault of children and those who refuse the health-destroying fake 'Covid vaccine'. One of the computer models involved came from the Cult-owned *Imperial College* and the other from Warwick University which I wouldn't trust to tell me the date in a calendar factory. The document states that both models presumed extremely high uptake

of the 'Covid vaccines' and didn't allow for 'variants'. The document states: 'The resurgence is a result of some people (mostly children) being ineligible for vaccination; others choosing not to receive the vaccine; and others being vaccinated but not perfectly protected.' The mendacity takes the breath away. Okay, blame those with a brain who won't take the DNA-modifying shots and put more pressure on children to have it as 'trials' were underway involving children as young as six months with parents who give insanity a bad name. Massive pressure is being put on the young to have the fake 'vaccine' and child age consent limits have been systematically lowered around the world to stop parents intervening. Most extraordinary about the document was its claim that the 'third wave' would be driven by 'the resurgence in both hospitalisations and deaths … dominated by *those that have received two doses of the vaccine*, comprising around 60-70% of the wave respectively'. The predicted peak of the 'third wave' suggested 300 deaths per day with 250 of them *fully 'vaccinated' people*. How many more lies do acquiescers need to be told before they see the obvious? Those who took the jab to 'protect themselves' are projected to be those who mostly get sick and die? So what's in the 'vaccine'? The document went on:

> It is possible that a summer of low prevalence could be followed by substantial increases in incidence over the following autumn and winter. Low prevalence in late summer should not be taken as an indication that SARS-CoV-2 has retreated or that the population has high enough levels of immunity to prevent another wave.

They are telling you the script and while many British people believed 'Covid' restrictions would end in the summer of 2021 the government was preparing for them to be ongoing. Authorities were awarding contracts for 'Covid marshals' to police the restrictions with contracts starting in July, 2021, and going through to January 31st, 2022, and the government was advertising for 'Media Buying Services' to secure media propaganda slots worth a potential £320 million for 'Covid-19 campaigns' with a contract not ending until March, 2022. The recipient – via a list of other front companies – was reported to be American media marketing giant Omnicom Group

Inc. While money is no object for 'Covid' the UK waiting list for all other treatment – including life-threatening conditions – passed 4.5 million. Meantime the Cult is seeking to control all official 'inquiries' to block revelations about what has really been happening and why. It must not be allowed to – we need Nuremberg jury trials in every country. The cover-up doesn't get more obvious than appointing ultra-Zionist professor Philip Zelikow to oversee two dozen US virologists, public health officials, clinicians, former government officials and four American 'charitable foundations' to 'learn the lessons' of the 'Covid' debacle. The personnel will be those that created and perpetuated the 'Covid' lies while Zelikow is the former executive director of the 9/11 Commission who ensured that the truth about those attacks never came out and produced a report that must be among the most mendacious and manipulative documents ever written – see *The Trigger* for the detailed exposure of the almost unimaginable 9/11 story in which Sabbatians can be found at every level.

Passive no more

People are increasingly challenging the authorities with amazing numbers of people taking to the streets in London well beyond the ability of the Face-Nappies to stop them. Instead the Nappies choose situations away from the mass crowds to target, intimidate, and seek to promote the impression of 'violent protestors'. One such incident happened in London's Hyde Park. Hundreds of thousands walking through the streets in protest against 'Covid' fascism were ignored by the Cult-owned BBC and most of the rest of the mainstream media, but they delighted in reporting how police were injured in 'clashes with protestors'. The truth was that a group of people gathered in Hyde Park at the end of one march when most had gone home and they were peacefully having a good time with music and chat. Face-Nappies who couldn't deal with the full-march crowd then waded in with their batons and got more than they bargained for. Instead of just standing for this criminal brutality the crowd used their numerical superiority to push the Face-Nappies out of the

park. Eventually the Nappies turned and ran. Unfortunately two or three idiots in the crowd threw drink cans striking two officers which gave the media and the government the image they wanted to discredit the 99.9999 percent who were peaceful. The idiots walked straight into the trap and we must always be aware of potential agent provocateurs used by the authorities to discredit their targets.

This response from the crowd – the can people apart – must be a turning point when the public no longer stand by while the innocent are arrested and brutally attacked by the Face-Nappies. That doesn't mean to be violent, that's the last thing we need. We'll leave the violence to the Face-Nappies and government. But it does mean that when the Face-Nappies use violence against peaceful people the numerical superiority is employed to stop them and make citizen's arrests or Common Law arrests for a breach of the peace. The time for being passive in the face of fascism is over.

We are the many, they are the few, and we need to make that count before there is no freedom left and our children and grandchildren face an ongoing fascist nightmare.

COME ON PEOPLE – IT'S TIME.

One final thought …

The power of love
A force from above
Cleaning my soul
Flame on burn desire
Love with tongues of fire
Purge the soul
Make love your goal

I'll protect you from the hooded claw
Keep the vampires from your door
When the chips are down I'll be around
With my undying, death-defying
Love for you

Envy will hurt itself
Let yourself be beautiful
Sparkling love, flowers
And pearls and pretty girls
Love is like an energy
Rushin' rushin' inside of me

This time we go sublime
Lovers entwine, divine, divine,
Love is danger, love is pleasure
Love is pure – the only treasure

I'm so in love with you
Purge the soul
Make love your goal

The power of love
A force from above
Cleaning my soul
The power of love
A force from above
A sky-scraping dove

Flame on burn desire
Love with tongues of fire
Purge the soul
Make love your goal

Frankie Goes To Hollywood

APPENDIX

Cowan-Kaufman-Morell Statement on Virus Isolation (SOVI)

Isolation: The action of isolating; the fact or condition of being isolated or standing alone; separation from other things or persons; solitariness
Oxford English Dictionary

The controversy over whether the SARS-CoV-2 virus has ever been isolated or purified continues. However, using the above definition, common sense, the laws of logic and the dictates of science, any unbiased person must come to the conclusion that the SARS-CoV-2 virus has never been isolated or purified. As a result, no confirmation of the virus' existence can be found. The logical, common sense, and scientific consequences of this fact are:

- the structure and composition of something not shown to exist can't be known, including the presence, structure, and function of any hypothetical spike or other proteins;
- the genetic sequence of something that has never been found can't be known;
- "variants" of something that hasn't been shown to exist can't be known;
- it's impossible to demonstrate that SARS-CoV-2 causes a disease called Covid-19.

In as concise terms as possible, here's the proper way to isolate, characterize and demonstrate a new virus. First, one takes samples (blood, sputum, secretions) from many people (e.g. 500) with symptoms which are unique and specific enough to characterize an illness. Without mixing these samples with ANY tissue or products that also contain genetic material, the virologist macerates, filters and ultracentrifuges i.e. *purifies* the specimen. This common virology technique, done for decades to isolate bacteriophages[1] and so-called giant viruses in every virology lab, then allows the virologist to demonstrate with electron microscopy thousands of identically sized and shaped particles. These particles are the isolated and purified virus.

These identical particles are then checked for uniformity by physical and/or microscopic techniques. Once the purity is determined, the particles may be further characterized. This would include examining the structure, morphology, and chemical composition of the particles. Next, their genetic makeup is characterized by extracting the genetic material directly from the purified particles and using genetic-sequencing techniques, such as Sanger sequencing, that have also been around for decades. Then one does an analysis to confirm that these uniform particles are exogenous (outside) in origin as a virus is conceptualized to be, and not the normal breakdown products of dead and dying tissues.[2] (As of May 2020, we know that virologists have no way to determine whether the particles they're seeing are viruses or just normal breakdown products of dead and dying tissues.)[3]

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1 Isolation, characterization and analysis of bacteriophages from the haloalkaline lake Elmenteita, KenyaJuliah Khayeli Akhwale et al, PLOS One, Published: April 25, 2019. https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0215734 – accessed 2/15/21

2 "Extracellular Vesicles Derived From Apoptotic Cells: An Essential Link Between Death and Regeneration," Maojiao Li1 et al, Frontiers in Cell and Developmental Biology, 2020 October 2. https://www.frontiersin.org/articles/10.3389/fcell.2020.573511/full – accessed 2/15/21

3 "The Role of Extraellular Vesicles as Allies of HIV, HCV and SARS Viruses," Flavia Giannessi, et al, Viruses, 2020 May

If we have come this far then we have fully isolated, characterized, and genetically sequenced an exogenous virus particle. However, we still have to show it is causally related to a disease. This is carried out by exposing a group of healthy subjects (animals are usually used) to this isolated, purified virus in the manner in which the disease is thought to be transmitted. If the animals get sick with the same disease, as confirmed by clinical and autopsy findings, one has now shown that the virus actually causes a disease. This demonstrates infectivity and transmission of an infectious agent.

None of these steps has even been attempted with the SARS-CoV-2 virus, nor have all these steps been successfully performed for any so-called pathogenic virus. Our research indicates that a single study showing these steps does not exist in the medical literature.

Instead, since 1954, virologists have taken unpurified samples from a relatively few people, often less than ten, with a similar disease. They then minimally process this sample and inoculate this unpurified sample onto tissue culture containing usually four to six other types of material – all of which contain identical genetic material as to what is called a "virus." The tissue culture is starved and poisoned and naturally disintegrates into many types of particles, some of which contain genetic material. Against all common sense, logic, use of the English language and scientific integrity, this process is called "virus isolation." This brew containing fragments of genetic material from many sources is then subjected to genetic analysis, which then creates in a computer-simulation process the alleged sequence of the alleged virus, a so called in silico genome. At no time is an actual virus confirmed by electron microscopy. At no time is a genome extracted and sequenced from an actual virus. This is scientific fraud.

The observation that the unpurified specimen — inoculated onto tissue culture along with toxic antibiotics, bovine fetal tissue, amniotic fluid and other tissues — destroys the kidney tissue onto which it is inoculated is given as evidence of the virus' existence and pathogenicity. This is scientific fraud.

From now on, when anyone gives you a paper that suggests the SARS-CoV-2 virus has been isolated, please check the methods sections. If the researchers used Vero cells or any other culture method, you know that their process was not isolation. You will hear the following excuses for why actual isolation isn't done:

1. There were not enough virus particles found in samples from patients to analyze.

2. Viruses are intracellular parasites; they can't be found outside the cell in this manner.

If No. 1 is correct, and we can't find the virus in the sputum of sick people, then on what evidence do we think the virus is dangerous or even lethal? If No. 2 is correct, then how is the virus spread from person to person? We are told it emerges from the cell to infect others. Then why isn't it possible to find it?

Finally, questioning these virology techniques and conclusions is not some distraction or divisive issue. Shining the light on this truth is essential to stop this terrible fraud that humanity is confronting. For, as we now know, if the virus has never been isolated, sequenced or shown to cause illness, if the virus is imaginary, then why are we wearing masks, social distancing and putting the whole world into prison?

Finally, if pathogenic viruses don't exist, then what is going into those injectable devices erroneously called "vaccines," and what is their purpose? This scientific question is the most urgent and relevant one of our time.

We are correct. The SARS-CoV2 virus does not exist.

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Dr. Andrew Kaufman, MD

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Across the range of subjects and positions of influence occupied mainly by people who don't strive to make things better it's the Media that I have always found the most frustrating and fascinating. Mainly because if the Media did their Jobs properly then so much of the negative things happening in the World simply would not be able to happen, because they would be exposed within a heartbeat.
Free Press and the Opportunities that the internet could have given would mean that the Media are able to expose things like never before and hold people to account for their actions. As we all know there are 'Untouchables' that walk among us, people the Media simply won't touch, expose or investigate and that leads to the dark underworlds that infest the establishment the World over.
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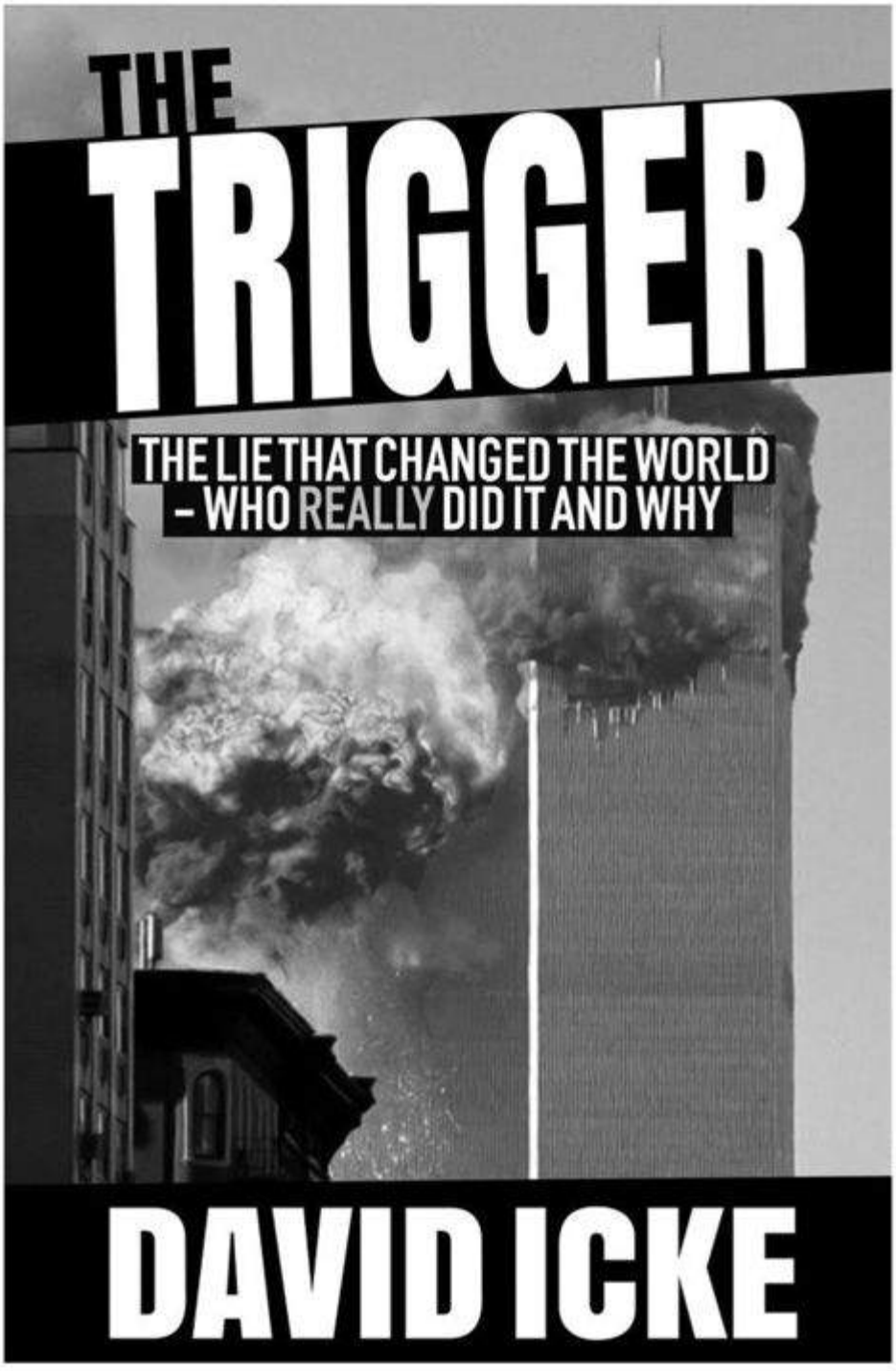
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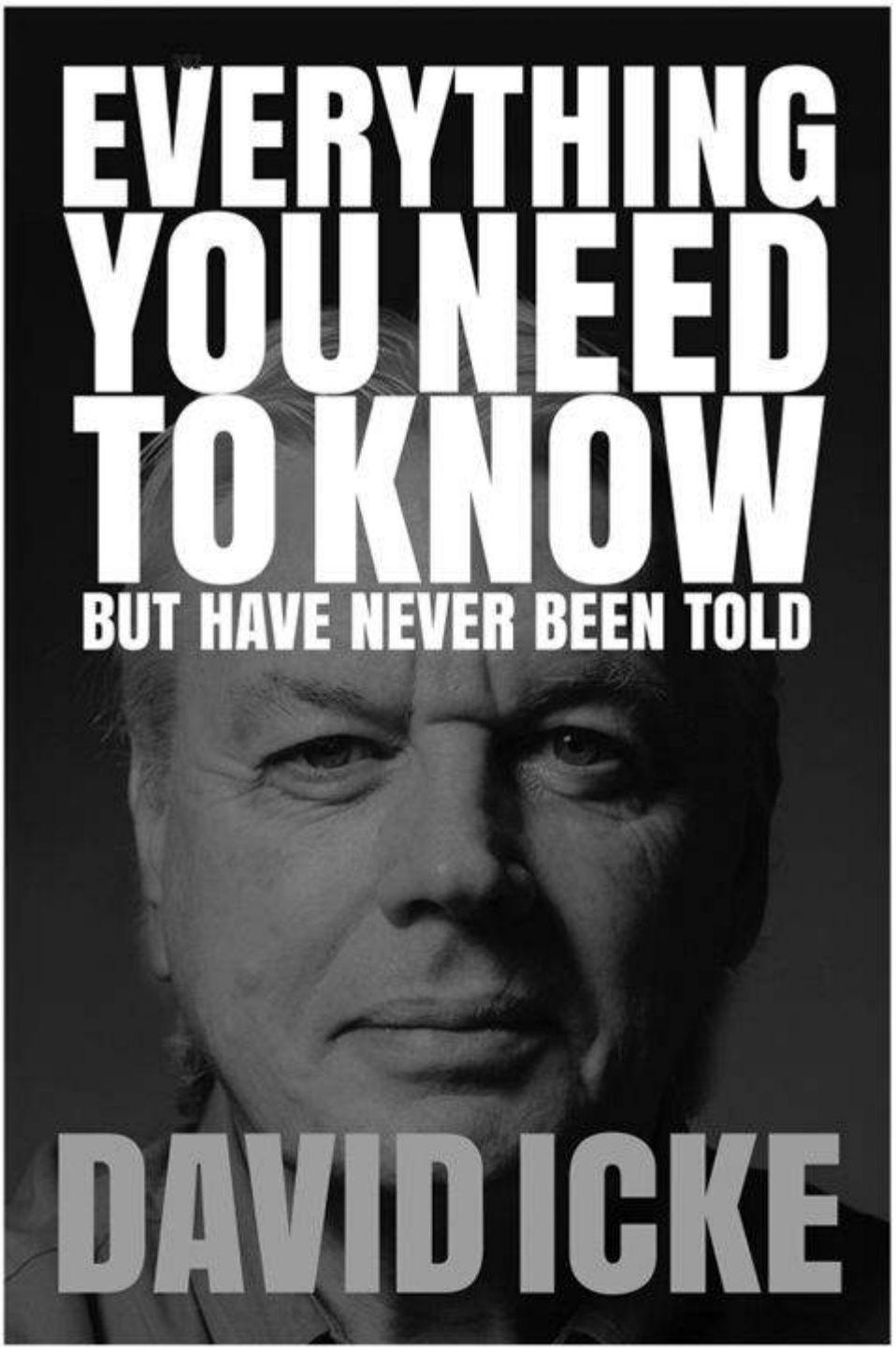
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