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Integrative Human Biochemistry

A Textbook for Medical Biochemistry

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This book is a tribute to the legacy of Leopoldo de Meis for his inspiration to younger generations. Thanks, Leopoldo.

Foreword: Leopoldo De Meis' Legacy—A Biochemistry Textbook with a Difference

This is a comprehensive and concise basic Biochemistry textbook for health science students. This readership is often overwhelmed by conventional textbooks, which cover many topics in great depth. Indeed, although this information is necessary for those aiming to become biochemists, it is excessively detailed for the interests of future nurses, physicians, and dentists. The authors—experienced teachers and researchers aware of the needs of health science students—have devised a book specifically for this community.

To this end, the book starts off with a description of the molecules of life and rapidly moves on to cover metabolism and related fields, such as the control of body weight. The book is therefore devoted to human metabolism. Given that its audience is health science students, only those topics considered of relevance for humans are presented. One of the hallmarks of current developments in the life sciences is the merge of classical disciplines. Consequently, the book encompasses pure biochemical information in the framework of related fields such as Physiology, Histology, and Pharmacology. The final chapters on the regulation of metabolism during physical activity and the control of body weight clearly reflect this multidisciplinary perspective.

The presentation of metabolism is organized around the concept of the generation and management of energy. Unlike most textbooks, here the synthesis of ATP is described first in a very detailed way, after which the metabolic pathways that feed ATP synthesis are addressed. This logical approach to presenting material was advocated by Leopoldo de Meis, one of the greatest Biochemistry teachers and educators of our time. In this regard, this book is a tribute to Leopoldo.

The structural aspects of macromolecules are consistently shown in the figures, and the fundamental notion that reactions are the result of molecular interactions is reiterated throughout the book. Given that in most university degrees Molecular Biology and Genetics are now taught in separate courses, the reader is provided with a description of nucleic acids, faithfully referred to as "Polymers of saccharide conjugates," in the chapter dealing with the families of biological molecules. However, the reader will not find information on DNA and RNA typical of conventional textbooks.

Another interesting feature of the book is the use of "boxes," which develop singular concepts in a more informal manner. This presentation technique is highly illustrative and reader-friendly. Furthermore, key experiments that have opened up new concepts are explained, thus helping students to appreciate that scientific knowledge derives from the work of researchers, some of which are depicted in caricatures. Finally, each chapter includes a set of up-to-date and well-chosen references, which will help those students wishing to delve further into specific fields.

In summary, this textbook provides a modern and integrative perspective of human Biochemistry and will be a faithful companion to health science students following curricula in which this discipline is addressed. Similarly, this textbook will be a most useful tool for the teaching community.

Barcelona, Spain

Joan Guinovart
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Preface

Traditional lecture classes in biological sciences are being challenged by modern forms of communication. Modern communication tends to be more visual and less interpretative in nature. In lectures, the didactics are changing vastly and rapidly; the deductive power of mathematics is complemented by the intuitive clarity of movie simulations, even if the first is fully embedded in the scientific method and the latter are mere artistic configurations of a faintly perceived reality. It is a general trend in modern societies that the most effective communication is more condensed and focused, contextualizes the information, and is disseminated across multiple media. Textbooks do not escape this reality. A modern scientific textbook to be effective should be a means of communication that needs to address specific issues of interest, place these issues in a broader interdisciplinary context, and make use of modern visualization tools that represent reality within the state of the art available in scientific research.

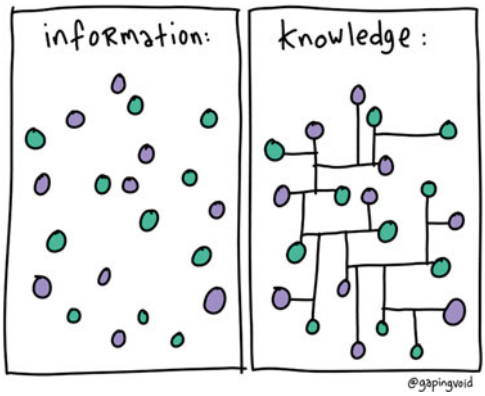
We have shaped this book based on many years of Biochemistry teaching and researching. We hope to stimulate other teachers to actively rethink biochemical education in health sciences and "contaminate" students with the passion for biochemical knowledge as an essential part of the indefinable but fascinating trick of nature we call life. "We're trying for something that's already found us," Jim Morrison would say.

Presentation of Book Structure

Our goal in this endeavor is not writing just another piece of literature in biochemistry. We aim at a different textbook. Biochemistry is defined as the study of the molecular processes occurring in living organisms, which means that it comprises the network of chemical and physical transformations that allow life to exist.

However, this intrinsic integrative nature of biochemistry may be lost if it is taught as lists of molecules' types and metabolic pathways. In this book, we intend to introduce the biochemistry world in an actual integrative way. For this, our option was to focus on human biochemistry, presenting the molecular mechanisms of

cellular processes in the context of human physiological situations, such as fasting, feeding, and physical exercise. We believe that this will provide to the reader not only information but knowledge (as very well represented in the cartoon from Hugh MacLeod's gapingvoid).



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The reader will find innovative approaches and deviations relative to the usual contents of classical textbooks. Part I deals with the importance of molecular-scale knowledge to reason about life, health, and disease (Chap. 1); the basic chemistry and physics of living systems (Chap. 2); and the systematization of biomolecules in chemical families, privileging molecular structure and dynamics instead of dealing with molecules as shapeless names (Chap. 3). Basic drug discovery concepts are presented to reinforce the importance of integrative biochemical reasoning. Drug discovery is a very important part of modern Medicinal Chemistry bridging biochemistry to Pharmacology and Biotechnology. Part I prepares the student for Part II, which is devoted to metabolisms. Part II starts with the fundamentals of regulation of series of reactions in which kinetic considerations are endowed with mathematical accuracy (Chap. 4), and, by extension, the key concepts in the regulation of metabolism (Chap. 5). To introduce energy metabolism, we first explore the mechanisms of ATP synthesis (Chap. 6) to create in the reader a need to know from where cellular energy comes from. The catabolism of major biomolecules follows naturally (Chap. 7). Metabolic responses to hyperglycemia (Chap. 8), hypoglycemia (Chap. 9), and physical activity (Chap. 10) are used to introduce and contextualize several metabolic pathways, and to illustrate the integrative interplay between different processes in different tissues. Finally, control of body weight and the modern metabolic diseases are explored (Chap. 11), placing biochemistry in a human health perspective, prone to be explored in later stages of health sciences students' training, when pathologies and clinical problems are addressed.

The option for the integrative view implied that sometimes complex topics have been reduced to their essence. This is the case of cholesterol synthesis, which is addressed but not described in detail, and the pentose–phosphate pathway, which

is presented in the context of fatty acid synthesis, although its other functions are summarized in a box. For the synthesis of purines and pyrimidines, the reader is referred to specialized literature. Vitamins are a heterogeneous group of molecules not directly related to their structure or reactivity; vitamins seen as a family of molecules is an anachronism and were not the theme of any section of the book. Also, the reader will not find in this book matters that are typically taught in Molecular Biology programs such as the replication, transcription, and translation of informative molecules.

It is also important to mention that biochemical nomenclature is a permanent challenge for the teacher and the student. The rich history and multidisciplinary nature of biochemistry have determined that nomenclature is not always clear or coherent. Coexistence of common and systematic names is frequent and different names have been consecrated by the use of different communities of biochemists. The most prominent example is the case of saccharides/sugars/carbohydrates. While all designations are common, carbohydrates is probably the one preferred by most professionals in different disciplines. Yet, this name relates to a profound chemical equivocation of "carbon hydrate": Many molecules of this family have a hydrogen:oxygen atom ratio of 2:1 as in water, which makes the empirical formula $C_m(H_2O)_n$. The illusion of an hydrate is obvious but has no chemical sense. Respecting the chemical accuracy we preferred the name saccharide in Part I, in which the chemical nature of biomolecules was presented and discussed, and reserved the name "carbohydrate" to discuss metabolic processes and dietary implications, for instance. The use of different names for different contexts and different implications is intrinsic to biochemistry.

Because biochemistry is made of biochemists and good ideas in addition to molecules, key historical experiments are used as case studies to ignite discussion and facilitate learning. Key historical experiments are excellent for classroom use, steering dynamic discussions between teachers and students. This is the perfect environment to teaching, learning, and showing that Biochemistry it is not only useful in shaping the future of humanity, it is also fascinating and appealing.

Urucureá, Brazil

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Contents

Part I
The Molecules of Life

Chapter 1
Introduction: Life Is Made of Molecules!

Studying molecules is the key to understanding life. A commonly accepted definition of life, known as the NASA (North American Space Agency) hypothesis, states that "Life is a self-sustained chemical system capable of undergoing Darwinian evolution" (Fig. 1.1). The link between molecules and life may be hard to explain, but it is simple to illustrate.

In this introduction we have selected three examples that are sufficient to show that knowledge on molecules is essential to reason about life itself, health, and disease:

1. Searching for the origin of life is a chemical "adventure" involving the molecules of primitive Earth and their reactivity.
2. Viruses are amazing molecular machines, too simple to be considered living beings for most researchers, but with a tremendous ability to interfere with the course of life, sometimes tragically.
3. The world of drug discovery and development consists of molecules being designed and synthesized and interacting with other molecules in silico, in vitro, and in vivo with the end goal of interfering with vital physiologic processes.

It is all about molecules. It is all about life.

1.1 Selected Illustrative Example #1: The Molecular Origin of Life

Nothing is better than trying to answer the question "what was the origin of life?" to realize that molecules are the key to life. Since the pioneering work of Aleksandr Oparin, the origin and evolution of life are elucidated based on the chemistry of molecules containing carbon. By introducing this concept, Oparin truly revolutionized the way science interprets life. Nowadays, there are two main hypotheses to explain the evolution of the complexity of molecular organization into what one today calls cells,

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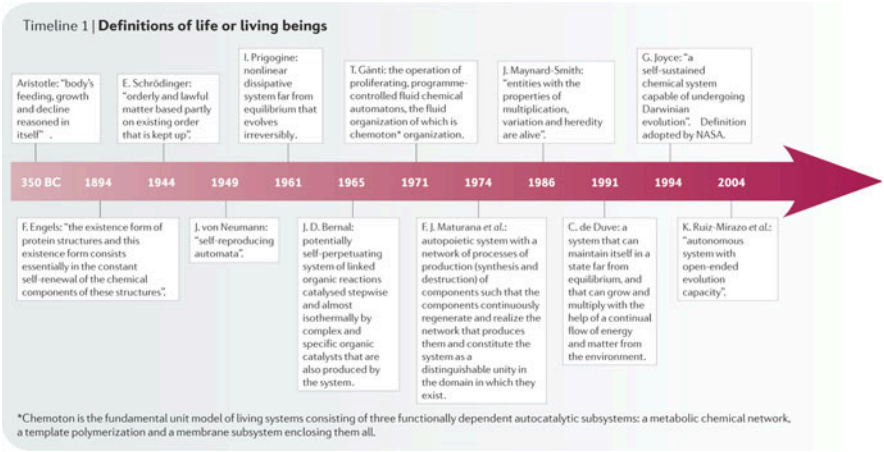
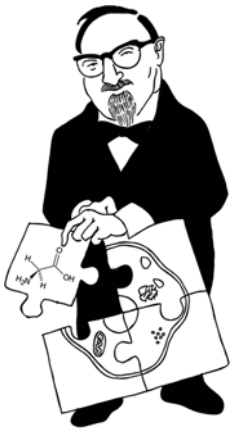


Fig. 1.1 Timeline for the definitions of life or living beings. Figure reproduced with permission from Moreva & López-Garcia, Nat. Rev. Microbiol. 7:306–311, 2009

the so-called "replicator" and "metabolism" hypotheses (Fig. 1.2). These hypotheses are based on two specific characteristics common to all living beings: Despite tremendous diversity among species, all life forms are organized in cells and all cells have a replicator polymer (DNA) and a membrane with restricted permeability (a "membrane" having lipids in its composition). Therefore, it is not surprising that the prevailing hypotheses to explain the origin of life are indeed models that elaborate on the appearance of the replicator polymer and compartmentalization. The replicator polymer is essential to transmit the molecular information inherited from the previous generation, and a membrane forming a compartment that separates the ancestral cell from its environment is essential to ensure that the molecules in this space can react among each other in a controlled and self-regulated way (a "proto-metabolism"), with minimal impact of fluctuations in environmental conditions. These two aspects are consensual among researchers who study the origin of life, but the details and chronological order of events that resulted in cells as we know today is far from being established.



Aleksander Oparin (1894 - 1980)

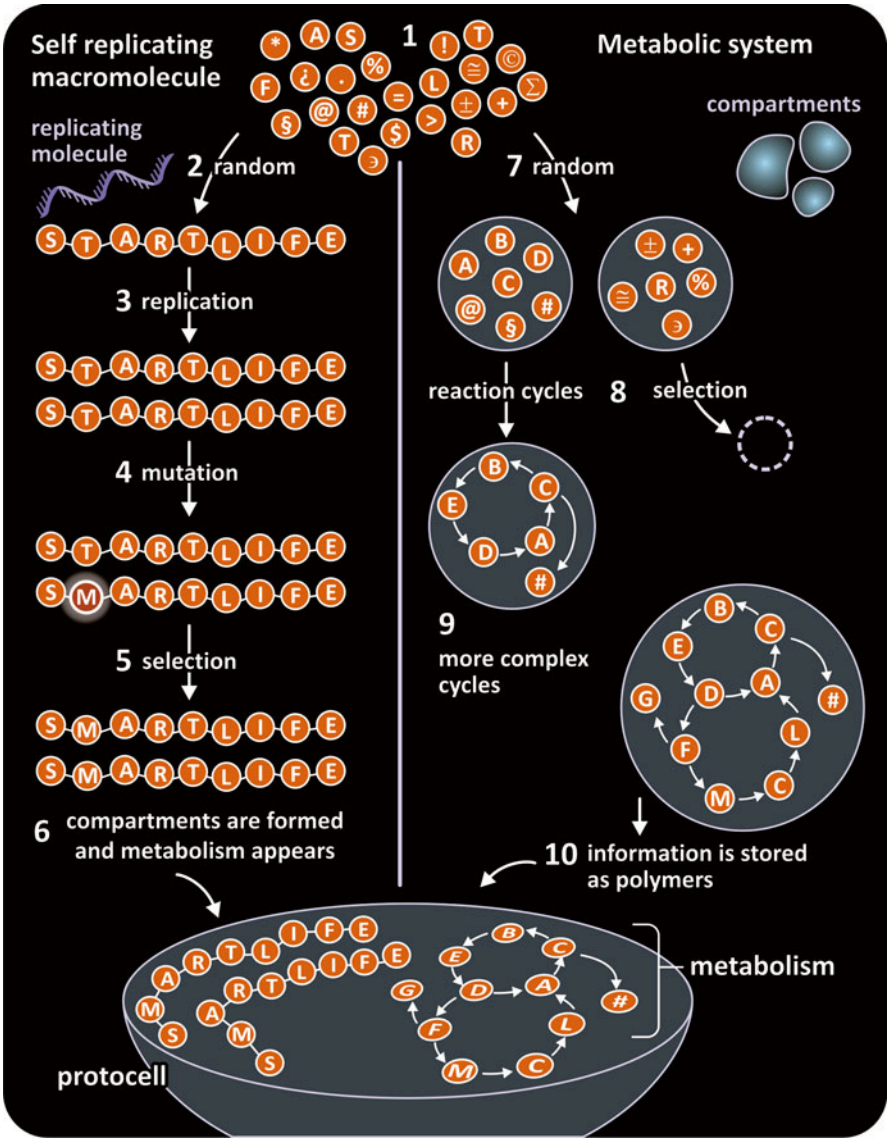


Fig. 1.2 Schematic representation of the replicator and metabolism hypotheses to describe the origin of life. Both models are molecular in nature and agree on the critical roles of a replicator molecule and compartmentalization but differ on the sequence of events. Figure reproduced with permission from Saphiro, Investigacion y Ciencia 371, 2007

1.1.1 The Replicator Hypothesis

According to the replicator hypothesis, life started with a molecule that was randomly formed but had the ability to replicate itself. It is an extremely unlikely event, hardly possible to occur twice in the universe, but one may work on the hypothesis that it has occurred. The obvious first "choice" for a replicator molecule is DNA, the ubiquitous replicator nowadays, but this leaves us in a paradox: proteins are needed to generate DNA and DNA is needed to generate proteins. What came first then? It may be that DNA had an ancestor with self-catalytic activity. RNA is eligible as such ancestor. RNA is not as chemically stable as DNA, so it is not so well suited to store information for long periods of time, but it can still constitute genetic material (many viruses, such as HIV or dengue virus, have RNA genomes). Concomitantly, RNA conformation dynamics enables catalytic activity, a perfect combination for the original replicator. The introduction of mutations and other errors in replication, in addition to other mechanisms, led to evolution and selection. How this process was coupled to the appearance of a metabolism is hard to conceive, but confinement of replicators into separated environments may have favored some chemical reactions that evolved in their restrained space to cause metabolism (Fig. 1.2).

1.1.2 The Metabolism Hypothesis

An alternative model skips the Achilles heel of the replicator hypothesis. Here, the origin of life in not dependent on a starting event that is nearly impossible to succeed. The key process was the confinement of small molecules that reacted among them. In some cases, organized ensembles of molecules may have formed stable reaction cycles that became increasingly complex. The result was the creation of metabolism and complex polymer molecules, including replicators (Fig. 1.2). Naturally, the boundaries of the confined environment where these reactions took place had to allow for selective permeation of matter. Permeation allowed growth and replication.

Nowadays, virtually all cell membranes are formed non-exclusively but mostly by lipids. Modern lipids are synthetic products of metabolism. So what could have been the predecessors of lipid membranes in the confinement of the first "protometabolic" reactions? Orevices in the outer layers of rocks are a possibility. Phospholipids or other surfactant molecules may have started as coatings that, due to their intrinsic dynamics and capability to expand into a film and seal, may have evolved into membranes. Lipids and other surfactants have the ability to form three-dimensional structures other than lamellae that may have contributed to confine chemical systems (Fig. 1.3).

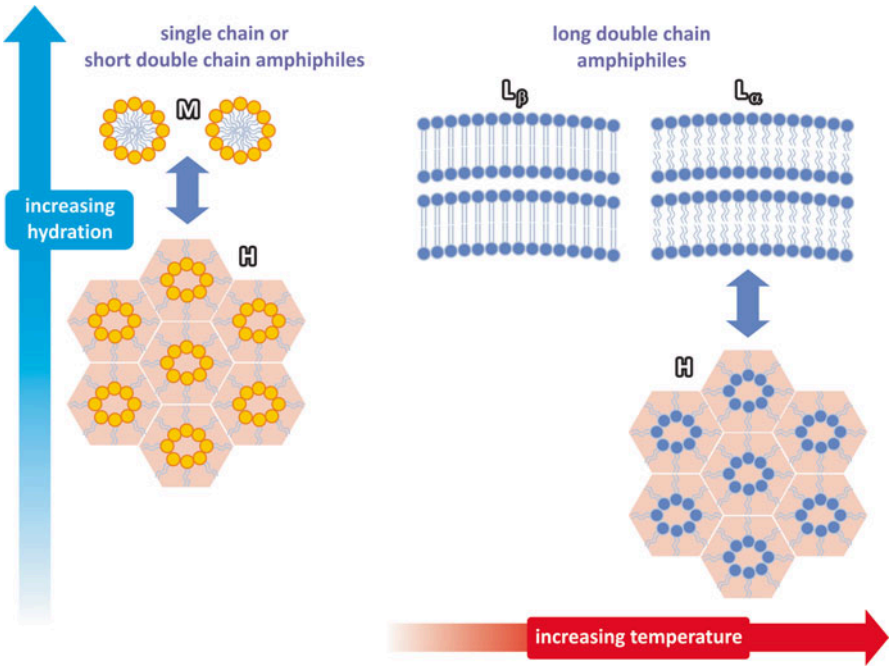


Fig. 1.3 The structure of lipid assemblies depends mainly on the degree of hydration and the molecular structure of lipids. Lipids may organize in different ways: rigid bilayers (L_β), fluid bilayers (L_α), micelles (M), or hexagonal (H) phases

Metabolism evolved towards self-regulation creating homeostasis, a situation whereby a balance exists. Small to moderate perturbations of such balance trigger responses that tend to reestablish the original, equilibrated balance. The ability of certain metabolites (intermediate molecules in a complex sequence of reactions in a living system) to activate or inhibit specific reactions in the metabolism was a major contribution to homeostasis (Fig. 1.4).

Nowadays, even the simplest cells, mycoplasma bacteria, for example, are extremely complex systems from the chemical/molecular point of view. Considering natural evolution, all metabolisms in all living cells are related by historical bonds and "metabolic maps" showing the main metabolic sequences in living beings can be drawn (Fig. 1.5). It is amazing that these complex series of reactions operate and do not conflict with each other. In reality, not all reactions depicted in (Fig. 1.5) occur in the same species and the ones that do occur in the same species may not be present in all cells. In case they co-exist in the same cell, they may not occur in the

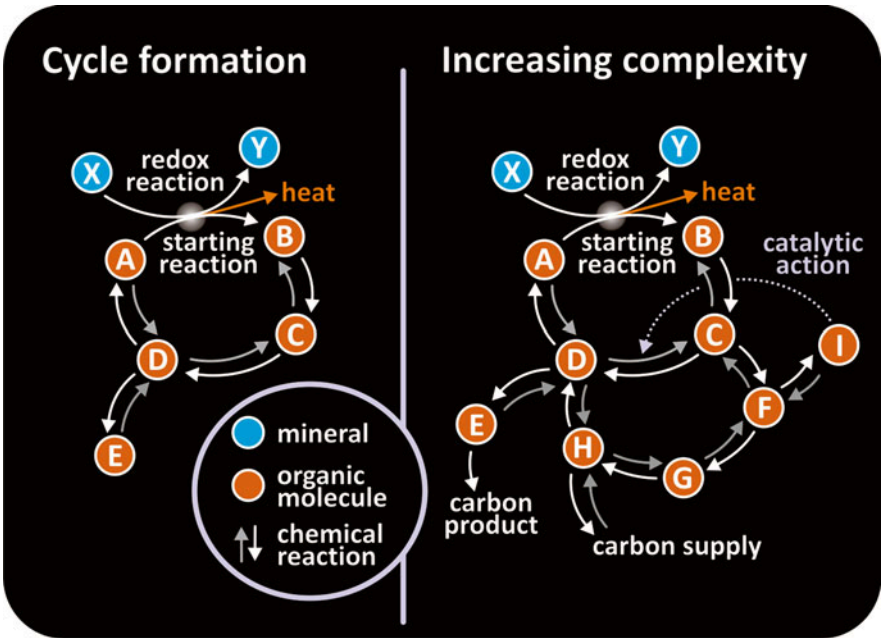


Fig. 1.4 The evolution of networks of chemical reactions. Simple cycles of reactions (*left*) may have evolved in complexity (*right*). The interference of certain metabolites on the course of reactions possibly resulted in self-regulated metabolisms. Figure reproduced with permission from Saphiro, Investigacion y Ciencia 371, 2007

same cell compartment and in case they do, they may not be working at the same time. "Complex" does not mean "confusing."

Because metabolic pathways (sets of metabolic reactions) have evolved from the same historical background, all molecules in all living cells are also related by historical links. Their common roots determined that, despite all the apparent molecular diversity, nearly all molecules in all cells can be grouped in few families. It is also intriguing at first glance that with so many chemical elements known to man (Fig. 1.6), cells rely heavily on very few of them: hydrogen, oxygen, carbon, and nitrogen are 99 % of the atoms that make a cell. How can this apparent puzzle be explained? Essentially, it all resorts to the common ancestor of all living cells in all living world: these were the most abundant elements in solution in the primitive ocean. These were the founding resources and life evolved from them.

We shall revisit in a more detailed manner the chemical nature of cells in Chap. 3.

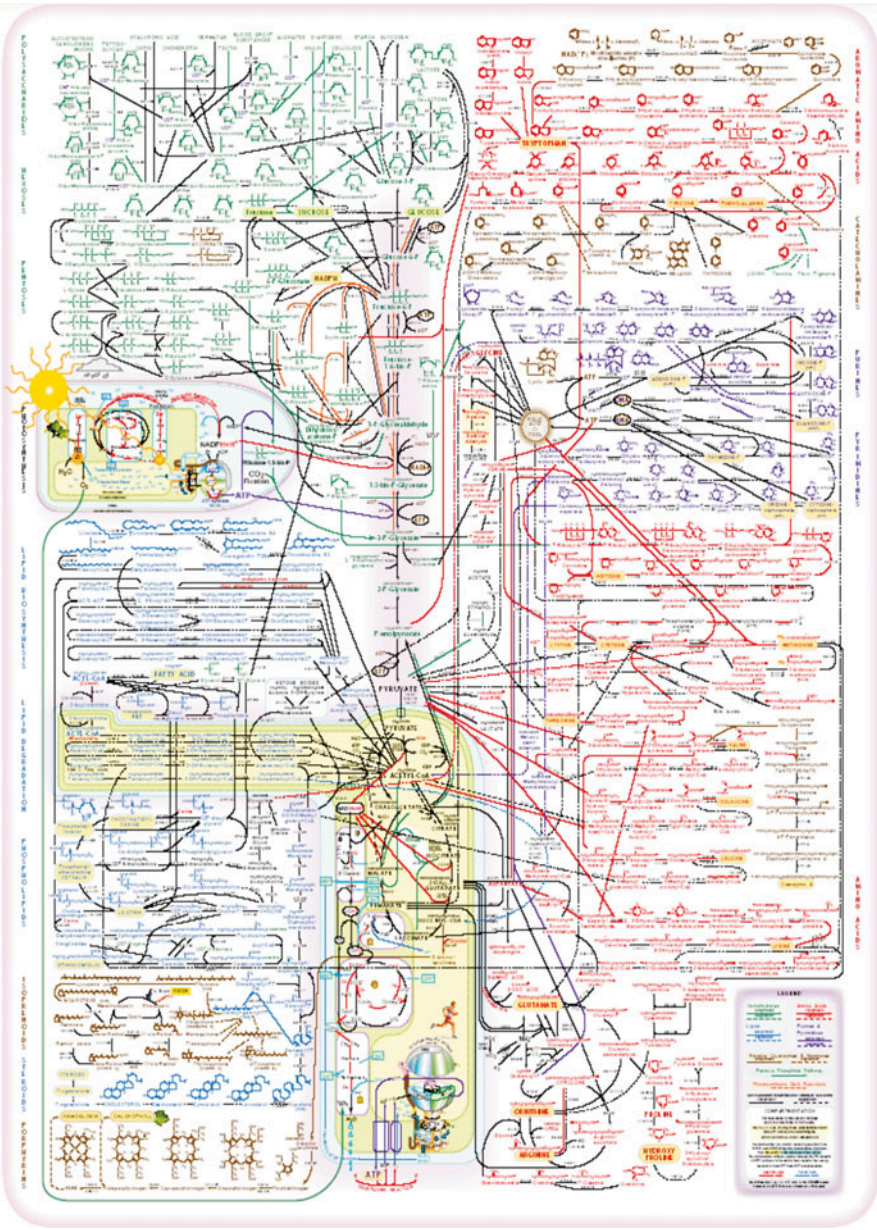


Fig. 1.5 A metabolic map showing a hypothetical cell, where the whole metabolism would gather many different sectorial metabolisms: amino acids, phospholipids, steroids, lipids, saccharides, etc. In reality, not all cells perform all sectorial metabolisms; those that occur in a certain cell may not occur in the same organelle and those that occur in the same organelle may not occur at the same time. The metabolism as a whole is usually so complex that in practice one tends to refer to "metabolisms" referring to the sectorial metabolisms in short. The word may be misleading because it may leave the impression that there are several independent metabolisms. Metabolisms are not independent of each other and they are highly correlated, even those occurring in different organs. The need for metabolic regulation extends to the whole human body. Figure reproduced with permission of IUBMB, International Union of Biochemistry and Molecular Biology

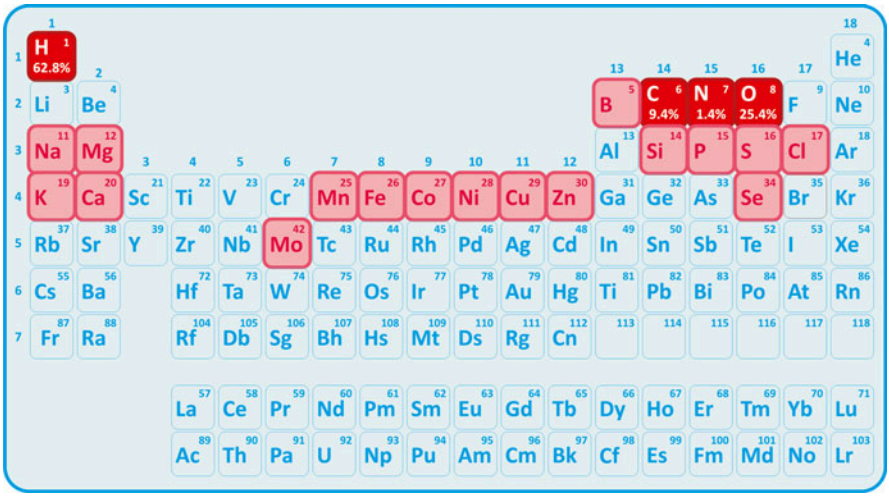


Fig. 1.6 Periodic chart of the elements, stressing the abundance of some in living beings (highlighted *red*). It should be noticed that very few elements are needed to "build" almost the totality of cells, and some elements are only present in trace amounts (highlighted *pink*). Yet, the elements that are rare may be absolute essential to life. Cobalt, Co, for instance, is part of vitamin B12 (see Box 3.8)

1.2 Selected Illustrative Example #2: Viruses, Molecular Machines Interfering with Life

Viruses are not considered by most researchers as living beings. They are on the boundary between living and non-living, able to interfere with homeostasis. They have similar molecular constituents compared to cells (proteins, lipids, nucleic acids, etc.), but there are important differences. Above all, viruses lack a metabolism of their own. Their simplicity is not a consequence of ancestry nor does it relate to any surviving form of primitive life. Instead, it is a consequence of parasitism and regressive evolution. Alternatively, viruses may have been part of the cells. Minimal genome sizes imply faster reproduction rates for viruses and are therefore an evolutionary advantage. One may argue that viruses lack a metabolism of their own but are physical entities that are able to self-replicate and evolve, thus living beings. Even so, it is questionable whether they may be considered as living because they do not replicate or evolve independently of cells. Virtually all parasites need a host to survive and multiply, but viruses are also not able to evolve independently: they are dependent on cells to evolve because they do not have their own machinery of molecular synthesis.

Virus–cell interactions are mostly physical in the early stages of the cellular infection as no chemical reactions are involved (no new covalent bonds are created or destroyed). Let's consider as an example the influenza virus, the virus that causes flu (there are three types of influenza viruses, A, B, and C, the influenza A virus being the major cause of seasonal flu). Influenza A virus is an enveloped virus, whose genome consists of eight single-stranded RNAs that encode

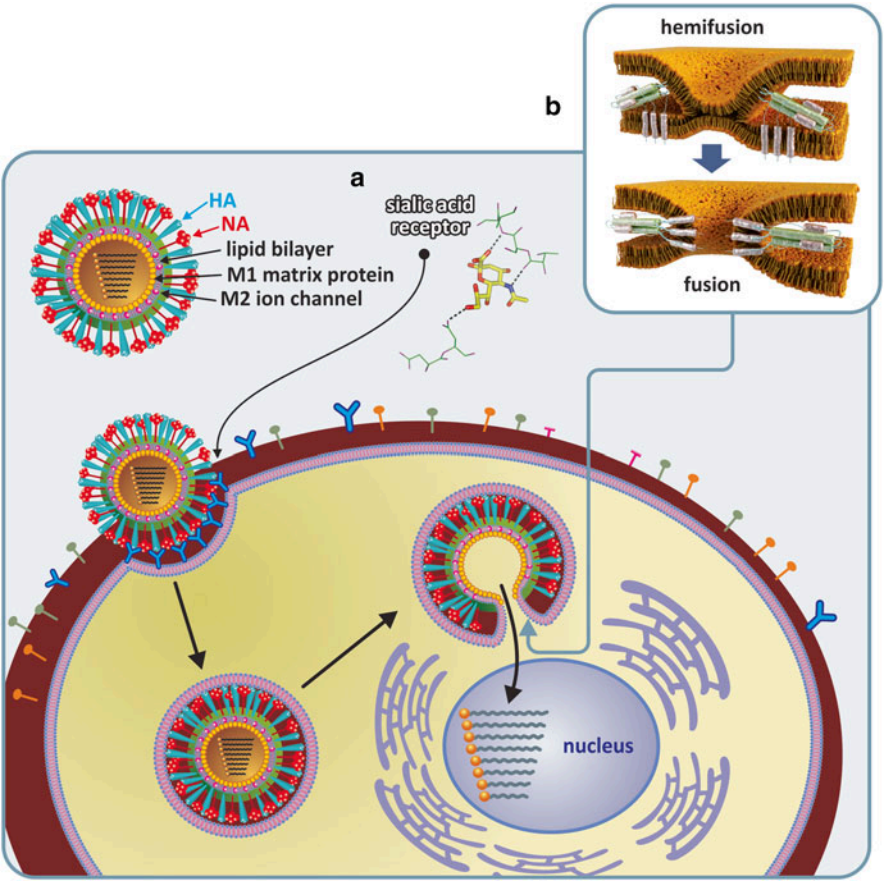


Fig. 1.7 Influenza virus entering a cell having sialic acid-containing receptor on its surface. The orientation, chemical nature, and distance of the binding amino acids of hemagglutinin A (HA) are such that sialic acid is able to engage in hydrogen bonds and other attractive forces. Panel (**a**) shows a zooming of the part of HA protein backbone contacting the sialic acid (protein carbon backbone in *green*; sialic acid carbon backbone in *yellow*). Upon acidification of endocytic vesicles inside the cell, HA undergoes conformational changes (not shown) that bring viral and cellular membranes in contact (Panel (**b**)) leading to the collapse of the membranes (named fusion) from which the viral content is released inside the cell

11 or 12 proteins (Fig. 1.7). The virus has the protein hemagglutinin A (HA) on its surface. This protein mediates virus entry into the host cells by binding to a saccharide, the sialic acid linked to molecules (glycans) present on cell surface, which are known as virus receptor. HA recognizes sialic acid due to a very precise combination of hydrogen bonds and ionic interactions, among others, between well-defined atoms on the protein and atoms on the sialic acid molecule (Fig. 1.7). These atoms, both the ones in the protein and in the saccharide, are at precise distances and orientations relative to each other so that a unique combination

of forces creates a strong binding between them. Influenza A virus may establish contact with many cells in the human body but will only bind to those having sialic acid-containing receptor on its surface (mainly cells of the upper respiratory tract epithelium). Consequently, these are the cells that can be preferentially infected by the virus. Virus–cell attachment (more precisely HA-sialic acid binding) induces virus internalization through endocytosis. The virus is enclosed in a vesicle in the cytosolic space. Upon acidification of the endocytic vesicle medium, HA is cleaved and undergoes conformational changes that result in the exposure of a terminal hydrophobic segment, called fusion peptide, to the endocytic vesicle membrane. Entropy balance then serves as a driving force (see Sect. 3.1) that promotes the fusion peptide into the endocytic vesicle membrane. Afterward, additional changes in the conformation of the protein will bring the viral envelope and the vesicle membrane together. They are both lipid bilayers, so they collapse. Ultimately, they fuse completely and the viral content is no longer separated from the cytoplasm. The viral RNA molecules proceed to the nucleus, where they are transcribed and replicated. The transcribed viral mRNAs are translated using the cellular protein synthesis machinery. The newly synthesized viral genome is packed by some of the viral proteins forming the nucleocapsid, whereas the viral surface proteins migrate to cell surface through the cellular secretory pathway. The nucleocapsid then associates to the surface proteins at the plasma membrane and new viruses bud from the cells ready to initiate another infection cycle.

When two different strains infect the same cell, the RNA of both may coexist in the nucleus. Scrambling of RNA originates new virus having random mixtures of the genetic material of both strains. The combined viruses may not be functional but occasionally a new strain of increased efficacy may be formed. For instance, it is possible that strains of avian or porcine flu combine with human flu to form new human flu strains. These events, combined with random mutations in viral proteins, may result in extremely lethal viruses. This was the case in 1918, when a flu strain, mistakenly named “Spanish flu,” killed hundreds of millions (!) of people across Europe and the USA (see Box 1.1). A mutation in a single amino acid in the HA-binding site to receptors in an avian virus was enough to make it able to infect human tissues (Fig. 1.8), a small change in a molecule with a tragic impact on mankind.

1.3 Selected Illustrative Example #3: Molecules as Tools, Drug Discovery, and Development

Designing new drugs that can be developed into new medicines demands knowledge on the role of different molecules in different pathologies. A molecular-level target is needed for the drug candidate, and the researcher needs to have an idea on how they are going to interact so that the target can be inhibited or activated. A drug candidate that is targeted to a protein, such as an enzyme or a membrane receptor,

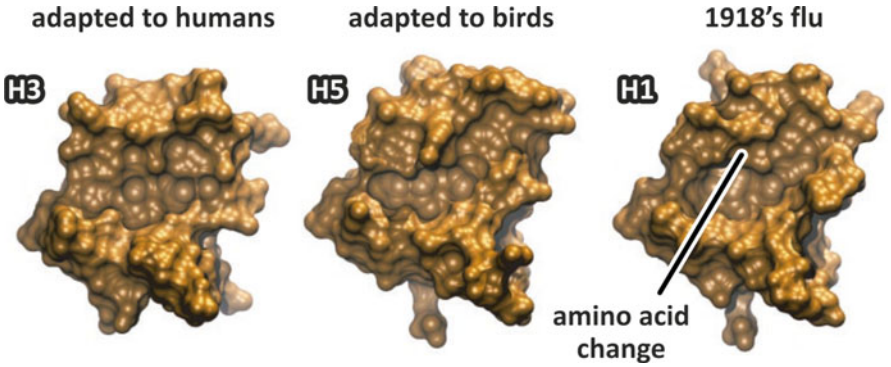


Fig. 1.8 Hemagglutinin 3 is adapted to human cells; Hemagglutinin 5 is adapted to birds. In 1918, a mutation in a single amino acid in the binding site of bird hemagglutinin made it able to bind human cells having sialic acid on its surface. This caused a tragic pandemic of flu among humans—the "Spanish flu" or "1918 flu." Figure reproduced with permission from Stevens et al., Science 303:1866–1870, 2004

Box 1.1: "Spanish Flu," Terrible and Almost Forgotten

Between April 1918 and February 1919, the world suffered the most severe pandemics of modern times. Probably it was the worst pandemics since the Black Death plague in the fourteenth century. Influenza, the virus causative of flu, infected hundreds of million people and killed, directly or indirectly, 50,000,000 to 100,000,000, figures so high that are hard to estimate. Europe was also being devastated by World War I. The mobility of the armies and the precarious medical assistance conditions helped to spread the disease. Moreover, the horrors of war and the censorship of the news from the fronts distracted the attention of mankind to the real dimension of the pandemics, which still remains largely ignored.

Despite the common name "Spanish flu," the disease did not start in Spain. Having a less tight censorship on the news because of its neutrality, Spain became a privileged source of information about the disease, which may have led to the impression that the disease was somehow related to Spain. In reality, the pandemic is believed to have started in the Kansas State region, in the USA in March 1918. The new virus strain caused sudden effects, killing in just a few days. In the worst cases, the patients suffered headaches, pain all over, fever, cyanosis, cough with blood, and nasal bleeding. Most deaths were associated to pneumonia, which was a consequence of opportunistic infection of the lungs by bacteria. The histological properties of the lungs were transformed, and there was accumulation of fluids that literally suffocated the victims, just as if they were drowning.

(continued)

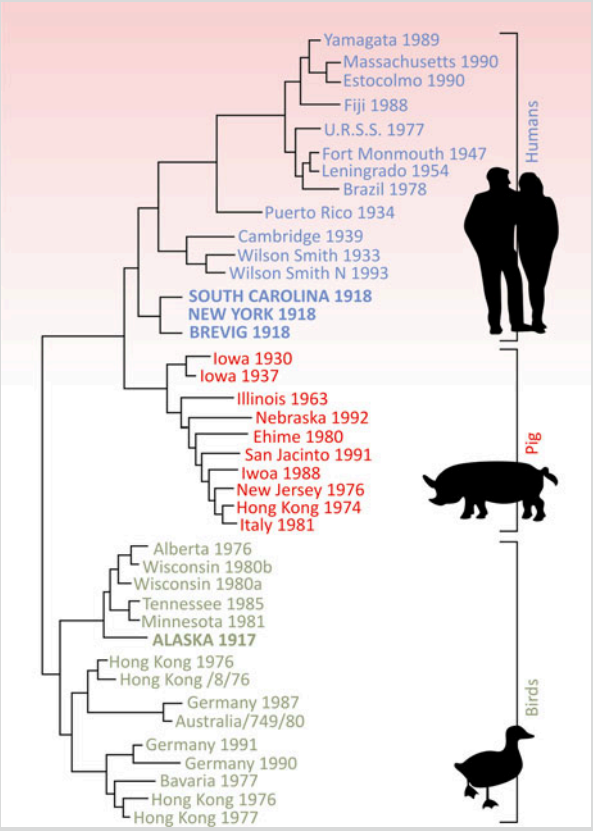
Box 1.1 (continued)

The electron microscope was invented in the 1940s. Before this technical breakthrough, it was very difficult to study viruses. Other breakthroughs have followed, such as the development of super-resolution optical microscopes and PCR (polymerase chain reaction) technique, but the molecular singularities of the 1918 virus are still a challenge. The quest for the sequence of amino acid residues of the proteins of the 1918 strain is a story of persistence and devotion. In 1940, Johan Hultin, a medical student, spent the summer in Alaska. He heard about Teller Mission, a small missionary settlement that literally disappeared in November 1918. Seventy-two victims of flu were buried in a common grave. Later Hultin matured the idea of recovering the 1918 flu virus from the bodies of the Teller Mission victims, presumably conserved in the Alaska permafrost. In the summer of 1951, he joined efforts with two colleagues from Iowa University, a virologist and a pathologist, and returned to Alaska to visit the former Teller Mission, meanwhile renamed Brevig Mission. With previous consent from the local tribe, Hultin obtained samples from lung tissue of some of the 1918 victims. The team tried to isolate and cultivate the virus using the most advanced techniques available, but they did not succeed. It was an extreme disappointment. Hultin quit his PhD studies and specialized in pathology. Forty-six years later, in 1997, he was retired, in San Francisco (California, USA), and read a scientific paper on a study of the genes of the 1918 flu strain obtained from 1918 to 1919 autopsies using PCR. Enthusiastically, Hultin resumed the intention of studying the samples from Teller/Brevig Mission. One of the Hultin colleagues from Iowa had kept the samples since 1951 until 1996! The samples had been disposed the year before! Hultin did not quit and asked permission to repeat the 1951 sample collection. This time he found the body of an obese young woman, whose lungs had been protected by the low temperatures and the layer of fat around them. The complete genome from the 1918 flu strain was obtained from these samples.

The hemagglutinin sequence of the 1918 strain (H1) was reconstructed from the genome of the virus. The sialic acid binding site suffered mutations in the amino acid residues relative to avian flu (H5) that enlarged the binding site, enabling the mutated viruses to bind and infect human cells. The modern studies on the phylogenetic tree of the flu viruses, which now include data from samples from South Carolina, New York, and Brevig, all from 1918, relate the origin of the virus to an avian strain found in a goose (Alaska 1917) (see figure). Although this hypothesis is not totally consensual among researchers, the fear that new unusually deadly flu strains adapted to humans evolve from avian flu strains persists and is a matter of thorough surveillance of health authorities around the world.

(continued)

Box 1.1: (continued)



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for instance, needs a binding site where it can react, or attach both strongly and selectively. "Selectively" means it will discriminate this site from all others in the same target or in any other molecule of the body. The uniqueness of the binding site is directed by the precise arrangement of the atoms in space. Ideally, only that site has the right atoms at the right distance, in the right orientation to maximize intermolecular attraction forces (see the example of HA-sialic acid binding in Fig. 1.8). Hydrogen bonding, ionic/electrostatic forces, van der Walls interactions, etc. all these are dependent on the spatial arrangement of elements of both drug candidate and target. The Beckett–Casy model for opioid receptors illustrates the basis of these principles (Fig. 1.9). In addition to the efficacy in binding to its target, drug

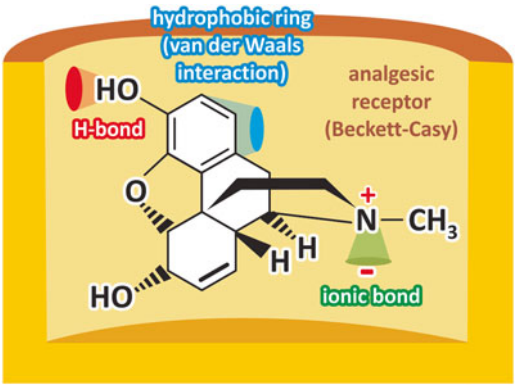

Fig. 1.9 Beckett–Casy hypothesis for the binding of an analgesic molecule (such as morphine, which is illustrated) to an opioid receptor. While the exact structure of the receptor is not known, the key interaction/attraction forces were identified: electrostatic attraction, H-bonds, and van der Waals interactions. The receptor is so specific for the ligand that chiral molecules (bearing the same chemical groups but with different orientation in space) do not fit

molecules cannot be exceedingly toxic or have significant other undesired effects, which are directly related to its reactivity and selectivity.

The same reasoning applies to complex therapeutic molecules such as antibodies. Let us now take one of the antibodies that target the protein gp120 on the surface of the human immunodeficiency virus (HIV) (Fig. 1.10). This protein is responsible for the binding to the receptors and co-receptors of the T lymphocytes, this being the first step of infection in acquired immune deficiency syndrome (AIDS). When an antibody binds to gp120, it may block the access of gp120 to the receptors and/or co-receptors, thus preventing infection. Anti-HIV antibodies are hopes for future therapeutics although the rate of mutations in gp120 and the presence of glycosylated groups on gp120 surface pose problems that are difficult to overcome.

Some researchers devote their work to antibody engineering, i.e., the manipulation of antibodies for a specific purpose. Some try to find the smallest portion of an antibody that is still active, so that antibody therapy can be made simpler and more cost effective. Manipulating antibodies demands knowledge on the molecular-level interactions they perform with their antigens. At this level, the forces that are responsible for selectivity and strength of binding are not different from those that small molecules (such as the opioids in Fig. 1.9) establish with their molecular targets, but the overall number of bonds (hydrogen bonding, electrostatic interactions, van der Waals interactions, etc.) involved may be higher, resulting in extreme selectivity and very strong binding forces.

The whole process of devising and studying drugs (frequently termed "pipeline") has three main stages: research, development, and registration (Fig. 1.11). The research stage is typically, but not exclusively, carried out at universities and academic research centers. During this phase, relevant targets for selected pathologies

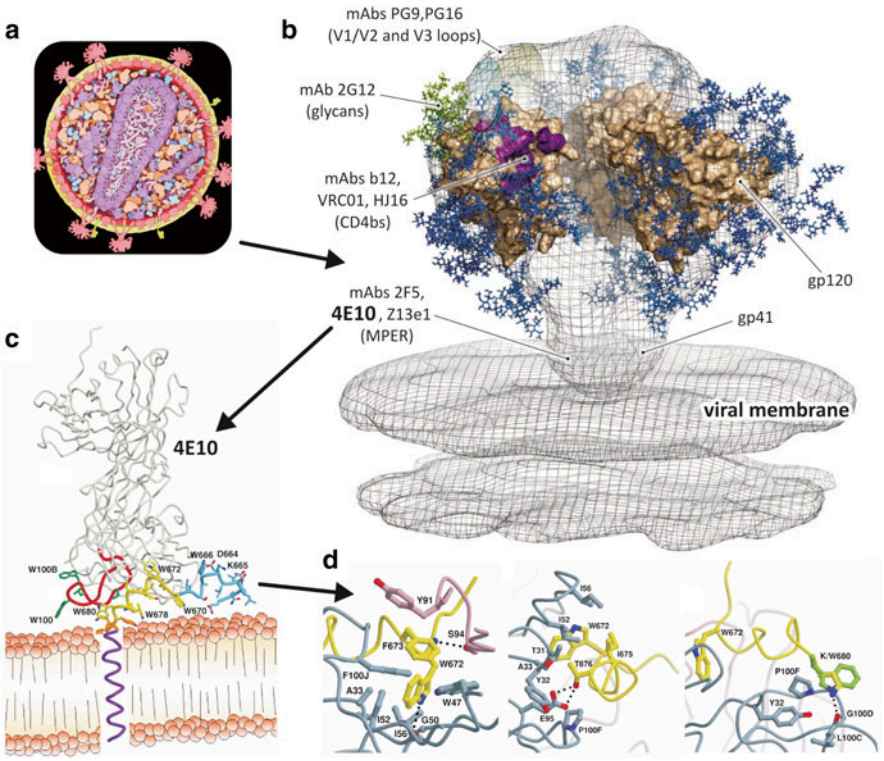

Fig. 1.10 (**a**) Illustration showing HIV particle, highlighting its capsid (*magenta*) surrounding viral nucleic acid and the envelope proteins (*pink*). Besides the capsid, the virion is loaded with several other proteins with different functions in the replication cycle (reproduced with permission from Goodsell, The Machinery of Life, 2009). (**b**) Broadly neutralizing monoclonal antibodies target specific motifs (epitopes) at the surface of the envelope proteins, gp120 and gp41. The model depicted was generated gathering scientific data from different sources. The contour of the envelope proteins and viral membrane is shown in *gray*; what is known from the structure of gp120 is shown in *colors*. The glycosidic (saccharidic) part of the protein is shown in *green* and *blue*. MPER stands for Membrane Proximal External Region and refers to the proteic part of gp41 nearest to the viral membrane (reproduced with permission from Burton & Weiss, Science 239:770–772, 2010). (**c**, **d**) Some antibodies, such as 4E10, target this region of the protein. At the contact points between the amino acid residues of antibody and gp41, attractive forces such as ionic, H-bonds, and van der Waals interactions contribute to a strong binding. The chemical nature and the spatial arrangement of the amino acids confer selectivity to antibody–epitope interaction. Figure reproduced with permission of Elsevier from Cardoso et al., Immunity, 22:163–173, 2005

are identified and a molecule to interfere with that target is selected. This molecule is a drug candidate that can be improved. Such molecule is termed "lead," and the process of improvement is termed lead optimization. Research stage takes several years (rarely less than five).

The preclinical development in the first step is the development stage and the last step before clinical trials. Preclinical studies consist in as many experiments in vivo

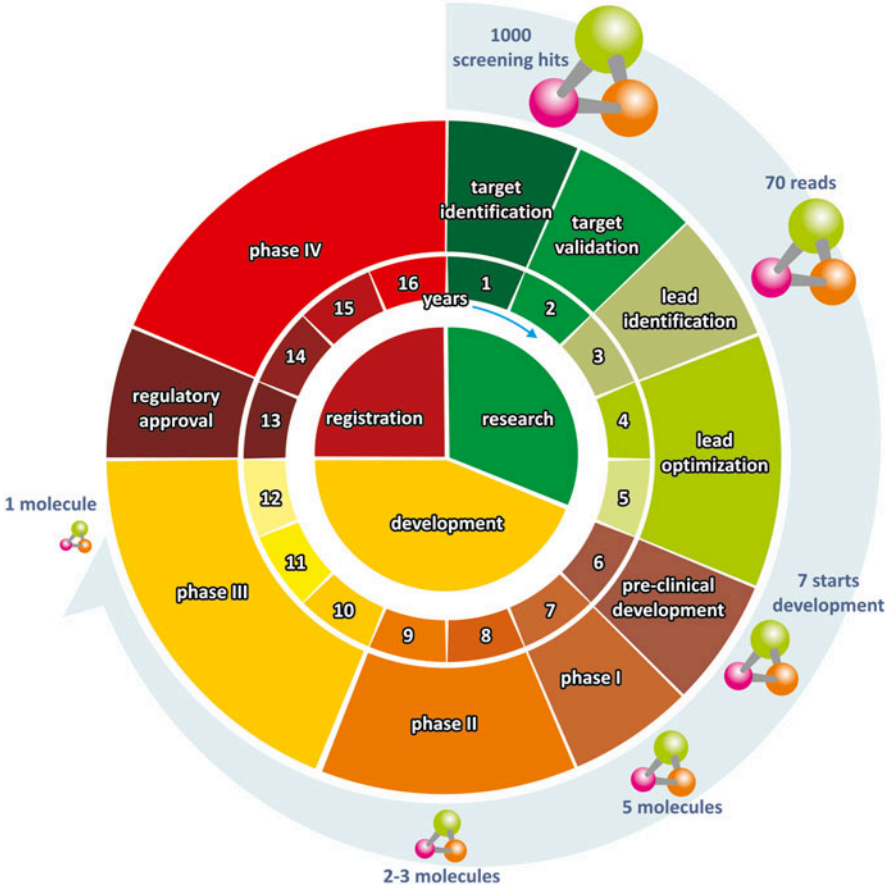

Fig. 1.11 The drug discovery and development process, generally termed "pipeline" in pharmaceutical industries. There are three main stages, research, development, and registration (*center*), distributed over several years (numerical timeline). Each stage is divided in subphases. During research phase, relevant targets for selected pathologies are identified and molecules to interfere with those targets are selected ("leads"). Research stage takes typically around 5 years. The development phase may proceed for the next 7 years, during which the drug leads are tested for safety and efficacy in carefully designed animal and human clinical trials. At the ending of each phase, there is evaluation of results; safety and/or efficacy issues may prevent further tests. Typically, for each nearly 1000 molecules starting the process, only one ends the last phase successfully. This ratio, named "attrition rate," is incredibly low. Moreover, not all molecules are granted approval to enter the market for regulatory reasons, and those that enter the market are still monitored afterward (Phase IV)

and in vitro (both in cells and artificial systems) as needed to ensure that a certain optimized lead is safe at a certain dosage range when prepared as a specific selected formulation using a defined mode of administration. The goal is to minimize risks to the lowest possible level when administrating the optimized lead to humans. Efficacy comes after safety in the priority list. This is the reason why the first tests in human (Phase I clinical trials) are performed in few healthy volunteers, not

patients. At Phase I clinical trials, it is safety that is tested, using conservative doses of the compounds under study. Drug tolerability, absorption, and distribution in the body and excretion are followed. Phase II clinical trials include patients and efficacy, besides safety, is also tested. The drugs are administered in up to several hundred individuals for several weeks or few months, typically. The dose range of the drug is improved during the trials. It should be stressed that all trials are scientifically controlled for the statistical significance of the results. The placebo effect (Box 1.2) is also accounted for in the trials. The process of designing clinical trials, data collection, and meaningful data analysis for reliable conclusions is, by itself, a complex discipline.

Phase III clinical trials are a replica of Phase II, but several thousands of individuals are enrolled and treatment may be extended in time. Phase III is thus a refinement of Phase II both in terms of efficacy and safety. Rare events such as unlikely undesired off-target effects that may have not been detected in Phase II are now more likely to be detected. Concern for rare undesired off-target effects that may jeopardize the safety of drugs, even to small and very specific subpopulations of patients, is always present, even after the drug has been approved as a medicine for clinical use. This is sometimes termed "Phase IV" and consists in screening how the drugs perform in the "real world," outside a tightly controlled environment.

Box 1.2: The Placebo Effect, the Power of Nothing
(based on "The Power of Nothing" by Michael Specter in The New Yorker December 12, 2011 Issue)

A placebo is a simulated or otherwise medically ineffectual treatment for a disease or other medical condition intended to deceive the recipient. Sometimes patients given a placebo treatment will have a perceived or actual improvement in a medical condition, a phenomenon commonly called the placebo effect.

For most of human history, placebos were a fundamental tool in any physician's armamentarium—sometimes the only tool. When there was nothing else to offer, placebos were a salve. The word itself comes from the Latin for "I will please." In medieval times, hired mourners participating in Vespers for the Dead often chanted the ninth line of Psalm 116: "I shall please the dead in the land of the living." Because the mourners were hired, their emotions were considered insincere. People called them "placebos." The word has always carried mixed connotations. Placebos are often regarded as a "pious fraud" because bread pills, drops of colored water, and powders of hickory ashes, for instance, may sometimes lead to a perceived improvement in patients.

The first publicly acknowledged placebo-controlled trial—and still among the most remarkable—took place at the request of King Louis XVI, in 1784. The German physician Franz Anton Mesmer had become famous in Vienna for a new treatment he called "animal magnetism," and he claimed to have

(continued)

Box 1.2 (continued)
discovered a healing fluid that could "cure" many ailments. Mesmer became highly sought after in Paris, where he would routinely "mesmerize" his followers—one of whom was Marie Antoinette. The King asked a commission of the French Academy of Sciences to look into the claims. (The members included the chemist Antoine Lavoisier, and Joseph Guillotin—who invented the device that would eventually separate the King's head from his body.) The commission replicated some of Mesmer's sessions and, in one case, asked a young boy to hug magnetized trees that were presumed to contain the healing powers invoked by Mesmer. He did as directed and responded as expected: he shook, convulsed, and swooned. The trees, though, were not magnetic, and Mesmer was denounced as a fraud. Placebos and lies were intertwined in the public mind.

It was another 150 years before scientists began to focus on the role that emotions can play in healing. During the World War II, Lieutenant Colonel Henry Beecher met with more than 200 soldiers, gravely wounded but still coherent enough to talk; he asked each man if he wanted morphine. Seventy-five percent declined. Beecher was astounded. He knew from his experience before the war that civilians with similar injuries would have begged for morphine, and he had seen healthy soldiers complain loudly about the pain associated with minor inconveniences, like receiving vaccinations. He concluded that the difference had to do with expectations; a soldier who survived a terrible attack often had a positive outlook simply because he was still alive. Beecher made a simple but powerful observation: our expectations can have a profound impact on how we heal.

There is also a "nocebo effect." Expecting a placebo to do harm or cause pain makes people sicker, not better. When subjects in one notable study were told that headaches are a side effect of lumbar puncture, the number of headaches they reported after the study was finished increased sharply.

For years, researchers could do little but guess at the complex biology of the placebo response. A meaningful picture began to emerge only in the 1970s, with the discovery of endorphins, endogenous analgesics produced in the brain.

Regulatory approval follows Phase III and precedes Phase IV and initiates the registration stage. The results of the development of the drug, from molecule to man, from bench to bedside, are submitted to regulatory agencies, which assess the results and conclusions of the whole clinical development based on the evaluation carried out by independent experts. The need for that specific new drug and how innovative it is when compared with existing drugs for the same purpose is also taken into consideration. The decision on allowing a specific molecule to be part of a new medicine or not belongs to these agencies.

The numbers associated to the difficulty in developing a successful drug, which is later incorporated in a new medicine, are absolutely impressive. For each 1–5 million "new chemical entities" (molecules tested for their pharmacological interest), only 1000 have positive results in vitro tests, from which only 70 are selected as leads, which are then optimized to form 7 drug candidates that enter clinical trials. Out of these 7, only 2–3 reach Phase III clinical trials and only 1 is approved by regulatory entities. The whole process takes around 15 years to complete (Fig. 1.11) and has an estimated total cost of several millions of USD for each approved new drug, on average. It is important to point out that these are very crude numbers that vary a lot for different areas of medicine, but they serve to illustrate the efforts needed to continuously fight against disease progress. Reducing the attrition rate (number of new chemical entities that fail during the drug development process), accelerating the whole process, and making it more cost effective are hugely demanding but urgently needed tasks.

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Chapter 2
The Chemistry and Physics of Life

Our idea of the interior of a cell at the molecular scale is often rather naïf. If one could see the interior of a cell with molecular resolution, one would not see an aqueous solution of molecules with the cellular organelles suspended. The molecular crowding, in particular the macromolecular crowding, inside a cell is such that the interior of a cell is more like a gel than a solution. Molecular packing is so dense that it is hard for macromolecules to diffuse freely. The ubiquitous presence of the cytoskeleton and macromolecular assemblies in a space that is highly restricted due to cellular organelles makes the interior of cells tightly packed (Fig. 2.1). Nevertheless, it is a highly hydrated environment, where solvation is made by water molecules (Fig. 2.1) and voids are filled by water that solubilizes ions and small molecules. Thus, virtually all exposed molecules in a cell are under the chemical and physical influence of water. The interior of a cell is not an aqueous solution, but the chemical reactions of the living cells are typical chemical reactions of aqueous solutions.

Life started in water and, chemically speaking, it is still dominated by water. Even the elemental composition of the molecules in a cell was determined by water. With few exceptions, the abundance of elements in a cell reflects the abundance of the same elements in the oceans (Fig. 2.2). Iron, phosphorus, or nitrogen is among the exceptions: they are more abundant in cells on average than in sea water due to their so-called chemical utility. The electronic structure of nitrogen (Fig. 2.2) makes it appropriate as an electron donor to establish dative covalent bonds, also known as dipolar or coordinate bonds. Phosphorus is an amazing element for its capabilities in coordination chemistry (Fig. 2.2). The chemistry of phosphate, PO_4^{3-}, is so useful to cells that phosphorylation/dephosphorylation is an ubiquitous mechanism to activate or inhibit enzymes or determine the reaction a molecule will undergo in a metabolic sequence. Iron may have once been abundant as soluble Fe^{2+} that was afterward oxidized to Fe^{3+} upon the appearance of molecular oxygen, O_2, in the Earth's atmosphere. Fe^{3+} formed oxides and hydroxides that precipitated, making iron less abundant in seawater. Nonetheless, iron was already being used by cells, and its "chemical utility" determined that cells kept this element at higher levels in their composition.

A.T. Da Poian, M.A.R.B. Castanho, *Integrative Human Biochemistry*,
DOI 10.1007/978-1-4939-3058-6_2

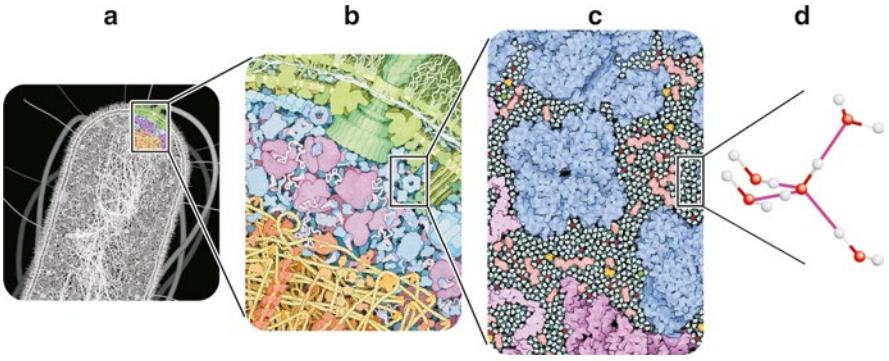


Fig. 2.1 Crowding in the molecular organization of cells. Even in simple cells such as bacteria (e.g., *E. coli*, **a**), cytoplasm is a dense tight packing of macromolecules (e.g., proteins and nucleic acids) and smaller molecules such as water, nucleotides, and amino acids (**b**). This situation is frequently referred to as "molecular crowding." The cell interior is thus more similar to a gel than to a solution. Nevertheless, virtually all the external surface of macromolecules, small molecules, and ions are in direct contact with water (**c**). Amino acids, saccharides, ATP, and many other small organic molecules are shown in *pink*. Metal ions are represented in *red*, phosphate ions are *yellow* and *orange*, and chloride ions are in *green*. Water molecules are *colored turquoise*. Although in many cases water molecules may be confined to the solvation shells (immediate layers of water surrounding other molecules), they impose many chemical and physical constraints to molecular organization and reactivity in cells. Water molecules are polar and prone to establish hydrogen bonds (**d**). Although the cell interior is not a solution, its chemistry and physics is dominated by water. Panels (**a**–**c**) are reproduced with permission from Goodsell, The Machinery of Life, 2009

Iron is able to participate in coordination chemistry organometallic complexes, hemoglobin being an example.

Elements that are very abundant on Earth's crust but not in the oceans such as aluminum (8.2 % of the atoms) or silicium (28 % of the atoms!) are also not abundant in cells because they are mostly part of insoluble oxides. Only 1 % of the total atoms in cells are not hydrogen (62.8 %), oxygen (25.4 %), carbon (9.4 %), or nitrogen (1.4 %). Yet, many elements that are only present in trace amounts may be part of molecules or processes essential to life, such as boron (B), cobalt (Co), cupper (Cu), manganese (Mn), or molybdenum (Mo).

So, water imposed severe constraints on the molecular evolution of cells. And still does! Water is a small but amazing molecule. In spite of its simplicity and abundance, water still fascinates chemists. In particular, its polarity and ability to establish hydrogen bonds are determinant to influence chemical reactions in regions where water serves as a major solvent, which means almost the whole cell (lipid membranes are the main exception). If it weren't for hydrogen bonding, for instance, water would not be a liquid at temperature and pressure ranges humans and most living beings are adapted to. Methane, with a molecular structure that can be compared to water, is not as nearly important as water in the history of life: methane boiling point is −162 °C (Fig. 2.3).

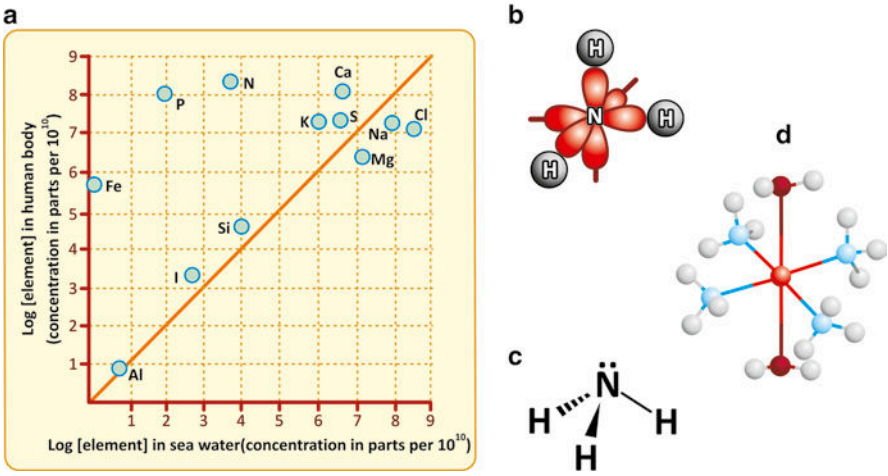


Fig. 2.2 Panel (**a**) shows that the abundance of the chemical elements in the human body matches the abundance of such elements in seawater, with few exceptions (reproduced with permission from Dobson et al., Foundations of Chemical Biology, 2001). Iron, phosphorus, and nitrogen are among such exceptions because they are very abundant in the human body. Elements such as carbon, hydrogen, and oxygen are not represented in the graph because they are extremely abundant. Nitrogen binds three hydrogens in ammonia (NH_3). The covalent bonds are formed with the *s* orbitals of H (*gray*) and a mix of the *s* (not shown) and the three *p* orbitals of N (*red*) (**b**). A fourth mixed *sp* orbital has electrons that are available to participate in dative (coordination) bonds. A simplified representation of the molecule represents these electrons as •• over N (**c**). Oxygen in water or dioxygen (O_2) molecules also have electrons available to participate in dative bonds, for instance, with metal ions. Panel (**d**) shows the tetraamminediaquacopper(II) cation, $[Cu(NH_3)_4(H_2O)_2]^{2+}$, in which a central Cu^{2+} ion accepts electrons from two water and four ammonia molecules. Binding of dioxygen to iron in hemoglobin follows the same principle

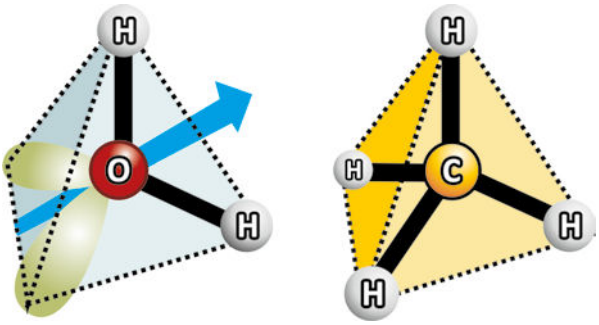


Fig. 2.3 Water vs. methane: similar molecular geometry but different polarity (charge asymmetry axis represented in *blue* from the slightly higher electronic density in the nonbonding O orbitals in green to lower electronic density in H) and different hydrogen bonding capabilities, which determines very different ebullition points: 100 °C to water, −162 °C to methane

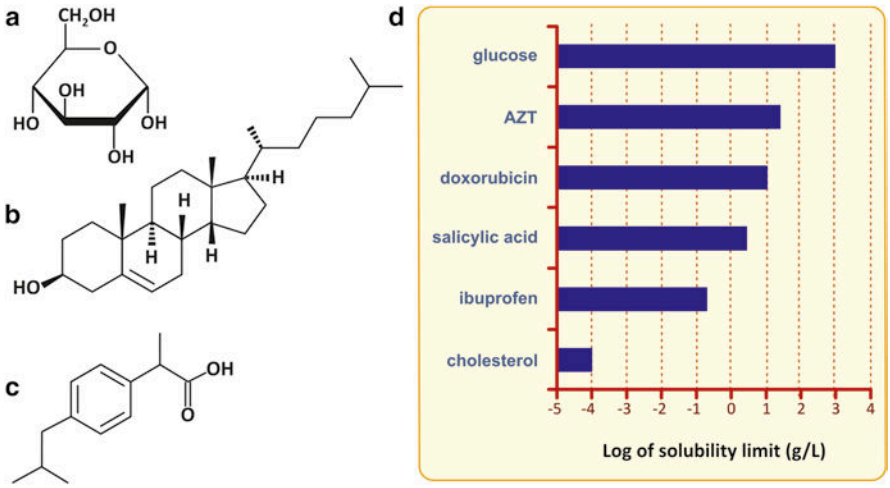


Fig. 2.4 Very hydrophilic molecules are polar, such as glucose (**a**). Like water, glucose can establish several hydrogen bonds per molecule. Its solubility in water is therefore very high, and glucose can be distributed freely in the human body when solubilized in the blood plasma. In contrast, cholesterol (**b**), having only a single polar group, the alcohol (–OH), is very hydrophobic (nonpolar) and is not soluble in aqueous media, such as blood plasma. Distribution of cholesterol and other nonpolar molecules in the human body requires special structures that emulsify these hydrophobic molecules in the blood, the lipoproteins. Some biological molecules and drugs, such as ibuprofen (**c**), for instance, may be intermediate cases and have limited solubility due to the simultaneous presence of hydrophobic and hydrophilic groups in the same molecule (ibuprofen has a phenyl ring and a carboxylic group), which is very important for their ADME: absorption, distribution, metabolization, and excretion by the human body. Solubilities of selected biological molecules and drugs are presented in (**d**)

Molecules that are polar, mainly those that can establish hydrogen bonding with water, can be distributed in the human body by simple diffusion, such as in blood and/or cerebrospinal fluids, while nonpolar molecules have low solubility and can only be distributed by special carrier systems, such as lipoproteins (see Sect. 3.1.2). Glucose (Fig. 2.4), for instance, distributes in the human body without a carrier, as its concentration in blood can reach very high values with no solubility problems. Cholesterol is the opposite as its solubility in aqueous environment is very low, so it would form crystals and precipitate if it was not kept in nonpolar environments such as the hydrocarbon core of lipid membranes or the interior of lipoproteins. Some pathological situations such as gallstones relate to the low solubility of cholesterol-related molecules, which leads to formation of aggregates (due to the so-called entropic effect; see Sect. 3.1) that in turn nucleate small crystalline structures that grow into "stones" that can measure up to a few centimeters.

Solubility is as important for pharmacology as it is for physiology. A drug that precipitates and crystallizes in the blood, or even in the stomach after being swollen, is hardly effective as it cannot be absorbed and/or distributed in the body.

Nonpolar drugs are usually mixed with other molecules in formulations that prevent drug aggregation in aqueous environment. Solubility is one of the key parameters considered to devise drug development strategies.

The tendency that drugs have to locate in aqueous or hydrophobic regions of tissues, such as lipoproteins, lipid bilayers, or adipose depots, is studied in pharmacology by measuring the partition of the drug between octanol and water, two immiscible solvents. Octanol is an organic solvent, largely apolar. In spite of the popularity of this method, octanol in still hardly similar to lipidic environments and more modern alternatives exist, like working with aqueous suspensions of lipid vesicles (Fig. 2.5).

The polarity of a given molecule is also determinant for its excretion. Polar molecules are easier to excrete for their solubility in blood and urine. The main strategy of the human body to eliminate xenobiotics (molecules that are not natural constituents of human tissues) consists in grafting hydroxyl (OH) groups, so that they become more polar and, therefore, more soluble in aqueous fluids. This is an efficient method that can be applied to a wide diversity of molecules, serving the purpose of low specificity for a broad protection of the human body against multiple toxic molecules. The molecular complex responsible for polyhydroxylation of different compounds is cytochrome P450, a big proteic complex that contains iron (Fig. 2.6).

Hydrophobicity (more accurately one would say the "entropic effect") is important not only for the absorption, distribution, and excretion of molecules, as discussed in this section, but also for holding together the most ubiquitous of all supramolecular non-covalent structures of cells: the lipid bilayer membranes. The importance of this issue is such that it will be kept for detailed explanation in a later section (Sect. 3.1)

2.1 The Basics of Chemistry in Cells and Tissues

The boundaries between chemistry and physics at the molecular scale are hard to establish. The interface between both is a rich scientific field referred to as physical chemistry or chemical physics. These disciplines deal typically with molecular structure and the way reactivity is affected by it. A functional distinction that is very practical for those working with molecules is to consider chemical reactions of all transformations of matter that involve formation or breakdown (or both) of covalent bonds. Transformations that do not involve alteration of covalent bonds are considered physical processes. Thus, light being absorbed by the molecules on the skin surface by a protective "sunscreen" cream constitutes a physical process, while UV radiation reaching the skin cells and damaging DNA due to covalent bond cleavage is chemistry. On a macroscopic scale, this functional frontier between chemistry and physics may lose intuitive sense: a plumber cutting a metallic or PVC tube would then be doing chemistry as he is actually destroying chemical bonds in doing so.

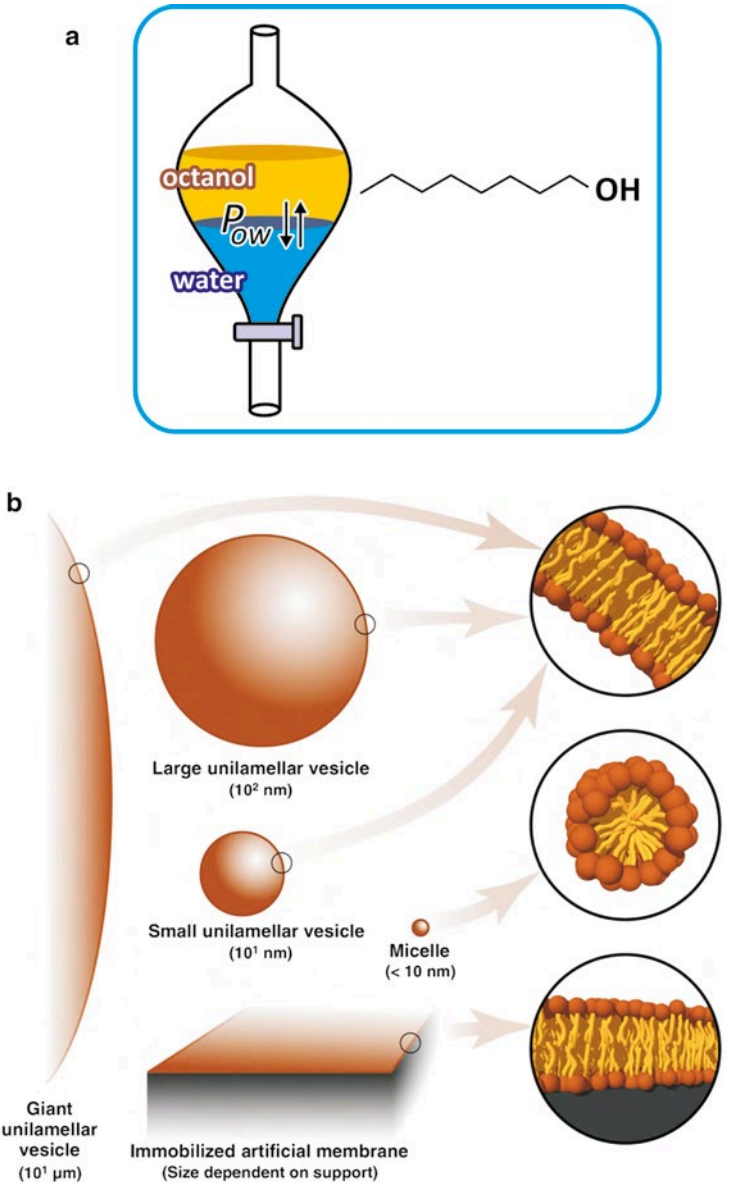


Fig. 2.5 (**a**) Octanol is an amphiphilic molecule with an acyl chain of eight carbons and a polar alcohol group (–OH). Its hydrophobicity prevents its miscibility with water. Depending on their own polarity, solutes will distribute more extensively to octanol or water. The ratio of the equilibrium concentrations of the solute in both phases is constant, regardless of the total amount of solute or the volume of each phase, which is the reason why this ratio is referred to as partition constant, P_{ow}. This parameter is used to estimate the tendency molecules under study (e.g., a drug candidate for a future medicine) have to interact with membranes and other lipidic structures, such as lipoproteins or lipid droplets. (**b**) More recent techniques use different approaches (artificial lipid bilayers in suspension). Despite the ubiquity and simplicity of the octanol/water approach, octanol is a poor replica of lipids. Lipid vesicles in suspension are lipid bilayers of very well-defined composition. Although they lack many characteristics of biological membranes (receptors, transporters, cytoskeleton anchorage, clustering of specific lipids, etc.), they are much more realistic as biological membrane mimetics than octanol. Figure b reproduced from Ribeiro et al., Trends Pharmacol Sci 31:449–454, 2010, with permission of Elsevier

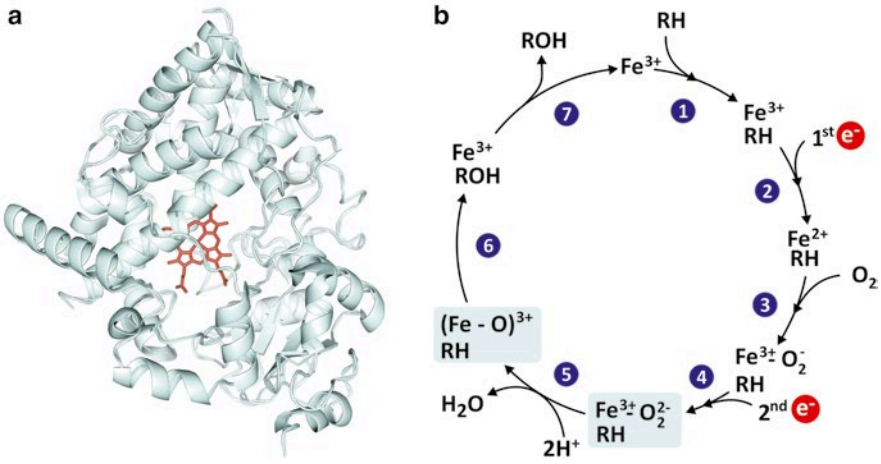


Fig. 2.6 The P450 cytochrome complex (**a**; PDB 1W0E) uses iron and oxygen to add an hydroxyl (OH) group to organic molecules (generically represented by RH in panel **b**) rendering it more water soluble, suitable to be excreted through urine. This is an important detoxifying mechanism in the human body

In practice, a clear definition of what is chemistry by opposition to what is physics is not needed or useful. Many professionals use both and do not really mind or think about naming what they are doing in terms of chemistry vs. physics classification.

The "chemical life" of cells is very rich and diverse. Many different kinds of reactions may occur. Probably all kinds of reactions occurring in aqueous medium that are described in the most complete organic chemistry textbooks can be found in cells. In this chapter, we will focus only on those most important to understand human metabolic regulation, the current core of human integrative biochemistry (i.e., biochemistry in relation to other disciplines such as histology, physiology, pharmacology, and even anatomy, so that a global perception of human body homeostasis is achieved). It should be stressed that such reactions are favored by the presence of water as solvent. As interaction with solvent molecules affects the electronic distribution of molecules, their reactivity is affected by the solvent. If natural evolution at molecular level was based on a different solvent (e.g., octanol), the "portfolio" of the chemical reactions of life would be different.

Oxidation–reduction reactions are among the most important reactions of the living world. As the name implies, oxidation–reduction reactions are those in which electrons are donated (oxidation) or received (reduction). Because electrons in cells do not remain isolated, individually, as they would in vacuum in outer space, they are transferred between molecules or ions and therefore oxidation and reduction coexist. They are thus referred to as oxidation–reduction reactions. Many different molecules may be oxidized or reduced. Some are particularly well adapted

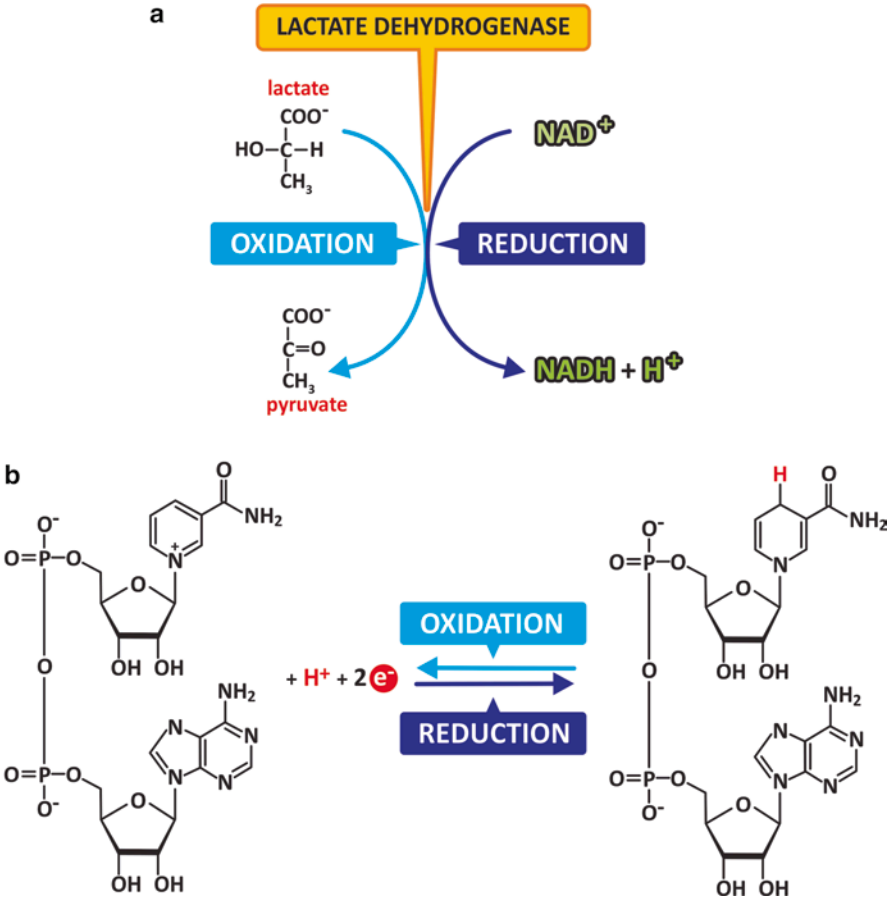


Fig. 2.7 Example of an oxidation–reduction reaction. Lactate is oxidized to form pyruvate (**a**). Its –OH group is transformed in a C=O (carbonyl) group, and electrons were transferred to NAD+ in the process, which was transformed in NADH (**b**)

as reducing agents, such as NADH (Fig. 2.7); others are particularly well adapted to be part of a chain of successive electron transfers, such as some metalloproteins. Metalloproteins have metallic elements in their composition that facilitate reception and donation of electrons. The electron transport system, for instance, has several of these proteins (see Sect. 6.2.3).

Acid–base reactions constitute another class of extremely important and ubiquitous reactions in the "living world." These are reactions in which a proton (H^+) is either donated (by an acid) or received (by a base). In aqueous environment, such as in the almost totality of the cell, H^+ does not exist as such because water molecules capture the proton forming H_3O^+ or donate a proton, forming OH^-, the

hydroxide anion. These chemical species (OH^-, H_3O^+, and H_2O) are all related and equilibrium among them may be reached:

$$2H_2O \rightleftharpoons H_3O^+ + OH^-$$

The equilibrium constant for this reaction is:

$$K_{eq} = \frac{\left[H_3O^+\right]\left[OH^-\right]}{\left[H_2O\right]^2}$$

Because $[H_2O]$ (molar concentration of water) is constant at any given temperature and pressure, the so-called ionic product of water, K_w, is used instead for its simplicity:

$$K_w = \left[H_3O^+\right]\left[OH^-\right]$$

Nearly at 25 °C, $K_w = 1 \times 10^{-14}$ mol^2 dm^{-6} (i.e., 1×10^{-14} M^2). This may seem rather pointless at a first glance, but it is from here that one can conclude that in pure water, pH value is 7. In pure water one ion of H_3O^+ is formed for each OH^-, therefore:

$$\left[H_3O^+\right]_{eq}\left[OH^-\right]_{eq} = 1 \times 10^{-14}\,M^2 \Leftrightarrow$$
$$\left[H_3O^+\right]^2_{eq} = 1 \times 10^{-14}\,M^2$$
$$\left[H_3O^+\right]_{eq} = 1 \times 10^{-7}\,M$$

Hence:

$$\left[H_3O^+\right]_{eq} = 1 \times 10^{-7}\,M \Leftrightarrow$$
$$-\log\left[H_3O^+\right]_{eq} = 7 \Leftrightarrow$$
$$pH = 7$$

So, in pure water, at temperature nearly 25 °C, the pH is 7.

The cytoplasm of cells is approximately at pH 7, although the pH could vary in certain cellular organelles. In the human body as a whole, the pH values of different environments are very diverse, from the extremely acidic gastric juice to the basic intestinal lumen medium (Fig. 2.8).

Other kinds of reactions such as addition elimination or nucleophilic substitutions are also very frequent, but acid–basic will continue to be the focus of our attention for the importance the control of pH has in homeostasis. Variations in pH cause variations in the protonation/deprotonation of proteins and other biological molecules, which in turn affect their function. Take the example of enzymes: protonation or deprotonation of chemical groups on the structure of the protein

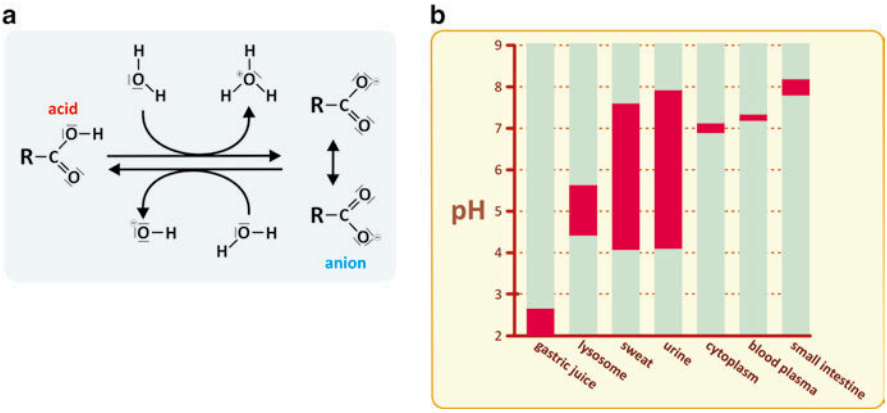


Fig. 2.8 (**a**) Protonation (right to left) and deprotonation (left to right) of a carboxylic acid. The pH of the medium depends on this kind of reactions. (**b**) A high variety of environments, having different pH can be found in the human body

causes variation in charge, leading to new sets of attractions and repulsions between different parts of the molecule that may cause that some segments of the protein contract and others become looser, impairing or facilitating the optimal function of enzymatic catalysis (this will become more clear in Sect. 3.3, where we address the structure of proteins in detail). The same impact on molecular structure and function applies for other biological molecules, such as polysaccharides. Therefore, stabilizing pH in order to guarantee proper structure and function of biological molecules is very important. This is not saying that the pH should be the same in all tissues or in all cells of the same tissue or in all organelles of the same cell. pH is actively controlled in different anatomical, histological, and cellular environments, but it is not the same in all cases. Blood plasma pH, for instance, is very strictly controlled and only allowed to vary in a very restricted range around 7.4. This is not surprising because the efficiency with which hemoglobin transports O_2 is very much dependent on pH. Nevertheless, CO_2, which is a molecule that has the potential to largely impact on pH (see next reaction scheme), diffuses freely in the blood.

$$CO_2 + H_2O \rightleftarrows H_2CO_3 \rightleftharpoons HCO_3^- + H^+, H^+ + H_2O \rightarrow H_3O^+$$

Then, how is it possible for our body to cope with CO_2 diffusion and still maintain a blood plasma pH tightly controlled, centered at pH 7.4? The answer resides on a deceivingly simple mechanism of pH control named "pH buffering" (Fig. 2.9).

pH buffers are no more than mixtures of weak acids or weak bases with their conjugated bases or acids, respectively. In practice, an aqueous solution of a weak acid or weak base in equilibrium is a pH buffer because these chemical species dissociate to a moderate extent forming mixtures that are pH buffers. The reason why these mixtures work in a way to maintain pH constant is related to the basic

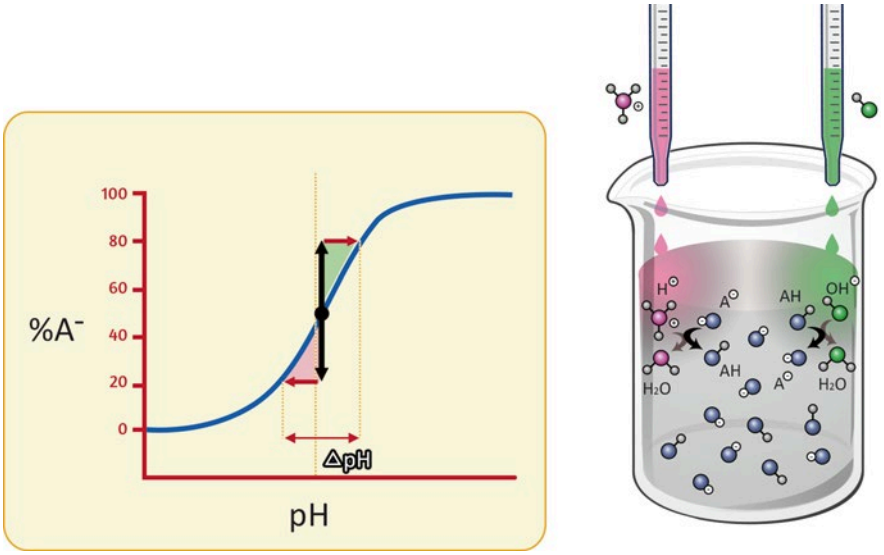


Fig. 2.9 How a pH buffer works. A pH buffer solution is a mixture of a weak acid (HA) with its conjugated base (A^-) or vice versa. When an acid is added to the solution, the deprotonated species (A^-) reacts with the added acid to form the protonated form (HA). Part of the H_3O^+ is thus consumed and the pH drop is thus attenuated. When the added solution is a base, part of the OH^- is consumed by reaction with HA, the acidic form of the buffer, and the rise in pH is thus attenuated. Additions of significant amounts of acids or bases to buffered solutions result in modest variation in pH as long as protonated (HA) and deprotonated (A^-) buffer species coexist

principles of chemical equilibrium. Consider a generic weak acid, represented by HA for the sake of simplicity, in aqueous solution:

$$HA + H_2O \rightleftharpoons A^- + H_3O^+$$

$$K_{eq} = \frac{\left[A^-\right]\left[H_3O^+\right]}{\left[HA\right]}$$

Upon the addition of a strong acid, HA′ (by definition strong acids have nearly complete dissociation), the concentration of H_3O^+ raises:

$$HA' + H_2O \rightarrow A'^- + H_3O^+$$

The newly formed H_3O^+ have an impact on the equilibrium of the weak acid, which will progress in the reverse order (formation of HA) in order to consume part of the H_3O^+. The extent of this consumption of H_3O^+ can be calculated based on the equilibrium constant, which remains unaltered: the concentrations of H_3O^+ and HA increase and the concentration of A^- decreases down to the point where K_{eq} is kept (see Box 2.1).

Box 2.1: pH Buffers and the Origin of the Henderson–Hasselbalch Equation

The initial situation, before strong acid addition is:

$$HA + H_2O \rightleftharpoons A^- + H_3O^+$$

$$K_{eq} = \frac{\left[A^-\right]\left[H_3O^+\right]}{\left[HA\right]}$$

After strong acid (HA′) addition $[H_3O^+]$ raises:

$$HA' + H_2O \rightarrow A'^- + H_3O^+$$

But HA dissociation equilibrium is the same in both cases, so:

$$K_{eq,i} = K_{eq,f} \Leftrightarrow \frac{\left[A^-\right]_i\left[H_3O^+\right]_i}{\left[HA\right]_i} = \frac{\left[A^-\right]_f\left[H_3O^+\right]_f}{\left[HA\right]_f} \Leftrightarrow$$

$$\frac{\left[H_3O^+\right]_f}{\left[H_3O^+\right]_i} = \frac{\left[A^-\right]_i}{\left[HA\right]_i} \Big/ \frac{\left[A^-\right]_f}{\left[HA\right]_f}$$

(i and f stand for the initial and final equilibria, respectively.)

This equation shows that as $\frac{\left[H_3O^+\right]_f}{\left[H_3O^+\right]_i} > 1$, the weak acid equilibrium is perturbed in a way to favor formation of HA, thus consuming H_3O^+. All HA′ is converted to H_3O^+, and in absence of the weak acid, the concentration of H_3O^+ would increase correspondingly. In the presence of the weak acid, part of the H_3O^+ is consumed by A^- to form HA, thus attenuating the drop in pH caused by the strong acid. This is the molecular mechanism of pH buffering (see also Fig. 2.9 for a pictured explanation).

If a strong base is used, the same principle applies, this time with the "intermediation" of the ionic product of water:

$$BOH \rightarrow B^+ + OH^-$$

$$OH^- + H_3O^+ \rightleftarrows 2H_2O$$

Also, if a weak base is used instead of a weak acid, the same buffering capacity exists.

The Henderson–Hasselbalch equation is a very simple and robust way to show how pH is expected to evolve when $[A^-]$ and [HA] are perturbed and change when new equilibria is formed upon the appearance of new acids or bases in solution. The Henderson–Hasselbalch equation

$$pH = pK_a + \log\frac{\left[A^-\right]}{\left[HA\right]}$$

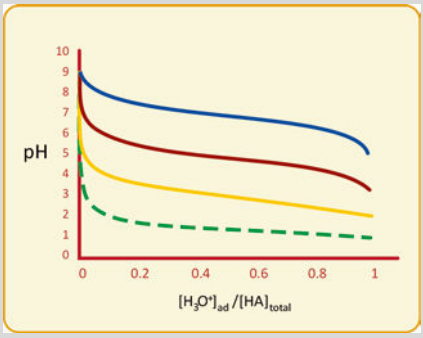
Box 2.1 (continued)

is no more than a simple rewriting of the equilibrium constant (here named K_a to stress it refers to an acid):

$$K_a = \frac{\left[A^-\right]\left[H_3O^+\right]}{\left[HA\right]} \Leftrightarrow \left[H_3O^+\right] = K_a \frac{\left[HA\right]}{\left[A^-\right]} \Leftrightarrow$$

$$-\log\left[H_3O^+\right] = -\log K_a - \log\frac{\left[HA\right]}{\left[A^-\right]} \Leftrightarrow \mathrm{pH} = \mathrm{p}K_a + \log\frac{\left[A^-\right]}{\left[HA\right]}$$

A graphical schematic representation of this equation (see figure) shows that pH varies very little when strong acids or bases are added to buffers, mainly when pH is within the $pK_a \pm 1$ range. This range is centered around the point where $[A^-] = [HA]$, as implied by the Henderson–Hasselbalch equation when $pH = pK_a$. Naturally, the efficiency of the buffer increases with [HA] because higher [HA] imply that more H_3O^+ of OH^- can be added to the solution before $[A^-]$ reaches zero, a point where the buffer mechanism becomes exhausted.



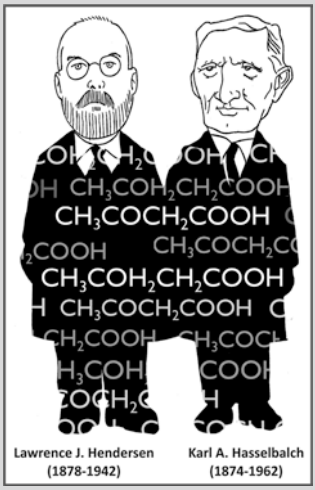
Changes in pH in a buffered solution are much less severe than they would be in the unbuffered solution. Examples of pH variation in a solution initially at pH 9 upon addition of a strong acid, with a total concentration of HA of 0.1 M, when the p*K*a of HA is 7 (*blue*), 5 (*red*), or 3 (*yellow*). The *dashed green curve* indicates the pH drop if HA was absent (unbuffered solution, $0 < [H_3O^+]_{ad} < 0.1$ M). The concentration of the H_3O^+ added to the solution is $[H_3O^+]_{ad}$ and is represented relative to the total concentration of HA used in the buffer ($[HA]_{total}$). The buffering zone (nearly horizontal pH variation lines) is mainly in the pH range that lies within $pK_a \pm 1$, and the buffering capacity (i.e., the limits of $[H_3O^+]_{ad}$ in the buffer zone) depends on $[HA]_{total}$. This example illustrates how big amounts in added H_3O^+ cause modest changes in pH in the range $pK_a \pm 1$. The case in which a base is added to an acidic solution is similar. OH^- concentration raises, which is concomitant with a decrease in H_3O^+, and the situation is symmetrical to the one obtained here (raise in pH instead of drop)

Although the Henderson–Hasselbalch equation is central in understanding fundamental pH buffer chemistry, its historical root is in medicine. Lawrence

(continued)

Box 2.1 (continued)

Henderson was a doctor interested in pH plasma alterations in pathology. He created the concept of acid–base equilibrium. Karl Hasselbalch studied the effect of CO_2 in hemoglobin with Christian Bohr (whom the Bohr effect was named after—see Sect. 3.3.3). The works of both doctors opened the way for the scientific study of respiratory and metabolic perturbation of blood plasma pH equilibrium, a significant breakthrough in the early twentieth century physiology.



2.1.1 Principal Biological Buffers

Generalization of the previously mentioned concepts and equations should be made cautiously for several reasons:

(a) From the point of view of scientific accuracy, activity coefficients should be used in addition to concentrations in all the abovementioned equations. Activity coefficients are used in thermodynamics to account for deviations from ideal behavior in a mixture of chemical substances. For practical reasons, it is assumed that activity coefficients do not alter equilibrium constants in the experimental conditions addressed.

(b) Water dissociates to form H_3O^+; therefore, H_3O^+ ions are always present in aqueous solutions. In the abovementioned equations and reasoning, the ionic product of water was never considered, which is valid in circumstances where the acidic species are present in concentration much higher than those involved in the ionic product of water $\left(\left[H_3O^+\right] = 10^{-7}\,\mathrm{M}\right)$.

(c) We have explored the concept of buffers in situations in which chemical equilibrium exists. This is not always the case in biological situations.

In spite of all these limitations, the general concept of buffer still applies to cells and organs. Equations should be applied judiciously to chemical problems in living systems, but the concept of pH buffer in vivo is still valid. Wherever weak acids or bases are present in cells or body fluids, they contribute to form a buffer. Ions such as $H_2PO_4^- / HPO_4^{2-} \left(pK_a = 6.8\right)$ are very important to buffer the cytoplasmic pH (notice that the pH of cytoplasm is about 7.0, well within the buffering range of $H_2PO_4^- / HPO_4^{2-} = pK_a \pm 1 = 6.8 \pm 1)$ but not plasma. H_2CO_3 / HCO_3^- is much more important to buffer plasma pH. The $–COO^-$ and $–NH_3^+$ groups present in plasma proteins also contribute to pH buffering but not as much as the hydrogenocarbonates $\left(H_2CO_3 / HCO_3^-\right)$: while proteins contribute to 24 % of the buffering capacity in plasma, H_2CO_3 / HCO_3^- contribute with 75 % (the remaining 1% is a modest contribution of $HPO_4^{2-} / H_2PO_4^-$). The prevalence of the H_2CO_3 / HCO_3^- is not surprising because CO_2 diffuses freely in plasma, regardless of a certain ability hemoglobin has to bind CO_2. Carbon dioxide is extensively converted to di-hydrogen carbonate by enzyme carbonate–dehydratase in erythrocytes:

$$CO_2 + H_2O \underset{\leftarrow}{\rightarrow} H_2CO_3.$$

H_2CO_3 is then present in plasma, where it is in equilibrium with hydrogen carbonate (also known under the popular name of "bicarbonate"), HCO_3^-:

$$H_2O + H_2CO_3^- \rightleftharpoons HCO_3^- + H_3O^+ pK_a = 6.1 \left(plasma\right)$$

HCO_3^- exists in plasma with typical concentrations in the 22–26 mM range.

It should be stressed that the H_2CO_3 / HCO_3^- equilibrium in vitro has $pK_a = 3.8$, quite different from the pK_a in plasma (6.1), which is said to be an apparent pK_a because it is under the influence of the enzymatic production of H_2CO_3. To account for the change in pH due to fluctuation in the plasma levels of CO_2, one can apply the Henderson–Hasselbalch equation to the multiple equilibria.

$$\begin{array}{l} CO_2 + H_2O \rightleftarrows H_2CO_3 \\ \underline{H_2CO_3 + H_2O \rightleftarrows HCO_3^- + H_3O^+} \\ CO_2 + 2H_2O \rightleftarrows HCO_3^- + H_3O^+ \end{array}$$

$$K_{a,apparent} = \frac{\left[HCO_3^-\right]\left[H_3O^+\right]}{\left[CO_2\right]}$$

The water concentration is constant and is thus incorporated in $K_{a,apparent}$; the variables are on the right-hand side of the equation, and the left-hand side of the equation is constant. It would be an unnecessary complication to have $[H_2O]$, which is constant, in the right-hand side of the equation.

Now the question arises as to what is the most appropriate way to express $[CO_2]$ because, at the pressure of 1 atmosphere and usual temperatures, CO_2 is a gas and molar units are not well suited for gases. $[CO_2]$ should then be converted to partial pressure, pCO_2:

$$\left[CO_2\right] = 0.03 \times pCO_2$$

for $[CO_2]$ in mM and pCO_2 in mmHg. Thus:

$$K_{eq,app} = \frac{\left[HCO_3^-\right]\left[H_3O^+\right]}{0.03pCO_2} \Leftrightarrow$$

$$pH = 6.1 + \log \frac{\left[HCO_3^-\right]}{0.03pCO_2}$$

This equation helps by anticipating the drop in pH when pCO_2 increases, but it should be emphasized that an increase in pCO_2 also causes an increase in $\left[HCO_3^-\right]$, mitigating the impact of the fluctuation of plasma levels of CO_2 in pH.

2.2 More than Only Chemistry: There Is Physics Too

Given the importance of chemical reactions in cells and in the organisms as a whole, one tends to forget the importance of physical processes in life. They are also important to understand life at the molecular level. Apart from all the issues related to the solubility of molecules in aqueous media, which were discussed in the beginning of this chapter, the interaction of light and biomolecules, for instance, is of utmost importance. This is clear when studying the vision of animals, the photosynthesis in plants or the bioluminescence of microorganisms, just to name a few examples. Regarding human biochemistry in the health sciences field, the topics of election are the early events of vision and the effects of ultraviolet (UV) light in tissues such as skin and hair. Other topics, such as the effects of radioactivity and other nonoptical radiation on humans, are left out of this book, although they are interesting and very relevant in particular situations.

When radiation interacts with matter, different kinds of phenomena may occur, such as absorption, scattering, or diffraction. Absorption implies that the energy of radiation matches the energy needed to change molecular states. This means that the energy of radiation is used in the change of the molecular configuration of nuclei and/or electrons so that the radiation extinguishes in the process (it is "absorbed"). This happens when a photon enters our eyes and triggers a radical change in the conformation of retinol in the retina, thereby initiating the physiological process that ends with visual perception. Scattering relates to the radiation that interacts with molecules and is not absorbed, but causes the molecules to emit secondary

radiation, having the same energy or not. This is what happens when light impinges on matter and is not absorbed: the radiation causes oscillations on the electronic cloud of molecules that in turn causes simultaneous emission of radiation, the scattered light. Scattering is responsible for the white color of milk, blue color of the sky, or reddish color of the sunset. Excessive light scattering in human eyes causes blurring in vision. Diffraction is a particular kind of scattering that occurs when radiation impinges on matter that has voids of size comparable to wavelength of the radiation. X-rays, for instance, are diffracted by molecular crystals because the distances between the nuclei, the chemical bonds length, are similar to its wavelength, i.e., in the order of tenth of nanometer. The spatial pattern of diffracted X-rays can be analyzed to reveal the 3D structure of crystallized molecules, even if they are quite big and complex, such as proteins (Fig. 2.10).

The complete range of frequencies (i.e., energies) of radiation known so far constitutes the so-called electromagnetic spectrum (Fig. 2.11a). It includes low-energy (low frequency, long wavelength) radiation, such as radio and television radiation, as well as high-energy (high frequency, short wavelength) radiation, such as X- and gamma rays, capable of ionizing molecules and disrupting covalent bonds. Very low-energy radiation hardly causes changes in molecules and therefore is not prone to produce effects in living organisms. High-energy radiation (starting from the far, high-frequency, UV radiation) is able to disrupt molecules by destroying chemical bonds and is therefore a potential hazard to the chemistry of life (an amazing exception is presented in Box 2.2). It is not surprising, therefore, that natural phenomena in living beings involving radiation occur in a limited range of energies, from microwaves to near (lower frequencies) UV. Even so, one has to wonder why many animal eyes, including human, uses a much smaller fraction of radiation, from about 400 nm (blue light) to about 800 nm (red light). The answer is simple and can be found in molecular evolution toward the optimized use of environmental resources: the 400–800 nm range accounts for the most abundant sun radiation reaching the surface of planet Earth (Fig. 2.11b). In addition, this radiation penetrates water down to tenths of meters. Naturally, this dictated the course of evolution of vision. The same happened with photosynthetic organisms.

In the human eye, rhodopsin, a membrane protein (opsin) that exists in retina rod cells, has a covalently bound retinal molecule, which adopts two stable isomeric forms: *cis* and *trans* (Fig. 2.12). When the protein-attached *cis*-retinal residue absorbs light, it converts to *trans*-retinal, this being the triggering event of vision. Not only retinal changes in shape, from a bent structure to a nearly linear arrangement, but it also detaches from opsin. All-*trans*-retinal (i.e., the "linear" retinal, with all double bonds in the *trans*-configuration) does not fit the pocket formed by the opsin transmembrane helices as the *cis* isomer does, causing tensions in the protein structure, which has to change conformation (shape) to adapt. A reduction in cyclic GMP results from this process, which in turn triggers a series of reactions that cause an electric current along the cell. The intensity of this current is proportional to the intensity of light that reached the retina. So, an electric impulse is created that is detected by ganglion cell and then the optic nerve. Nerve fibers reach the

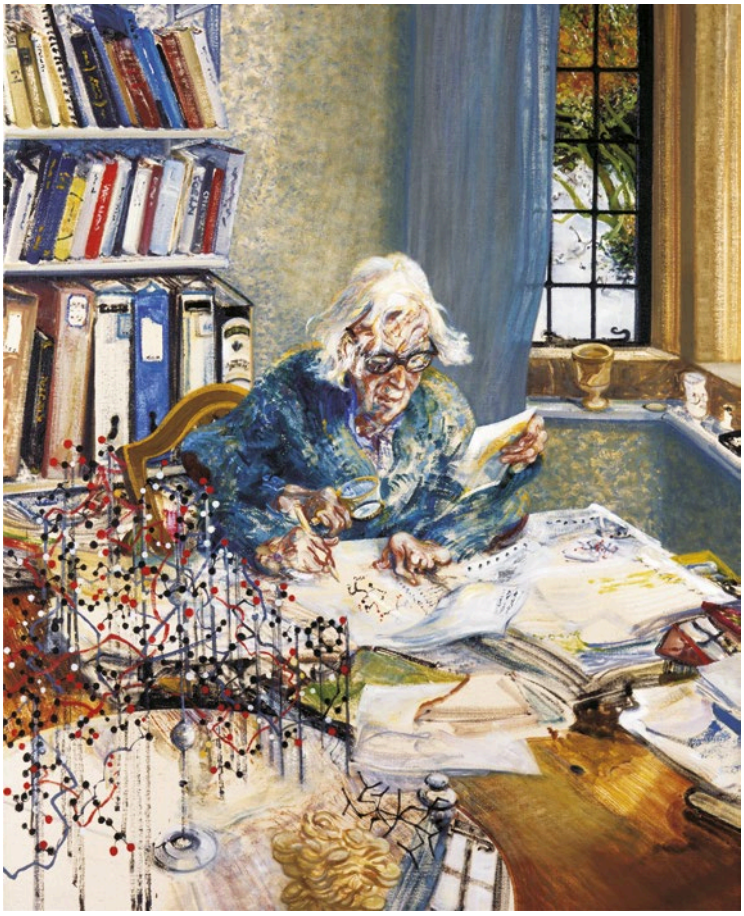


Fig. 2.10 Dorothy Mary Crowfoot Hodgkin in her study at home portrayed by Maggi Hambling (oil on canvas, 1985). The four-handed scientist representation symbolizes her unusual working and entrepreneurial capacity. Dorothy Hodgkin made extremely valuable contributions to the advancement of biochemistry. She revealed the structure of cholesterol, penicillin, insulin, and vitamin B_{12}, among other molecules. She also contributed to the refinement of X-ray diffraction spectroscopy to unravel the structure of proteins. A structural model of insulin is on the *left*. Reprinted with the permission of the National Portrait Gallery, London, UK

back of the brain (occipital lobe), where images are created. This region is called the primary visual cortex. Some of the visual fibers extend to other parts of the brain to help to control eye movements, the responses of the pupils and iris, and behavior.

The effects of UV light on the skin and hair constitute other examples on how the radiation–matter interaction is important in natural processes. In practical terms, UV is divided in three categories, A to C, according to their biological effects. UV-A is the lowest energy class (320–400 nm). UV-C is the highest energy range (200–290 nm) and is, to a great extent, filtered by the ozone layer in the atmosphere.

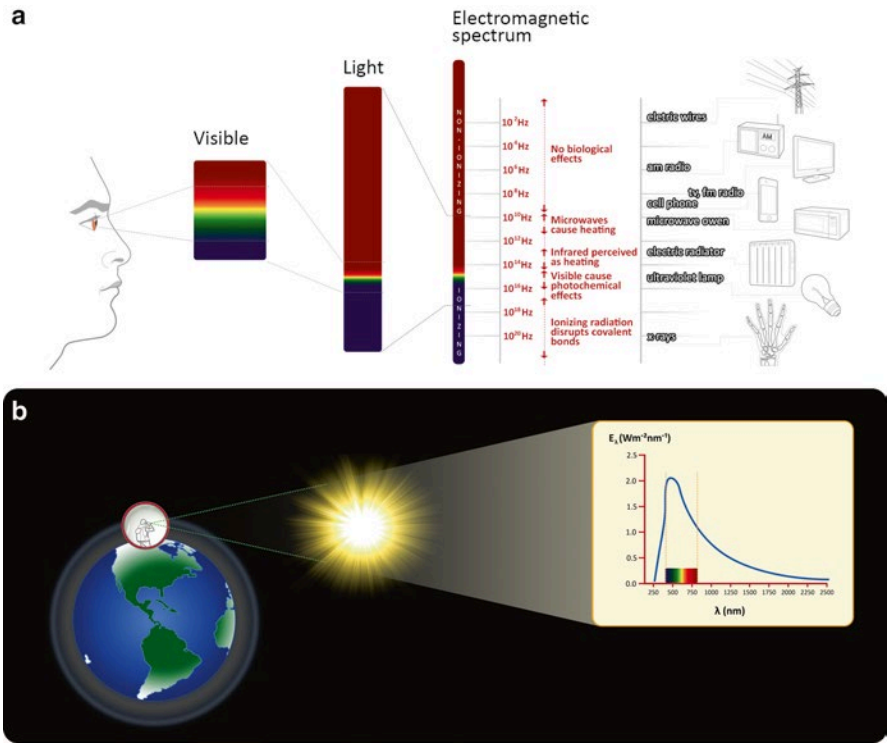


Fig. 2.11 (**a**) The electromagnetic spectrum with the typical frequencies of radiation, devices that use them, and their typical biochemical/physiological effects. The narrow range of optical radiation (in practical terms, the radiation that can be conducted using lenses and mirrors), to which human eyes are sensitive, is highlighted. (**b**) Spectrum of total solar radiation power that reaches Earth surface. The maximum power range is coincident with optical radiation one calls "visible light," the radiation many animal eyes, including human, have adapted to. This radiation penetrates water down to tenths of meters, which was also important for the evolution of vision

A part of the UV-B radiation is also absorbed by ozone. Having sufficient energy to break chemical bonds in the nucleic acids of exposed cells in the skin layers near the surface, UV-C and, to a less extent, UV-B are serious health hazzards regarding skin tumors, mainly in prolonged exposure to sun and other UV sources. Whenever the ozone layer is threatened, the risk of skin cancer raises. Nevertheless, the immediate, most noticeable effects of UV-B are erythemas, such as those associated to "sunburns" in short-term solar overexposure. Being moderate to high-energy radiation, UV-C and UV-B interfere with many molecular processes. The consequence of this is that their penetrance is shallow in the skin (they are totally absorbed in the outer layers of skin—epidermis and nearby dermis; Fig. 2.13), where they are responsible for some naturally occurring biochemical processes, such as the conversion of the amino acid tyrosine in melanin (a sun radiation–protection pigment that colors the skin), but also by some lesions such as the detachment of epidermis from dermis ("sunburn blisters"). UV-A penetrates deeper in the skin because it is not so

Box 2.2: The Toughest Cell on Earth

In 1956 Arthur Anderson, working in Oregon (USA), was studying if canned food could be sterilized using high doses of gamma radiation. Despite the fact that he was using doses enough to destroy all forms of life known to man to sterilize meat, the food subsequently spoiled. A bacterium, *Deinococcus radiodurans*, was isolated and found to be responsible for this result. Later studies revealed that it is extremely resistant to ionizing radiation of different frequencies, desiccation, and oxidizing agents. While a bacterium like *E. coli* can stand up to 200–800 Gy, *D. radiodurans* can stand up to 5000 Gy. Human cells have much lower tolerance levels: below 5 Gy.

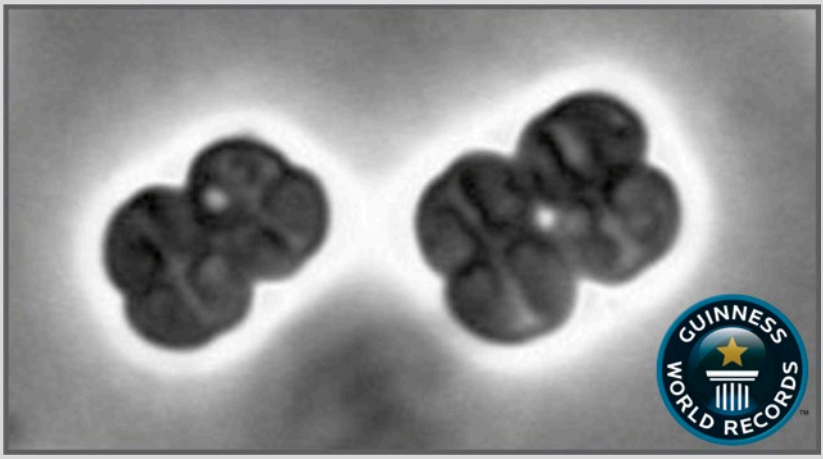
From the chemical point of view, the DNA composition of *D. radiodurans* is not different from that of other organisms. The key to resistance is not the chemical nature of DNA but the efficacy of the repair mechanisms. Radiation interacts with the chemical bonds of the DNA similarly in all cells, causing occasional breakings in these bonds when its energy is high enough. It is impossible for the vast majority of cells to cope with very frequent and simultaneous breaking of the DNA covalent bonds. *D. radiodurans*, however, has its DNA packed in toroids and multiple copies of the genome, usually 4–10. The whole repair machinery enables the reconstruction of the complete genome from shattered bits in hours. Bacterium-to-bacterium transfer of DNA may also play a role in increased resistance.

One interesting question that arises from the amazing properties of *D. radiodurans* is "Could a bacterium like this have developed in a planet where there are no environments with high doses of ionizing radiation?" It has been suggested that the origin of *D. radiodurans* is extraterrestrial because it has acquired resistance to a set of very harsh physical–chemical environmental conditions, like the ones expected in Mars. However, the bacterium is genetically, biochemically, and microbiologically very similar to other bacteria, and there is no other evidence for Martian forms of life. As dehydration and radiation cause very similar types of DNA damage, it is possible that resistance to radiation is a side effect from selective pressure toward resistance to dehydration. *D. radiodurans* is extremely well adapted to dryness.

The remarkable biochemical properties of *D. radiodurans* may make it a powerful ally to clean contaminated radioactive areas, in which the bacterium can survive and operate. This kind of approach is named bioremediation. Radioactive toxic waste would be processed by bacteria that would naturally colonize high risk areas.

Box 2.2 (continued)

The extreme resistance of *D. radiodurans* inspired researchers and science communication professional, who frequently call it "Conan bacterium." In 1998 the Guinness Book of World Records listed *D. radiodurans* the "most radiation resistant lifeform…."



Deinococcus radiodurans (credits: Sandra P. Santos and Célia Romão, ITQB-UNL, Portugal)

energetic, reaching the core of the dermis. It also stimulates the formation of melanin ("suntan"), which provides some protection to the skin against the effects of solar radiation. Nevertheless, contrary to popular belief, the extent of this protection is very limited (Sun Protection Factor, SPF, of about 2 to 4, far below the minimum recommended SPF of 15 for direct skin exposition to sun radiation).

The SPF is determined from the UV exposure time that is needed for the appearance of a minimal erythema (i.e., erythema after 24 h). Specifically, it is the ratio of the time needed for a minimal erythema in protected skin over the time needed in unprotected skin. It is determined indoors with a light source that is meant to reproduce the noontime sun. The SPF is mainly useful to have a quantitative scale of UV-B protection when sunscreens are used. A sunscreen with an SPF of 10 filters 90 % of the UV-B light, for instance (in other words, a person that would have sunburn after a 20 min exposition to sun, using a sunscreen of SPF 10, has the same effect after 200 min). Sunscreen creams, lotion, sprays, gels, or other topical formulations have organic molecules in their composition that absorb UV light. Notice that the most common of these compounds have aromatic rings in their structure (Fig. 2.13), which are groups that absorb UV light, and/or inorganic metallic compounds (zinc oxide, for instance) that scatter UV light (the scattering efficacy is for UV than for visible light).

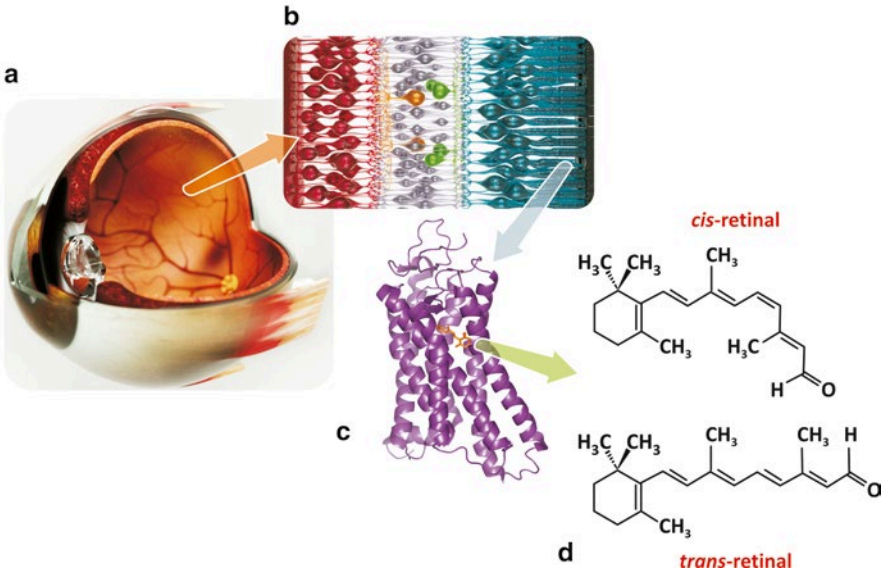


Fig. 2.12 Anatomical cross-section of the eye (**a**), highlighting the retina (**b**), where rod cells are located. Sensory rhodopsin (**c**; PDB 2I35) binds retinal (**d**), which changes conformation with light absorption

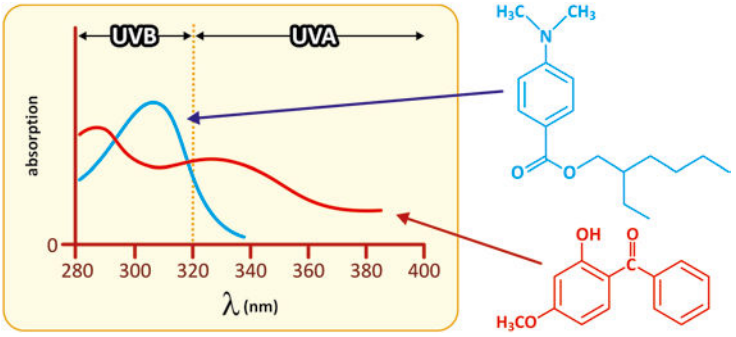


Fig. 2.13 Two UV-absorbing molecules commonly found in sunscreen formulations: octyldimethylaminobenzoate (OD-PABA), in *blue*, and 2-hydroxy-4-methoxybenzophenone (HM-BZP) in *red*. The chemical group responsible for the absorption of radiation ("chromophore") is the aromatic (benzenic) ring, which is present in both molecules and many other components of sunscreens as well

Although the effects of UV radiation on the skin usually monopolize one's attention, the biochemistry involved in the damage caused by UV radiation on other structures such as hair and eyes is worthy of consideration. Hair is an extremely well-adapted cellular organization: it conjugates flexibility and strength. The hair has an external proteic cuticle, an inner core named medulla, and an intermediate

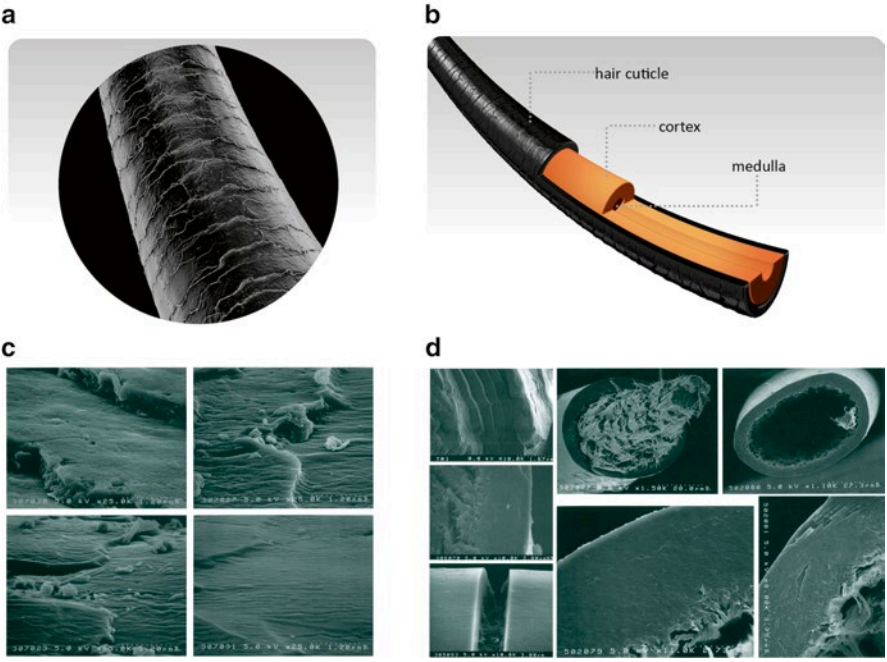


Fig. 2.14 Hair surface as seen under the electronic microscope (**a**) reveals scales. The external layer of the hair shaft, named cuticle (**b**), is formed of proteic plaques that slide over each other due to the lubrication of lipids, which makes the hair flexible. Intense UV exposure leads to "fusion" of the plaques in the whole cuticle (**c**, **d**). "Fused" cuticles lose flexibility and break easily (**d**). Panels (**c**) and (**d**) were reproduced from Ruetsch et al., in *Comprehensive Series in Photosciences* 3:175–205, 2001, with permission from Elsevier

layer called cortex (Fig. 2.14). The cuticle is formed of proteic plaques that are lubricated by the presence of lipids. The plaques are rigid, but lubrication allows flexibility. Exposure to UV light, mainly UV-B, causes "fusion" of these plaques, turning the cuticle in a rigid non-flexible structure as a whole. The hair then becomes prone to breakage. Hair with broken ends loses its natural appearance, and UV protection of the hair is one of the main topics in cosmetic research. Loss of coloration due to oxidation of melanin pigments caused by the UV radiation is another factor that contributes to the loss of natural appearance.

While the solar UV-B radiation can be blocked and prevented from entering and damaging our eyes using glasses (not necessarily using dark lenses because UV rays are colorless, so transparent materials may be efficient in blocking UV light), one should ask ourselves how natural molecular evolution coped with the unavoidable exposure of eyes to UV radiation. The answer lies in the same concept as chemical sunscreens one uses to protect skin. All light enters the eye through the crystalline (also named "lenses," Fig. 2.15). There are natural molecules that absorb UV light that are present in the crystalline. To ensure an efficient filtering of the whole range

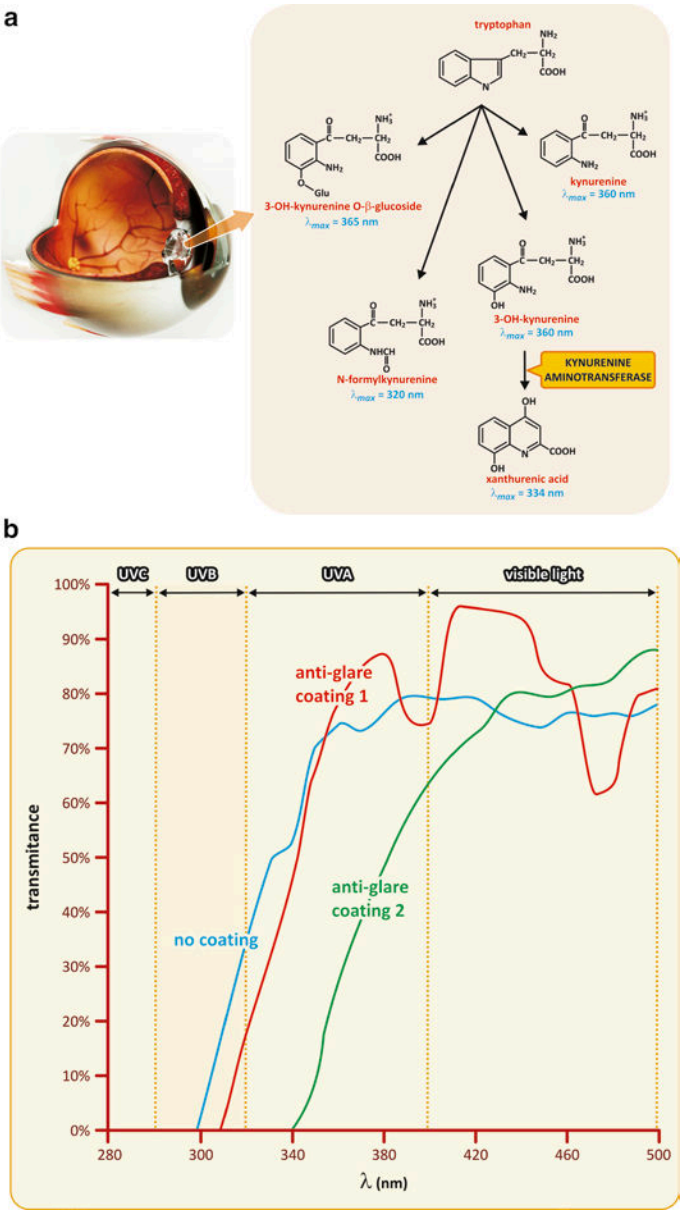


Fig. 2.15 Natural eye preventive protection against UV consists in the presence of a family of tryptophan derivatives in the crystalline (**a**). Each of these molecules absorbs UV light at a slightly different wavelength range. Acting together, they are able to filter a broad range of UV light entering the eye. Wearing glasses may be a form to reinforce the protection of eyes against UV radiation. Depending on the materials used in their manufacture, lenses may absorb UV and/or visible light. Transparent lenses do not interfere significantly with visible light but may filter, at least partially, UV light. Sunglasses having good quality lenses usually filter a substantial fraction of visible light (therefore the term "dark glasses" also used for sunglasses) and most UV light. Panel (**b**) shows the example of light filtering by vitreous material having two different possible coatings (transmittance is the fraction of light intensity that crosses the material)

of UV light, human eyes' molecules evolved to create a family of compounds derived from tryptophan, an amino acid that enters the constitution of proteins. All these molecules absorb UV light at slightly different wavelength ranges. The effect of the sum of all the spectra is a generalized blocking of UV radiation at the entrance of the eye. This is the natural barrier to UV radiation that protects the retina.

It should be stressed that cells have biochemical processes that repair the damage caused by the UV radiation on nucleic acids and other molecules. As this section focuses on light–matter interaction, this subject will not be further addressed, but it is important to keep in mind that even when molecular and cellular lesions occur due to the action of radiation, repair mechanisms exist. Pathological situations arise when both preventive and repair mechanisms fail to meet all the insults radiation exposure imposes.

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Chapter 3
The Families of Biological Molecules

Diversity is essential to the sustainability of living systems. This is true for species in ecosystems as it is for molecules in cells, tissues, and organisms. Yet, the same way different species are linked by common ancestors and may be grouped in taxonomic classes according to common characteristics they share, molecules may be grouped in classes and classified according to common chemical and physical characteristics. One of such characteristics is solubility in water (in other words: how polar atoms are distributed in the 3D structure of molecules). One class of biological molecules, the lipids, includes only low water solubility ("hydrophobic") molecules, this being the characteristic that defines this class. Other classes include molecules that are mostly moderately or highly soluble in water and can be recognized for the dominant presence of specific chemical groups: OH in saccharides (also referred to as "carbohydrates") and a combination of amino and carboxyl groups in amino acids. Lipids, saccharides, and amino acids may combine with molecules of their own class to form either polymers (molecules formed by successively covalently attaching smaller molecules), such as polysaccharides and proteins, or supramolecular assemblies (organized arrangements of molecules that are in contact but are not covalently attached), such as the lipid bilayer of cell membranes. It is common to find molecules and supramolecular assemblies that combine elements from different classes, such as nucleotides, which contain saccharides. Proteins are extremely versatile in this regard because protein interactions with saccharides, lipids, and nucleic acids (nucleotide polymers) are ubiquitous in virtually all cells.

Figure 3.1 depicts the basic principles that support the organization of biological molecules in different classes.

A.T. Da Poian, M.A.R.B. Castanho, *Integrative Human Biochemistry*,
DOI 10.1007/978-1-4939-3058-6_3

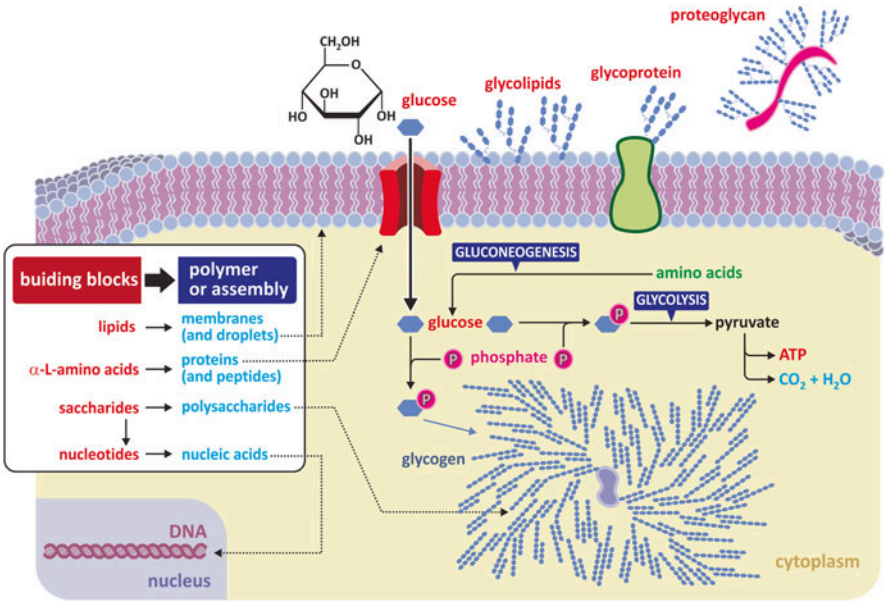


Fig. 3.1 Three classes of fundamental molecules ("building blocks") are enough to organize in different families most biological macromolecules (polymers) and large supramolecular assemblies (such as lipid bilayers and lipid droplets). Nucleic acids are polymers of nucleotides, which have saccharide residues in their composition. Proteins are polymers that are commonly combined with molecular residues of other classes such as saccharides (glycoproteins or proteoglycans when the saccharidic content is high). Glycolipids (glycosylated lipids—lipids with saccharide groups attached) are also common in membranes. Both building blocks and polymers/assemblies are important in the structure and functioning of cells, constituting the vast majority of matter in a cell (excluding water). Glycogen is a branched saccharide polymer (polysaccharide). Branching occurs at every 10 glucose monomer residues approximatly (branching unrealisticly highlighted in the figure for illustrative purposes)

3.1 Lipids and the Organization of Their Supramolecular Assemblies

Lipids are highly hydrophobic molecules that nonetheless may have polar chemical groups in their composition. Because one portion of the structure of the molecule is polar and the other is non-polar, the molecule is referred to as amphiphilic, which stresses its dual nature: the polar part will tend to interact with water and other polar molecules, and the other will tend to minimize its interaction with water and other polar molecules. Nevertheless, it is important to bear in mind that lipids are molecules in which hydrophobicity predominates, even if they are amphiphilic. This is a qualitative definition with no clear boundaries in terms of molecular structure, which is nonetheless a useful working definition because hydrophobicity grants lipids the ability to organize in supramolecular assemblies that are very distinctive from polar molecules. Take lipid bilayers as example: they are very extensively

organized supramolecular assemblies that are very stable and yet do not involve covalent bonds between lipid molecules. Lipids spontaneously self-associate in aqueous environments, and amphiphilic lipids in particular may self-associate in a very organized way. This results from the so-called hydrophobic effect, although the most appropriate term would be "entropic effect."

The entropic effect is a corollary of the second law of thermodynamics, which in one of its possible statements implies that all physical and chemical events tend to evolve in a way so that total entropy ("disorder") increases. Consider Fig. 3.2; strongly amphiphilic molecules of generic cylindrical or rectangular cuboid shape will spontaneously form a bilayer to minimize the contact of nonpolar regions with water. The driving force for this event may be counterintuitive at first glance: the bilayer is the arrangement that corresponds to the most disordered system. This may seem absurd because we tend to focus our attention in the solute (the lipids, in this case) and forget the solvent (water); yet, the gain in entropy refers to both. The lipids become ordered relative to each other, but the contact of hydrophobic groups with water molecules imposes restrictions to the orientational freedom of water, which is very costly in terms of entropy.

Broadly speaking, diacyl lipids (i.e., lipids containing two acyl—aliphatic—chains) with polar "heads", such as most of the lipids found in cell membranes, have a generic shape with the characteristics depicted in Fig. 3.2, and the entropic effect

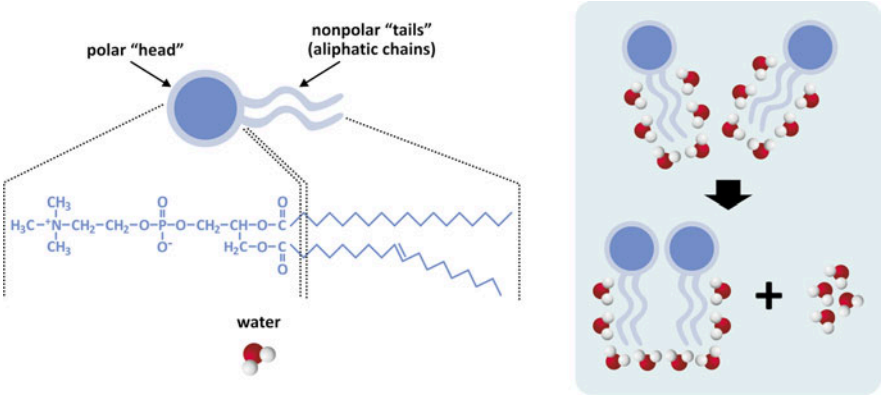


Fig. 3.2 Simplistic representation of two molecules of lipids having two aliphatic (hydrocarbon) chains and a polar "head." Polar heads usually contain phosphate (phospholipids) and other polar groups (*left panel*). Nonpolar regions of lipids tend to associate to each other because fewer molecules of water get exposed to the aliphatic chains (*right panel*). Exposed aliphatic chains force molecules to orient their oxygen atom away from the hydrocarbon constituents, i.e., they force a certain degree of order to the solvent. When two lipid molecules associate, less water molecules are forced to order and although the lipid molecules become more ordered relative to each other, the whole molecular systems (water included) becomes more disordered, in agreement with the entropic formulation of the second law of thermodynamics. Thus, this is named the entropic effect (occasionally imprecisely referred to as "hydrophobic effect"). The same principle applies when 3, 4, 5, …, *n* molecules form large assemblies of lipids, such as lipid bilayers (see Sect. 3.1.1)

compels these molecules to an ordered and parallel self-association. This is the physical process that sustains the stability of cell membranes as we shall see in Sect. 3.1.1. Now we will look more closely into the chemical structure of diacyl lipids: although these lipids tend to assemble as bilayers, the differences in their chemical nature determine which chemical processes they are able to participate in (e.g., cell signaling) and their tendency to associate to membrane proteins and/or form specific domains in the membrane. Most triacyl lipids occurring in nature, such as triacylglycerols (often referred to as triglycerides), lack a bulk polar "head" group and therefore do not display the propensity to form ordered supramolecular structures such as membranes (Fig. 3.3). Instead, they self-associate disorderly, forming aggregates, such as the lipid droplets found in cells at the site these lipids are synthesized (hepatocytes) or stored (adipocytes). Figure 3.3 shows histological images of the adipose tissue in which the presence of lipid aggregates occupying almost all cellular volume is detected.

Fatty acids can be considered as the basic unit common to most lipids in human cells. They consist of a linear unbranched aliphatic chain ("tail") with a carboxyl group (Fig. 3.4a). The aliphatic chain can be saturated (i.e., having all the carbon–car-

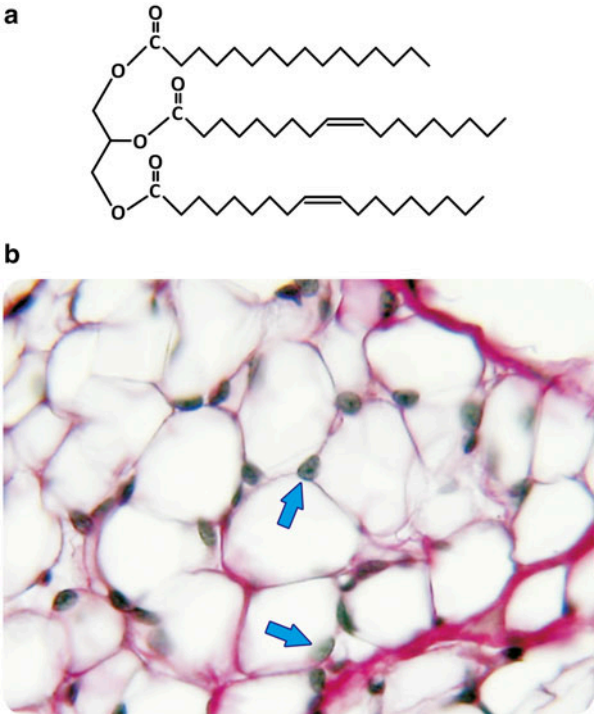


Fig. 3.3 Triacylglycerols lack bulk polar "head" groups, which keeps them from self-assembly into bilayers (**a**). Triacylglycerols aggregate in disordered agglomerated structures that can be detected in histological preparations of adipocytes (**b**). In panel (**b**) the lipids were extracted leaving white empty areas; arrows point to the nucleus. Figure reprinted with the permission of Instituto de Histologia e Biologia do Desenvolvimento, Faculdade de Medicina, Universidade de Lisboa, FMUL

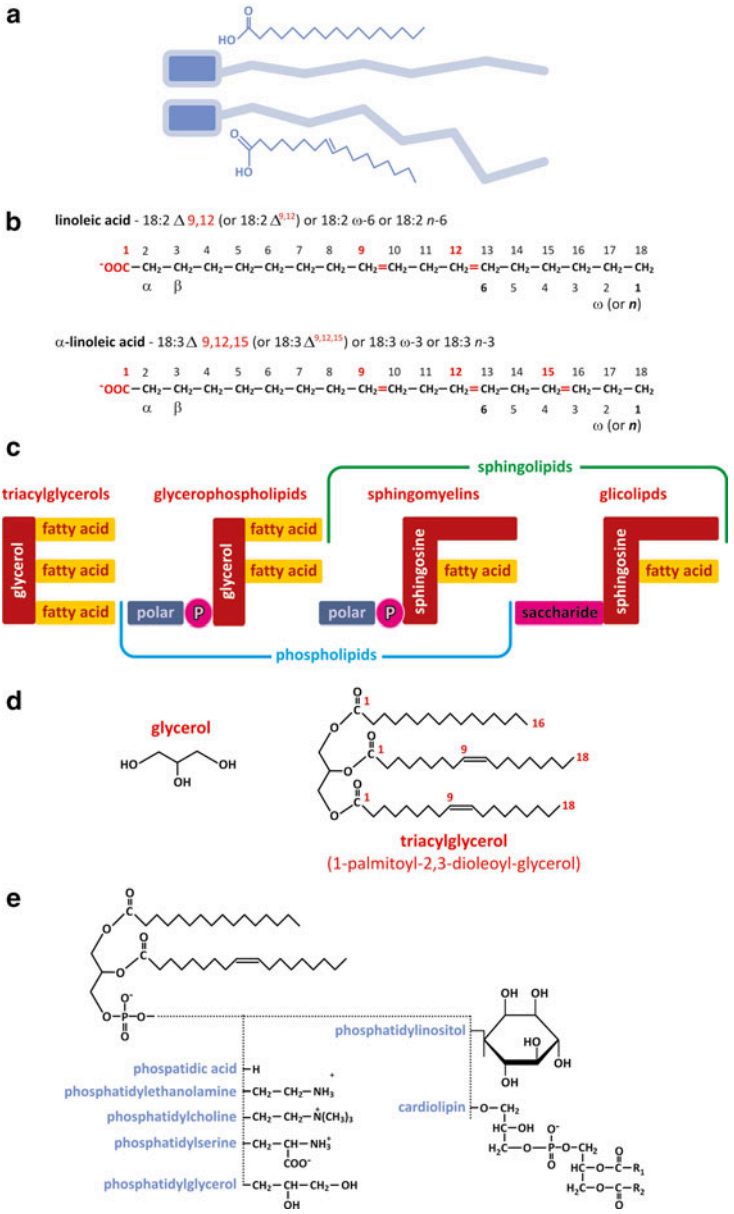


Fig. 3.4 (**a**) Pictoric representation of free fatty acids, highlighting the polar carboxylic group in *blue* and the nonpolar aliphatic chain in *gray*; double bonds impose constraints in the conformation of the chain, preventing stretching and rotation around the carbon skeleton, for instance. (**b**) Illustrative examples of the application of different nomenclature systems. Recent systems (numbering carbons from the carboxylic group) are more precise and descriptive, but older systems (Greek lettering and *ω*—numbering from the non-carboxylic ending carbon) are still widely used in some disciplines as nutrition. Trivial (nonsystematic) names (Table 3.1) were never abandoned for their "user-friendly" nature. (**c**) Fatty acids form ester bonds with other molecular structures to form more complex lipids such as triacylglycerols [detailed example in panel (**d**)], glycerophospholipids, or sphingolipids. Fatty acid residues are present in all. In sphingomyelins the polar group attached to phosphate is either choline or ethanolamine [panel (**e**)]. The example of specific triacylglycerol in (**d**) is 1-palmitoyl-2,3-dioleyl-glycerol. (**e**) Glycerophospholipids form different classes depending on the chemical nature of the "polar head" (fatty acid chains in this example are illustrative)

bon bonds reduced to simple bonds, without double or triple bonds) or unsaturated. Most fatty acids in humans have an even number of carbons, ranging from 10 to 28. Fatty acids are important molecules in the energetic metabolism of the organism, and they can be found either in "free" form (unattached to other molecules) or attached to other molecular structures through ester bonds (Fig. 3.4d, e). Free fatty acids differ from each other in the number and position of double bonds they have and in the length (in practice, the number of carbons) of the chains. The diversity attainable by changing these characteristics is virtually infinite, but, in practice, not all combinations are detected in nature (Table 3.1 displays examples of the most frequent fatty acids found in the human cells). Naturally, the nomenclature of fatty acids highlights their characteristic differences in terms of number and position of double bonds and chain length (Fig. 3.4b). The most recent nomenclature systems are very descriptive of these characteristics, although older, traditional nomenclature systems are widely used. The recent nomenclature identifies the carbon atoms in a chain by number, starting consecutively from the carbon of the carboxyl group (see Fig. 3.4b). Double bonds, if existent, are identified by the number of the carbon of lowest order forming the bond. The ω, or n, nomenclature numbers the carbons in reverse order (see Fig. 3.4b). Another nomenclature, still popular among many biochemists, identifies carbons by Greek letters in alphabetical order (α, β, γ, δ…) starting from the carbon adjacent to the carboxyl group (see Fig. 3.4b). A process of metabolic degradation of lipids is titled "β-oxidation" because of the importance of carbon β.

Glycerolipids such as diacylglycerols and triacylglycerols result from the chemical conjugation of a glycerol molecule with fatty acids through sterification (Fig. 3.4). Yet, glycerolipids may also conjugate other groups besides fatty acids. Usually a phosphate group is used to bridge glycerol to a polar group, forming glyc-

Table 3.1 Nomenclature of the most common fatty acids found in humans, both saturated (only simple carbon–carbon bonds) and unsaturated

Number of *C* (chain length)	Saturated	Unsaturated	Number double bonds	Nomenclature system		
				Δ	ω	n
14	Myristic					
16	Palmitic	Palmitoleic	1	16:1 Δ9	16:1 $\omega-7$	16:1 $n-7$
18	Stearic	Oleic	1	18:1 Δ9	18:1 $\omega-9$	18:1 $n-9$
		Linoleic	2	18:2 Δ9,12	18:2 $\omega-6$	18:2 $n-6$
		α-Linoleic	3	18:3 Δ9,12,15	18:3 $\omega-3$	18:3 $n-3$
		γ-Linoleic	3	18:3 Δ6,9,12	18:3 $\omega-6$	18:3 $n-6$
20	Arachidic	Arachidonic	4	18:4 Δ5,8,11,14	18:4 $\omega-6$	18:4 $n-6$
24	Lignoceric					

There are trivial names and descriptive abbreviations. Trivial names usually refer to the neutral (protonated) form of the acid, but one should bear in mind that at most physiological pHs (including plasma pH, 7.2–7.4), the acidic groups are unprotonated (pH above 4–5). Abbreviated nomenclatures highlight the number of carbon atoms in the aliphatic chain and number and position of unsaturated carbon–carbon bonds. Carbon numbering varies depending on adopting modern or older (ω, n) systems. Older systems are still widely used in some fields such as nutrition

erophospholipids (Fig. 3.4). The phosphate binds both the glycerol and the polar group through phosphodiester bonds, which are ubiquitous in biochemical processes for their importance in molecular structure and energetics.

Glycerophospholipids are named according to the fatty acids attached to glycerol and the chemical nature of the polar group (Fig. 3.4e).

Sphingolipids constitute another class of lipids present in many human cells, being responsible for rigid domains in the membranes. Figure 3.5 shows that although these lipids are similar in structure to glycerophospholipids, they are chemically different. These differences are responsible for the formation of rigid bilayers when mixed with phospholipids and cholesterol.

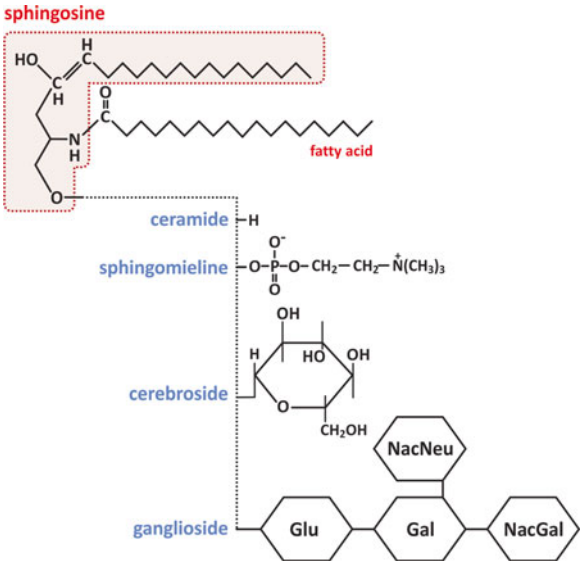


Fig. 3.5 Molecular structure of sphingolipids. As with glycerophospholipids, sphingolipids form different classes depending on the chemical nature of the "polar head" (the fatty acid chain in this example is illustrative). Glu - glucose, Gal - galactose, NacGal - N-acetylgalactosamine, NacNeu - N-acetylneuraminic acid

Sterols, of which cholesterol in an example (Fig. 3.6), are extremely hydrophobic alcohols. These molecules are characterized by a flat rigid system of carbon rings, cyclopentaphenanthrene, and have some remarkable properties:

1. Only one small polar group present (OH in cholesterol; C=O in the equivalent ketones).
2. A very flat molecule formed of four coplanar rings.
3. When placed in lipid bilayers, it acts as a fluidity regulator (not too rigid to compromise dynamics and not too fluid to compromise barrier integrity).

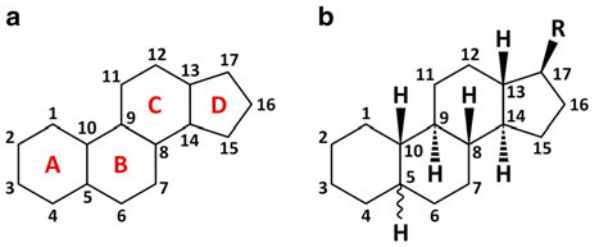


Fig. 3.6 Cyclopentaphenanthrene [panel (**a**)] is the basic structure of steroids [panel (**b**)], in which R is a generic group. The numbering system of carbons is highlighted. Cyclopentaphenanthrene is extremely rigid, planar, and hydrophobic. Sterols are a subfamily of steroids having an alcohol group in carbon 3 (see Table 3.2). Sterols can be found in the lipid membranes of animals, fungi, and plants but not bacteria. Sterols are the targets of some anti-infectious drugs in humans (this issue is readdressed in Fig. 3.9)

Cholesterol is a vital molecule to humans (Box 3.1). In addition to its properties in membranes, it takes part in other biochemical phenomena such as the synthesis of some hormones. Nevertheless, the seriousness of problems associated to its ingestion in excessive amounts in unhealthy diets, associated to its very low solubility in aqueous media, turns this important molecule into a potential killer. Curiously, although cholesterol is essential to many species in nature, including humans (Table 3.2), its synthesis is relatively recent in evolutionary terms as it uses molecular oxygen, which was only present in atmosphere after the advent of photosynthesis.

Box 3.1: Cholesterol: A Hero with Bad Reputation
High concentration ("level") of cholesterol circulating in the blood is a major risk factor for cardiovascular disease, which affects a significant fraction of the whole population in many countries. The campaigns to prevent cardiovascular disease are often centered in reducing dietary cholesterol, which gives the impression that cholesterol is some kind of poison or toxin that should be banned. The persistent unhealthy doses of cholesterol in diet are indeed harmful, but this must not create the illusion that cholesterol is by itself harmful to cells. Virtually all molecules in excessive doses are harmful and cholesterol is not an exception. Moreover, human body is so dependent on cholesterol that it synthesizes its own cholesterol and has homeostatic mechanisms to regulate its production in connection with several metabolic processes.

(continued)

Box 3.1 (continued)

The way the human body works at biochemical and physiological levels is crucially dependent on cholesterol. This extremely hydrophobic molecule intervenes in three major processes in humans:

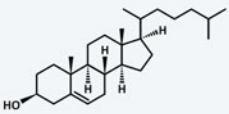
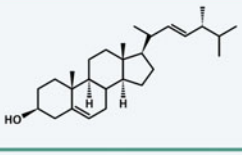
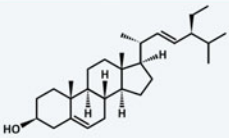
(a) It contributes to the balance of physical–chemical properties of lipid membrane dynamics in cells; this includes the plasma membrane and the intracellular membranes.
(b) It is a part of the bile acid synthesis; bile acids are important to absorb the lipids existing in food after ingestion.
(c) It is also a part of the synthesis of vitamin D and hormones as important as estrogen in women and testosterone in men.

Because of its hydrophobicity, the solubility in aqueous media such as blood is very low. There are specialized structures, lipoproteins, which incorporate cholesterol and form stable emulsions in aqueous medium. When cholesterol is not contained in these structures and/or lipoproteins deteriorate due to oxidation, depots are formed in the endothelial tissue that forms blood vessels. These depots are typical of atherosclerosis. In the most extreme cases, the blood vessels may be blocked and neighboring tissues are not irrigated. Lack of nutrients and oxygen (ischemia) may cause severe lesions in tissues. Vulnerable atherosclerotic plaques may also detach and clog other vessels. Any of these conditions is called infarction. Myocardial infarction, commonly known as a heart attack, is one example. Cerebral infarction is another example, commonly known as stroke.

Different lipoproteins transport cholesterol to different destinations (see Sect. 3.1.2). Cholesterol associated to HDL (high-density lipoproteins) can be disposed to form bile acids, whereas the cholesterol associated to other lipoproteins such as LDL (low-density lipoproteins) cannot. This has been referred to as good ("HDL-associated") and bad ("non-HDL-associated") cholesterol. The distinction simplifies medical communication to lay audiences but is absolutely nonsense from the biochemical point of view. Cholesterol synthesis, distribution, and transformation can be considered a whole and interconnected process in the human body.

The most eloquent demonstration of how cholesterol is indispensable is the Smith–Lemli–Opitz syndrome. This is a rare disease characterized by failure to thrive; mental retardation; visual problems; physical defects in hands, feet, and/or internal organs; increased susceptibility to infection; and digestive problems, among others. Smith–Lemli–Opitz syndrome is a genetic disease caused by a defect in cholesterol synthesis, namely, deficiency of the enzyme 3β-hydroxysterol-Δ7-reductase, the final enzyme in the sterol synthetic pathway that converts 7-dehydrocholesterol (7DHC) to cholesterol (the complete pathway of cholesterol synthesis is described in Box 8.8). This results in low plasma cholesterol levels and elevated levels of cholesterol precursors, including 7DHC.

Table 3.2 Naturally occurring sterols

name	structure	occurrence
cholesterol		animals
ergosterol		fungi
sitosterol		plants

3.1.1 The Structure of Biological Membranes

The concept illustrated in Fig. 3.2 can be extended to the association of many lipid molecules. The entropic effect will cause the molecules to self-associate orderly, ultimately forming bilayers of lipids with a hydrophobic core (Fig. 3.7). If these bilayers are extensive enough for the overall structure to bend, then the bilayer

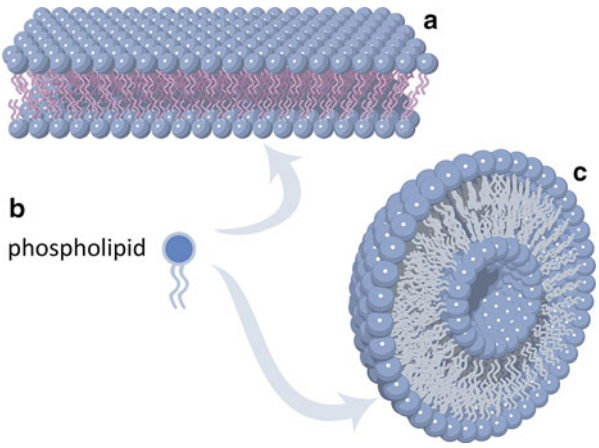


Fig. 3.7 Lipid molecules with a nearly cylindrical shape as a whole, such as phospholipids (**b**), tend to self-associate orderly due to the entropic effect (Fig. 3.2). Extensive self-association forms lipid bilayers (**a**) that ultimately bend and curve to self-seal into vesicles (**c**). Vesicles are the simplest prototype of cell membranes

curves allowing the sealing of the hydrophobic borders. All hydrophobic aliphatic chains become protected from water because the only areas in direct contact with the surrounding aqueous environment are the external and internal ("luminal") surfaces formed by polar head groups of the lipids. This is depicted in Fig. 3.7.

Lipid vesicles form spontaneously and they are the simplest models of biological membranes. Amazingly, there is not a single cell known in nature that does not have their membranes formed with lipid bilayers to some extent. Diversity arises from the different kinds of lipids used and from their combination with other molecules, but the structural arrangement of the membrane itself is configured by lipid bilayers. Some cells (e.g., bacteria, fungi, and plants) may also have a cell wall in addition to the cellular membrane, which serves for structural and/or protection purposes.

In fact, lipid membranes are relatively malleable and fragile. Yet, such malleability and apparent fragility are extremely important characteristics from a dynamic point of view: membrane division and fusion, for instance, are favored as these processes do not imply covalent bonds to be broken or formed among lipids, and lipid bilayers are flexible. Membrane fusion is advantageous in many biological circumstances (Fig. 3.8). In biotechnology, mainly in pharma and cosmetics, lipid vesicles are valuable tools due to their properties (Box 3.2).

Because lipid bilayers are the basic structure of cell membranes, several drugs directed to bacteria and fungi target the organization of lipid bilayers of these pathogens. Several antimicrobial peptides, for instance, are cationic so they bind and disrupt the lipid membranes of bacteria, which are highly anionic. Polyene antibiotics such as nystatin B and amphotericin B bind specifically to ergosterol disrupting the selective membrane permeability and ultimately causing the lysis of fungi (Fig. 3.9) because ergosterol only exists in the membrane of fungi.

While lipid vesicles depicted in Fig. 3.7 are formed only by lipids (a single pure lipid or a mixture of lipids), biological membranes are often composed of lipids, proteins, and saccharides. How these components organize in the membrane has been the subject of intensive scientific research over the years (Fig. 3.10). Nowadays, a biological membrane of a human cell is regarded as a lipid bilayer having on heterogeneous distribution of lipids both in each layer and among layers. This heterogeneity leads to the formation of specific domains of lipids having defined functions. Rigid platforms, for instance, may serve to anchor proteins on the membrane (Fig. 3.11). The cell membrane is directly connected to the cytoskeleton through an array of proteins, the so-called cytoskeleton anchors. The outer surface of cell membranes may have lipids and proteins that are glycosylated (i.e., covalently attached to saccharides) contributing to a rich chemical diversity on the surface of cells (Fig. 3.10).

The lipid composition of cell membranes varies a lot from species to species, from organelle to organelle in the same cell, and from the inner leaflet to the outer leaflet in the same membrane (Fig. 3.12). This variety of compositions grants the necessary diversity to membranes so that they are specific for certain functions, in spite of the common feature of all membranes of all cells: in the end they are all constructions based on lipid bilayers.

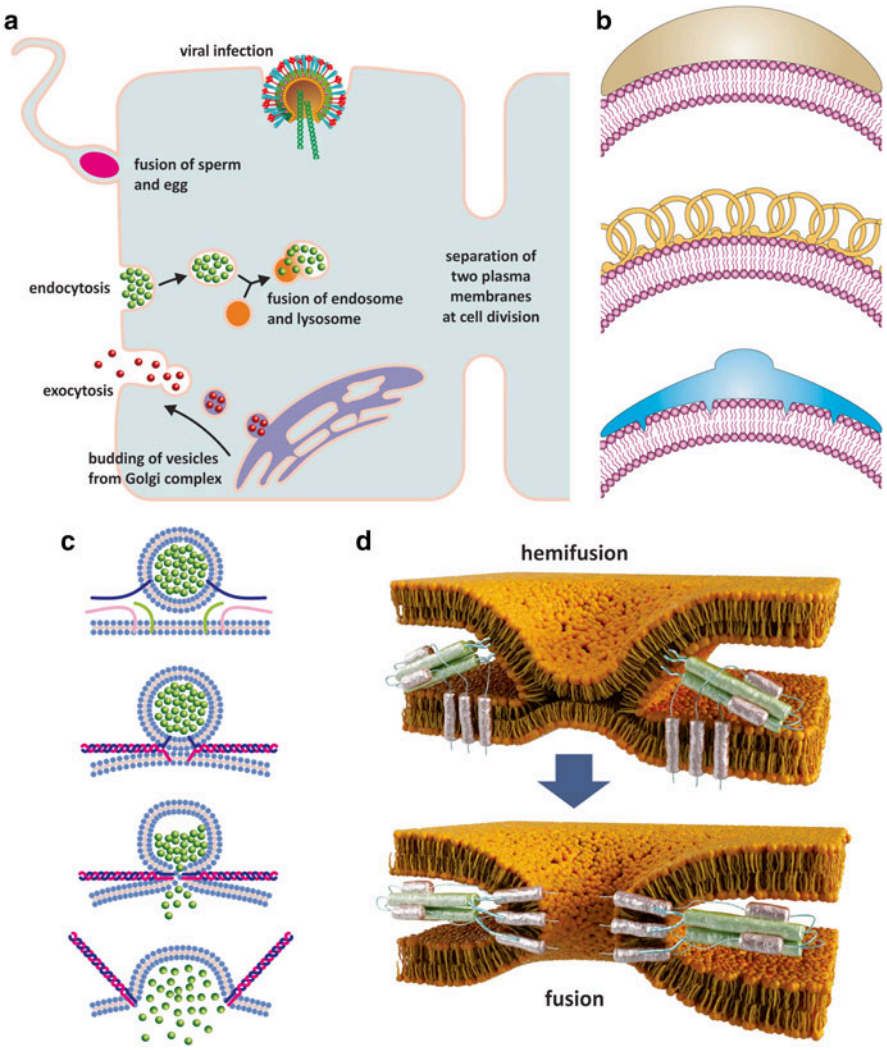
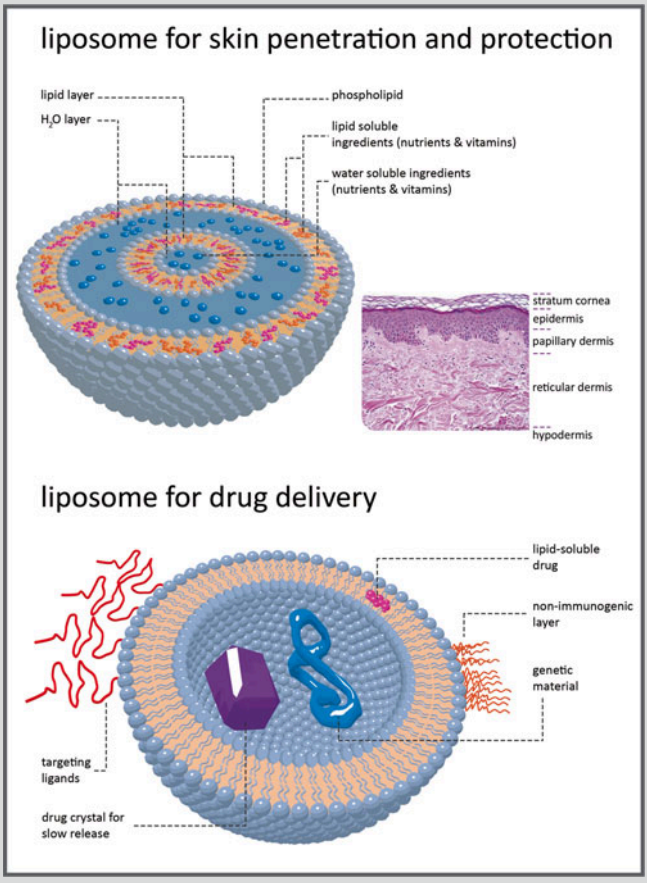


Fig. 3.8 Lipid membrane fusion occurs in nature associated with a plethora of cellular events (**a**). Some of these events such as endocytosis or budding of vesicles from the Golgi complex require inducing a curvature in membranes so that vesicles are formed. This induction is accomplished by the action of specialized proteins (**b**) that are curved and adhere to the surface of lipid bilayers (**b**, *top*), stabilize spontaneous curvatures (**b**, *middle*), or insert into one leaflet of the bilayer only thus forcing the membrane to bend (**b**, *bottom*) (reproduced with permission from Zimmerberg & Kazlov, Nature Rev Mol Cell Biol 7: 9–19, 2006). Membrane fusion implies that two different bilayers come together (**c**, **d**), which is due to the action of proteins that insert the two bilayers and promote conformational changes that lead to the contact of the lipid bilayers. First, a common bilayer is formed from the mix of the two membranes (hemifusion state), and then total fusion merges the two entities that were initially enclosed in their own membranes. Panel (**c**) shows the example of membrane fusion during neurotransmission, namely, a neurotransmitter being released at a synapse. The proteins responsible for fusion are named SNAREs (represented by *green*, *pink*, and *blue filaments*). Panel (**d**) shows the details of hemifusion and fusion of an envelope virus such as influenza virus, HIV, or dengue virus with the target cell. In this case viral proteins at the surface of the virus insert the membrane of the target cell and undergo conformational changes ending in fusion and consequent release of viral contents in the cytosol

Box 3.2: Lipid Vesicles in Pharma and Cosmetics

For many decades, lipids were considered relatively inert biologically, with functions of storage of energy in the adipose tissue or constitution of a matrix for cell membranes. Thus, in general, there was little interest in research to discover the properties, structures, biosynthetic pathways, biological utilization, and other functions of lipids. In the present, the situation is completely opposite. Lipids are regarded as important biological molecules that in addition to being energy stores and membrane components, participate in the regulation of many biochemical processes in cells and endocrine physiological regulation in the human body. Moreover, lipids are now important tools in pharma and cosmetic industries because they can be used in formulations that distribute and deliver drugs or other biologically active molecules in the human body. In most of these formulations, the lipids self-assemble in bilayers that form extensive vesicular systems, liposomes (see figure), able to encapsulate molecules having desirable functions. Liposomes are very versatile systems as hydrophobic molecules may be accumulated in the lipid areas, and hydrophilic molecules may remain solubilized in the aqueous spaces inside vesicles.

In cosmetic applications, the liposomes may be part of formulations to be applied topically in skin. The lipids help in the diffusion of the formulation through the outer layers of skin. Moreover, the simple fact that lipids and water are forming an emulsion will help the formulation to hydrate the desired areas of the skin.



(continued)

Box 3.2 (continued)

Liposomal encapsulation can substantially improve the action of a drug, such as the decreased toxicity observed with amphotericin B (see Fig. 3.9). Conventional amphotericin B has been generally considered the drug of choice for many types of systemic fungal infections. These infections are a major threat to those whose immune systems are compromised, such as patients undergoing chemotherapy for cancer, bone marrow transplant recipients and AIDS patients. However, amphotericin B is very toxic, thus limiting its utility. For these patients, who have a high rate of morbidity and mortality, there is a dosage form distinct from conventional amphotericin B, which consists of amphotericin B complexed with two phospholipids in approximately a 1:1 drug-to-lipid molar ratio: L-α-dimyristoylphosphatidylcholine (DMPC) and L-α-dimyristoylphosphatidylglycerol (DMPG), present in a 7:3 molar ratio. Doxorubicin is another example. Liposomal doxorubicin is designed to target to tumor cells and spare healthy tissue, maintaining efficacy while reducing toxicity. Conventional doxorubicin, drug commonly used to treat cancer, is limited by its potential for causing a variety of severe side effects, particularly irreversible heart damage.

Researchers are developing innovative liposomes with refined drug delivery properties to be part of future medicines. Some have their surface modified with proteins and other selected polymers to target selected cells. Synthetic phospholipids are suitable for specific applications in liposome targeting and gene therapy. Gene therapy is based on the efficient delivery of genes to their intended targets. Researchers have successfully put DNA into liposomes and have achieved fusion of these liposomes to cells. Scientists have also succeeded in protecting these liposomes from degradation and are able to modulate their circulation time.

Fig. 3.9 (continued) sides of the polyene rings face the lipids and the polar sides face each other forming a hydrophilic pore (**a**, *right*). Filipin is not so selective for ergosterol when compared to cholesterol. Amphotericin B, therefore, it is more toxic to human cells. Panel (**b**) (*right*) shows atomic force microscopy images of pores created by filipin in cholesterol-containing bilayers (*arrows*). Large structures in the surface of the lipid bilayer (**b**, *center*) are filipin aggregates. Panel (**c**) *left* also shows atomic force microscopy images but of an individual bacterium (*E. coli*). Exposure of the bacterium to the cationic amphipathic peptide BP100 caused the collapse of the bacterial membrane. The bacterial membrane is anionic, thus attracting electrostatically the peptides, which then aggregate on the lipid bilayer causing perturbation and increasing permeability. This perturbation may be caused by formation of pores or by unspecific destruction of the lipid organization (**c**, *right*, shows the action of $rBPI_{21}$, a peptide derived from bactericidal/permeability-increasing protein potentially useful against meningitis). (Figures in panel (**b**) *right* are reprinted with permission from Santos et al., Biophys J. 75:1869–1873, 1998. Figures in panel (**c**) *left* are reprinted with permission from Alves et al., J. Biol. Chem. 285:27536–27544, 2010. Figures in panel (**c**, *right*) are reprinted with permission from Domingues et al., PLoS ONE, 4:12, e8385, 2009

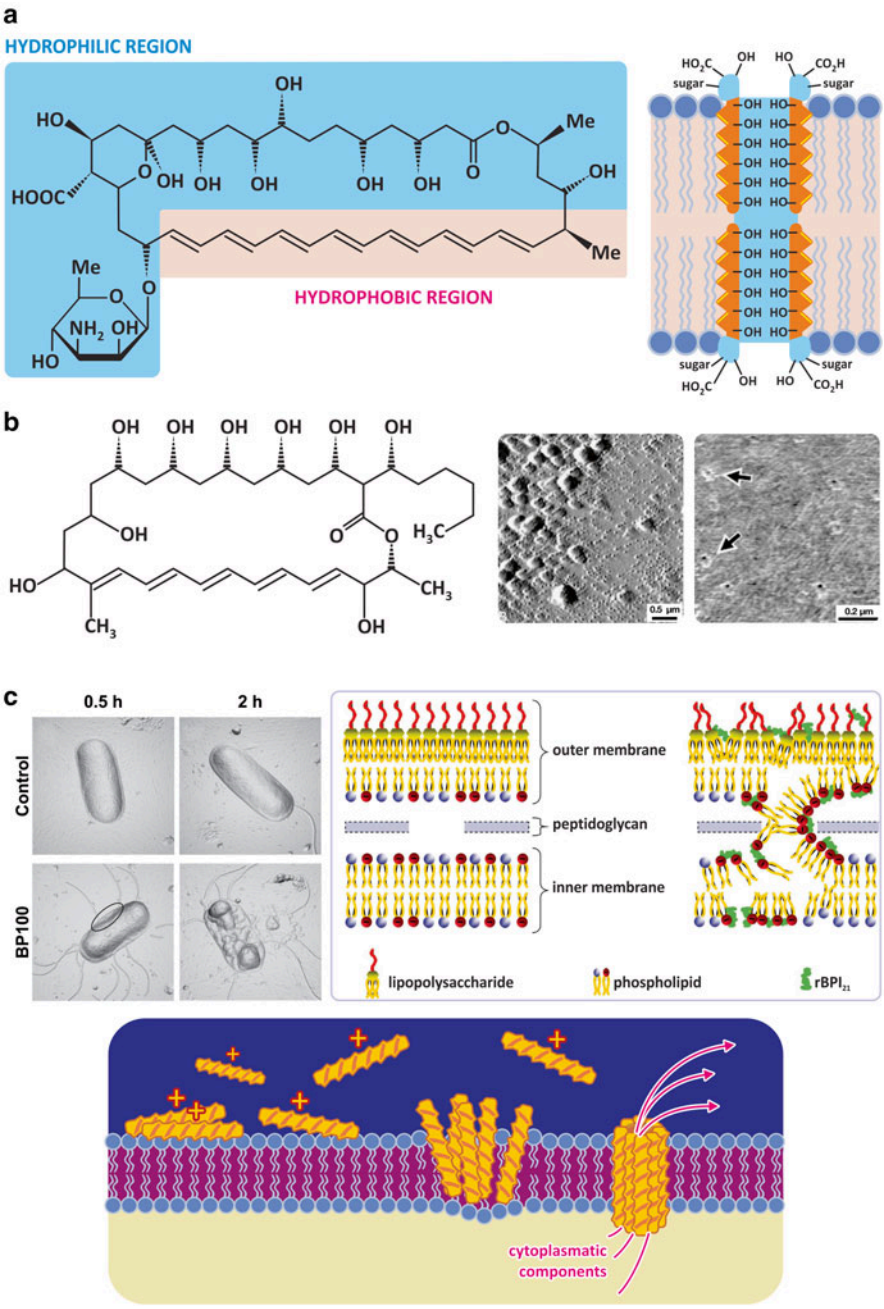


Fig. 3.9 Some drugs such as amphotericin B (**a**), a fungicide used in the treatment of infections with *Candida* sp. among others, or filipin (**b**), a fungicide also toxic to human cells and therefore not used in therapies, and cationic amphipathic peptides with antibacterial properties (**c**) target cell membranes. Amphotericin B binds to ergosterol forming ordered complexes in which the hydrophobic

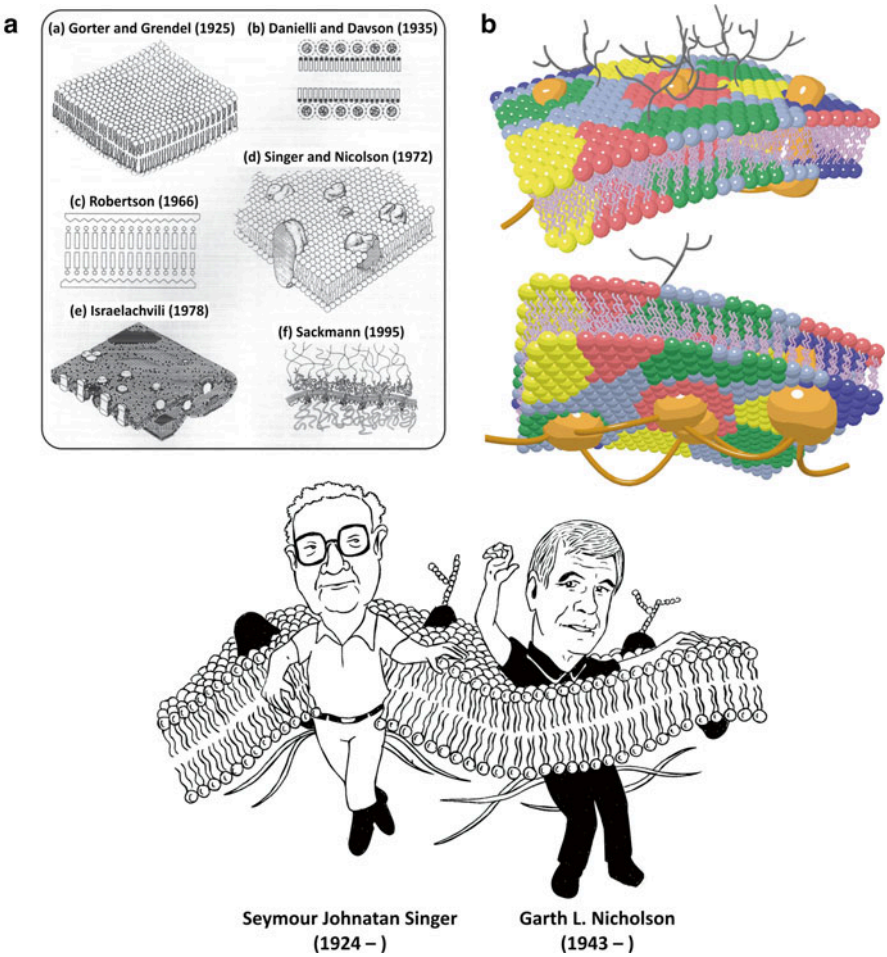


Fig. 3.10 (**a**) Historical evolution of the concept of cell membranes (according to Ole Moritsen; reprinted with permission from Biol. Skr. Vid. Selsk. 49:7–12, 1998). The lipid bilayer was first identified, followed by the discovery that proteins interact with lipids. The concept that there are integral proteins embedded in the lipid bilayer forming a dynamic structure came with the fluid mosaic model by Singer and Nicolson in 1972, which is still accepted as the basic framework of membrane structure. Nevertheless, from then on, the organization of cell membranes has been continuously unraveled. Lipids are now known to self-associate in lateral domains of different composition, and some membrane proteins are bound ("anchored") to the cytoskeleton (A(e) and A(f)). The modern view on cell membranes is schematized in (**b**), in which lipid colors represent heterogeneous lipid compositions. In the outer surface of cell membranes (**b**, *top*), glycosylated (i.e., saccharide-containing, *black*) lipids and proteins are present with different functionalities (see Sect. 3.2). Cytoskeleton and cytoskeleton-binding proteins are represented in *brown*

Fig. 3.11 (continued) segregated areas are clear, and the height profile along the line seen in the *top image* (*bottom graph*) shows that the segregated areas are higher, therefore corresponding to more rigid areas of the membrane. Epifluorescence images of lipid bilayers having one of the lipid tagged with a fluorescent dye confirm segregation of both lipids (**b**, *right*). Figure reprinted with permission from Franquelim et al., J. Am. Chem. Soc. 130:6215–6223, 2008; and Franquelim et al., Biochim. Biophys. Acta. 1828:1777–1785, 2013. These more rigid areas of the membrane are more adapted to anchor proteins and membrane receptors (**c**). Sphingolipids and cholesterol, for instance, further enhance these characteristics. Proteins covalently attached to lipids are typically found in these rigid platforms

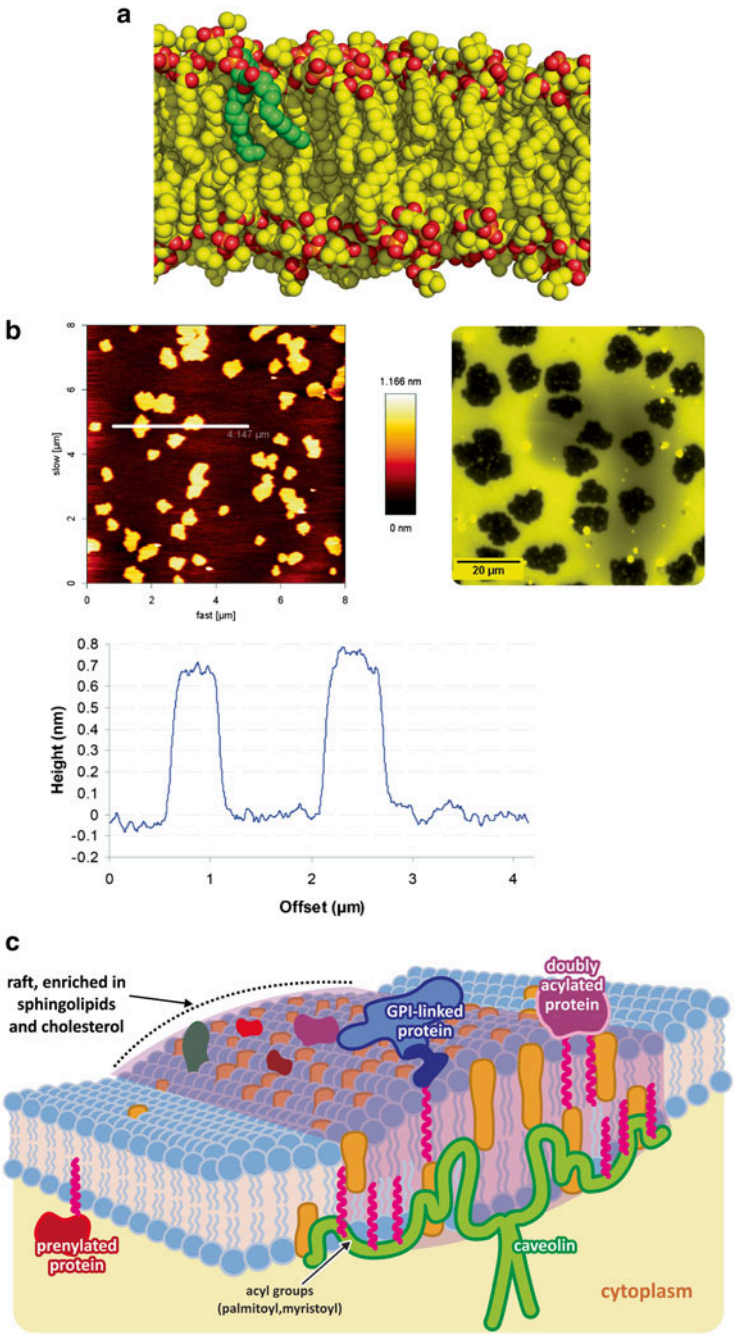


Fig. 3.11 Heterogeneity in lipid membranes. Although phospholipids are frequently depicted in an oversimplified form with ordered stretched aliphatic chains, as in Fig. 3.2, in reality most molecules in lipid bilayers in physiological conditions in cells have very flexible and dynamic acyl chains. Panel (**a**) shows molecular dynamics simulation of lipids with one single lipid in *green* to highlight the bent conformation (courtesy of Dr. Claudio Soares, ITQB-UNL, Portugal). Lipids with longer and saturated chains adopt stiffer and linear conformations as they interact more tightly with each other. Mixing stiff and fluid lipids results in partial segregation of the lipids. The atomic force microscopy image of a mixture of a fluid unsaturated lipid (palmitoyl-oleyl phosphatidylcholine, POPC—50 % molar) with a saturated rigid lipid (dipalmitoyl-phosphatidyl choline, DPPC) is shown in panel (**b**, *left*);

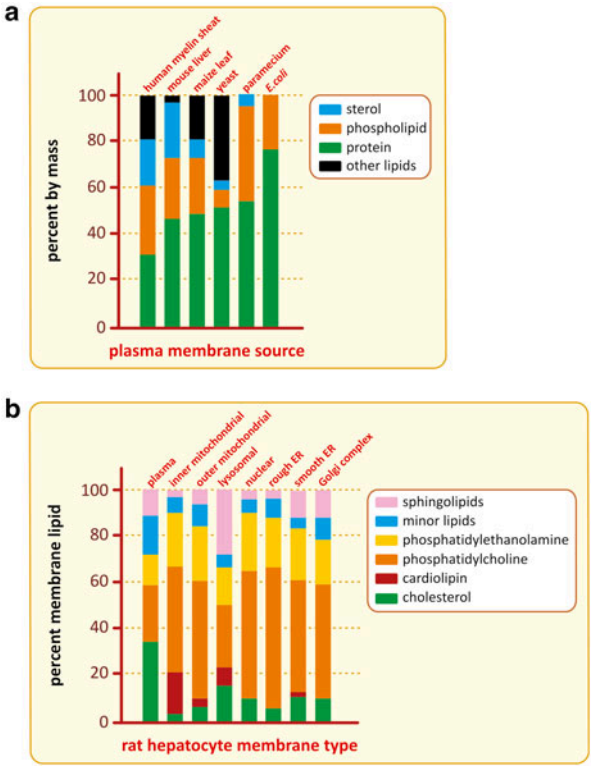


Fig. 3.12 Although all membranes in cells in nature are constructions based on lipid bilayers, the membrane composition varies a lot among species (*top*), among organelles of the same cell (*bottom*), and even between the two leaflets of the same membrane (not shown)

Because lipid bilayers are hydrophobic barriers, hydrophilic molecules such as glucose cannot freely transverse them, but channels and transporters in their membranes overcome the limitation. Channels and transporters are proteins specific for certain molecules or ions that facilitate or enable the translocation of such molecules or ions across the membranes. This subject will be revisited in Sect. 5.3.1, after careful consideration of protein structure.

3.1.2 The Structure of Lipoproteins

Lipoproteins are organized assemblies of lipids and proteins covering a wide range of sizes and densities (Fig. 3.13). These assemblies have a lipidic core formed mainly by triacylglycerols and cholesterol esters, covered by a monolayer of phospholipids. The global arrangement is largely determined by the entropic effect as the monolayer of phospholipids minimizes the contact between apolar components and water molecules in blood (Box 3.3). Lipoproteins transport lipids between different tissues. There are proteins inserted in the lipid layer, exposed at the surface of the lipoprotein, as shown in Fig. 3.14.

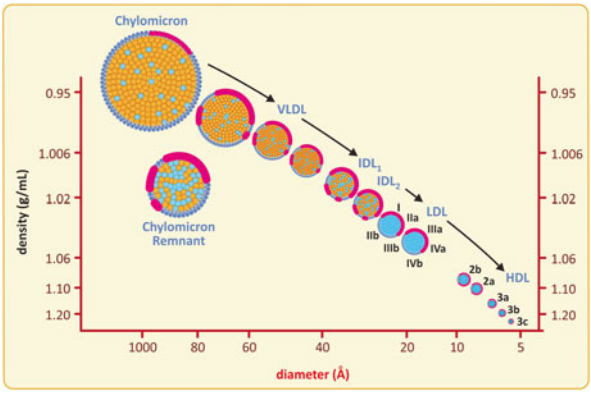
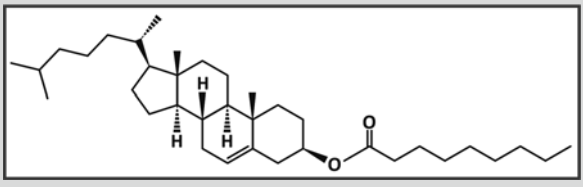


Fig. 3.13 Lipoproteins are grouped according to their densities and sizes, although the most common nomenclature refers to density (*HDL* high-density lipoprotein, *LDL* low-density lipoprotein, *VLDL* very-low-density lipoprotein). Chylomicra are the structures formed with dietary lipids in the enterocyte (intestines) and released in plasma

Box 3.3: Lipoproteins: The Burden of Lipid Transport

Lipids have extremely low solubility in aqueous media. Therefore, as a consequence of the entropic effect (see Sect. 3.1), when placed in aqueous medium, they tend to self-associate. Most phospholipids, having a hydrophilic "head" and two hydrophobic acyl chains, tend to pack side by side and form bilayers. Cholesterol is not prone to form very organized supramolecular assemblies itself but is able to insert in the lipid bilayers and contribute to its stability. Triacylglycerols ("triglycerides") and esters of cholesterol (cholesteryl esters; see figure) do not have the amphipathic properties and structural requirements to form lipid bilayers. Instead, triacylglycerols and cholesteryl esters amalgamate in an aggregate having no polar surface. These aggregates tend to be spherical, the geometry that minimizes the surface area exposed to the solvent. The lipid aggregates tend to grow until free lipid is nearly absent unless a phospholipid monolayer covers the surface of these aggregates, forming an entropically favorable interface. The phospholipid monolayers stabilize the lipid aggregates and an emulsion is formed. Emulsion means the lipids are heterogeneously distributed in microscopic scale because the lipids are clustered in aggregates but homogeneously distributed in macroscopic scale since the aggregates are evenly disseminated in the solvent.



Example of a cholesteryl ester: cholesteryl nonanoate. The molecule is composed of a cholesterol moiety and a fatty acid moiety (nonanoic acid in this case)

Box 3.3 (continued)

In human body, very large lipid aggregates are found in the cells of the adipose tissue (adipocytes), occupying almost all cytoplasmic space. It is a storage place. Smaller aggregates are found emulsified in blood, in association with specific proteins. These smaller aggregates are dragged by blood and serve as lipid transporters. In both cases, the aggregates are covered by monolayers of phospholipids having the polar groups exposed to aqueous environment and the acyl "tails" in contact with the lipids. The ensemble formed by the emulsified lipid aggregate covered with a phospholipid monolayer and associated to specific proteins is named lipoprotein (see Fig. 3.14 in the main text). The proteins themselves are named apolipoproteins. The lipoprotein as a whole is the lipid carrier entity; apolipoproteins bind to specific receptors so that lipids are delivered to target cells only.

Lipoproteins vary among them in the proportion of triglycerides/cholesteryl esters/protein, which directly interferes in their compactness and density. Early studies on the properties of lipoproteins achieved separation of several classes of lipoproteins based on their different densities, so the density-based nomenclature was naturally adopted, from high-density lipoproteins (HDL) to very-low-density lipoproteins (VLDL) and chylomicra (see below table). There is a concomitant change in volume but it is not given importance regarding lipoprotein classification. It is also worth highlighting that apolipoproteins are also divided in classes (A, B, C, etc.) and HDLs are the only lipoproteins not bearing apolipoproteins B, which is a distinctive feature.

Properties of plasma lipoproteins

Plasma lipoproteins	Density (g ml^{-1})	Diameter (nm)	Apolipoprotein	Physiological role
Chylomicron	<0.95	75–1200	B48, C, E	Dietary fat transport
Very-low-density lipoprotein	0.95–1.006	30–80	B100, C, E	Endogenous fat transport
Intermediate-density lipoprotein	1.006–1.019	15–35	B100, E	LDL precursor
Low-density lipoprotein	1.019–1.063	18–25	B100	Cholesterol transport
High-density lipoprotein	1.063–1.21	7.5–20	A	Reverse cholesterol transport

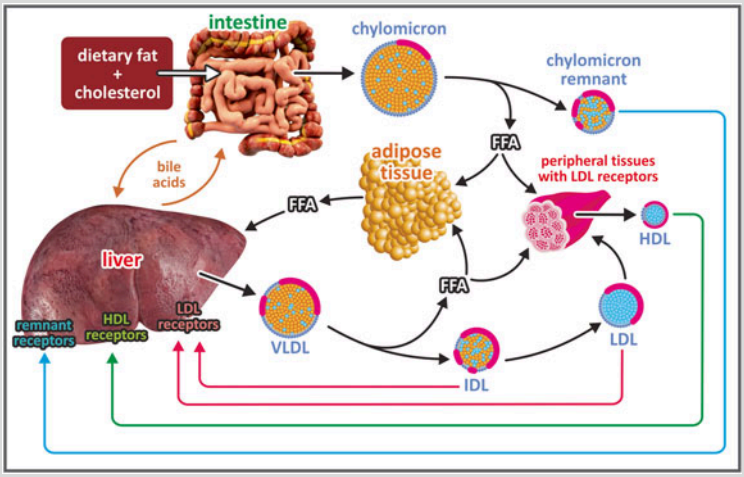
TAG triacylglycerol, *CE* cholesterol ester, *C* free cholesterol, *PL* phospholipid, *P* protein

During digestion, the lipids are partially degraded and emulsified in the intestinal lumen by bile acids, molecules similar to cholesterol but having several polar groups. Lipids and other nutrients are taken up by the intestinal

(continued)

Box 3.3 (continued)

cells, enterocytes (see figure). Chylomicra are formed in these cells and released in the blood. Eighty to ninety percent of the lipids in chylomicra are triglycerides, which account for their low density. The remaining cargo is free cholesterol (1–3 %), cholesteryl esters (3–6 %), and phospholipids (7–9 %). Chylomicra circulate in the blood, where degradation of their triacylglycerides into free fatty acids occurs. Part of these fatty acids is delivered to adipose tissue and peripheral tissues. The chylomicra remnants bind to liver cells having specific receptors. The excess of nutrient incorporated after digestion is converted to lipids in the liver, where they form VLDLs in a process similar to the assembly of chylomicra in the intestine. VLDLs are released from the liver cells. In the bloodstream, they are depleted of free fatty acids from triacylglycerols, resulting in the intermediary density lipoproteins (IDLs) and low-density lipoproteins (LDLs), which transfer lipids to peripheral tissues bearing LDL receptors. Liver cells having LDL receptors also bind LDL. In contrast, HDLs transport cholesterol from peripheral tissues to the liver, where there are cells having specific receptors for HDL. The cholesterol is then used to synthesize bile acids. HDLs are the only lipoproteins that dispose of cholesterol. This characteristic renders the name "good cholesterol" to HDL-associated cholesterol in public health campaigns for lay audiences. This name makes no sense on biochemical grounds but helps to spread the message that in cardiovascular risk evaluation, it is important to differentiate between cholesterol that is being removed and cholesterol that is being incorporated. Interestingly from the biochemical point of view, cholesterol to be disposed is associated to lipoproteins having no apolipoprotein B and cholesterol to be incorporated is associated to apolipoprotein B-containing lipoproteins.



Origin and fates of plasma lipoproteins

(continued)

Box 3.3 (continued)

LDLs are degraded inside the cells after being taken up by endocytosis. The LDL receptor is segregated in the endocytic vesicle, which then divides in two: one empty vesicle having the receptors in the membrane returns to the surface of the cell, and the other vesicle has the proteins and lipids of the lipoproteins and fuses with the lysosome. LDL-derived cholesterol may then be used either in cell membranes, or as a precursor to synthesize steroid hormones or bile acids, or simply be stored as cholesteryl esters. The exact destination of cholesterol in the cell depends on the type of cell and its metabolic state. Dietary cholesterol suppresses the synthesis of cholesterol by the body, and high free cholesterol levels inhibit the synthesis of LDL receptors. Cellular uptake is thus inhibited in the presence of excess cholesterol, and the level of LDL in the blood increases. Moreover, the LDLs take more time to be uptaken and circulate in blood for longer periods. This increases the chances of having the LDL exposed to oxidative agents such as NO, hydrogen peroxide, or the superoxide ion. Oxidized LDLs are then removed from circulation by macrophages, but the macrophages get their properties severely altered, becoming the so-called foam cells. Foam cells accumulate in the walls of endothelia, releasing growth factors and cytokines that stimulate the migration of smooth muscle cells that proliferate in the site of accumulation of foam cells and form collagen matrices. This consists of the deposition of atherosclerotic plates, which poses a severe cardiovascular risk.

Interestingly, the proteins responsible for triacylglycerol conversion into fatty acids in the heart (enzymes heart lipoprotein lipases) have a much higher affinity for triacylglycerols than the corresponding proteins in the adipose tissue. The affinity parameter, $1/K_M$, which will be addressed in Sect. 4.2.1 is about tenfold higher in the heart. During starvation the levels of plasma triacylglycerol drop, but delivery of fatty acids from triacylglycerols is kept in the heart even when suppressed to the adipose tissue.

Different classes of lipoproteins differ in density (due to differences in the relative amounts of proteins, phospholipids, cholesterol, triacylglycerols, and cholesterol esters in their composition), size, and specific proteins associated (see Table in Box 3.3). Nevertheless, these classes are named after the differences in density only, which relates to the most practical property that can be used for their separation in different fractions (see Fig. 3.13). Lipoproteins formed in the intestine with dietary lipids are known as chylomicra, and the remaining classes range from very-low-density lipoproteins (VLDLs) to high-density lipoproteins (HDLs). Intermediate-density lipoproteins (IDLs) and low-density lipoproteins (LDLs) are in between.

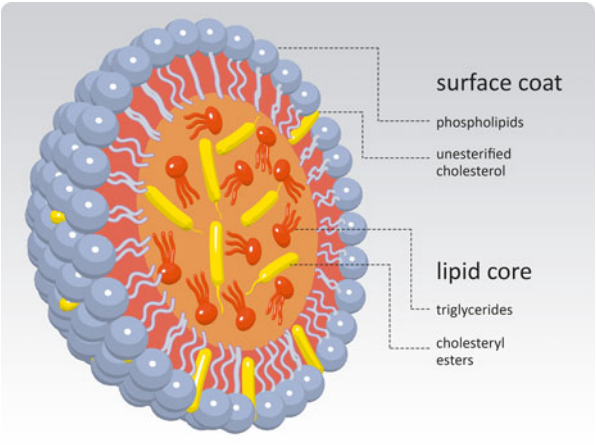


Fig. 3.14 Global generic structure shared by all classes of lipoproteins. A monolayer of phospholipids and cholesterol covers a lipid droplet of triacylglycerols and cholesterol esters. There are proteins at the surface (not shown), the apolipoproteins, which are specific of each class of lipoproteins and serve for cell recognition, i.e., interaction with specific receptors in cells

The different classes of lipoproteins have different functions and different target tissues for their action (see Box 3.3). Target recognition depends on the specificity of the proteins present on the lipoprotein surface (referred to as apolipoproteins to highlight that only the proteic part is being addressed) for well-defined receptors.

Other lipidic supramolecular assemblies, such as lipid droplets in the liver and lipid depots in adipose tissue, exist. Lipid depots may occupy most of the volume of an adipocyte, the adipose tissue cells (see Fig. 3.3), confining the cytoplasm to a very small fraction of the cell. These lipid depots are also covered by phospholipids that are stabilized as a consequence of the entropic effect.

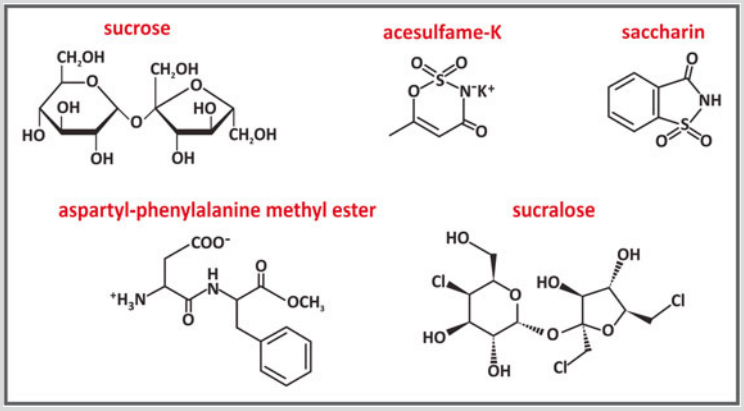
3.2 Saccharides and Their Polymers and Derivatives

Saccharides, different from lipids are extremely polar, therefore, hydrophilic, molecules. They are linear aldehydes or ketones with hydroxyl groups bound to the carbons that do not form the carbonyls (C=O). Many of these molecules have only C, H, and O in their composition and fit the formula $(CH_2O)_n$. This spurious characteristic consecrated the designation "carbon hydrate", which is still widely used to identify saccharides despite its total inadequacy in chemical terms: not all saccharides obey to $(CH_2O)_n$ and this does not reflect an hydration of carbon, only a specific molar proportion between C, H, and O atoms. Referring to saccharides as "sugars" is equally inadequate and misleading. "Sugar" is related to a property, sweetness, which not all saccharides possess and extends to molecules other than saccharides, such as peptide sweeteners (Box 3.4). Saccharides

Box 3.4: Sweeteners and Sugar Substitutes

The problem of popularization of high caloric diets stimulated the search for sugar substitutes. Sucrose, the most commonly used sugar in cooking, is a natural sweet molecule from which a certain amount of energy can be used by the human body after metabolization. However, there are molecules known as high-intensity sweeteners that have manyfold the sweetening power of sucrose. Saccharin, for instance, is approximately 300-fold sweeter than sucrose when equal quantities are compared. Aspartame and acesulfame-K are approximately 200-fold sweeter than sucrose. For sucralose the ratio raises to an impressive 600-fold. A specific chemical modification in aspartame, advantame, grants an impressive 20,000-fold increase in sweetness relative to sucrose. Therefore, much less mass of sweetener is needed to achieve the sweet taste of a food or beverage. Even though the "caloric content" of a unit mass of the molecule may be equivalent to sucrose in some cases, the total amount used is several orders of magnitude less and the total calories in the diet drops drastically.

The chemical structure of sucrose and most artificial sweeteners are very different. Sucrose is a disaccharide composed of the residues of the monosaccharides glucose and fructose. Sucralose is prepared from sucrose via the substitution of three hydroxyl groups for three chlorides. Saccharin and acesulfame-K have much different structures. Aspartame is the methyl ester of the dipeptide L-aspartyl-L-phenylalanine.



The molecular structure of sweeteners must be such that they bind to a specific receptor molecule at the surface of the tongue. The receptor is coupled to a G protein (see Sect. 5.4), which dissociates when the sweetener binds to the receptor. This dissociation leads to the activation of an enzyme

(continued)

Box 3.4 (continued)

that triggers a sequence of events resulting in signals that are carried to and interpreted by the brain. The sweetness perception depends on fine details of the interaction between receptor and sweetener. The importance of molecular shape to sweetness is illustrated by the case of aspartame, as its stereo isomer, L-aspartyl-D-phenylalanine methyl ester, has a bitter, not a sweet, taste.

There has been a long and continuous controversy about the impact of artificial sweeteners on health, which has driven a lot of research on the possible toxicity of their metabolic products. Saccharin has been very controversial and banned in some countries. In the body, aspartame is broken down into/absorbed as products that include aspartate, phenylalanine, and methanol. Phenylalanine is toxic to individuals with phenylketonuria, a genetic disease wherein individuals cannot process phenylalanine. Products containing aspartame must therefore be labeled for phenylalanine. Aspartame's breakdown products—phenylalanine and aspartate, as well as methanol and its breakdown products formaldehyde and formate—are a matter of debate. Regardless of the controversies and limitations in their use, artificial sweeteners have an important role in the improvement of the quality of life of diabetics, who are limited in the consumption of sucrose and other saccharides.

or oses are therefore the preferred nomenclatures for biochemists although "carbon hydrates", or carbohydrates, in short, and "sugars" are commonly used in closely related areas such as nutrition, for instance.

Saccharides are the most abundant biomolecules and owe this ubiquity to their reactivity and structural plasticity, which enable a great variety of functionalities, including energetic storage, cell communication, and cell protection against mechanical aggressions and dehydration. In order to understand such structural plasticity and the functionalities arising from it, one has to start with the basic chemistry and reactivity of saccharides. Although this is a wide and complex area in the realm of biochemistry, we will devote ourselves to the understanding of the most important saccharides in human biochemistry only. One will stick to the basics of this fascinating world for the sake of clarity and focus on processes that are foundational for other medical disciplines such as histology and physiology.

The simplest conceivable saccharides have three-carbon chains, i.e., they are trioses ("tri" for three carbons, "ose" for saccharide). Depending on the position of the carbonyl group, C=O, which may be terminal (aldehyde) or not (ketone), the saccharide is an aldose or a ketose. In the specific case of trioses, only glyceraldehyde and dihydroxyketone are possible (Fig. 3.15). But even in these cases, two kinds of common chemical reactions in nature are possible: esterification and reduction (Fig. 3.16).

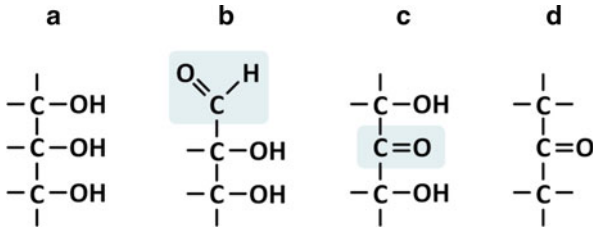


Fig. 3.15 Glycerol [(**a**); also in Fig. 3.4d] is related to an aldehyde, glyceraldehyde (**b**), which has a ketone isomer, dihydroxyketone (**c**), which in turn is related to a ketone (**d**), dimethyl ketone. The aldehyde and ketone groups of glyceraldehyde and dihydroxyketone are highlighted in the chemical structures (**b**) and (**c**), respectively, by a *shadowed box*

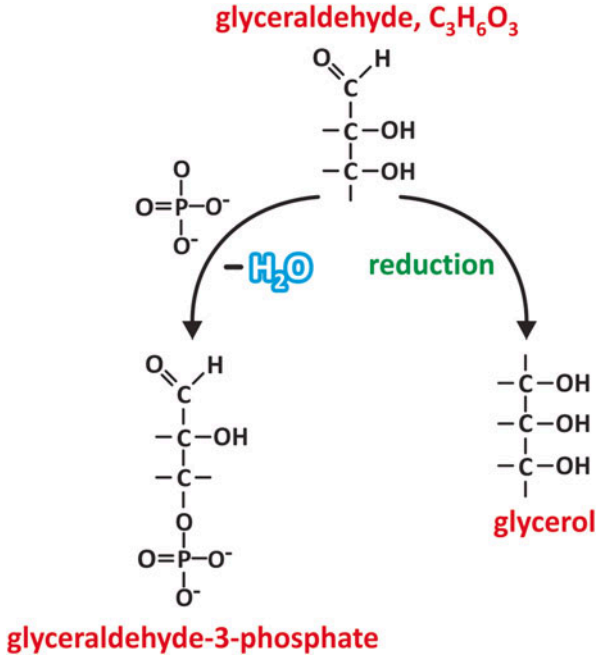


Fig. 3.16 Glyceraldehyde may be reduced to glycerol upon chemical reduction of the C=O group in carbon 1 (carbons are numbered starting with the one from the carbonyl group, similarly to fatty acids, in which carbons are numbered starting in the carboxyl group). Phosphoric acid (HPO_4^{2-}) may react with carbon 3, for instance, to form an ester, glyceraldehyde-3-phosphate. These are examples of very frequent reactions involving saccharides

The chemical structure of glyceraldehyde deserves close attention as its central carbon (C2) has four different substituents (i.e., it is bound to four different atoms or groups of atoms), being referred to as a chiral carbon or chiral center. Imagine the permutation of the H and OH substituents, for instance. A different molecule results from this switch (Fig. 3.17).

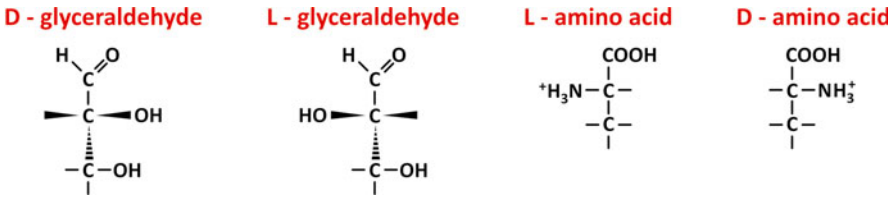


Fig. 3.17 L- and D-glyceraldehydes are mirror images. The same nomenclature was extrapolated to amino acids (alanine is shown as example)

Although having the same elemental composition and very similar structure, both molecules cannot overlap because the orientation in space of the H and OH substituents is different. The difference is clear if the molecule is represented in a 3D perspective. A closer look reveals that both molecules are mirror images of each other, i.e., they are enantiomers. To distinguish between both enantiomers of glyceraldehyde, one is named "L," and the other is named "D." These labels were arbitrarily assigned by Emil Fischer but are used to name saccharides and amino acids by extrapolation from glyceraldehyde (Fig. 3.17): D stands for dextro (right in Latin) and refers the structure having the OH group in the chiral carbon to the right when it is projected toward the observer; L stands for levo (left in Latin) and refers the structure having the OH group in the chiral carbon to the left when it is projected toward the observer.

Hermann Emil Fischer
(1852 –1919)

In chemistry research, there are two other nomenclature conventions for enantiomers independent from each other: the *R* and *S* system, which is based on a classification of the substituent group based on the atomic number of atoms bound to the central atom (chiral center), or the + and − system based on optical activity, i.e., on the direction of rotation of incident plane polarized light. Symbols + and − are sometimes replaced by *d*- (dextrorotatory) and *l*- (levorotatory) but *d*- and *l*- are easy to confuse with D- and L- and prone to misunderstanding. Both systems are more robust than Fischer's D- and L- because they are not dependent on the compari-

son with glyceraldehyde. Chemists tend to use *R/S* or +/− but biochemists are still "attached" to the D/L system for a simple reason: chiral diversity among biological saccharides and amino acids is very restricted. By far the most abundant saccharides in human biochemistry are D-. Interestingly L- is the preferred form in amino acids. Natural evolution favored one form specifically probably because it is simpler to have only one form as the building block for saccharide polymers (polysaccharides) and amino acid polymers (proteins). A small protein with 100 amino acid residues that could be D- or L- would have 2100 different possible isomeric structures. Because only L-amino acids are used, only one isomeric form is allowed. Why specifically L-amino acids and D-saccharides and not the other forms? It is not clear; probably it originated from originally random processes that later propagated and converged by evolution into the specific enantiomers found in nature nowadays.

When longer carbon chains are considered, more complex saccharides are possible depending on the:

1. Length of carbon chains
2. Position of the carbonyl group in the carbon chain
3. Number and location of chiral centers

Although many different saccharides can be found in human body, pentoses and hexoses are the most frequent in metabolic processes so we will now focus on these molecules, namely, ribose and glucose (Fig. 3.18).

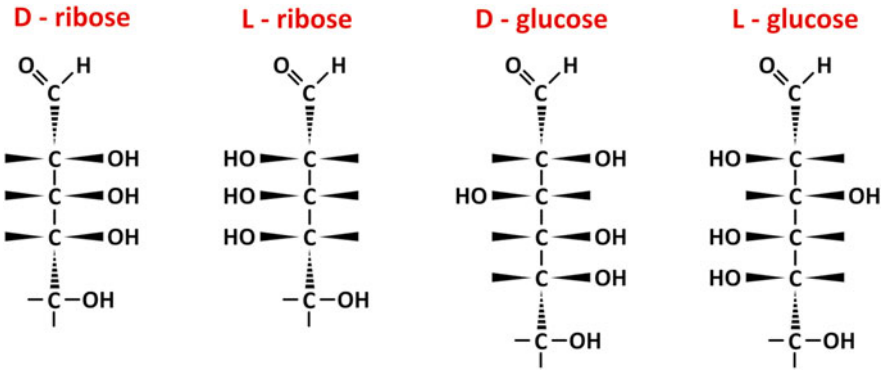


Fig. 3.18 D- and L-isomers of ribose (pentose) and glucose (hexose). D-forms are the more relevant forms in nature

Aldoses such as glyceraldehyde, ribose, and glucose react with water, forming a hydrate. This happens at the C=O group because the oxygen atom attracts the electrons leaving the C atom deficient in electrons, therefore prone to interact with the electrons of water oxygen (Fig. 3.19). The reaction is reversible, so

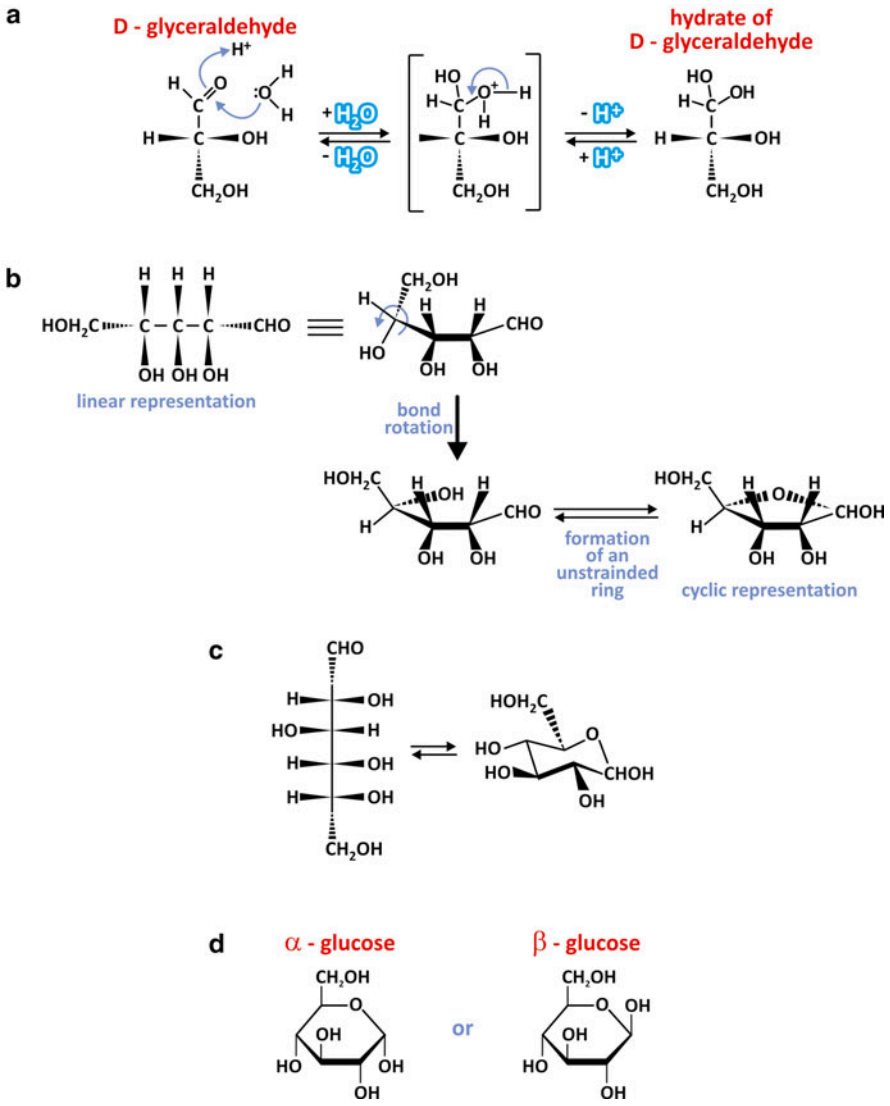


Fig. 3.19 Hydration of glyceraldehyde forms a hydrate (**a**). This reaction is reversible so glyceraldehyde coexists with its hydrate. The carbon originally present as a carbonyl group is the only carbon with two bonds to oxygen. A similar reaction may occur intramolecularly in pentoses (**b**) and hexoses (**c**). Panel (**b**) shows in detail the reaction of C=O (C1) with the OH group in C4, analogous to hydration. A cyclic pentose is thus formed. As with hydration, the reaction is reversible and both forms coexist, although the cyclic form is more abundant. The cyclic form of the hexose glucose is not planar as ribose is, as the molecular hexagon is flexible and adopts other conformations, such as the "chair" conformation (**c**). Cyclization results in the formation of two anomers (**d**) because the OH group formed at C1 may be placed on two different sides of the newly formed molecular ring: in the α-anomer the OH group in C1 is in the opposite side of the plane of the ring relative to the terminal carbon, C6 (–CH_2OH) and in the β-anomer they are both at the same side. Both the "chair" (**c**) and the more simplistic planar (**d**) representation of hexoses is used in this book

aqueous solution of aldoses contains mixtures of their aldehyde and hydrate forms. Nevertheless, pentoses and hexoses may react intramolecularly in a way that is similar to hydration. Because the carbon chain is able to bend and is dynamic (similarly to saturated aliphatic chains in lipids), the carbonyl group may contact alcohol groups (OH—hydroxyl) in the same molecule and react with it. The result is the formation of a cyclic molecule by the conversion of the carbonyl group in a hemiacetal group (Fig. 3.19). The cyclization is reversible, and in cells, the cyclic forms of ribose and glucose coexist with the linear forms, but the cyclic forms are dominant. Upon cyclization, two enantiomers are formed because the hydroxyl group in C1 may be linked to any of the two sides of the plane of the ring: in the α-anomer the OH group in C1 is in the opposite side of the plane of the ring relative to the terminal carbon, C6 ($–CH_2OH$), and in the β-anomer, they are both at the same side. Glucose adopts a "chair" conformation at variance with the strict planar ring of ribose (Fig. 3.19), but α and β anomers exist the same way. The existence of the enantiomers has drastic implications in the polymerization of hexoses.

3.2.1 From Monomers to Polymers: Polysaccharides

Saccharides such as hexoses and pentoses may react with each other forming chains that may reach considerable size. Molecules built from the association of smaller molecules of a kind are generally named polymers, and polymers made of unit saccharides such as ribose or glucose are named polysaccharides. The units forming polysaccharides are referred to as monosaccharides. A covalent association of two monosaccharides is a disaccharide. Association of "few" monomers forms "oligosaccharides"; the size boundary between oligosaccharides and polysaccharides is not well defined.

Two monosaccharides may associate by dehydration. Take the example of two glucose molecules forming a maltose molecule (a disaccharide) by dehydration (Fig. 3.20). C4 in one molecule and C1 in the other become covalently attached by an acetal linkage or *O*-glycosidic bond. Water resulting therefrom is formed with the oxygen previously attached to C1. The reverse process is the hydrolysis of maltose into two glucose monomers, which although thermodynamically favorable, is a very slow reaction. In practice, when degrading enzymes are not present, the process my be be so slow that it is irrelevant.

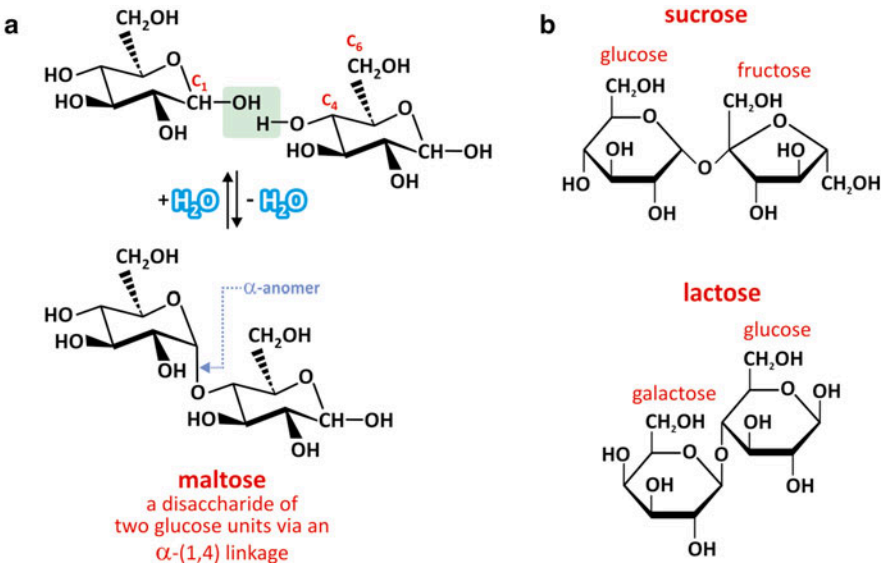


Fig. 3.20 Two glucose molecules may associate covalently by dehydration (**a**). When C1 in α-glucose (α anomer) reacts with C4 in another glucose, maltose is formed. Maltose is thus a disaccharide formed by linking two glucose molecules through an acetal or *O*-glycosidic bond. This bond is named "α-(1,4)" to stress that C1 in the α anomer binds to C4 in the other molecule. Sucrose and lactose (**b**) are other examples of disaccharides. Both are formed from the conjugation of different constituent monosaccharides: glucose and fructose in the case of sucrose and galactose and glucose in the case of lactose. Sucrose involves an α-(1,2) bond, whereas lactose involves a β-(1,4) linkage

The stereo chemistry (i.e., the spatial orientation of the chemical groups in the molecule) is very important because the covalent linkage of two molecules imposes restrictions on the way molecules can move in space. Depending on whether monomers are α or β anomers, different degrees of restriction arise. The flexibility of the conjugate is very much dependent on the enantiomers because the interaction between molecular groups in the disaccharide is very different (Fig. 3.21a). This effect is amplified in large polymers; polysaccharides may have a wide range of flexibilities, from extremely stiff and straight to coiled and deformable depending on the enantiomers used. Cellulose, for instance, is a glucose polymer formed with β(1,4) bonds that is extremely resistant mechanically, whereas amylose is an example of a flexible polymer formed by a α(1,4) backbone (Fig. 3.21b, c). Cellulose properties determined its evolutionary selection toward structural functions in plants, forming cell walls, which impacts in the macroscopic properties of wood. Amylose is a component of starch, a molecule that curls forming helices and is stored in plants for use in the energetic metabolism. Starch is the most common polysaccharide in human diet. Cellulose and amylose are striking examples of how apparently small details may actually determine profound differences in molecular properties and structures and therefore also in function.

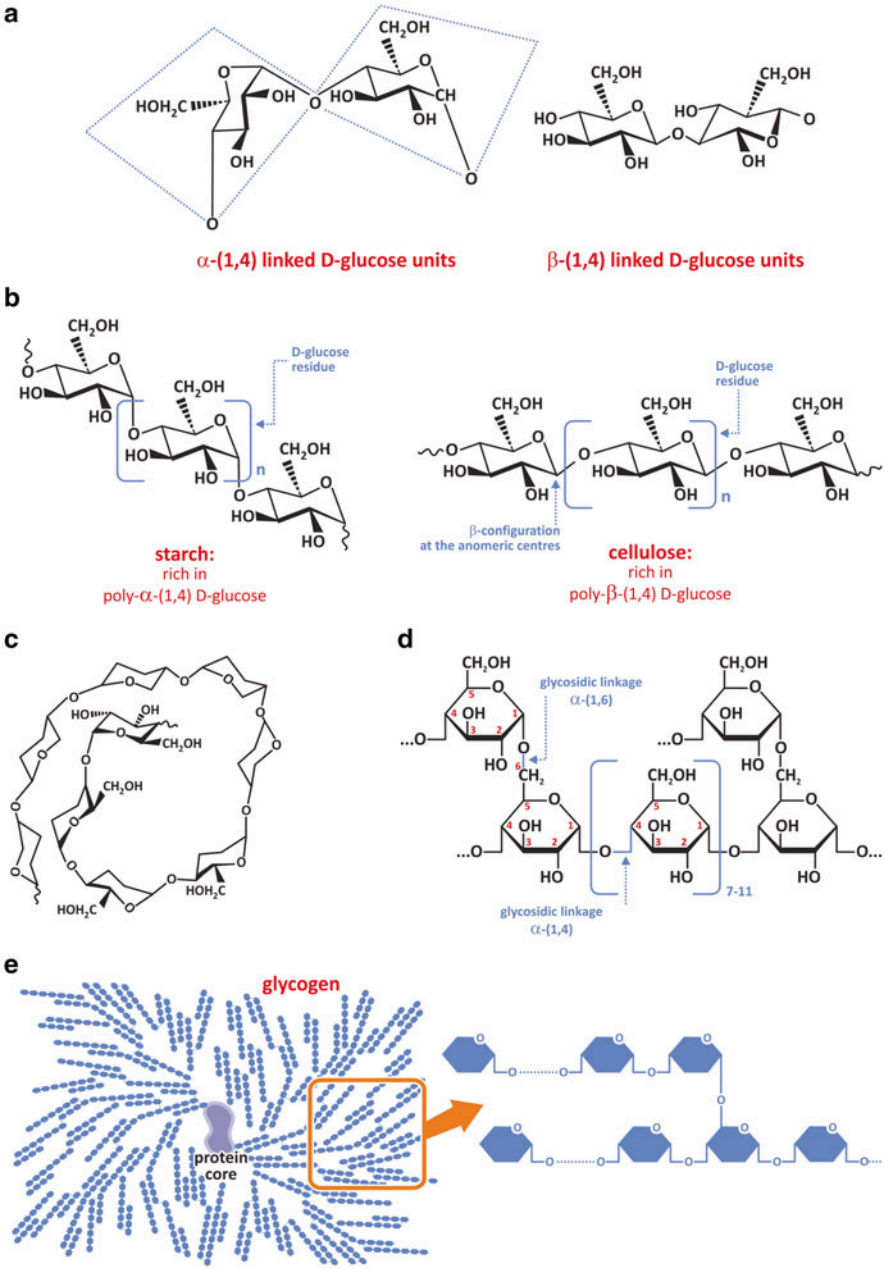


Fig. 3.21 Two monosaccharides such as D-glucose forming a disaccharide (**a**) have very different restrictions to articulate and to move depending on whether the glycosidic bond is α-(1,4) or β-(1,4). When several monomers bind to form a polymer (**b**), successive α-(1,4) or β-(1,4) bonds confer distinct properties to the polymer: α-(1,4) bonds enable bending between monomers, which results

Carbon 6 is also available for reaction so linear polymers formed of C1–C4 chains may branch when C1–C6 bonds are also formed (Fig. 3.21d). Amylose turns into amylopectin when β(1,6) links are formed. Glycogen is the human storage polysaccharide and is very similar to plant amylopectin (Fig. 3.21e). They differ only in the frequency of branching and average size of α(1,4) segments. The advantage of having glycogen as energy storage relative to a linear (unbranched) polysaccharide relates to the fact that glycogen is enzymatically degraded by saccharide hydrolysis of the terminal units. A branched molecule has several termini which can all be degraded at the same time, making glucose readily available at high rate.

Depending on their chemical composition and stereochemistry, polysaccharides found in nature have one of three functions: (1) structural/mechanical protection, (2) energy storage, and (3) water-binding (protection against dehydration). Several examples are listed in Table 3.3. Hyaluronic acid is particularly interesting for its involvement in connective tissue. Its interest extends from biochemistry to histology. Hyaluronic acid, a polysaccharide with sulfate groups (SO_4^{2-} has similar properties to PO_4^{2-}), forms an extracellular mesh with collagen, making up a flexible but resistant hydrated histological structure (Fig. 3.22).

Table 3.3 Examples of the function of polysaccharides found in nature: structural (Str), energy storage (Sto), water-binding hydration (Wat) (D-Glc, D-glucose; D-GlcNAc, *N*-acetyl-D-GLUCOSAMINE; D-GlcUA, D-glucuronic acid)

Polysaccharide	Monosaccharide 1	Monosaccharide 2	Bond	Branching	Location	Main function
Bacteria						
Peptidoglycan	D-GlcNAc	D-MurNAc	β-(1,4)	–	Bacterial wall	Str
Dextran	D-Glc	–	α-(1,6)	α-(1,3)	Capsule	Wat[a]
Animals						
Chitin	D-GlcNAc	–	β-(1,4)	–	Insects, crabs	Str
Glycogen	D-Glc	–	α-(1,4)	α-(1,6)	Liver, muscles	Sto
Hyaluronic acid	D-GlcUA	D-GlcUA	β-(1,4) β-(1,3)	–	Connective tissue	Str, Wat

[a]Capsular materials like dextrans may be overproduced when bacteria are fed with saccharides to become reserves for subsequent metabolism

Fig. 3.21 (continued) in curled polymers such as amylose (**c**) and the stiffer β-(1,4) links between monomer favor linear straight polymers, such as cellulose. Therefore, cellulose is found in structural elements of plants, while amylose is used by plants as energy storage. Humans also use a poly-α-(1,4) saccharide as energy storage. Periodic α-(1,6) branching every 10 residues, approximately, further enables a globular organization of this polysaccharide (**d**). The final result is a regularly branched polymer of D-glucose named glycogen (**e**). Glycogen synthesis is initiated by a protein and elongation requires several enzymes (see Sect. 8.2)

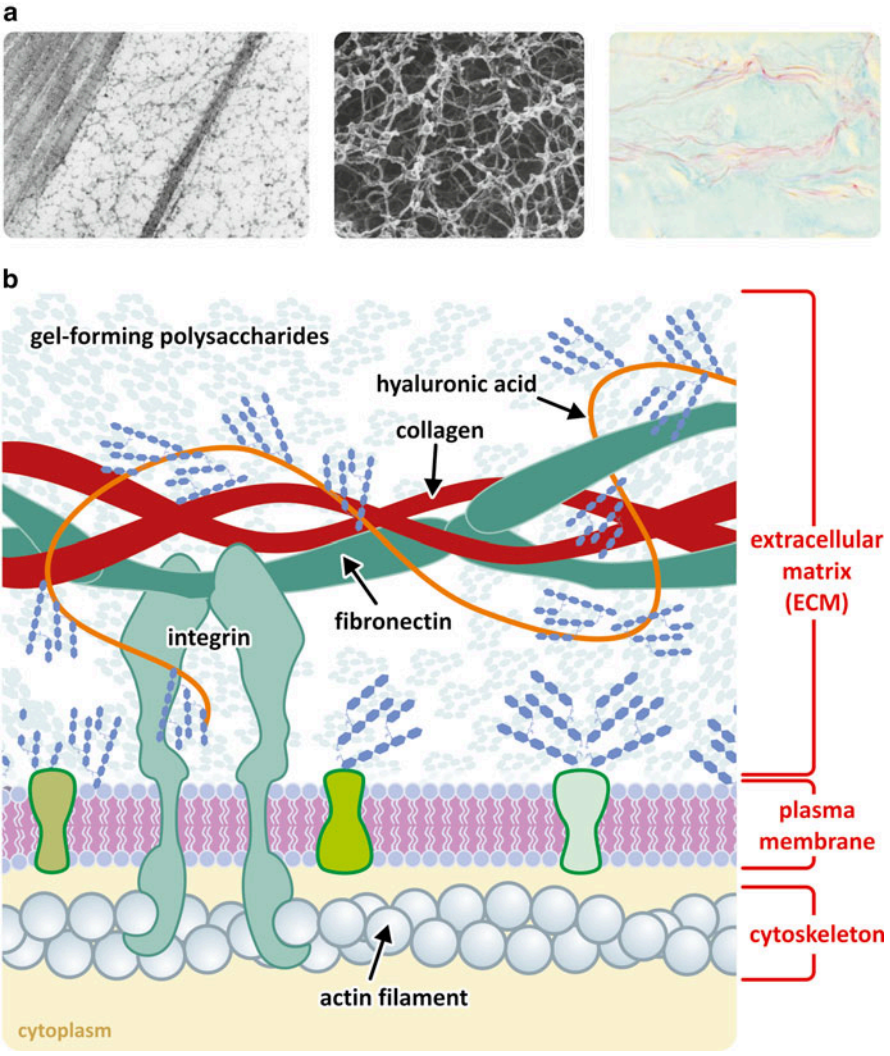


Fig. 3.22 The extracellular matrix has hyaluronic acid in its composition. (**a**) Histological preparations of cock crest highlighting the hyaluronic acid matrix (*left*, conventional electron microscopy; *center*, platinum–carbon replica; *right*, preserved blue-dyed hyaluronic and extracellular heavily glycosylated proteins, the "proteoglycan matrix"). Figure reprinted with the permission of Instituto de Histologia e Biologia do Desenvolvimento, Faculdade de Medicina, Universidade de Lisboa, Portugal. (**b**) Schematic representation of the molecular organization of extracellular matrix, which is composed of gel-forming saccharides attached to a backbone of hyaluronic acid that is intertwined among collagen fibrils. Polysaccharides form gels due to the high density of H bonds. These gels confer a moderately rigid structure and mechanical protection to cells and retain water, which prevents desiccation of the tissues

3.2.2 *Molecular Conjugates of Monosaccharides*

We have seen in previous sections that saccharide monomers offer diverse possibilities of reaction and so they are molecules that form many conjugates in nature. The most important derivatives are phosphate esters. Phosphoric acid is able to form up to three ester bonds (Fig. 3.23), although the tri-esters are not commonly found in nature. Yet, diesters are important and enable saccharide phosphates to form polymers (e.g., nucleic acids) or bridge saccharides with other organic molecules.

Phosphate groups forming esters are anionic in aqueous environment in the most common biological pH ranges. This means that neutral molecules, such as glucose, become charged when esterified with a phosphate. The consequence is an increase in solubility in water and a decrease in the ability to cross lipid bilayers, for instance. This is deemed important as glucose metabolism starts by forming glucose phosphate (Fig. 3.23a).

Phosphate ester hydrolysis is a spontaneous but very slow process, which makes it under enzymatic control in cells. In addition, many chemical processes occurring in cells, such as condensation of polymers with formation of water, are unfavorable processes (there is "excess" water in most cell environments); enzymes speed the reaction but do not shift the equilibrium toward condensation. The use of phosphate derivatives of the monomers in the process of condensation facilitates the reaction as phosphates are so-called good leaving groups: they alter the reactivity of transient chemical species in the course of the mechanism of reaction.

ATP (adenosine triphosphate, Fig. 3.23b) is among the biological molecules that are saccharide derivatives and involves a phosphate ester. A phosphate diester bond bridging two other phosphates is another interesting characteristic of this molecule. The energy involved in the phosphate–phosphate bonds makes this molecule pivotal in energetic metabolism. Divalent cations such as Mg^{2+} are usually associated to ATP and other molecules having diphosphate groups. This reduces electrostatic repulsion between the oxygen atom of water and the negative charge of phosphate groups, facilitating the hydrolysis of phosphate derivatives.

Probably not so famous as ATP, but equally important in biochemistry, is coenzyme A (Fig. 3.23c). This is a relatively small but complex molecule. Amazingly, coenzyme A has phosphate and saccharide groups but owes its reactivity to a terminal thiol (–SH) group. This thiol group may bind an acetyl residue through a thioester bond, but may also bind a fatty acid, forming acyl-CoA, which is involved in lipid metabolism.

Nicotinamide adenine dinucleotide (NAD^+) is another interesting case of saccharide derivative that also contains phosphates. NAD^+ intervenes in redox reactions as it may accept and donate electrons, changing from NAD^+ to $NADH + H^+$ or vice versa. One extra phosphate group turns NAD^+ into $NADP^+$, which has similar redox properties but can only bind to specific enzymes that usually do not bind NAD^+. This implies that there are specific metabolic roles for $NADP^+$, distinct from NAD^+. The phosphates are involved in enzyme recognition but not in the redox activity itself (Fig. 3.23d). The same happens with flavin adenine dinucleotide (FAD and $FADH_2$; Fig. 3.23e).

Nucleotides themselves deserve closer attention because they polymerize to form the so-called nucleic acids. They will be left for further discussion in the next section. To finalize, it should be stressed that many therapeutic drugs are also sac-

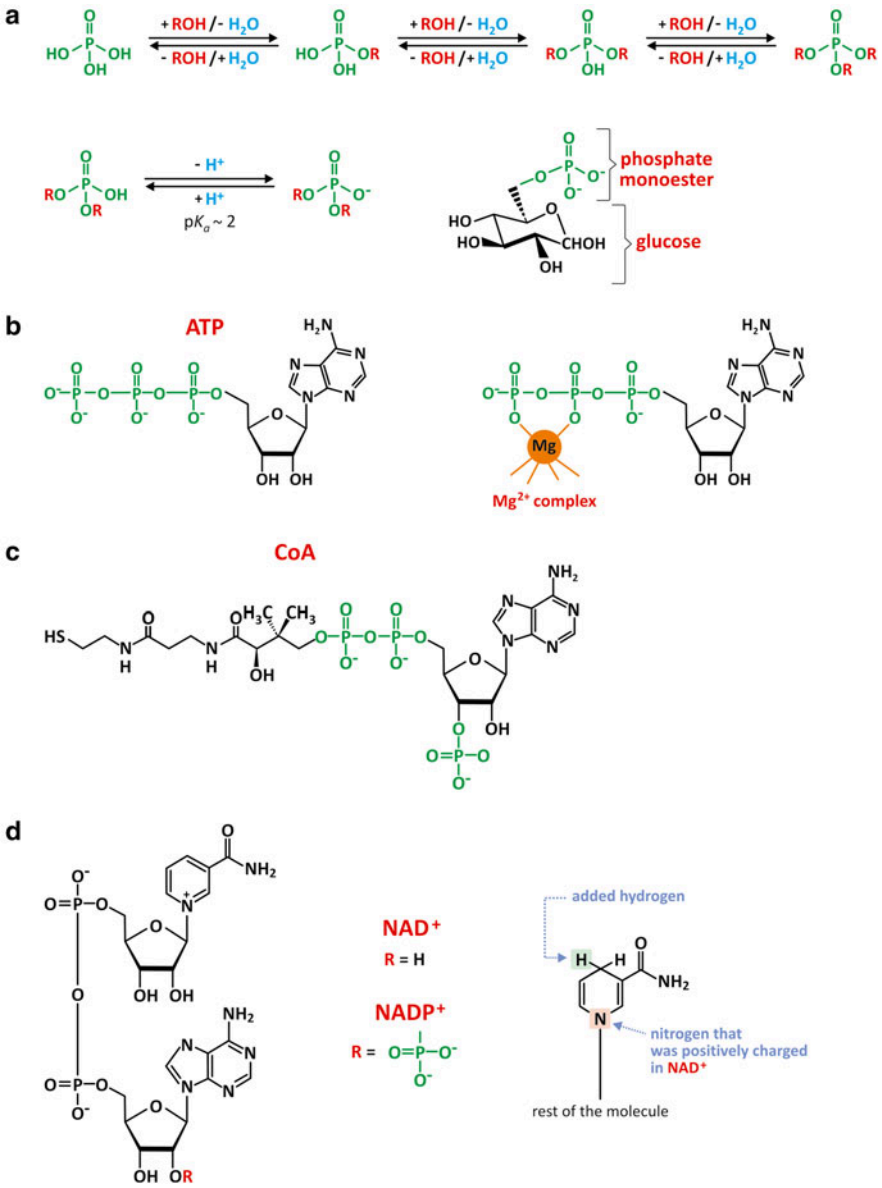


Fig. 3.23 Phosphates are able to form esters or diesters bridging two organic molecules (**a**). Phosphate confers an anionic charge to the newly formed chemical entity because the ionization of the phosphate group occurs at pH > 2, increasing its solubility in aqueous medium and decreasing its ability to translocate across lipid membranes. This is the case for glucose-6-phosphate, which is "trapped" in the cytosol of cells, where it will be processed in different metabolic pathways. Adenosine triphosphate, ATP (**b**), and coenzyme A, CoA (**c**), are important biological molecules with a saccharide residue bound to a phosphate group. ATP also contains a phosphodiester bond, very important for its reactivity in cells. CoA has a couple of phosphate groups bound to

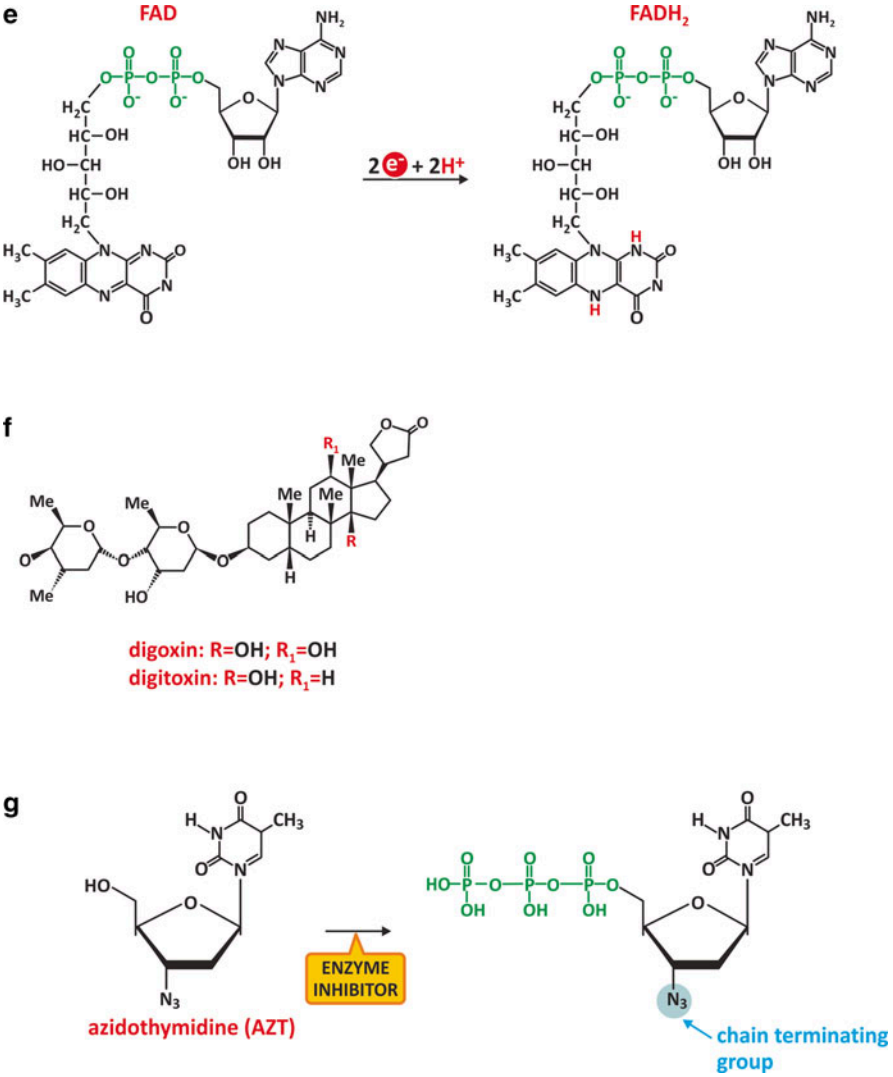


Fig. 3.23 (continued) each other, but its reactivity in cells is dictated by the sulfhydryl group (-CSH). Nicotinamide adenine dinucleotide (NAD^+) is another saccharide derivative with saccharide residues bound to phosphates. NAD^+ may be reduced to NADH (**d**). Redox reactions of NAD^+/NADH take place in a specific cyclic residue of the molecule, involving a nitrogen atom (*right*). NAD^+/NADH phosphate, $NADP^+$/NADPH (**d**), also participates in redox reactions in human metabolism. NADH and NADPH cannot be distinguished by their reducing properties because the phosphate group that distinguishes them does not interfere with the nitrogen atom that affords the redox properties. Yet enzymes use specifically NADH or NADPH and so there is no redundancy between these molecules. FAD and $FADH_2$ (**e**) are molecules similar to NAD^+ and NADH in that both constitute adenine nucleotides and their role in metabolic redox reactions. Digoxin and digitoxin (**f**) are examples of drugs with monosaccharides in their structure; more specifically, three residues are specifically combined as part of a unique structure. Another example of drug that is a saccharide derivative is azidothymidine (AZT), which is converted to a triphosphate in cells (**g**) and is able to insert in the active center of the reverse transcriptase of HIV because it is structurally similar to the natural substrate. However, the natural substrate does not have the N_3 group. The presence of this group blocks the conversion of the viral RNA into DNA

charide derivatives, such as digoxin (Fig. 3.23f), used in the treatment of heart conditions. Azidothymidine (AZT) is another example. It is an analogue of thymidine that may inhibit the action of reverse transcriptase of HIV. It was the first drug used in the treatment of AIDS. Cellular enzymes convert AZT into the effective 5-triphosphate form (Fig. 3.23g). Once bound to reverse transcriptase, the azide group, N3, is responsible for chemical inhibition. Inspired by the success of AZT (Fig. 3.24), many nucleosides are now under development to create new inhibitors of HIV reverse transcriptase to fight AIDS.

3.2.3 Molecular Conjugates of Oligosaccharides

It is worth stressing that the combination of saccharide monomers may generate a big diversity of products when compared to amino acids (Fig. 3.25). Two glucoses, for instance, can bind via 6 carbons in each monomer, thus being able to form 36

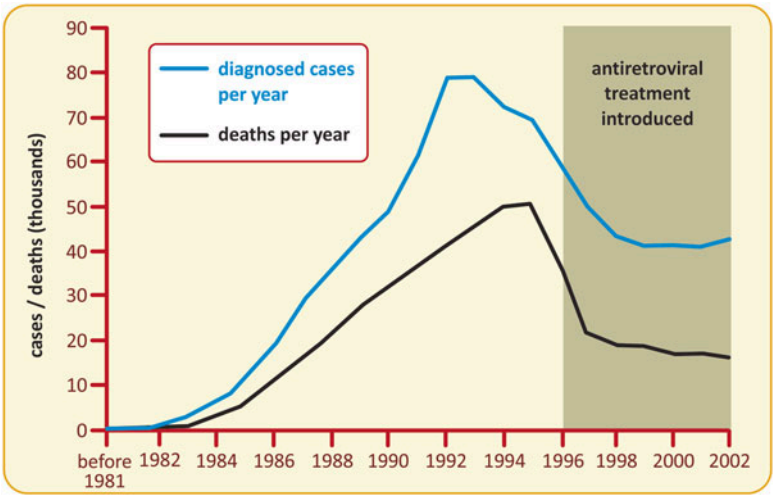


Fig. 3.24 Preventive campaigns highlighting the need to change risk behaviors had a strong impact in the spreading of AIDS in the USA, with a marked decrease in the number of diagnosed cases and deaths per year after 1993-95. The use of AZT and other drugs had a very positive additional effect in the reduction of AIDS-caused mortality

Fig. 3.25 (continued) Saccharide tags are covalently bound to lipids, usually rigid lipids such as ceramide for a better anchoring to the membrane (**b**). Glycolipids (i.e., associations of saccharides and lipids) determine blood groups, for instance (see Box 3.5). The same principle applies to oligosaccharides attached to proteins, i.e., glycoproteins (**c**). The side chain of the amino acid asparagine may react with a saccharide by dehydration forming an *N*-glycosidic bond (analogous to an *O*-glycosidic bond, but involving *N* instead of *O*). Likewise, the side chain of the amino acid serine may react with a saccharide forming an *O*-glycosidic bond. The oligomeric sequences of saccharides attached to protein IgG is shown in panel (**c**) *bottom* as an example

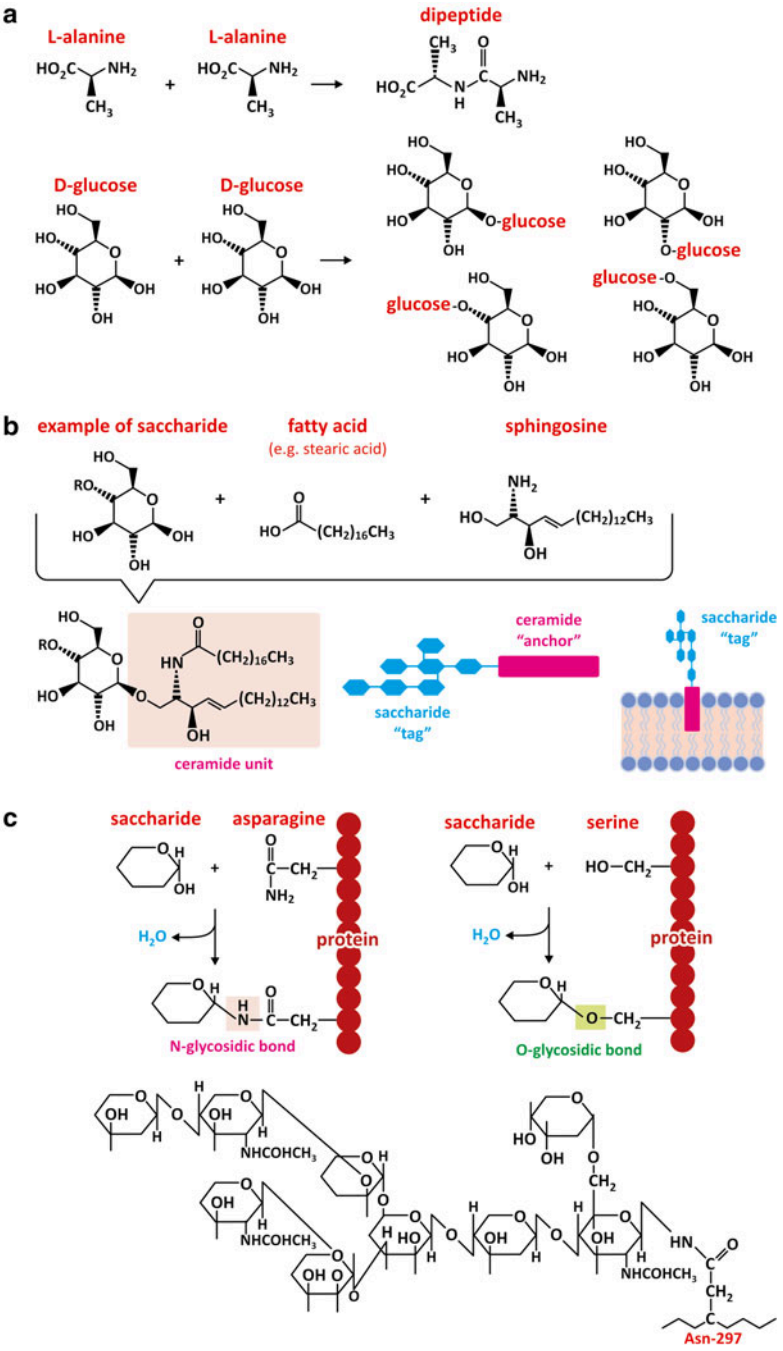
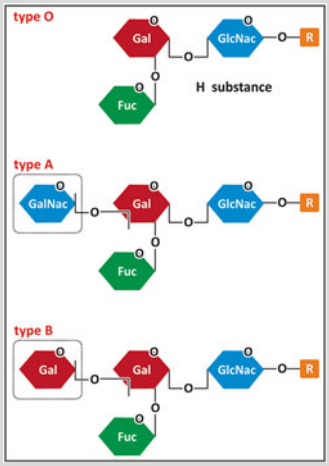


Fig. 3.25 A combination of two amino acids generates one single dimer, but there are several ways that two monosaccharides can combine to form a disaccharide (**a**). Saccharides are better suited to form highly specific structures at the surface of cell membranes (**b**) or proteins (**c**).

different molecules. Considering the anomers, the diversity increases. It is not surprising that oligosaccharides are present in the surface of cells as receptors of unique structure (see an example in Box 3.5) while amino acids form polymers (proteins) having domains with few restricted well-defined structures. Moreover, monosaccharides or oligosaccharides are frequently found in nature attached to proteins.

Box 3.5: The ABO Blood Groups

There are different blood groups according to different immunogenic molecules present in erythrocytes. The most important classification of blood groups is based on three antigens, A, B, and O that form 4 groups: A, B, O, and AB—the ABO blood groups. The ABO blood group antigens are oligosaccharide chains attached to proteins and lipids located in the outer surface of erythrocytes. One single residue of a small oligosaccharide determines whether the antigen is A, B, or O (see figure).



Fuc represents the monosaccharide fucose, Gal, galactose, GalNAc, N-acetylgalactosamine, and GlcNAc represents N-acetylglucosamine

The immune system of an individual produces antibodies against the ABO antigens not present in his own erythrocytes. Individuals in A group will have antibodies against B and vice versa. Type O, the most common, does not contain the last residue, which is the antigen, in its structure (in fact the original nomenclature was 0—zero—but became the letter O). So, individuals in blood group O will have both anti-A and anti-B antibodies. Individuals in blood group AB are rare and, naturally, have no anti-A and no anti-B antibodies. This has tremendous implications in blood transfusions as a patient cannot receive erythrocytes against which he/she has antibodies. AB individuals

(continued)

Box 3.5 (continued)

can, in principle, receive blood from any donor; O individuals can donate blood to any individual; A and B individuals can only donate and receive blood to/from individuals belonging to the same blood group.

It is believed that ABO antibody production is stimulated when the immune system contacts in foods or in microorganisms with the saccharide antigens that are absent in the erythrocytes. The functions of the ABO blood group antigens are not known. Individuals who lack the A and B antigens are healthy, suggesting that any function the antigens have is not important, at least not in modern times.

Hemolytic disease of the newborn (HDN) is a serious medical problem that occurs almost exclusively in infants of blood group A or B who are born to group O mothers. This is because the anti-A and anti-B formed in group O individuals tend to be of the IgG type, which can cross the placenta. HDN tends to be relatively mild mainly because fetal erythrocytes do not express adult levels of A and B antigens. However, the precise severity of HDN cannot be predicted.

3.2.4 Polymers of Saccharide Conjugates: Nucleic Acids

Nucleotides that compose deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) are formed by 2-deoxyribose or ribose, respectively, linked to a heterocyclic base, a purine (adenine, guanine) or a pyrimidine (cytosine and uracil or thymine), and a phosphate group attached to carbon 5 of the ribose residue. To avoid ambiguity with numbering of carbons of the heterocyclic base, the carbon numbers of the ribose and deoxyribose are identified with a prime: phosphate ester linkage occurs at C5′ (Fig. 3.26). The physical and chemical characteristics of the heterocyclic bases are extremely important as they are determinant for the way nucleotide polymers (nucleic acids) organize. The bases are planar, cyclic, aromatic molecules with N and O atoms able to participate in hydrogen bonding in the plane of the ring. The bases are low polarity groups poorly solvated, so both faces of the plane of the base rings will be fairly hydrophobic and thus subject to significant entropic effects.

There are four different possible nucleotides in RNA and DNA. RNA is formed by adenosine-5′-monophosphate (AMP), guanosine-5′-monophosphate (GMP), cytidine-5′-monophosphate (CMP), and uridine-5′-monophosphate (UMP). DNA is formed by deoxyribose, which is denoted by the prefix d in dAMP, in dGMP, in dCMP, and in dTMP. dTMP stands for thymine-5′-monophosphate using deoxyribose; DNA does not contain dUMP.

Because nucleotides are phosphate monoesters, they can form additional ester links to other alcohols, such as the OH groups in other nucleotides. In other words, they can polymerize by dehydration reactions. Nucleic acids are formed by phosphodiester bonds between C5′of one nucleotide and C3′ of another nucleotide (Fig. 3.26c). The result is a linear polymer having the heterocyclic bases and the

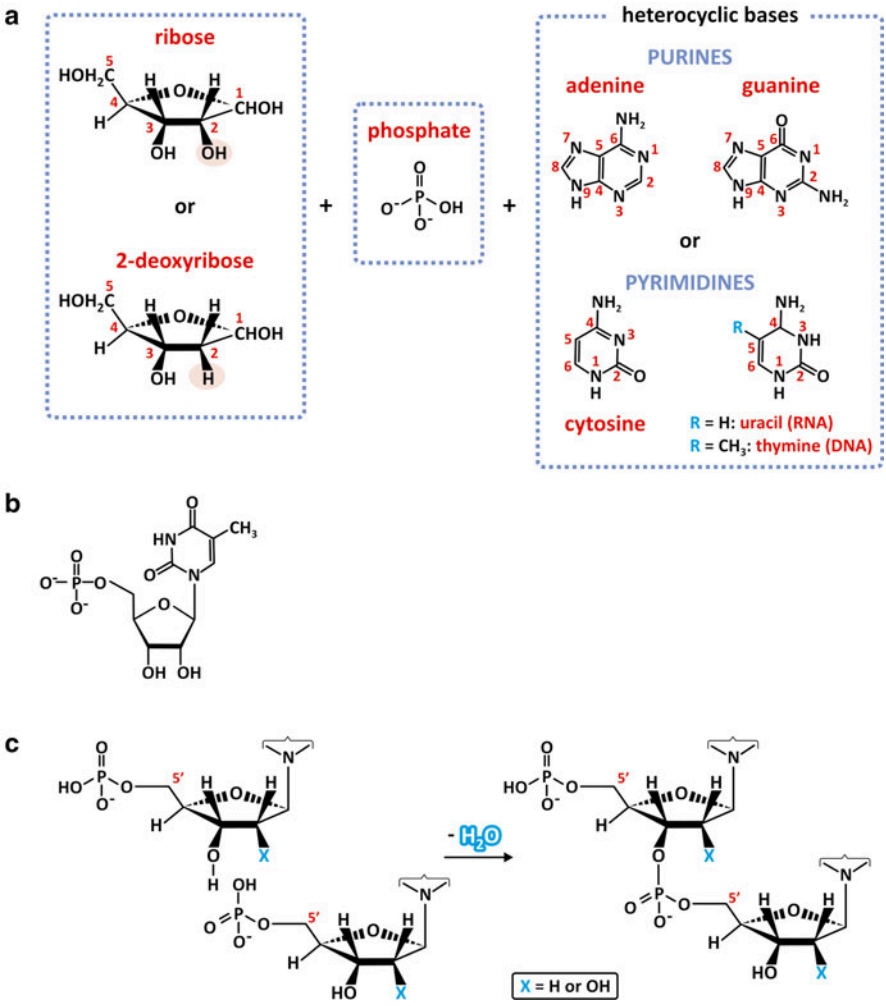


Fig. 3.26 Nucleotides are formed with ribose or 2-deoxyribose, phosphate and a heterocyclic base, purine (adenine or guanine) or pyrimidine (cytosine, uracil, or thymine) (**a**). The phosphate group forms a phosphodiester bond in C5 and the heterocyclic base binds to C1. The nucleotide deoxythymidine phosphate is shown as an example (**b**). A dimer of nucleotides may be formed by dehydration, which creates a phosphodiester linkage between the monomers via C5′ and C3′ (**c**). In RNA X=OH, in DNA X=H

phosphate groups in opposing sides, the phosphate groups being anionic (Fig. 3.27). Some simplified representations of nucleic acids pinpoint this characteristic (e.g., Fig. 3.27b), which remains elusive when the nucleic acid is simply represented by a sequence of letters identifying the nucleotides (T, thymine; C, cytosine; G, guanine; A, adenine; U, uracil). By convention, nucleic acid sequence is written from the C5′ to the C3′ endings, 5′ → 3′ (Fig. 3.27).

Unlike polysaccharides, nucleic acids are amphiphilic molecules (Fig. 3.27) so the entropic effect will be a significant driving force for folding in aqueous environ-

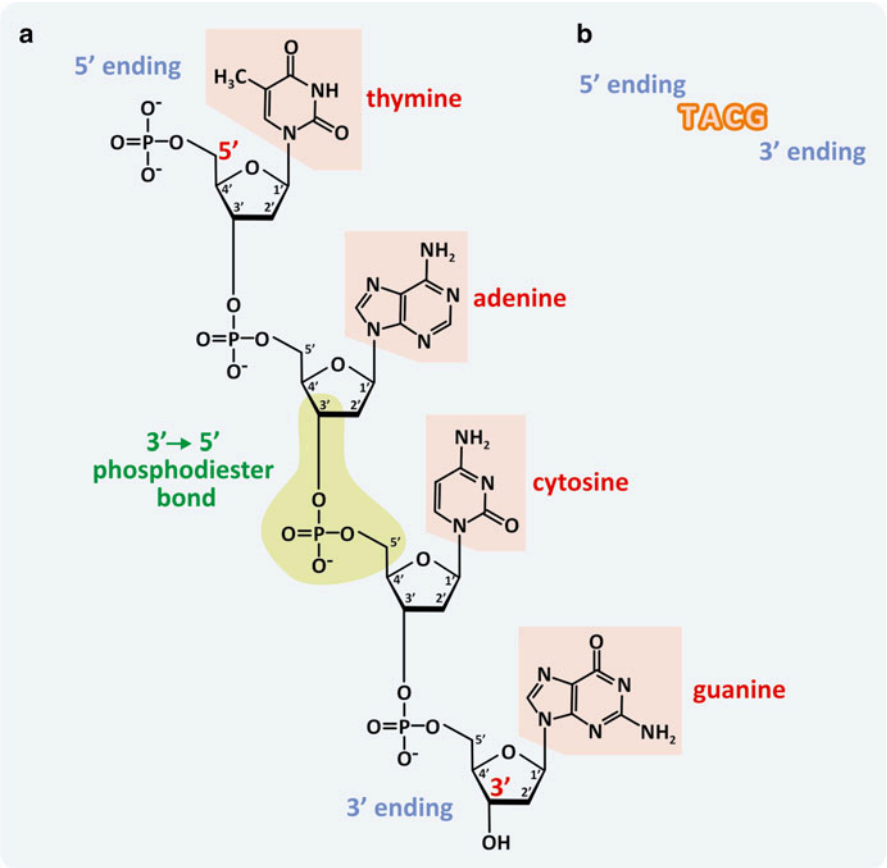


Fig. 3.27 Natural polymers of nucleotides are named nucleic acids. Desoxyribonucleic acid (DNA) has 2-deoxyribose residues and uses guanine, cytosine, adenine and thymine but not uracil (**a**). Ribonucleic acid (RNA) has ribose residues and uses guanine, cytosine, adenine and uracil but not thymine. Both polymers are formed by C3′–C5′ phosphodiester bonds. For the sake of simplicity, the chemical structure of the monomers is usually omitted, and other forms of presenting the nucleotide residues sequence are preferred. The simplest and most common form represents the nucleotides by a one-letter code (the first letter of the base name: G, A, C, T or U). Which end is the free, C5′ or C3′, is not explicitly mentioned but it is established by convention that the sequences are presented in the directions 5′ to 3′ (**b**)

ment. The heterocyclic bases will tend to nucleate to minimize their contact with water molecules. The crystal structure of transfer RNA (tRNA) shows that the bases stack parallel to each other, which is favored by their strictly planar structure. In addition, the nucleic acid tends to twist along its major axis forming a helix that exposes the phosphates to the aqueous medium and has the bases stacking in its core. In addition, most of the helical regions in tRNA consist of two sequences of the same RNA chain running in opposite directions with bases in opposite sequences contacting each other close enough and with the adequate stereochemical arrangement to establish hydrogen bonding between them. This adequate arrangement only occurs if purines pair with pyrimidines, as in pairs A–U and G–C (Fig. 3.28). This

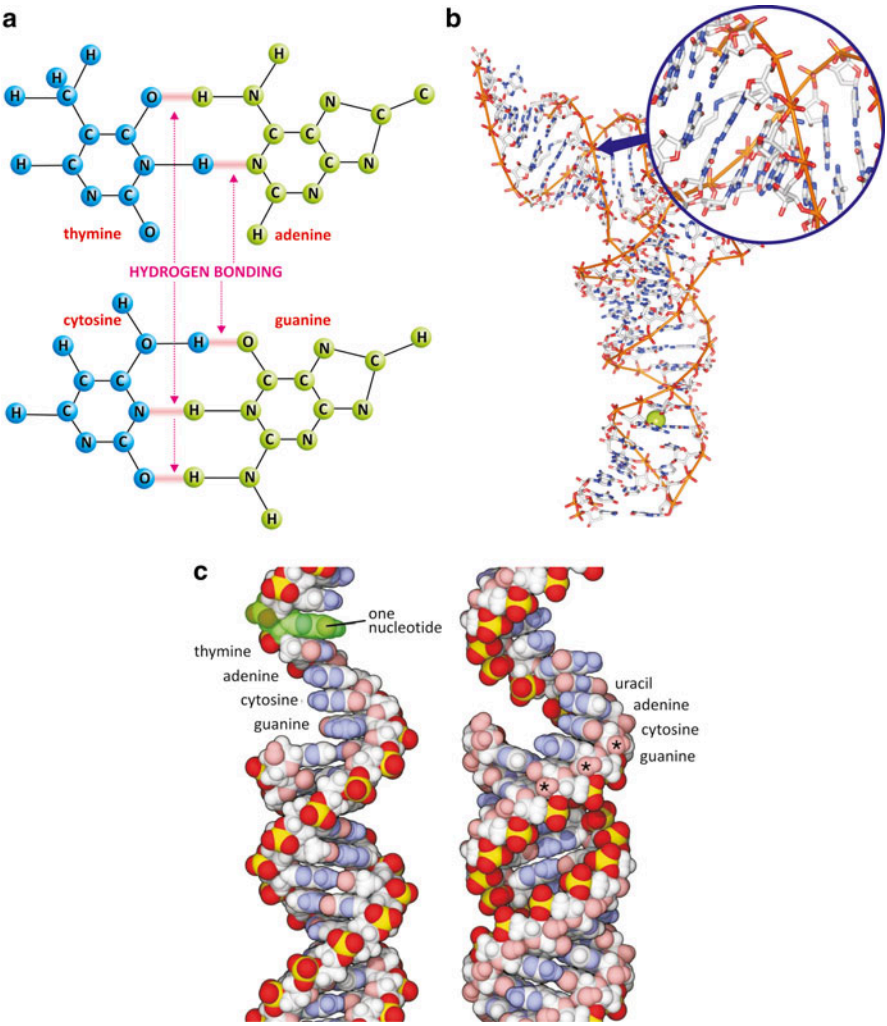


Fig. 3.28 Heterocyclic bases are flat. Hydrogen bonding occurs in the plane of the rings. Purines and pyrimidines are able to interact because of the match in the number and orientation of H doning/acceptor groups, forming pairs T-A and C-G (**a**), which is known as Watson–Crick base pairing. Because bases are so flat, relatively hydrophobic on both sides, and undergo base pairing, nucleic acids may bind complementary sequences of nucleotides in the same polymer or from a different polymer. The entropic effect will cause this arrangement to twist around its long axis forming a double helix in which the polar parts of the molecule, phosphate and pentose residues, are exposed to the aqueous medium shielding the relatively hydrophobic bases. In the center of this helix, the bases stack parallel to each other and are slightly rotated relatively to each other. This kind of organization can be found even in some domains of the transfer RNAs (**b**; PDB 2TRA). The OH groups present in C2′ groups of RNA (*asterisk* in panel (**c**) *right*) but not DNA (panel (**c**) *left*) have structural implications in the conformation of nucleic acids as these groups contribute to shield the core of the double strands from the aqueous environment. Panel (**c**) was reproduced from Goodsell, The Machinery of Life, 2009

is known as Watson–Crick base pairing. Hydrogen bonding occurs at the edges of heterocyclic bases, in the plane of the rings.

In regions in which the two opposing antiparallel sequences of tRNA have a considerable array of complementary base pairs, both RNA sequences fold into a helical structure to keep the parallel stacked base pairs in the core surrounded by the pentose and phosphate esters backbone. This is the double helix structure frequently associated to DNA but equally present in some regions of the RNA molecule. Ribosomal RNA structure is similar to that of tRNA (Fig. 3.28). It is also worth stressing that DNA polymers may have complementary RNA polymers, associate with them and even fold into helices. However, the alcohol group, OH, at C2′ makes the structure of RNA less compact due to its volume and polarity.

The structure of RNA is not only less compact, but it is also less chemically stable. The OH group at C2′ is close to the phosphate diester bond with which it can react to hydrolyze RNA (Fig. 3.29). Because RNA molecules have transient functionalities and are not stored for very long periods in the cells, this limitation of RNA is not a problem. DNA is less prone to hydrolysis because it lacks the OH group in C2′, being the molecule that natural evolution selected to store genetic information for longer periods.

Some drugs target DNA taking advantage from the parallel stacking of heterocyclic bases. Notably, most of these molecules are composed of hydrophobic planar heterocyclic groups able to intercalate the base pairs of DNA (Fig. 3.30). Some of these molecules are used in the treatment of cancer because they prevent cell multiplication.

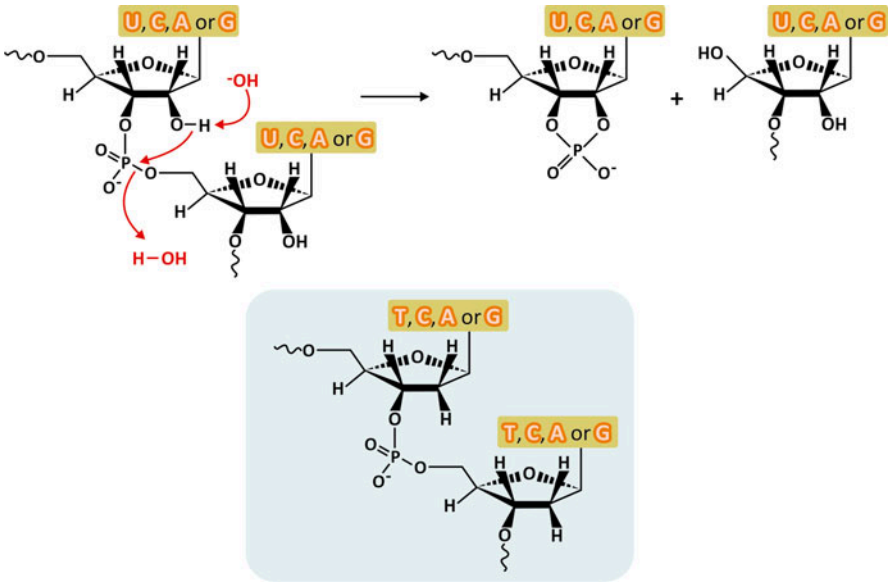


Fig. 3.29 Intramolecular hydrolysis of the phosphodiester bonds of RNA caused by a base (*top*). The absence of a hydroxyl group at C2′ increases the hydrolytic stability of DNA relative to RNA (*bottom shaded structures*)

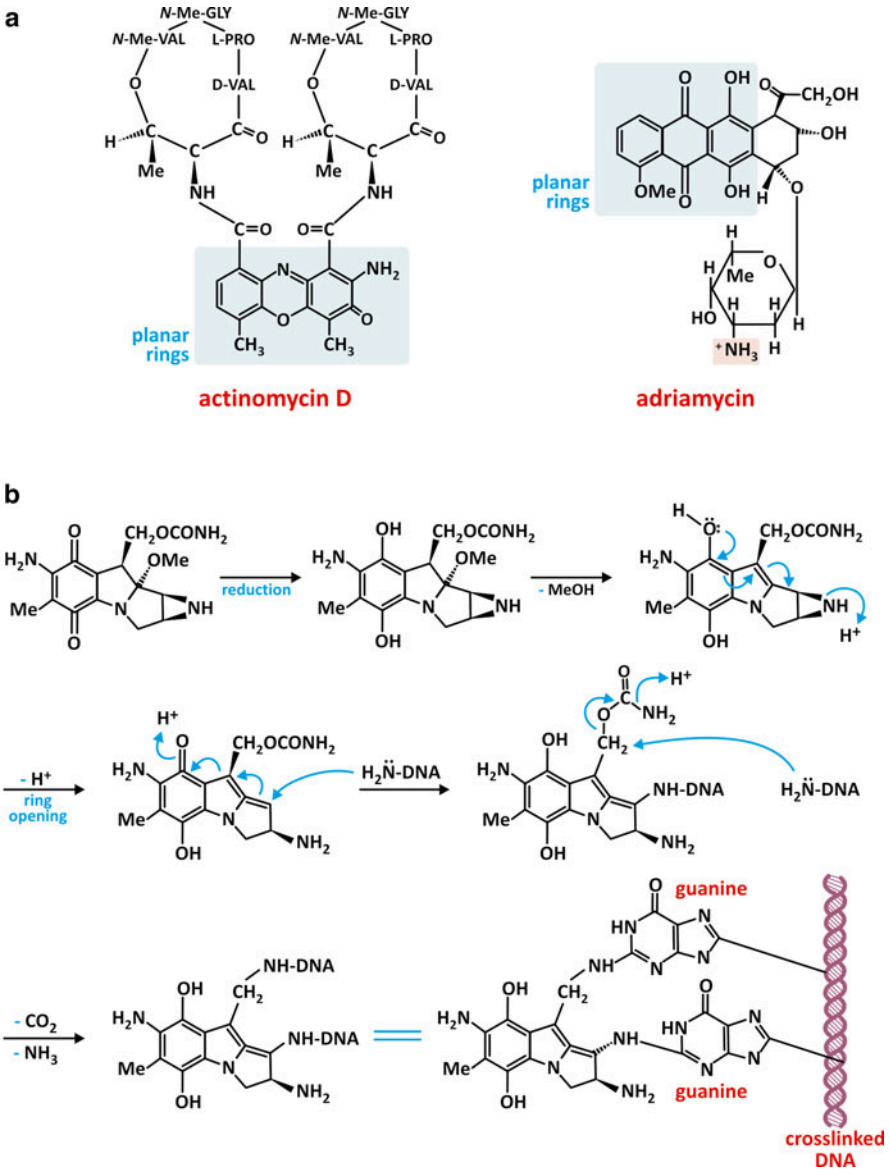


Fig. 3.30 Actinomycin D is an antibiotic with anticancer activity (**a**). It binds DNA because it has a flat polycyclic and relatively hydrophobic group able to intercalate the stacked bases of DNA, preventing RNA synthesis. Adriamycin (**a**) is also an anticancer drug that operates with the same mechanism of action: intercalation of a polycyclic flat hydrophobic group between the base pairs of DNA, preventing cell proliferation. Mitomycin C (**b**) has a different mechanism of action: it is a DNA cross-linker by covalently linking two guanines. The direct contact with these nucleotide residues is possible because this drug is a polycyclic flat and relatively hydrophobic molecule

3.3 Amino Acids and Their Polymers: Peptides and Proteins

Chemically speaking amino acids are molecules that simultaneously have carboxyl (–COOH) and amine ($–NH_2$) groups. Biochemists focus on α-amino acids, in which these groups are bound to the same terminal carbon (the so-called α-carbon in older organic chemistry nomenclatures), because naturally occurring proteins are polymers of α-amino acids. These amino acids have the structure depicted in Fig. 3.31. Besides the amino and carboxylic acid groups, the α-carbon (also named central carbon) attaches a hydrogen atom and another group, the so-called side chain that is represented by R. In nature R is one of 20 more common groups with few exceptions, which are usually derivatives of these 20 groups.

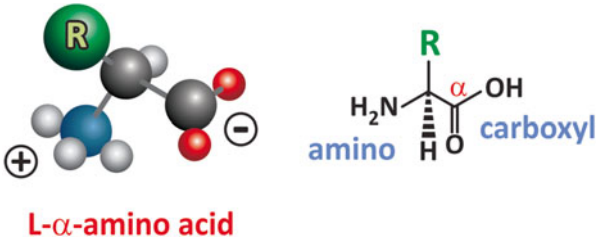


Fig. 3.31 The structure of α-amino acids. Depending on pH, in aqueous solution the amino group may be protonated and the carboxylic acid deprotonated, which makes amino acids potential zwitterions, i.e., bearing two opposite charges. Being weak bases and acids, amino acids have the ability to constitute buffers themselves (see Sect. 2.1.1)

Another interesting peculiarity of naturally occurring amino acids besides being α-amino acids is that they are almost exclusively L-enantiomers as the α-carbons are chiral centers. The other enantiomer is named D. The L and D nomenclature for the stereochemistry of the amino acids was established by Emil Fisher in analogy with glyceraldehyde, which also has a single chiral center with two possible enantiomers. Another nomenclature, more complex and following modern rules, exists to describe the stereochemistry of amino acids, but the predominance of L-amino acids and the simplicity of the L vs. D system resulted in the long-term longevity and universality of this system.

One simple empirical rule to distinguish L- from D-enantiomers is to adopt the perspective of the chemical structure of the amino acid along the H-αC axis (Fig. 3.32). The groups COOH, NH_2, and H appear projected as the vertices of a triangle. You can now recognize "corn" written clockwise in L-enantiomers or counterclockwise in D-enantiomers. This is the CORN rule of thumb.

The chemical nature of the lateral chain, R, is determinant for biochemical processes in which amino acids participate and for the structure that proteins adopt when such amino acids are present. Broadly speaking, amino acids can be grouped in four different categories based on polarity and acidic/basic nature of R (Fig. 3.33): acidic, basic, neutral polar, and neutral nonpolar. Other classification systems are based on the chemical nature of R: hydrocarbons, carboxylic acids, amides ($–CONH_2$), acyclic nitrogen containing, hydroxyl, sulfur containing, and nitrogen heterocycles. Figure 3.33

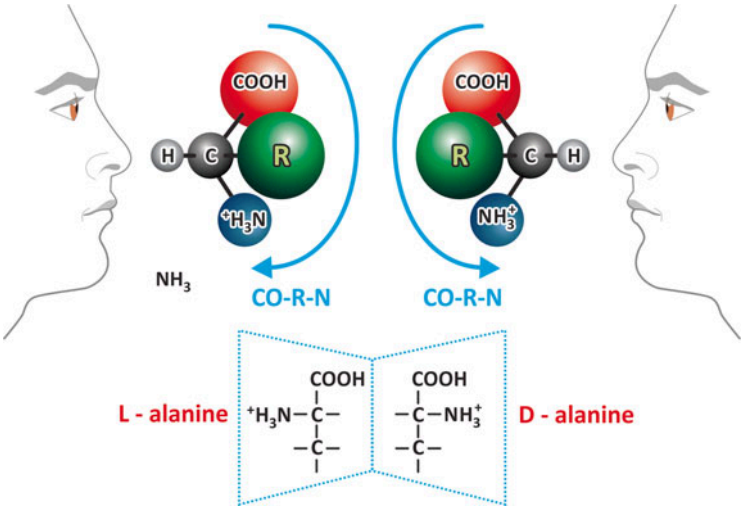


Fig. 3.32 The L vs. D nomenclature revealed by the CORN rule of thumb. When R=H (this happens in glycine, the simplest amino acid), chirality does not exist as two equal substituents (H, in this case) are attached to the central carbon. The example of L- and D-alanine is presented highlighting that they are mirror images

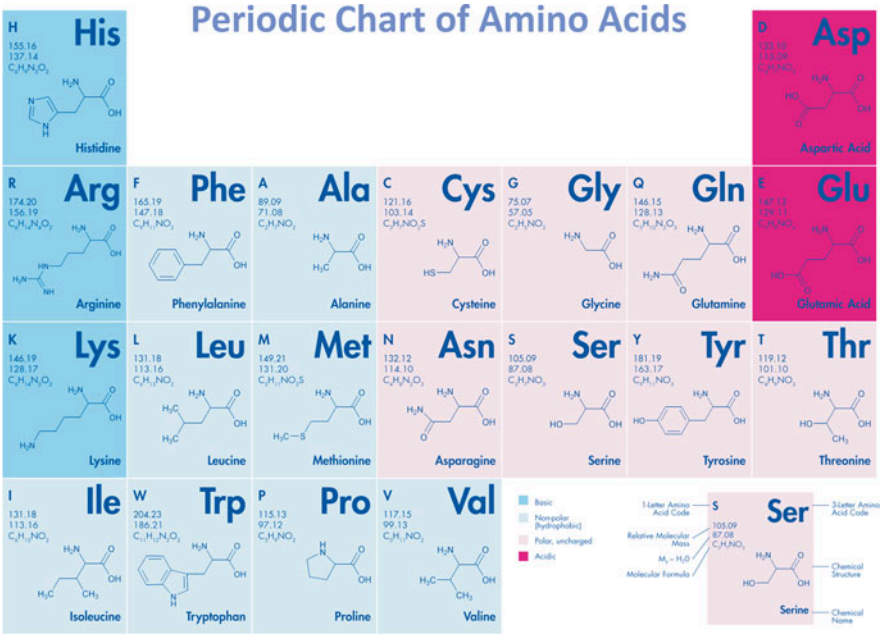


Fig. 3.33 Periodic chart-like arrangement of the natural amino acids. Figure reprinted with the permission of Bachem, Bubendorf, Switzerland

includes grouping of the amino acids according to polarity and charge and shows the chemical nature of the side chains. Table 3.4 clarifies the relationship between the names of amino acids in full and the three-letter and one-letter code abbreviated nomenclature. It also summarizes the most relevant properties of amino acids.

Table 3.4 Natural amino acid nomenclature (three-letter and one-letter code) and main properties. An essential amino acid cannot be synthesized *de novo* and therefore must be obtained from diet

Nomenclature rationale for one-letter code	Amino acid	Three-letter code	One-letter code	Main properties
First letter is unique	**Cys**teine	Cys	**C**	Thiol side chain susceptible to oxidization to form disulfides
	Histidine	His	**H**	Essential amino acid with imidazole side chain. The imidazole side chain has a pK_a of approximately 6.0, which implies that relatively small shifts in most frequent physiologically relevant pH values will change its average charge, which in turn may impact significantly on protein structure
	Isol**eu**cine	Ile	**I**	Essential amino acid isomer of leucine. Chiral side chain
	Methionine	Met	**M**	Side chain possesses a S-methyl thioether, which may be a source of sulphur for cartilage healing. It has been suggested that methionine is able to strengthen the structure of hair and nails because its side chains may cross react
	Serine	Ser	**S**	Residues of serine are found in some phospholipids (besides proteins)
	Valine	Val	**V**	Essential amino acid. Like Leu and Ile, has a branched side chain
First letter not unique. Most frequent amino acids have priority	**Ala**nine	Ala	**A**	D-Alanine occurs in bacterial cell walls and in some peptide antibiotics. Side chain is very small (methyl group)
	Glycine	Gly	**G**	Side chain consists in H, making Gly the only achiral and the smallest possible amino acid
	Leucine	Leu	**L**	Essential branched side-chain amino acid
	Proline	Pro	**P**	The amine nitrogen is bound to two alkyl groups forming a cyclic side chain, which gives proline an exceptional conformational rigidity compared to other amino acids. When proline is involved in a peptide bonding, its nitrogen is not bound to any hydrogen, meaning it cannot act as a hydrogen bond donor, causing a disruption of α-helices and β-sheets
	Threonine	Thr	**T**	Essential amino acid. Chiral side chain. The hydroxyl group in the side chain is prone to glycosylation and phosphorylation

(continued)

Table 3.4 (continued)

Nomenclature rationale for one-letter code	Amino acid	Three-letter code	One-letter code	Main properties
First letter not unique and less frequent: letter with phonetic similarity or side chain chemical nature	**Arg**inine	Arg	**R**	The guanidinium group in the side chain is positively charged at physiological pH ranges therefore prone to binding negatively charged groups. This group has also the ability to form multiple H bonds
	Asparagi**n**e (side chain contains N)	Asn	**N**	Its side chain is curiously an amide (like in peptide bonds). Ows its name to asparagus because it was first detected in aspargus juice
	Aspartate	Asp	**D**	Together with glutamic acid, aspartate is an acidic amino acid because of the carboxylic group in the side chain
	Glutamate	Glu	**E**	In addition to its role in proteins and amino acid metabolism, in neurosciences glutamate is a very important neurotransmitter
	Glutami**n**e	Gln	**Q**	Its side chain is curiously an amide (like in peptide bonds) formed by replacing the side-chain hydroxyl of Glu with an amine functional group.
	Phenylalanine	Phe	**F**	Essential amino acid with a benzyl side chain, which makes it fluorescent and neutral
	Tyrosine	Tyr	**Y**	Tyrosine has a phenol group in the side chain, which makes it fluorescent. More importantly, the phenol group functions as a receiver of phosphate mediated by protein kinases (so-called tyrosine kinases) resulting in alterations in the activity of the target protein.
	Tryptophan (side chain with double ring)	Trp	**W**	Essential amino acid having a fluorescent indole functional group in the side chain. The indole group is bulky and hydrophobic, so Trp is commonly found in lipid-contacting domains of proteins, such as transmembrane regions of membrane proteins or fusion domains of viral proteins
Nearest first letter	**Lys**ine	Lys	K	Essential amino acid. Like in Arg, Lys side chain participates in hydrogen bonding and is cationic at physiological pH range, therefore prone to binding negatively charged groups
Unknown	(Unknown amino acid)	–	X	Unidentified amino acids in peptide or protein structure are generically represented by X

It should be kept in mind that the ionization states of amino acids vary with pH, so depending on pH, amino acids may have different net charges. The example of histidine is presented in Fig. 3.34. Histidine is peculiar as the side chain changes ionization ($pK_a \sim 6$) not far from the range of plasma and cytoplasmic pHs. The intermediate value of the neutrality range (from pH 6 to 9), the so-called isoelectric point, pI, is 7.6, within the range of plasma and cytoplasmic pH range, which happens only for histidine.

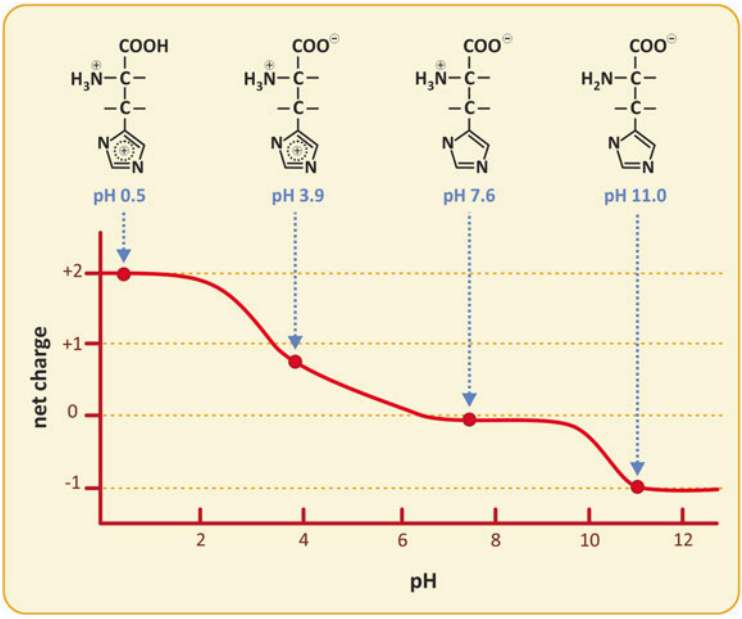


Fig. 3.34 Variation of the net charge of His with pH. The molecule has three ionizable groups, one acidic and two basic. Therefore, allowed global charges range from −1 to +2. However, in most common physiological pHs, the global charge is nearly nil

3.3.1 From Monomers to Polymers: Peptides and Proteins

Amine and carboxylic groups may react, forming amide bonds (Fig. 3.35). Amide bonds connecting several amino acids form a peptide. Many amino acids connected through amide bonds form a protein. There is no precise limit to separate the number of amino acid monomers in peptides and proteins although 30 is usually taken as a reference value.

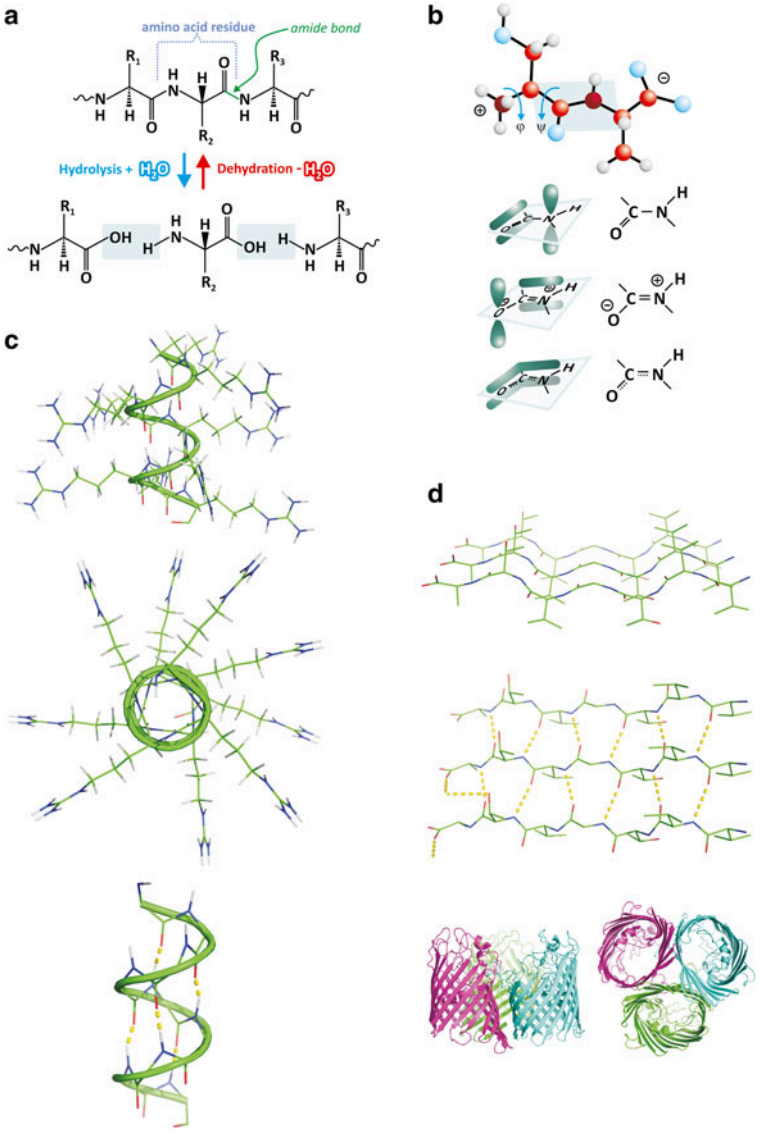


Fig. 3.35 Dehydration reactions among amino acids lead to polymerization through amide (also known as "peptide") bonds (**a**). The CO, CN, and NH bonds are coplanar because of the electronic distribution among the connected OCN set of atoms (**b**). This implies that when the polymeric chain folds, flexibility is limited and specific arrangements tend to be adopted, which include α-helices (**c**) and β-sheets (**d**). Whether a certain sequence of amino acid residues adopts the conformation of α-helix, β-sheet, or any other depends largely on the amino acids involved, their order, and environmental factors such as solvent polarity, pH, and temperature. Both α-helices and β-sheets are conformations that enable the occurrence of frequent intramolecular hydrogen bonding and externalize the location of side chains [(**c**) and (**d**)]. Proteins may be formed almost exclusively of α-helices, such as hemoglobin (see figure 3.42), or β-sheets, such as porin (**d**, bottom) or be a mixture of both. Images of porins are a courtesy of Dr. Claudio Soares, ITQB-UNL, Portugal

Among biochemists, amide bonds forming peptides or proteins are generally referred to as peptide bonds. Because peptide bonds are very planar (C=O, C–N, and N–H bonds are coplanar) due to electron distribution limitations imposed by specific molecular orbitals and have the R groups in close vicinity, the chain of peptide bonds forms a polymer that is not freely flexible. It articulates with spatial constraints, which means that the polymeric chain tends to adopt fixed angles between its amide groups; these angles are the ones that allow accommodating the side chains of the amino acids and adapting the orientation of the amide bonds to each other (Fig. 3.35). As shown in Fig. 3.33, there is a wide diversity of side chains in charge, polarity, and size. All these parameters influence the way a protein folds to cope with the electrostatics, hydrogen bonding, entropic effects (hydrophobicity), and occupation of 3D space. In the end, all these factors determine that amino acid polymers have two different favored kinds of regular conformations: α-helices and β-sheets. Many other folds exist but are not as common because these two are the ones that better accomodate the stabilization of amino acid sequences.

In a α-helix, the peptide bond sequence (i.e., the peptide or protein "backbone") adopts a helical structure projecting the side chains, R, to the exterior of the helix. It is a very stable structure because there are almost no constraints to spatially accommodate R and because the vast array of C=O and N–H groups in the backbone interact strongly through frequent hydrogen bonds. Many proteins, such as hemoglobin, are composed of several helical segments in their amino acid residues sequence. To facilitate protein representation and reading, helical segments are usually represented as a helical ribbon or a cylinder. This highlights the conformation of the segments, although it overlooks what specific amino acids are involved.

β-sheets are extended conformations that turn in specific points resulting in several linear amino acid residue sequences antiparallel to each other. Like in helices, this enables frequent hydrogen bonding in the protein backbone and projection on the side chain groups to the exterior of this compact arrangement. A certain degree of bending is allowed, and big extensions of β-sheets are usually associated to very stable proteic structures, such as membrane pores. β-sheet representation is usually done with straight ribbons and/or arrows (Fig. 3.35d).

The complete protein structure is described in three or four levels. The primary level is simply the sequence of amino acids that compose the protein, conventionally counted from the free amine terminal to the free carboxyl terminal. This elucidates the chemical nature of the protein but tells us little about what are the domains engaging α-helices, β-sheets, or none, which form the secondary-level structures. α-helices and β-sheets from different parts of the same protein tend to interact with each other toward mutual stabilization by means of electrostatic forces, hydrogen bonding, and entropic effects contributions (Fig. 3.36). The tertiary level arises there from α-helices, β-sheets, and other local arrangements that organize in space to form the protein structure itself. Occasionally, there are different parts of the

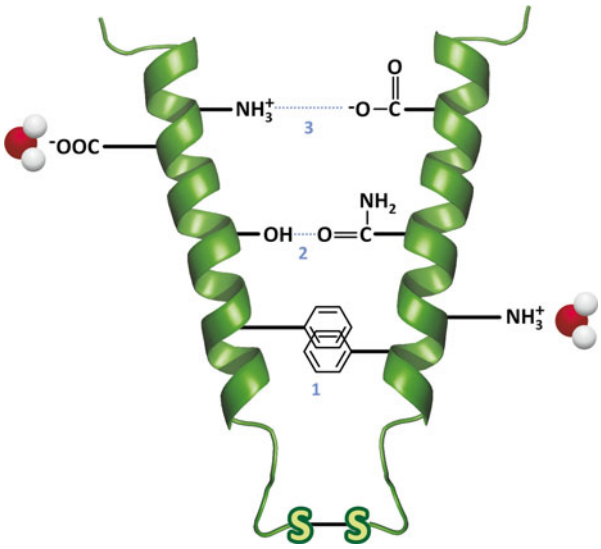


Fig. 3.36 Secondary-level structures such as helices interact intramolecularly or intermolecularly through electrostatic forces (3), hydrogen bonding (2) or entropic effect factors that result in exposure of polar groups such as $–COO^-$ and $–NH_3^+$ to aqueous solvent, and association of hydrophobic groups with minimal exposure to the aqueous environment (1). Two Cys residues in contact may react through the thiol groups (–SH) in the side chains forming disulfide bonds (S–S) that strongly contribute to the stabilization of the structure of proteins (see as an example the structure of insulin in Fig. 3.37)

global geometry of the protein that form fairly independent and separable parts, frequently having specific dynamics and specific functions. These are known as domains. An upper level exists for proteins that associate with other proteins, equal or not, to form organized protein assemblies: the quaternary-level structure. The different levels for protein structure are illustrated in Fig. 3.37, using as example insulin, whose structure was discovered by Dorothy Hodgkin, whom also discovered the structure of cholesterol (see Fig. 2.10).

Hydrogen bonding is frequently the strongest non-covalent factor in keeping the tertiary and quaternary levels of the structure of proteins. Enolase is a good example. Although there are no covalent bonds between both proteins in the dimer,

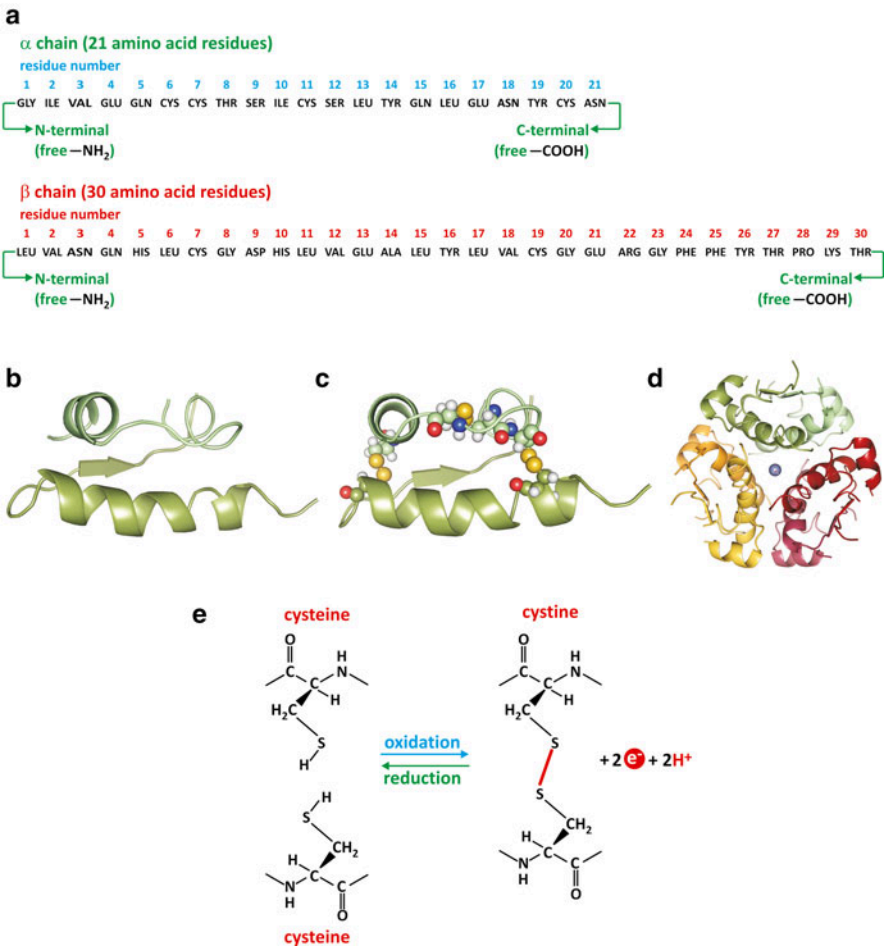


Fig. 3.37 The hormone insulin, from primary to quaternary-level structure. (**a**) Amino acid (three-letter code) sequence, the primary-level structure. (**b**) Segments engaging α-helical secondary-level structure are represented as helical ribbons. (**c**) The protein folds into a tertiary-level structure that is stabilized by disulfide bonds (*yellow* in the protein structure). Disulfide bonds are the result of oxidation of two thiol (–SH) groups to form an S–S bond (**e**). It is common that Cys residues react this way in extracellular proteins. (**d**) Six insulin monomers associate forming a homohexamer, the quaternary-level structure. The quaternary-level structure is stabilized by the presence of two zinc ions (central sphere) and due to contacts between hydrophobic surfaces of monomers (entropic effect). Insulin is stored in the pancreatic beta cells and secreted into the bloodstream in the form of aggregates of these compact hexamers. Upon dilution in the blood, insulin dissociates, and the active form is believed to be the monomer

hydrogen bonds are frequent (Fig. 3.38). Altogether, the sum of all hydrogen bonds creates a strong network of adhesion forces in the contact surface of the proteins. Hydrogen bonds are directional; they occur in a well-defined direction between chemical groups at a definite distance; this further contributes to maintain the structure of proteins. The extreme contribution of hydrogen bonding to polymer structure

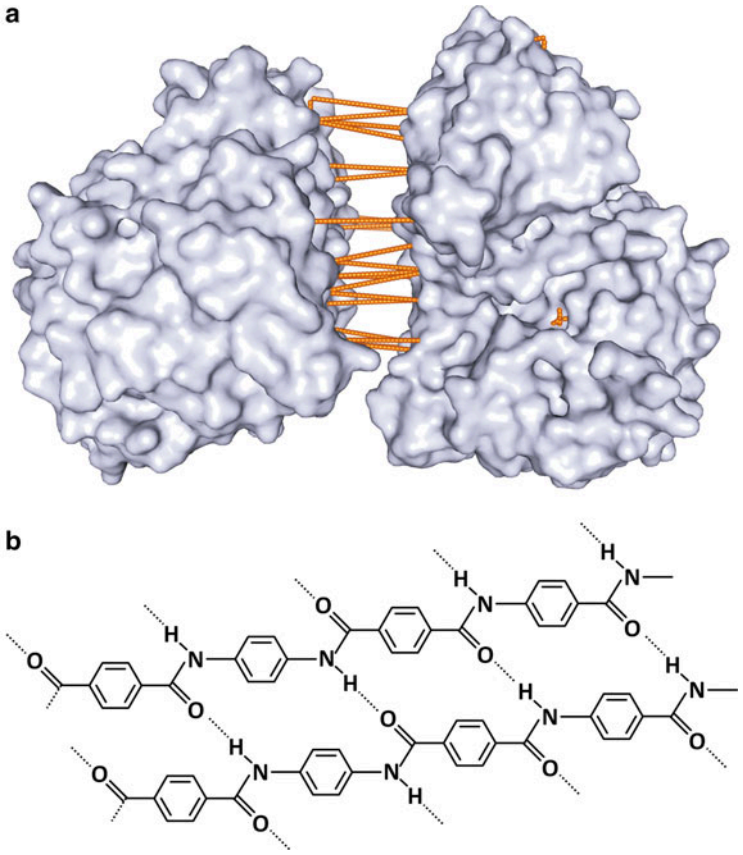


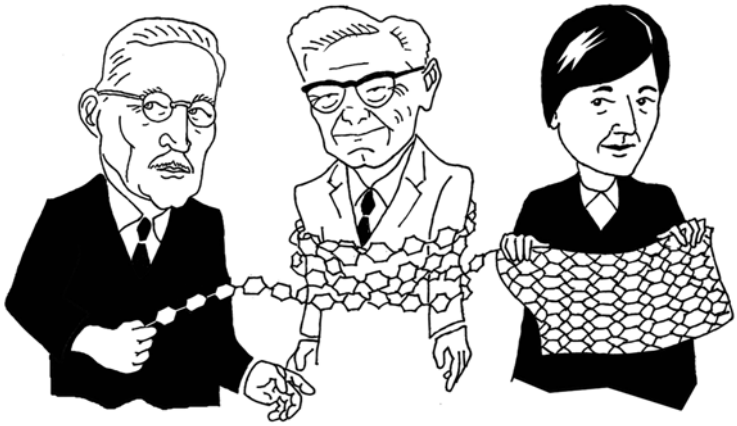
Fig. 3.38 Examples of hydrogen bonding contribution to polymer structure. (**a**) Enzyme enolase (PDB 1IYX) is a dimer in which both subunits are attached by a dense array of hydrogen bonds in the contact surface between them. (**b**) The structure of an amide polymer (such as proteins) commercially known as Kevlar. It involves a dense network of hydrogen bonds, which confers high resistance, and Kevlar is used in protective materials such as helmets and bullet proof vests. Compare the molecular-level details of Kevlar and β-sheets in proteins; there is a parallelism between the resistance of Kevlar and the extreme stability of aggregates formed by the juxtaposition of β-sheets in amyloid plaques (see Box 3.6)

may not be intuitive, but one should bear in mind that Kevlar, an extremely resistant material used in protective items such as bulletproof vests, owes its properties in part to hydrogen bonding (Fig. 3.38).

Kevlar was named after its inventor, the chemist Stephanie Louise Kwolek, whom had planned to attend a medical school but started a temporary job in chemistry and finally quit a medical career. The historic parallelism between artificial polymeric materials and biological molecules dates back to 1920, when Hermann Staudinger proposed that rubber and other polymeric molecules such as starch, cellulose, and proteins are long chains of short repeating molecular units linked by covalent bonds, a disruptive concept at that time. Staudinger used the term macromolecule

("makromoleküle") for the first time, a term now very popular among biochemists. Paul Flory, a chemist pioneer of the studies of three-dimensional organization of polymers and its relation to dynamics, also worked for the rubber industry during certain periods of his career. His work opened the field to the establishment of structure–function relationships in macromolecular biochemistry. Flory was awarded the Nobel Prize in Chemistry in 1974 "for his fundamental achievements, both theoretical and experimental, in the physical chemistry of macromolecules."

Thinking of natural protein fabrics, such as silk and spider webs, and artificial fabrics made of nylon and other polymers helps us realize that in molecular world the boundaries between nature and human artifacts are extremely tenuous.



As mentioned before, there are also covalent contributions to the tertiary level of structure of proteins, namely, disulfide bonds (or "bridges") and attachment of metal or other non-proteic groups to more than one amino acid residue. Disulfide bonds are formed by oxidation of two contacting Cys thiol (-SH) groups originating an S–S bond between the Cys residues (cystine). Cell cytosol is a relatively strong reducing environment, and the contribution of disulfide bonds to the stabilization of cytosolic proteins is limited. However, in other circumstances, disulfide bridges form and are strong stabilizers of protein structure at the tertiary level. Insulin, a peptide hormone, is an example (see Fig. 3.37).

Metals can bind multiple ligands and covalently link different amino acid residues in a protein, therefore also contributing to stabilize a tertiary-level structure. Frequently, metals bind to the thiol group of Cys. In the electron transfer chain proteins, several metallic complexes are present, which in addition to chemical functions also contribute to the stability of the proteins (Fig. 3.39; see also Sect. 6.2.2).

When one refers to quaternary-level structure, one usually refers to proteins that associate with high specificity and well-defined function, such as hemoglobin. This does not include pathological cases in which aggregation of proteins leads to loss of function and increase in toxicity. Extensive tertiary-level alterations are observed when amyloid fibers form upon aggregation of proteins or when prions trigger

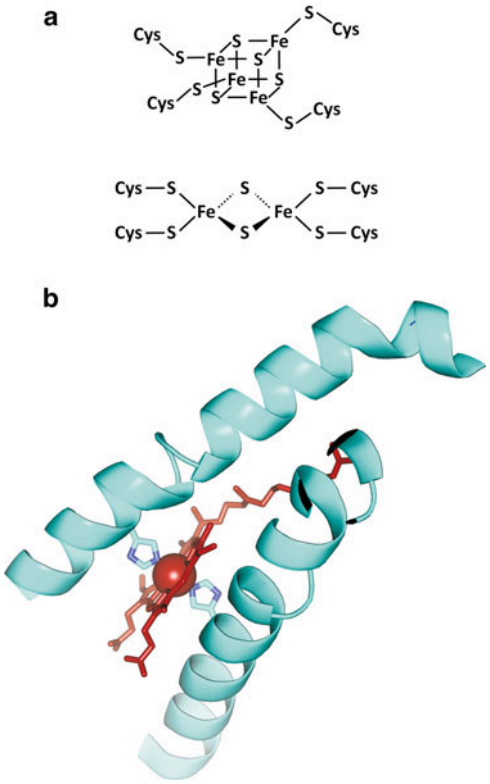


Fig. 3.39 Metal complexes, such as iron–sulfur centers (**a**), are common among the proteins of the electron transfer chain. Iron complexes with sulfur atoms but also with the thiol group of the side chain of Cys residues, resulting in stabilization of the structure of the proteins where they insert. The nitrogen atoms in the side chains of His are also prone to complex formation with metals. Panel (**b**) shows a detail of complex formation with metals in complex IV (PDB 1OCC) of the electron transfer system (see also Sect. 6.2.3). An iron ion (*red sphere*) complexes simultaneously the N atoms of two His side chains (*blue*), stabilizing the tertiary-level structure of the protein. It also binds to a nonprotein molecule, the heme a (*red organic structure*), which is also associated to complex IV

conformational changes of native proteins (see Box 3.6), for instance. These are referred to as protein-folding diseases. Folding is the expression used to comprise secondary- and tertiary-level structure altogether.

3.3.2 Structure and Function in Proteins

Proteins can adopt many different structures at the tertiary level, from extended rods to compact globules. Extended proteins may associate in fibers and globular proteins may have flexible domains able to bind other molecules. This gives the impression that proteins with extended conformation, like keratin, collagen, or silk fibroin, are adequate to maintain the structure of tissues or biomaterials, whereas globular proteins intervene in dynamical processes, which explains why enzymes

Box 3.6: Amyloids and Prions: When Misfolding Turns into Disease
The relationship between the structure and function of proteins has been one of the main issues of modern biochemistry for decades. Mutated proteins may have important changes in their structure and may thus display a defective function. However, the knowledge that proteins without mutations can fold in diverse forms, some of them pathogenic, is recent. Protein folding is the key to important diseases such as Alzheimer's, in which massive stacks of β-sheet-folded proteins accumulate in the brain. These stacks form plaques of insoluble protein in the extracellular tissue, which cannot be broken down by enzymes. When these plaques were found for the first time, they were described as related to saccharides and named amyloids. Although the chemical nature of the plaques is now known not to be related to saccharides, the name "amyloid" is still used and the group of diseases is known as amyloidoses.

Amyloid plaques grow with an ordered structure forming long filaments (fibrils). There are about 20 different proteins that can act as the building blocks of these fibrils, each of which is associated with a different disease. In so-called systemic amyloidoses, the precursors of these plaques are transported through the bloodstream from their point of origin to their point of deposition. Localized amyloidoses are of great clinical significance, as they mainly affect the central nervous system, the extracellular tissue of which is particularly susceptible to damage.

Transmissible spongiform encephalopathies (TSEs), which include mad cow disease (bovine spongiform encephalopathy; BSE) and Creutzfeldt–Jakob disease (CJD) in humans, are forms of amyloidoses in which the diseased brain degenerates to a porous sponge-like structure. These diseases appear when human proteins called prions misfold. The human prion is a component of the membrane of healthy nerve cells (called PrPc) that may misfold in a particular way. Amazingly, the misfolded prion may induce misfolding in a neighboring prion if contact among both molecules occurs. This has the appearance of an infection-like process in which the misfolded molecule "infects" the "healthy" molecule. "Infectious" prions can be transmitted in the diet, triggering a domino effect on healthy prions.

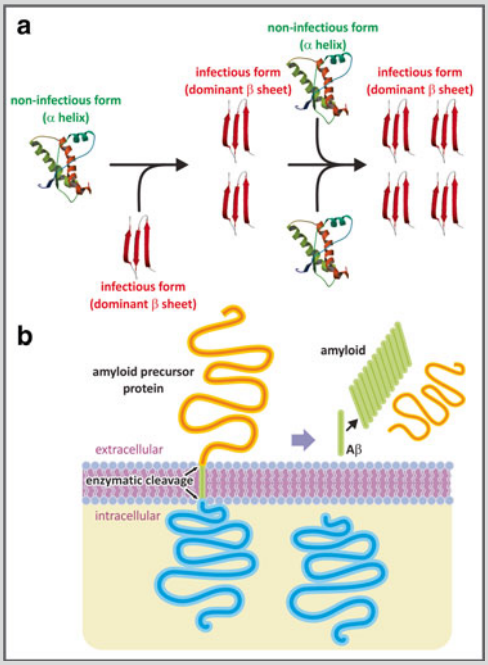
In Alzheimer's disease β-amyloid plaques are formed by cleavage of the amyloid-precursor protein (APP) by two different enzymatic activities, which release peptide fragments that are 40 or 42 amino acids long. When these peptides fold into β-sheets and aggregate, fibrils are formed, surrounding neurons and causing damage. This does not happen when the same peptides fold differently. It is only in β-sheets that hydrophobic amino acids are exposed, and they rapidly bind to hydrophobic groups of other peptides due to the entropic effect. The β-sheet structure, being highly ordered, is prone to regular stacking, ultimately leading to fibril formation.

In panel (a) of the following figure, a prion protein domain PrP(121–231) is shown in the noninfectious form (mouse, PDB 1AG2). The infectious form, having a structure not known in detail but dominated by beta strands

(continued)

Box 3.6 (continued)

(tentatively depicted; red), may induce conformational changes in the noninfectious protein to produce a replica of itself. The process may amplify and aggregates of the infectious form thus accumulate in pathological conditions. In Alzheimer's disease, β-amyloid plaques are not formed by an infection-like propagation of the proteins dominated by beta-strand domains. Panel B shows cleavage of the APP with concomitant release of peptides (green) that fold into β-sheets and aggregate.



are globular proteins. While this is generally true, it should also be acknowledged that the frontier between both is not always clear. For instance, actin is a globular protein that binds other actin molecules to form quaternary-level fibers in the cytoskeleton and muscle contractile system. Actin is an example of a globular protein having structural functionality (Fig. 3.40; see also Sect. 10.1.1). Another interesting

Fig. 3.40 (continued) exposed in collagen and form cross-links that strengthen the collagen fibers. The chemical process of this cross-linking reaction between the endings of the Lys side chains depends on vitamin C (ascorbic acid). Lys residues are also involved in cross-links of elastin (**d**), a connective tissue protein of unusual elasticity. The cross-links involve four Lys residues forming a desmosine arrangement. Actin (**e**) is a globular protein that self-associates forming fibers important for muscle contraction. Myosin (**f**), on the other hand, is a combination of an extended domain with a globular domain ("head") having catalytic activity. The extended domain associates to other proteins forming fiber-like oligomers responsible for muscle contraction (see Sect. 10.1.2) Hyp - Hydroxyproline

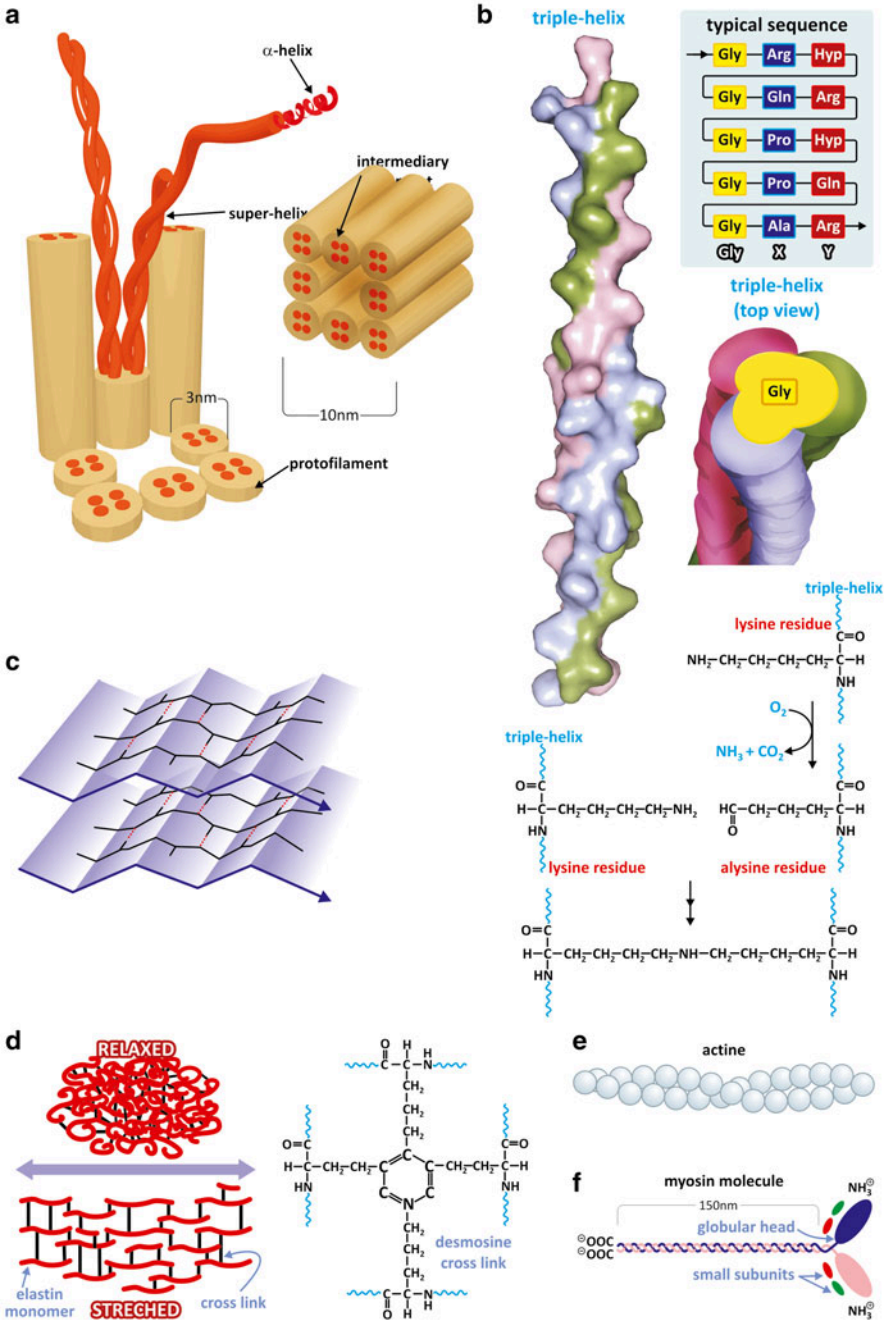


Fig. 3.40 Proteins such as keratin (**a**) and collagen (**b**, PDB 1BKV) adopt string-like conformations (very extended helices) that associate to form fibers that stabilize structures such as hair or nails (keratin), or the connective tissue (collagen). Silk fibroin (**c**) forms very extensible Gly- and Ala-rich β-sheets that associate in an antiparallel fashion forming a very resistant biomaterial such as spider silk. It is important to realize that collagen has a Gly residue at every third position of its amino acid sequence. Gly is a very small amino acid because the side chain is H. This creates a line along the helix surface where two other similar helices may dock in close contact. Lys side chains are

molecule that challenges the classical dichotomy of structural/extended vs. functional/globular proteins is lung elastin. When relaxed, elastin is a globular protein but it stretches allowing lung expansion. Elastin molecules are interconnected covalently by the side chain of four Lys residues: cross-linked desmosine bonds (Fig. 3.40). In this way, the continuous alteration between the globular and extended conformation of elastin confers to the lung the ability to expand and contract without histological lesions. Finally, one should stress the fact that there are also proteins that have both extended and globular domains. This is the case of myosin, another central protein in muscle contraction, in which an extended domain forms oligomeric fibers (Fig. 3.40; see also Sect. 10.1.1).

Besides tight parallel packing in collagen fibers, proteins bind covalently to other proteins in different fibrils of collagen through side chains of Lys residues (Fig. 3.40). When these strong covalent meshing of collagen is disrupted, the properties of the connective tissue are very much affected causing diseases (Box 3.7). Likewise, mutations of the Gly residues impact dramatically on collagen structure and connective tissue function. This has a particular effect on bones from early age: They loose resistance and break easily. This disease is known as osteogenesis imperfecta, Lobstein syndrome, or, more commonly, "brittle bone disease."

Box 3.7: Scurvy: An Example of a Pathology Directly Associated to Protein Structure

Scurvy is a pathology characterized by fatigue, anemia, gingivitis (gum disease), and skin hemorrhages caused by diets with a prolonged deficiency of ascorbic acid (vitamin C). It was a frequent disease of sailors in long voyages during the pioneering intercontinental discoveries of the fifteenth century. Many men died until it was discovered that scurvy could be cured and prevented by consuming citrus, such as oranges, lemons, and limes. The Portuguese sailor Vasco da Gama was the first European to lead a fleet that reached India by sea, linking Europe and Asia and the Atlantic and the Indic. The drama of scurvy in his first trip to India (1497–99) is eloquently described in an epic poem by Luís de Camões, in *The Lusiads* (1572):

"And 'twas that sickness of a sore disgust,
the worst I ever witness'd, came and stole
the lives of many; and far alien dust
buried for aye their bones in saddest dole.
Who but eye-witness e'er my words could trust?
of such disform and dreadful manner swole
the mouth and gums, that grew proud flesh in foyson
till gangrene seemed all the blood to poyson:
"Gangrene that carried foul and fulsome taint,
spreading infection through the neighbouring air:
No cunning Leach aboard our navy went,
much less a subtle Chirurgeon was there;
but some whose knowledge of the craft was faint
strove as they could the poisoned part to pare,

(continued)

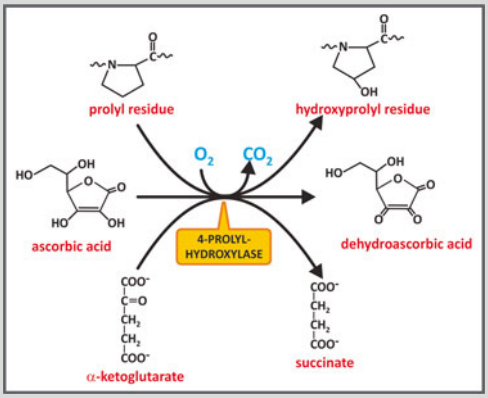
Box 3.7 (continued)

as though 'twere dead; and here they did aright; —
all were Death's victims who had caught the blight.
"The Lusiads" (Canto V, 81 and 82) version reproduced here was translated to English in 1880 by Richard Burton

Nearly two thirds of the sailors of the entire fleet of Vasco da Gama died during the trip, although documents from that time clearly show that the by Portuguese sailors knew that a diet based on fruits and other unprocessed foods was a treatment for scurvy. A manuscript from a pilot in the fleet of Pedro Álvares Cabral, discoverer of Brazil in 1500, says that a diet of fresh foods, including sheep, chicken, ducks, lemons, and oranges, was used to heal scurvy.

It was only in the eighteenth century that the Scottish doctor James Lind related scurvy to diets poor in citrus on a reasonably scientific way. Ascorbic acid was discovered by the biochemist Albert Szent-Gyorgyi (Born Hungarian, later US citizen), who was awarded the Nobel Prize in Medicine or Physiology in 1937 (Szent-Gyorgyi also performed important studies on muscle contraction; see Box 10.1). Ascorbic acid takes part of in biochemical pathways, the synthesis of collagen being one of them. Specifically it is mandatory in protein hydroxylation, which is a posttranslational modification in which a hydroxyl group (–OH) is added to a protein residue. Collagen is naturally hydroxylated in healthy individuals. Ascorbic acid is also mandatory in the biosynthesis of carnitine. Impaired synthesis of carnitine and collagen accounts for the common symptoms of scurvy.

The primary defects behind rotten or loose teeth, rigid tendons, or cartilage fragility observed in scurvy reside in the connective tissue. Without ascorbic acid, collagen is not hydroxylated, and a nonfibrous, defective incomplete collagen is formed instead of fibrous collagen due to the impairment of cross-linking. The enzyme prolyl 4-hydroxylase, for instance, hydroxylates a Pro residue using an iron atom that is oxidized in the process. Ascorbic acid is needed to reduce the iron and make the enzyme active again (see figure).



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Box 3.7 (continued)

Lysyl hydroxylases are also operative. Hydroxylation of Pro and Lys residues, both exposed in the triple helix of collagen, favors intermolecular adhesion interactions by hydrogen bonding and further chemical modifications, which are strengthened with age (this is one of the reasons why meat from young animals is tenderer than from older animals).

Carnitine is involved in the transport of fatty acids into the mitochondria, where they are oxidized (see Sect. 7.4.3). Ascorbic acid is used by two different enzymes in the carnitine biosynthesis. Without ascorbic acid, production of carnitine declines and fatty acids cannot be used as energy source. This leads to fatigue, which is one of the symptoms of scurvy. Curiously, fatigue appears prior to other symptoms. This may be explained by the fact that the enzymes in carnitine biosynthesis require higher concentrations of ascorbic acid to function (they have "lower affinity" for ascorbic acid) when compared to hydroxylases.

Other pathologies, such as the Ehlers–Danlos syndrome (EDS) and osteogenesis imperfecta (OI), are genetic pathologies associated to deficiencies on protein–protein interactions in collagen. A fraction of the Lys residues in collagen react with one another forming covalent cross-links in collagen fibers. In some forms of EDS, this cross-linking is impaired, rendering the skin less firm and less resistant, hyperelastic. Collagen contributes to the mechanical strength of the skin, joints, muscles, ligaments, blood vessels, and visceral organs. In OI, replacement of Gly residues in the collagen amino acid residue sequence destroys the capacity of the protein to assemble in perfect triple helices because all other residues are bulkier than Gly. This leads to an extremely severe condition that is characterized by alterations in the physical properties of collagen and perturbations in the biochemical processes involving collagen homeostasis. The relationship between the collagen fibrils and hydroxyapatite crystals when bones are formed is altered, causing brittleness. For this reason, OI is also known as "brittle bone disease."

One remarkable property of many globular proteins is the ability to both bind other molecules and change conformation upon binding. Taking adenylate cyclase as example, binding of molecules such as ATP or ADP causes very mobile domains of the protein to change position (Fig. 3.41). This often leads to dynamic distortions of the ligand molecules because of the contact with amino acid residues. The results may be such that covalent bonds are formed or broken, thus chemically transforming the ligand molecule into a product. In practice, the protein action is to increase the rate of transformation of the ligand in the product. If only the ligand, not the protein, is chemically transformed in this process, this can be seen as an enzymatic catalysis, i.e., increase of the velocity of chemical reactions caused by proteins, the enzymes. In these cases, the ligand (reactant) is named substrate.

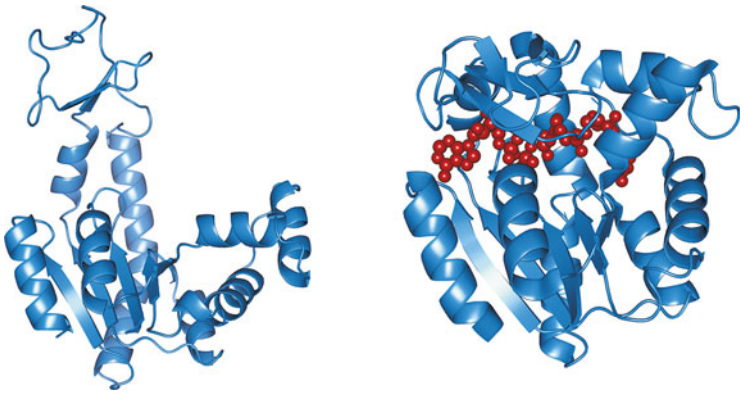


Fig. 3.41 Adenylate cyclase (or adenylyl cyclase), unbound (PDB 4AKE; *left*) and bound (PDB 1AKE; *right*) to a dinucleotide analogue (*red*). Upon binding to the dinucleotide analogue, the mobile domains adapt by changing position

3.3.3 Cooperative Interplay Between Tertiary-Level and Quaternary-Level Structure

As discussed in the previous section, the tertiary-level structure of a protein is determined and maintained by arrays of sites in which attractive or repulsive forces between groups of atoms exist. This is a relatively delicate balance. When a significant number of such "force spots" are altered, the conformation of the protein adapts by adopting a different tertiary-level structure, which corresponds to the new balance of forces. Likewise, in cases in which a quaternary-level structure exists, the changes in the conformation of one protein monomer at the surface of contact with other monomer may impose alterations in the "force spots" (hydrogen bounds, electrostatic repulsion and attraction, entropic factors, etc.) so that the second monomer changes conformation to adapt. In practice, this means that conformational changes in one protein may be transmitted to a neighboring protein that is in contact with it. In other words, tertiary-level structural changes may be transmitted and amplified to other proteins throughout the quaternary-level structure. Hemoglobin is a good example. It is formed by two subunits, α and β, forming a dimer that associates to other dimer—a tetramer that is in fact a dimer made of dimers. Dimers are numbered 1 and 2; therefore, hemoglobin is a tetramer of four subunits β1, α1, β2, and α2 (Fig. 3.42). α – β attractive forces are stronger than 1–2 attractive forces, but both are sufficient to transmit to neighboring monomers conformational changes. This affects the affinity of the hemoglobin monomers to oxygen. Each hemoglobin monomer is covalently associated to a non-proteic

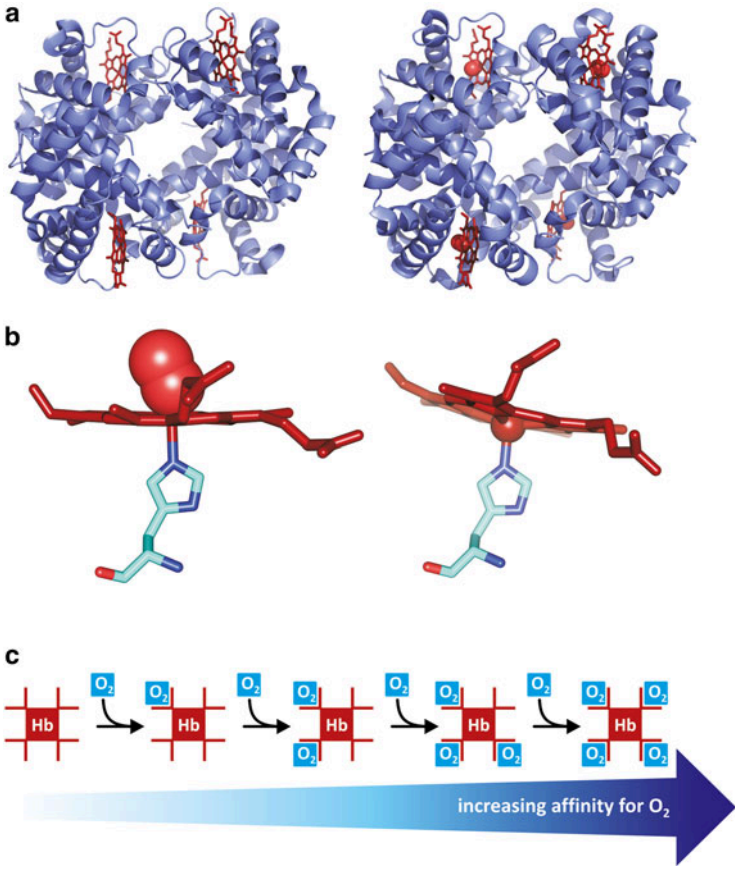
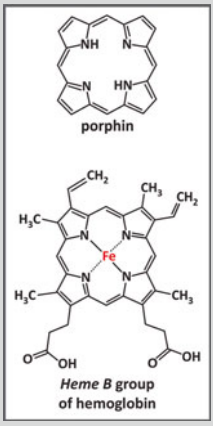


Fig. 3.42 Hemoglobin is a tetramer (**a**), each subunit containing a heme group (*red*). Human deoxyhemoglobin (*left*; PDB 2HHB) and oxyhemoglobin (*right*; PDB 1GZX) show subtle but important changes in conformation. Binding of molecular oxygen to the heme group [(**b**), *left*; PDB 1HHO] causes a shift in the orientation of the heme relative to the His residue when compared to the heme in deoxyhemoglobin [(**b**), *right*; PDB 4HHB). This slight distortion in the position of the heme leads to a variation in the conformation of the protein, which propagates to neighboring monomers in the tetramer. The neighboring monomers acquire higher affinity for O_2 (**c**)

group, i.e., a prosthetic group, of the porphyrin family (Box 3.8). In this case the porphyrin binds in its center an iron ion, forming a heme. The iron ion complexes with the heme through four bonds in the plane of the porphyrin and to a His residue side chain orthogonally to the heme plane. Another orthogonal bond, opposite to His is established with small molecules having electron donor atoms, such as O_2 or CO. When an O_2 molecule binds to the iron in the heme, the position of the iron slightly shifts, which in turn affects the position of the His residue. When the His

Box 3.8: The Importance of Heme Groups in Proteins.



Many natural proteins are associated to prosthetic groups of similar chemical nature called porphyrins. Porphyrins are macrocyclic compounds related to porphin (see figure). Hemoglobins, for instance, bind porphyrin groups, such as heme *b* (see figure). Hemes are porphyrins that bind iron ions in the center. The remarkable capacity to bind metallic ions of charge +2 or +3 in the center of the ring may explain the success of these molecules during natural selection and subsequent ubiquity of porphyrins in nature. The porphyrin macrocycle has 26 delocalized (π) electrons in total, being classified as aromatic from a chemical point of view. This system of delocalized electrons extends to the nitrogen atoms and is available to bind the cationic metals. It is also responsible for the intense absorption bands in the visible region of electromagnetic radiation. This is the reason why compounds with porphyrins, such as hemoglobin and chlorophyll, are intensely colored. Heme-containing proteins, hemoproteins, and other metal-containing proteins, metalloproteins, due to their unique electronic properties, are adequate for the transient binding of diatomic gases that occurs during their transportation in blood and electron transfer (i.e., electron donation and reception to and from other compounds). It is possible that hemoproteins evolved from ancient proteic forms, whose function was electron transfer in sulfur-based photosynthesis in the ancestors of cyanobacteria before molecular oxygen existed in atmosphere.

In addition to the peculiar intrinsic properties of hemes and other porphyrin–metal associations, the interaction between the porphyrin and the amino acid residues at the site where it inserts in proteins is of extreme importance. Variations in the shape, volume, and chemical composition of the binding site, in the mode of heme binding, and in the number and nature of heme–protein

(continued)

Box 3.8 (continued)
interactions result in significantly different heme environments in proteins having different biological roles. The outcome is a fine-tuning of the heme properties. Take the 3D structure of the hemoglobin chain as an example. The position of a His residue is such that its protonation interferes with oxygen release from the iron ion. Acidification of the medium causes the protonation of His, which in turns facilitates the release of oxygen. This is known as Bohr effect (see Fig. 3.44 in main text) and is not a simple curiosity: In the pulmonary vasculature, the pH is higher than in the peripheral tissues because the pH is affected by the local abundance of CO_2; therefore, the Bohr effects help in increasing the efficacy of binding oxygen in the lungs and releasing in peripheral tissues.

The porphyrin groups are equally important to stabilize protein structure and resistance to proteolysis, although these properties are frequently overlooked. Even in cases in which the porphyrins are not covalently bound to the protein, the interplay between amino acid residues and the non-proteic groups is very specific.

residue is pulled, the whole structure of the protein changes slightly. This change in conformation induces a change in conformation of the neighboring monomers. As a consequence, the neighboring monomers acquire increased affinity to bind an oxygen molecule. So, binding of O_2 to a monomer increases the chances that a second O_2 molecule binds to another monomer in the hemoglobin tetramer relative to a monomer in a tetramer without bound O_2 molecules. This is called positive cooperativity, i.e. several entities influencing each other favouring a certain event.

Hemoglobin monomers interact cooperatively to bind up to four oxygen molecules. Myoglobin, a protein abundant in muscles, also binds O_2, but this protein occurs as a monomer, in contrast to hemoglobin. Comparing hemoglobin to myoglobin makes the effect of cooperativity clear. The fraction of myoglobin binding O_2 relative to total myoglobin increases nearly linearly with partial pressure of oxygen up to saturation. To be more precise, the variation is hyperbolic. In contrast, hemoglobin binds O_2 critically at a triggering narrow concentration range, in which it reaches saturation. Hemoglobin changes from highly unsaturated to almost saturated in a narrow O_2 partial pressure interval. Interestingly, the narrow interval of transition to near saturation corresponds to the partial pressure of oxygen found in peripheral tissues (Fig. 3.43), away from the lung alveoli. Therefore, cooperativity among the hemoglobin monomers enables hemoglobin to saturates with oxygen in the lungs and delivers its cargo to peripheral tissues. Myoglobin would not be adequate for this function as it is almost saturated in both situations. Myoglobin is fit for oxygen storage in muscle cells. Release of oxygen occurs when the consumption in mitochondria is such that the cell is almost depleted in oxygen (Fig. 3.43).

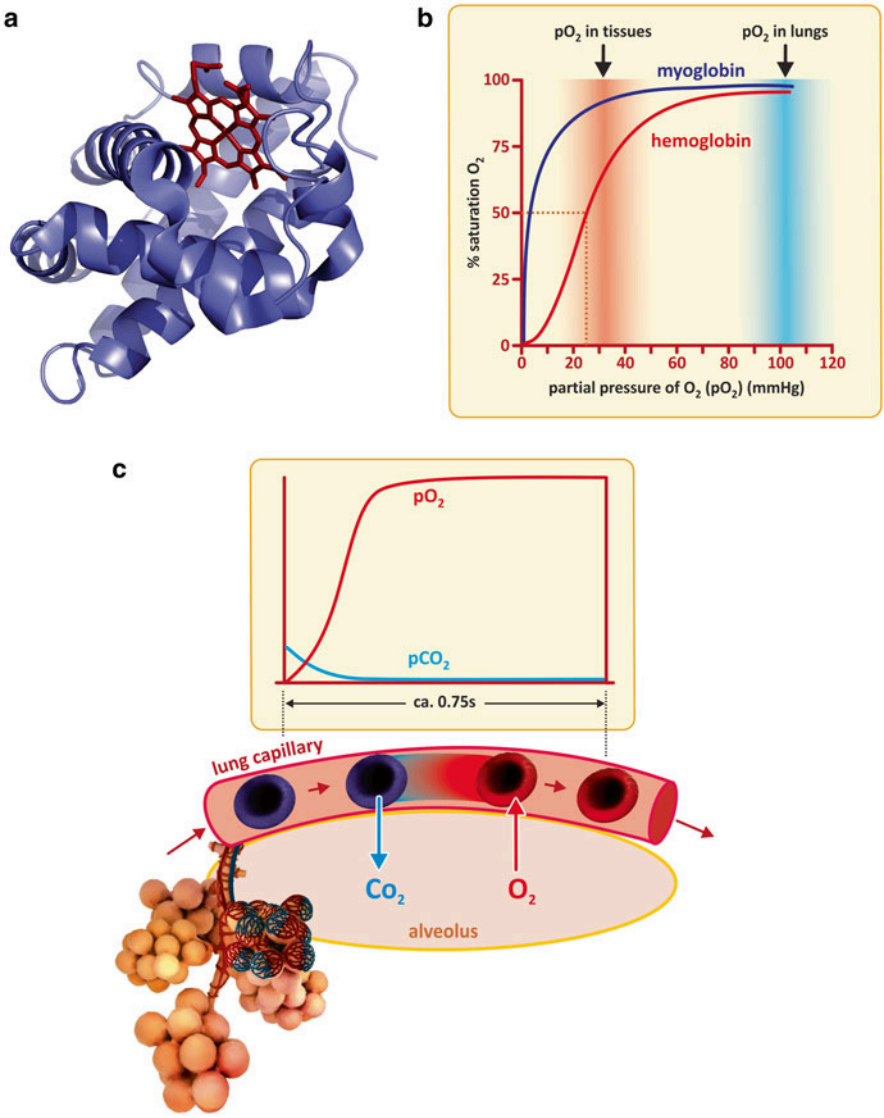


Fig. 3.43 Myoglobin (**a**) is a monomeric oxygen-binding protein. Like a hemoglobin monomer, it also binds oxygen through a heme group (*red organic structure*). The absence of cooperativity in myoglobin when compared to hemoglobin implies distinct binding capacities at different oxygen partial pressures, pO_2 (**b**). Hemoglobin has an abrupt transition from low to high binding when pO_2 changes from values typical from peripheral tissues to values typical of the lungs. As the erythrocytes pass adjacent to alveoli, O_2 and CO_2 diffuse freely across arterial and lung cells driven by partial pressure gradients (**c**)

It is worth stressing that hemoglobin transports most, but not all, oxygen used in tissues. Oxygen, like carbon dioxide, is a small, hydrophilic molecule, which easily dissolves in aqueous media. Although being hydrophilic, it is a very small molecule and diffuses freely in tissues. This is the reason why cells do not need oxygen transporters or channels. The same happens with CO_2 but it has lower affinity for hemoglobin. In addition, CO_2 is converted to HCO_3^- that equilibrates with H_2CO_3 (see Sect. 2.1.1). Therefore, plasma CO_2 transport is not dependent on a specific protein.

Although the direct binding of CO_2 to hemoglobin is not significant, hemoglobin is very important in the chemistry and physiology of CO_2 in the human body as the protein itself is a weak base or weak acid depending on pH. In the peripheral tissues, in which CO_2 is present at higher partial pressure, CO_2 diffuses to plasma and therefore in erythrocytes. Carbonic anhydrase then converts CO_2 to H_2CO_3 that acidifies the medium. Acid pH leads to the protonation of hemoglobin, which has lower affinity for O_2. Near the lung alveoli, plasma CO_2 diffuses to the alveoli due to the gradient in the partial pressure of CO_2 (Fig. 3.43). This drop in plasma partial pressure of CO_2 in plasma causes carbonic anhydrase to convert H_2CO_3 in CO_2, therefore shifting the equilibrium HCO_3^-/H_2CO_3 toward the consumption of HCO_3^- and H^+. The slight drop in pH causes the deprotonation of hemoglobin, which has higher affinity for oxygen. Thus, there is a coupling between pH and the efficiency of oxygen capture, transport, and release (Fig. 3.44). The coupling of hemoglobin structure with pH is known as the "Bohr effect."

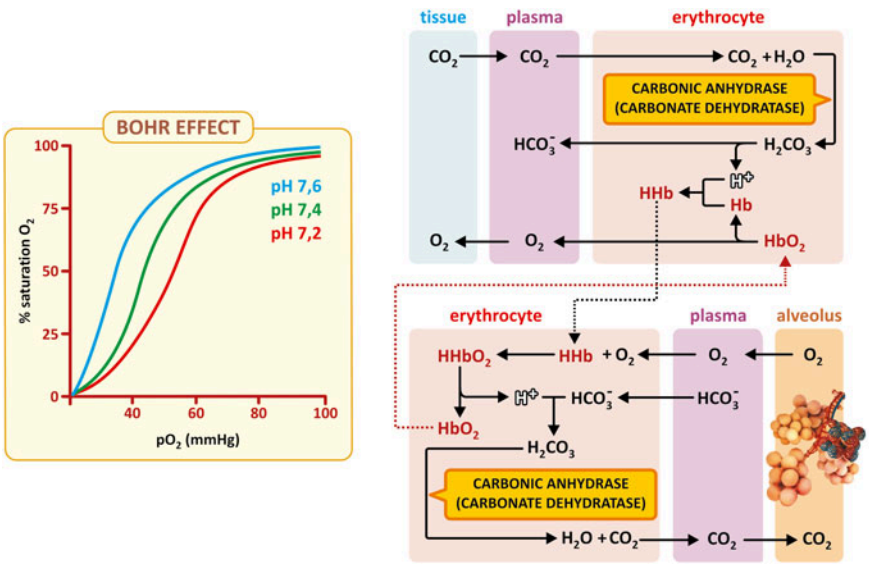


Fig. 3.44 The Bohr effect associated to hemoglobin. CO_2 levels in blood influence the transport of O_2 by hemoglobin through plasma pH because protonation/deprotonation of hemoglobin affects cooperativity in O_2 binding. Lower pH (found in tissues due to the release of CO_2 in plasma) favors hemoglobin protonation and unbinding of oxygen. Hb - Hemoglobin, HHb - protonated hemoglobin

In the same way oxygen binding, transport, and release by hemoglobin are affected by pH, binding of 2,3-bisphosphoglycerate (2,3-BPG) also influences oxygen fixation in a way such that the binding curve of oxygen by hemoglobin is shifted toward higher partial pressures of oxygen (Fig. 3.45). This is far from being a simple curiosity: 2,3-BPG forms from 1,3-BPG, a metabolite of the glycolysis pathway (see Sect. 6.1.3). When glycolysis is highly active, 2,3-BPG is formed and release of O_2 from hemoglobin becomes more effective. This is convenient for the cell as higher glycolytic activity implies, in principle, a higher demand for oxygen by the human cells. The fixation of oxygen in lungs remains unaffected.

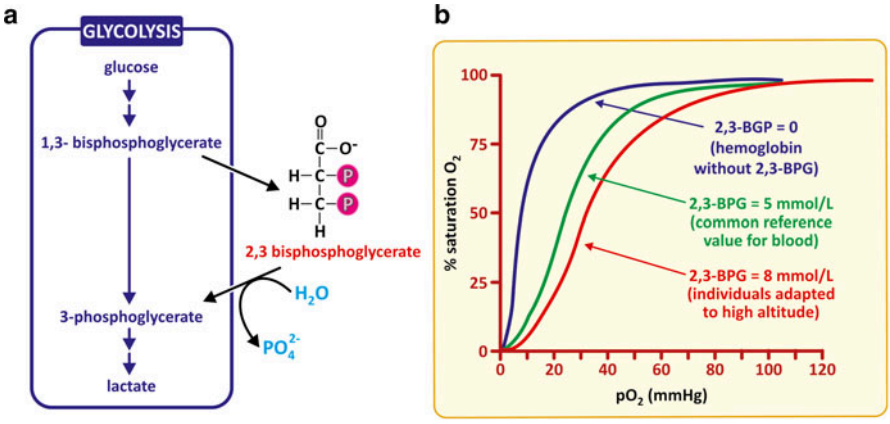


Fig. 3.45 2,3-BPG forms from 1,3-bisphophoglycerate, an intermediate metabolite of glycolysis; it is therefore, a chemical signal of glycolytic activity (**a**). 2,3-BPG binding to hemoglobin affects cooperativity in such a way that the O_2 binding curves are shifted in a way that O_2 release in peripheral tissues is facilitated, but the O_2 captured in the lungs is not affected (**b**)

3.3.4 Enzymes

The previous section showed how dynamic the binding of proteins to other molecules may be. In the case of hemoglobin, oxygen binds to the iron of the heme group. However, frequently more complex molecules bind directly to side chains of certain amino acid residues. Specific sets of amino acid residues in a protein may precisely locate and orient in space and have the correct physical properties (charge, polarity, hydrogen donor/acceptor groups, etc.) to specifically bind molecules that establish attractive forces with them (Fig. 3.46). The electronic clouds of these molecules are distorted by the contact with the amino acid residues, which in turn adapt their tertiary-level structure to the presence of the molecules. This mutual adaptation between protein and bound molecules frequently weakens some chemical bounds of the molecules, which may thus be destroyed. Likewise, formation of other bonds is possible. The result is that the molecule that bound to the protein is

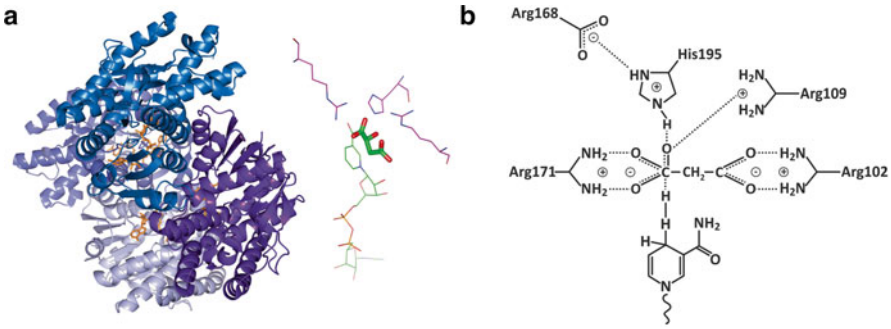


Fig. 3.46 Malate dehydrogenase (PDB 2DFD) is a homodimeric enzyme ([A]) that catalyzes the oxidation of malate to oxaloacetate concomitantly with the reduction of NAD^+ to NADH [highlighted in *orange* in (**a**)]. A specific set of amino acids of the enzyme has the right properties (charge, H-binding ability, etc.), the right location, and the right orientation to simultaneously fit the malate molecule (**b**). This set of residues forms the so-called active site (or active center) of the enzyme. NAD^+ binds to other site, specific for it, in close vicinity to the active site and participates in the oxidation of malate facilitated by the action of the amino acids [(**b**), *bottom structure*]

converted in a different molecule. If the resulting product dissociates from the protein and the protein returns to the same state as before binding the original molecule, then the protein is an enzyme, i.e., a proteic natural catalyst. The initial molecule that binds the enzyme and undergoes a chemical reaction is said to be a substrate, as previously mentioned in Sect. 3.3.2.

Because the enzymes interact with the substrate and facilitate its conversion to products, they increase the velocity of chemical reactions enormously, typically, above 10^7-fold. In some cases, the increase may be 10^{17}-fold, which is a figure difficult to conceive intuitively. Considering that a 2×10^8-fold increase in the velocity of a relaxed walk (~1.5 ms^{-1}) would leave us traveling at the speed of light (~3×10^8 ms^{-1}), this intuitive perception becomes clearer. 10^{17}-fold is more than the difference between a relaxed walk and 100 million faster than the speed of light in vacuum. One will see in Chap. 4 that enzymes accelerate reactions but cannot turn impossible reactions in possible ones. However, enzymes turn very slow reactions (so slow that in practice they seem unable to occur) into fast reactions. So, in practice it is almost like if an impossible one was transformed into a possible reaction by the intervention of an enzyme.

3.3.4.1 The Importance of Studying Enzymes

Enzymes are interesting molecules because the dynamics of their tertiary-level structure implies catalytic activity, which has shaped life as it exists today. Even viruses need enzymes to be effective. Because enzymes are so proficient in speeding reactions, controlling the activity of enzymes is, in practice, controlling the course of chemical reactions in a cell. This is an essential piece to impose order in the chemistry of the cells. Also controlling the activity of enzymes ensures that certain

reactions only occur to a significant extent *when* and *where* the enzyme is inside the cell. This prevents conflicting reactions in a well regulated cell and permits certain reactions to be coupled. Imagine substrate A and substrate B; now imagine that enzyme E_A converts A in B and a second enzyme, E_B, converts B in C. The simultaneous presence of both enzymes in the same cell compartment has the practical consequence that A is converted in C. This coupling of reactions may reach considerable complexity, with many substrates, reactions, and enzymes being involved, sometimes with branched and cyclic reaction sequences (recall Figs. 1.3 and 1.4). Such sets of reactions are referred to generally as "metabolisms." Regulation of metabolism is largely dependent on enzymes. The mechanisms of metabolic regulation are extremely important and will be addressed in Chap. 5. A regulated metabolism is a sine qua non condition for a particular state of organisms we call "health."

Yet, the structure–activity relationship in enzymes and their significance in metabolic regulation is only part of the importance of studying enzymes. Enzymes can operate outside cells and be used in industrial processes in pharma, food, or detergent processing and manufacturing, for instance. More importantly, in biomedical sciences and clinical practice, they can be used as valuable tools for diagnosis. When enzymes that were supposed to be confined in cellular compartments in specific tissues are found with increased levels in plasma, this is a sign of tissue lesions with rupture of cell membranes (and consequent leakage of enzymes to the plasma). The death of cells in tissues implies a constant flow of intracellular contents to the plasma, but in non-injured tissues this occurs to a very limited extent. A severe lesion in the liver, heart, or other organ leads to unusually high increased levels in the plasma of enzymes that are specific for that organ. Prostatic specific acid phosphatase (PSAP, Fig. 3.47), for instance, is an enzyme produced by the prostate that can be found in

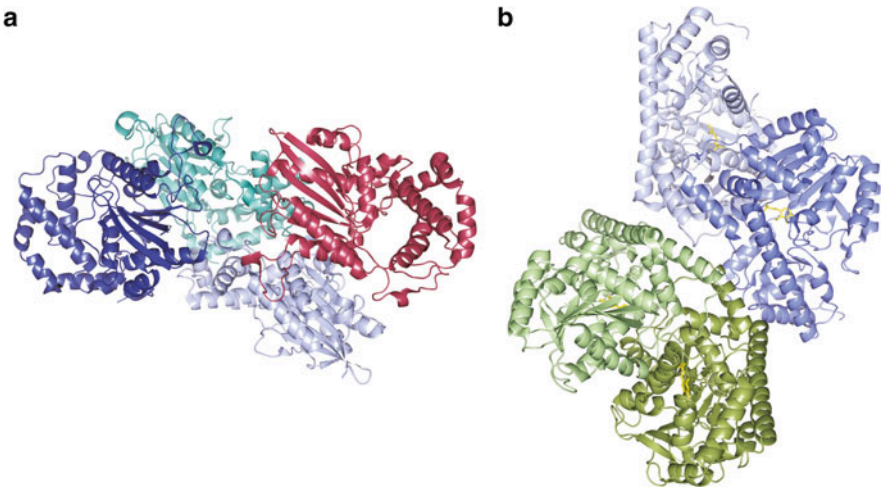


Fig. 3.47 Human prostatic specific acid phosphatase, PSAP [(**a**); PDB 1cvi), and aspartate aminotransferase, ASP [(**b**); PDB 3II0], are markers of prostate cancer and hepatitis, respectively. Pyridoxal 5-phosphate is a cofactor that is shown bound to ASP (*yellow*)

high amounts in the blood of men who have prostate cancer. A short number of other diseases cause moderate increased levels of PSAP, but only direct lesion of the prostate such as that provoked by tumors in this organ causes high levels of the protein in plasma. PSAP is then classified as a marker for prostate cancer.

Being highly irrigated (see Box 8.1) and particularly exposed to the action of drugs and other exogenous chemicals and viruses, the liver is an organ that suffers frequent insults that lead to the presence of hepatic enzymes in plasma. Two transaminases, alanine transaminase (ALT) and aspartate transaminase (AST), are markers of lesions frequently assayed in blood samples when hepatitis, poisoning, or alcoholic liver disease is suspected. However, one should bear in mind that these enzymes are also present in other organs, albeit in smaller concentrations. A full diagnosis is composed of data that takes into consideration not only biochemical analysis but also the symptoms, the history, lifestyle, and other diagnostic results.

The enzymes lactate dehydrogenase (LDH) and creatine kinase (CK) are of particular interest because they are markers of heart muscle lesion. Although these enzymes also exist in muscles other than the heart, there are differences in the amino acid composition that enable detection of the heart variants. Enzymes with similar activity and extensive structure homology are known as isoenzymes. For instance, three CK isoenzymes have been discovered: CK-MM or CK3, found mostly in skeletal muscle; CK-BM or CK2, found mostly in myocardium; and CK-BB or CK1, which is concentrated in the lungs and brain. Because of this distribution of CK isoenzymes, a pulmonary embolism is associated with elevated levels of CK-BB. On the other hand, an acute myocardial infarction is associated with elevated levels of CK-MB, and injuries of skeletal muscle cause elevated levels of CK-MM. LDH isoenzymes are tetramers, both in the heart and other muscles. These tetramers may disassemble and reassemble in the form of mixed heterogeneous tetramers because the structure of the monomers is very similar. In any case, the presence of dominant heart isoenzymes can be detected in plasma in case of myocardial infarction. The plasma enzyme changes in acute myocardial infarction are shown in Fig. 3.48. CK-MB isoenzyme peaks first, AST next, and LDH last.

There are also nonenzymatic markers that are used in the diagnosis of acute myocardial infarction: myoglobin and two cardiac troponins, troponin I (cTnI) and troponin T (cTnT). CK-MB and the heart LDH isoenzyme are the most important ones due to their heart specificity. Cardiac troponins are also important as their serum levels are frequently elevated during the first hours of acute myocardial infarction, even at a time when CK and CK-MB activities are still within the reference range, but are not fully established as the enzymatic markers.

While the scientific and clinical discipline of studying enzymes for direct clinical diagnosis, clinical enzymology, is expanding and gaining importance, it is curious to mention that dead brain tissue does not release into the blood any significant amounts of enzymes. Despite the frequency of cerebral infarcts (strokes), no test for

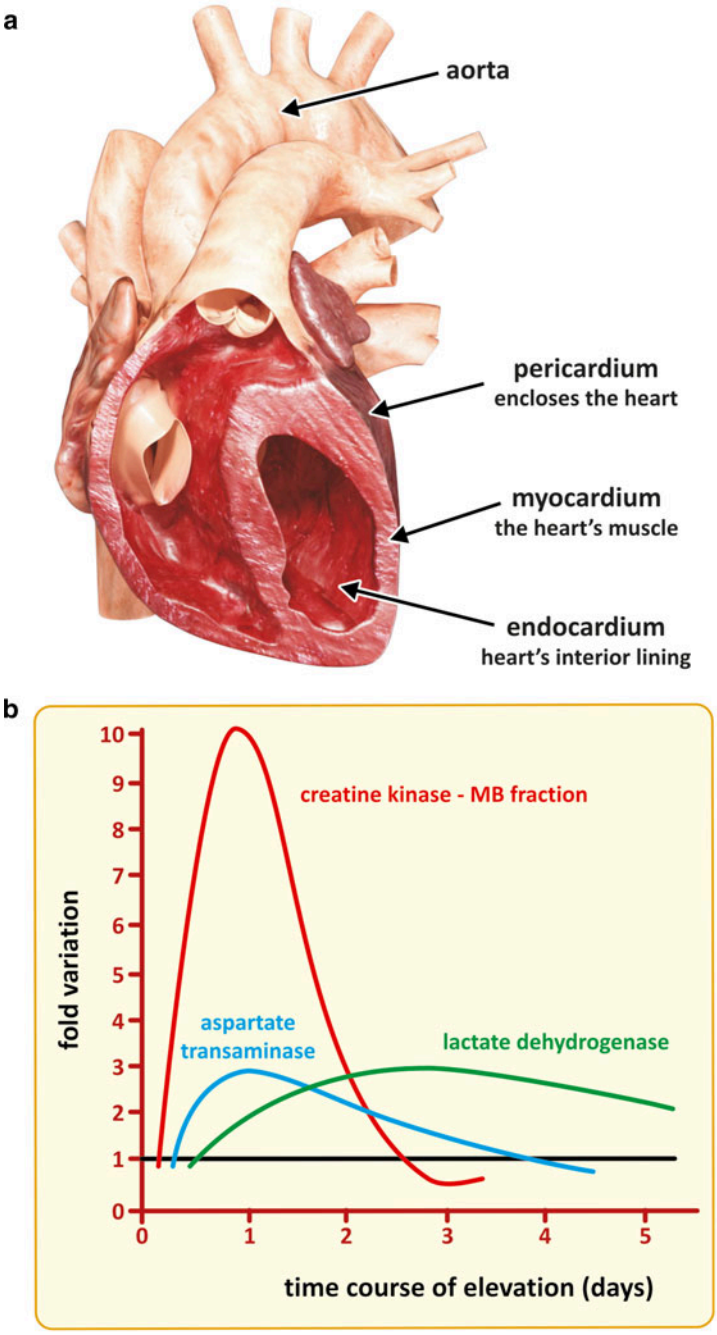


Fig. 3.48 Myocardium is the heart's muscle (**a**). Myocardium infarction involves partial tissue death (necrosis) caused by a local deficit of oxygen supply, as a consequence of an obstruction of the tissue's blood flow. Cardiac muscle enzymes, such as CK-MB, AST, and LDH, appear in the blood after the infarct (**b**). The combined information of CK-MB and LDH allows to estimate the time of the infarct, which in turn helps devising a therapeutical strategy. CK - creatine kinase, AST - Aspartate transaminase, LDH - Lactate dehydrogenase

brain enzymes is currently available due to the blood–brain barrier (BBB, Fig. 3.49), the network of capillaries that irrigates the central nervous system. The cells of these capillaries are connected by tight junctions and adhesion molecules that severely restrict the diffusion of hydrophilic macromolecules into the cerebrospinal fluid (CSF). Small gas molecules, such as O_2 and CO_2, diffuse passively through the barrier, and some nutrients and hormones are actively transported with specific proteins (this will be revisited, for instance, in Box 9.3, which discusses the glucose transport through the BBB).

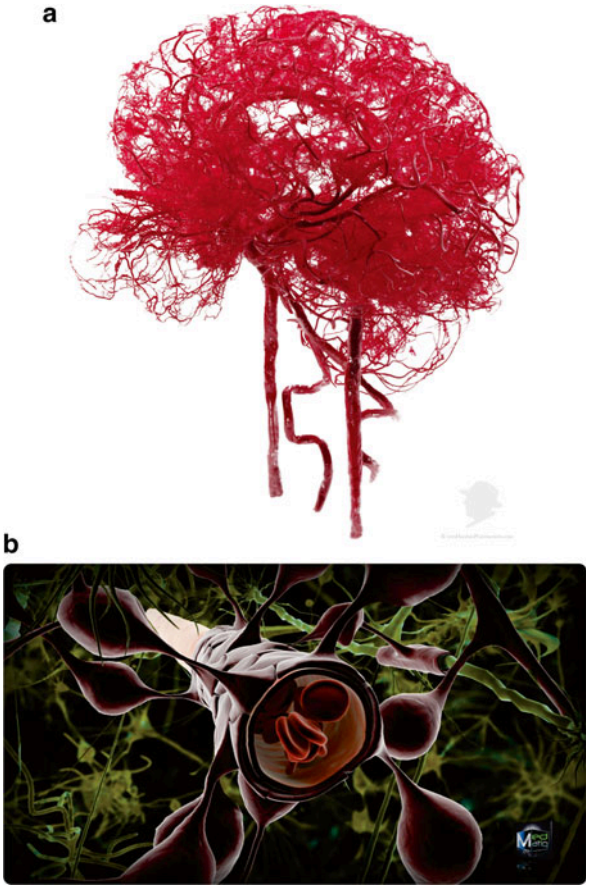


Fig. 3.49 The blood–brain barrier (BBB) is the network of brain capillaries (*top*). This photograph shows the result of a technique used to conserve anatomical structures named plastination applied to the human BBB (reprinted with permission of von Hagens Plastination, Germany; © www.vonHagens-Plastination.com). The network of very thin arteries that penetrate the brain forms a very reticulated mesh. The capillaries are associated to a thick basement membrane and astrocytic end feet (brown cells covering the endothelium; *bottom panel*). Passage of molecules across the endothelial cells of the BBB is highly selective. Enzymes released from nerve cells upon a stroke cannot reach the blood, a fact that makes diagnosis very difficult. Bottom panel is a Ben Brahim Mohammed work reproduced under the Creative Commons license

3.3.4.2 The Nomenclature of Enzymes

Because an enzyme is very specific for the substrate or for a family or related molecules of very similar structure, it is very unique. In the early days of metabolic studies, enzymes were named individually, one by one, with no concern for general rules of nomenclature. With time, the diversity of names and multitude of criteria to indentify newly discovered enzymes was such that the lack of a nomenclature that could be used worldwide was indering to the progress of enzymology (the scientific discipline devoted to study enzymes). The International Union of Pure and Applied Biochemistry (IUPAB), an international organization emerging from the joint efforts of many national societies of biochemists around the globe, appointed a working group to propose general rules that could be used to classify and identify enzymes. The result was a nomenclature based on the kind of reaction catalyzed by the enzyme consisting of:

- A name based on the contraction of "substrate + suffix ase" (e.g., urea + ase = urease, an enzyme that catalyzes a reaction with urea). This name has some flexibility.
- A rigid four-number code preceded by EC (for "Enzyme Commission"), which is unique for each enzyme (or sets of isoenzymes). The numbers refer to a family of enzymes and three successive subfamilies (Box 3.9). EC 5.2.1.3, for instance, identifies an enzyme from family 5 ("isomerases—catalyzes an isomerization reaction), first subfamily 2 ("cis-trans isomerization"), and the total code identifies specifically retinal isomerase, an enzyme involved in vision (see Sect. 2.2). Part of the whole tree of enzyme nomenclatures is presented in Box 3.9.

Box 3.9: Enzyme Classification and Nomenclature

IUBMB, the International Union of Biochemistry and Molecular Biology, is the organization responsible for recommendations on the nomenclature and classification of enzymes. Enzyme classification and strict nomenclature rules allow the unambiguous identification of enzymes. A working group, named Enzyme Commission, was established in 1956 to propose a universal classification and nomenclature system. Nearly 659 enzymes were known by then and the chaos in enzyme naming was clear. Nowadays more than 5500 enzymes are known, and it would be virtually impossible to communicate in enzymology if an official universal classification and nomenclature systems had not been established.

In 1961, the Enzyme Commission presented its report, in which enzymes are divided in six classes according to the reaction they catalyze. Classes and three levels of subclasses are numbered. The Enzyme Commission thus identified enzymes through a four-number code preceded by the letters EC to

(continued)

Box 3.9 (continued)

clearly identify that the numeric code corresponds to the classification set by the Enzyme Commission. The Enzyme Commission itself has been renamed but the classification system is still the same. The initials EC have remained although the commission they refer to has not.

Besides the numeric EC code, a name is also used because names are more intuitive and immediate than numeric codes. The most commonly used name for the enzyme is preferred, provided that it is unambiguous, but there are alternative systematic names that attempt to describe unambiguously the catalysis. Systematic names consist of two parts. The first contains the name of the substrate or, in the case of a bimolecular reaction, of the two substrates separated by a colon. The second part, ending in -ase, indicates the nature of the reaction, e.g., oxidoreductase, oxygenase, transferase (with a prefix indicating the nature of the group transferred), hydrolase, lyase, racemase, epimerase, isomerase, mutase, and ligase.

In practice, the enzyme classification and nomenclature stem from the classification of enzyme-catalyzed reactions, not from protein structures. A single protein may have two or more EC numbers if it catalyzes two or more reactions. This is the case, for example, for two proteins in *Escherichia coli*, each of which catalyzes the reactions both of aspartate kinase and of homoserine dehydrogenase. It may also happen that two or more proteins with no detectable evidence of homology catalyze the same reaction. For example, various different proteins catalyze the superoxide dismutase reaction and share a single EC number, EC1.15.1.1. This latter case is relatively rare, but it is almost universal that proteins catalyzing the same reaction in different organisms, or sets of isoenzymes in one organism, are homologous, with easily recognizable similarities in sequence.

Take Class EC 1 of enzymes, oxidoreductases, as example. This class contains the enzymes catalyzing oxidation reactions. Since the oxidation of one group must be accompanied by the reduction of another, they are grouped together as oxidoreductases. The systematic enzyme name is in the form "*donor*/*acceptor* oxidoreductase." The substrate that is being oxidized is regarded as being the hydrogen donor. The name is commonly "*donor* dehydrogenase." Although the term reductase is sometimes used as an alternative, it is important to remember that the recommended name does not define the equilibrium position of the reaction or the net direction of flux through the enzyme in vivo. The term "*donor* oxidase" is used only when O_2 is the acceptor.

Enzyme Classes

There are six classes of enzymes:

EC 1: Oxidoreductases catalyze reactions in which a substrate donates one or more electrons to an electron acceptor, becoming oxidized in the process.

(continued)

Box 3.9 (continued)

EC 2: Transferases catalyze reactions in which a chemical group is transferred from a donor substrate to an acceptor substrate.

EC 3: Hydrolases catalyze reactions in which a bond in a substrate is hydrolyzed to produce two fragments.

EC 4: Lyases catalyze non-hydrolytic reactions in which a chemical group is removed from a substrate leaving a double bond.

EC 5: Isomerases catalyze one-substrate one-product reactions that can be regarded as isomerization reactions.

EC 6: Ligases catalyze the joining together of two or more molecules coupled to hydrolysis of ATP or an analogous molecule. These enzymes are also sometimes called synthetases, a name that was already in use before creation of the original Enzyme Commission.

In reality all of the enzymes in classes 1–3 satisfy the definition of transferases. However, as these three classes are all large compared to the other three groups, it is convenient to break them into three classes and to reserve the name transferase for enzymes that are not oxidoreductases or hydrolases.

Enzyme Subclasses

Each of the six classes is divided into subclasses on the basis of the salient differences between the enzymes in the class. In **EC 1**, for example, the subclasses define the type of substrate acted on:

EC 1.1: Acting on the CH–OH group of donors
EC 1.2: Acting on the aldehyde or oxo group of donors
EC 1.19: Acting on reduced flavodoxin as donor
EC 1.97: Other oxidoreductases

This last subclass is numbered **EC 1.97** because it is provisional. In due course the enzymes it contains may be reclassified more appropriately. The original report had two subclasses **EC 1.99** and **EC 1.98** that were removed when sufficient information was available to place the enzymes they contained elsewhere.

Classes **EC 3–5** are divided into subclasses on the basis of types of substrate, in much the same way as in **EC 1**. In **EC 2**, however, it was more useful to emphasize the nature of the transferred group. So, for example, we have:

EC 2.1: Transferring one-carbon groups
EC 2.2: Transferring aldehyde or ketone residues
EC 2.3: Acyltransferases
EC 2.8: CoA-transferases

(continued)

Box 3.9 (continued)

In **EC 6** the division into subclasses is made on the basis of the type of product:

EC 6.1: Forming carbon–oxygen bonds
EC 6.2: Forming carbon–sulfur bonds
EC 6.3: Forming carbon–nitrogen bonds
EC 6.4: Forming carbon–carbon bonds
EC 6.5: Forming phosphoric ester bonds

Enzyme Sub-subclasses

The subclasses are divided into sub-subclasses in much the same way as the way the subclasses themselves are defined. For example, **EC 1.16** (oxidoreductases oxidizing metal ions) contains two sub-subclasses:

EC 1.16.1: With NAD^+ or $NADP^+$ as acceptor
EC 1.16.2: With oxygen as acceptor

As with the numbering of subclasses, 99 (or a smaller number if necessary) is used for sub-subclasses containing a miscellaneous group of enzymes. For example, subsection **EC 1.6** contains oxidoreductases acting on NADH or NADPH, and within this there is **EC 1.6.99** for miscellaneous acceptors.

There are also sub-subsubclasses so that each enzyme is identified by four different numbers. The division of sub-subclasses into sub-subsubclasses follows the same rationale as before. An exhaustive visit of the 4th level of classes is not justified here.

Final note: *Text based on "Enzyme Classification and Nomenclature" by S Boyce and K Tipton (Encyclopedia of Life Sciences, 2001) and "Current IUBMB recommendations on enzyme nomenclature and kinetics" by A, Cornish-Bowden (Perspectives in Science, 2014, 1, 74–87)*

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Part II
The Interplay and Regulation of Metabolism

Chapter 4
Introduction to Metabolism

Cells are made up of molecules, but cells are not simple mixtures or assemblies of molecules. If thrown to a test tube separately or in a random mixture, the whole set of molecules of a cell would interact physically and react chemically, but would not spontaneously form a cell. Cells form and exist because the molecular events that create and maintain cells are highly ordered. The sequence and specific place of events and the flows of matter and energy are such that the cell is able to preserve stability and evolve by adaptation to a certain extent, as addressed in Sect. 1.1. In Part II, we will focus on some of the most important chemical reactions occurring in different tissues of the human body and their coordination so that one understands how the changes of matter and transfer of energy enable the human body to exist, move, adapt to external challenges, and reproduce. This ensemble of chemical reactions is called metabolism.

Because the whole metabolism is such a complex array of chemical reactions, biochemists tend to study and concentrate on subsets of reactions separately. The easiest form to understand and explain metabolism is to divide it in parts depending on the chemical nature of the molecules involved, i.e., according to the families of molecules addressed in Part I. So, for practical reasons and for the sake of simplicity, the whole metabolism, which is a single highly complex network of chemical reactions and physical events, is regarded as a sum of "metabolisms": the metabolism of saccharides, the metabolism of amino acids, the metabolism of lipids, etc.

The part of metabolism more directly related to nutrient absorption and ATP production is the "energy metabolism." The human body continuously transforms molecules, sometimes forming higher molar mass products, others breaking down molecules into smaller molar mass entities. Typically, these situations correspond to incorporate mass and energy from nutrients, or consuming such mass and energy in the absence of nutrient intake, respectively. The "metabolisms" of the first kind are known as anabolic (construction), the latter being known as catabolic (degrading). So, "amino acid catabolism," for instance, refers to the breakdown of amino acids into smaller molecules, as opposed to amino acid synthesis (amino acids anabolism).

A.T. Da Poian, M.A.R.B. Castanho, *Integrative Human Biochemistry*,
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Amino acids, in turn, may polymerize and form proteins: protein anabolism, in contrast to the breakdown of proteins, amino acids resulting therefrom (protein catabolism). Subsets of reactions that are active in both catabolic and anabolic conditions are "amphibolic" (the prefix "amphi" referring to its dual nature). This is the case of the tricarboxylic acid (TCA) cycle, also known as Krebs cycle (see Sect. 7.2).

An example of generic metabolism is schematized in Fig. 4.1. No matter how complex the metabolism may seem at first glance, the interpretation of a scheme of consecutive reactions is simple and depends on the identification of five key factors:

1. The reactants that originate the process
2. The end products, regardless of being formed in intermediate reactions or the final reaction
3. The branching points, i.e., steps where the sequence of reactions may follow different courses
4. The irreversible reactions
5. The specific reactions that are catalyzed by enzymes that are finely regulated and so have the ability to highly accelerate segments of the metabolism, or not

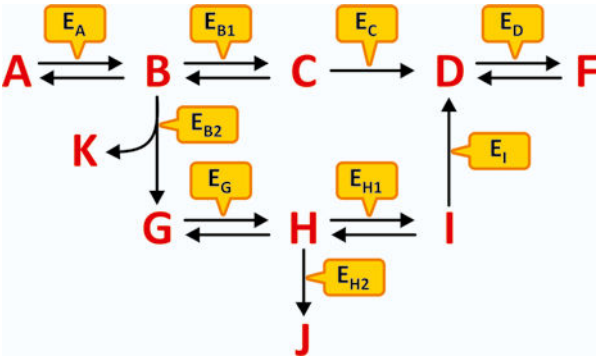


Fig. 4.1 Hypothetical schematic metabolism involving metabolites A to K and enzymes E_x (subscript X in E_x represents the substrate, X = A to K). The metabolism is "fed" by A and has K, F, and J as "end products." An external source of G may also lead to the formation of J and F, but not K. An external source of D can only lead to the formation of F. However, if E_D is not present or not operative, F will not be formed in any circumstance. Likewise, if E_{B2} is not present or not operative, K, G, H, J, and I will not be formed even in the presence of high concentration of B. If E_{B1} and E_{B2} are never active or inactive at the same time, F is always formed but not J; the reactions' scheme assures the permanent formation of F but the selective formation of J when the control of the reaction course is performed by alternate states of activity of E_{B1} and E_{B2}

These five key factors in the interpretation of a metabolism will help the reader not to look to a metabolism as a chaotic ensemble of chemicals, enzymes, and reaction arrows. Metabolism is an appealing and richly informative text on the organization of life, should we be able to interpret it.

Enzymes are key protagonists of metabolisms together with metabolites (i.e., the intermediate reactants/products of the reactions of metabolisms). Not only enzymes

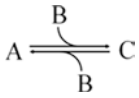
greatly accelerate reactions, they are the entities that make metabolisms happen in specific directions. Their importance and action is better illustrated if one considers first a hypothetical metabolism without enzymes (described in Sect. 4.1) and the same set of reactions with enzymes (described in Sect. 4.2).

4.1 Consecutive Reactions Without Enzymes

Consider the simple chemical reaction:

$$A + B \rightleftharpoons C$$

which in terms of a more amenable representation to biochemists may be written as:



to highlight the conversion of A into C with the intervention of B. Nevertheless, one should not underestimate the importance of B in the balance of energy and mass of the reaction, even if B is H_2O or a small ion, for instance, and A and C are big complex molecules. A, B, and C are all equally important to study the course and velocity of the reaction, i.e., the thermodynamics of the reaction. To better understand the basic principles that govern the chemical reaction, we will start with very simplistic formulations. One will assume that A and B are mixed at a specific instant in time ($t=0$) and the reaction initiates forming C. As soon as C is formed, it will also start converting to A + B, but in the beginning only few molecules of C exist, so the velocity of the conversion of A + B into C is larger than the opposite conversion, C to A + B. Naturally, in this condition, the concentration of C will increase until the point in which the velocities of both reactions will match: the velocity with which C is formed equals the velocity with which it is consumed. The reaction takes place because A and B are continuously transformed into C and vice versa, but the concentrations of A, B, and C present in solution do not change. This is the point of equilibrium. At this stage it is important to dissociate the extent of the reaction (i.e., the fraction of A and B that was transformed to reach the equilibrium) and the velocity of the reaction, which is related to the time needed to reach the equilibrium. A very extensive reaction is represented by:

$$A + B \rightarrow C$$

and named "irreversible" (therefore written with a one-way arrow), but this tells us absolutely nothing about the velocity of the reaction. In fact, the conversion of A and B into C may be so slow that in practical terms C is not formed. So, to study chemical reactions, one needs to address both the extent and the velocity of reactions. A favorable reaction has a high degree of conversion of reactants in products;

a fast reaction reaches the equilibrium in a short time. Both aspects are independent of each other, and so it is not surprising that they can be modulated by separate means in metabolism.

The extent of a reaction is described by the equilibrium constant, which in practical terms is calculated from the concentrations at equilibrium:

$$K_{eq} = \frac{[\mathrm{C}]_{eq}}{[\mathrm{A}]_{eq}[\mathrm{B}]_{eq}}$$

It is obvious that unfavorable reactions have low K_{eq} and favorable reactions have high K_{eq}. K_{eq} varies from nil to infinity. The determinants that contribute to the value of K_{eq} for a given reaction (i.e., how favorable it is) relate to the balance of both released energy and order of the whole environment in which the reaction takes place. Neither is intuitive, but both can be experimentally demonstrated. Chemical reactions tend to consume more reactants as the products are more stable ("less energetic") than reactants and the environment becomes more disordered with the presence of the products (i.e., closer to a random organization). The degree of disorder is generically referred to as entropy. The balance of energy and entropy are so deeply rooted in thermodynamics that they are the subject of the first and second laws of thermodynamics, respectively, as the founding principles of this discipline. The exact quantitative weighting between the balance of released energy as heat (the enthalpy, ΔH) and balance of entropy (ΔS) is provided by the parameter ΔG, the difference of the Gibbs energy during the chemical reaction:

$$\Delta G = \Delta H - T\Delta S$$

Assuming that the temperature, T, does not change in the process, ΔG is related to the concentration of reactants and products:

$$\Delta G = \Delta G^0 + RT \ln \frac{[C]}{[A][B]}$$

In equilibrium $\Delta G = 0$; therefore,

$$\Delta G^0 = -RT \ln K_{eq}$$

While thermodynamics may reach complex formulations to explain the interaction of energy with matter, we shall stick to those most important in medical biochemistry. Previous equations on ΔG are useful because they provide information about the spontaneity of reactions. If $\Delta G < 0$ (exergonic reaction), the reaction will proceed spontaneously until equilibrium; if $\Delta G > 0$ (endergonic reaction), the reaction will not be spontaneous; if $\Delta G = 0$, the reaction is at equilibrium. Whether ΔG is positive, negative of nil depends largely on the concentrations of reactants and products, and the reaction will continue until these concentrations reach equilibrium

($\Delta G=0$). $\Delta G_{eq}{}^{0}$ is related to the concentrations at equilibrium specifically and so provides information on the final extent of reaction (i.e., how far the reaction goes until equilibrium):

$$\Delta G^0 \ll 0 - high\ extent\ of\ reaction\ \left(high\ \ K_{eq}\right)$$
$$\Delta G^0 \gg 0 - low\ \ extent\ of\ reactions\ \left(low\ \ K_{eq}\right)$$

Box 4.1 illustrates these concepts for a simple reaction. Figure 4.2 shows two examples of how ΔH may dominate over ΔS and vice versa and be determinant for the extent of the reaction, i.e., for ΔG. However, in most biological processes related to the use of nutrients, ΔH is large compared to $T\Delta S$, which makes ΔH, the caloric "value" of food, an approximate measure of the total energy that can be used by the human body.

Isolated reactions such as the ones considered before are rarely relevant in metabolism as metabolism consists of consecutive reactions. So let us now consider the reaction

$$\mathrm{C} \rightleftharpoons \mathrm{D} + \mathrm{E}$$

Box 4.1: The Basic Thermodynamics of the Simplest Reaction

In the very simple case of one single reactant (R) being transformed in one single product (P):

$$R \rightleftharpoons P$$

the equilibrium constant is:

$$K_{eq} = \frac{[P]_{eq}}{[R]_{eq}}$$

and

$$\Delta G^0 = -RT \ln K_{eq} = -RT \ln \frac{[P]_{eq}}{[R]_{eq}}$$

It is obvious that:

$$\Delta G^o < 0 \quad \text{if} [P]_{eq} > [R]_{eq} \left(K_{eq} > 1\right)$$
$$\Delta G^o > 0 \quad \text{if} [P]_{eq} < [R]_{eq} \left(K_{eq} < 1\right)$$

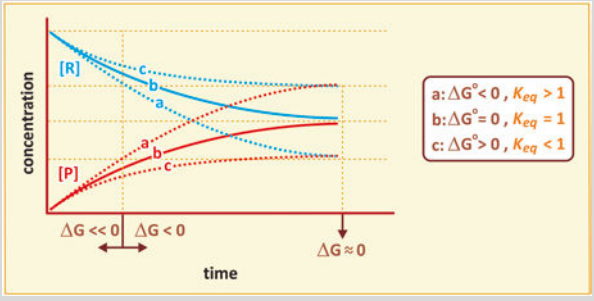
(continued)

Box 4.1 (continued)
The reactions with highest K_{eq} have the most negative ΔG^0_{eq}. If the equilibrium is not yet attained, two situations are possible:

1. $$[R] > [R]_{eq} \left(i.e. [P] < [P]_{eq}\right)$$
$$\Delta G = \Delta G^0 + RT \ln \frac{[P]}{[R]} = +RT \ln \left(\frac{[P]}{[R]} \frac{[R]_{eq}}{[P]_{eq}} \right)$$
which is obviously negative. The reactions will proceed with transformation of R in P until equilibrium, i.e., $\Delta G < 0$ implies spontaneous transformation of reactants in products.

2. $$[R] < [R]_{eq} \left(i.e. [P] > [P]_{eq}\right)$$
This is the opposite case, in which $\Delta G > 0$ and the spontaneous process is the reverse reaction, i.e., the transformation of the product P, in the reagent R.

The relationship between ΔG, ΔG^0, and the course of reaction is represented in the figure below.



coupled to the first reaction, $A + B \rightleftharpoons C$, to form:

$$A + B \rightleftharpoons C \rightleftharpoons D + E$$

At equilibrium, it is obvious that the concentrations of A and B are related to the concentrations of D and E because C takes part in both reactions. In term of equilibrium constants:

$$K_{eq,1} = \frac{[C]_{eq}}{[A]_{eq}[B]_{eq}}$$
$$K_{eq,2} = \frac{[A]_{eq}[B]_{eq}}{[C]_{eq}}$$

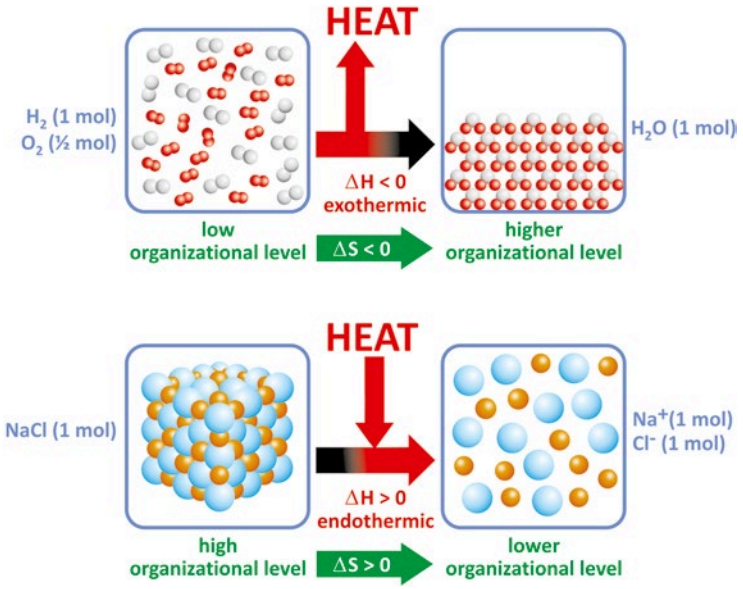


Fig. 4.2 Some reactions occur because the released energy, ΔH, compensates for the decrease in ΔS (**a**), or the increase in disorder compensates for the heat consumption (**b**)

(1 and 2 refer to the first and second reactions in the sequence).
Therefore,

$$K_{eq,1}K_{eq,2} = \frac{[\mathrm{D}]_{eq}[\mathrm{E}]_{eq}}{[\mathrm{A}]_{eq}[\mathrm{B}]_{eq}}$$

which is the apparent equilibrium constant of the abbreviated form ("sum of reactions") of the reactions $\mathrm{A}+\mathrm{B} \rightleftharpoons \mathrm{C}$ and $\mathrm{C} \rightleftharpoons \mathrm{D}+\mathrm{E}$ above:

$$\mathrm{A}+\mathrm{B} \rightleftharpoons \mathrm{D}+\mathrm{E}$$
$$K_{eq,app,1,2} = K_{eq,1}.K_{eq,2}$$

The amazing consequence is that $\Delta G°$ of the global process is simply the sum of ΔG of the two consecutive reactions:

$$\Delta G^0_{eq,1,2} = -\ln K_{eq,app,1,2} = -\ln\left(K_{eq,1}K_{eq,2}\right) = -\ln K_{eq,1} - \ln K_{eq,2} = \Delta G^0_{eq,1} + \Delta G^0_{eq,2}$$

The consequences of metabolism are immense because it is implied that the conversion of C in D+E may be extremely unfavorable, but the extent to which A+B is converted to C "pushes" the reaction toward the formation of D+E through the increase in the concentration of C. This can be generalized to any set of consecutive reactions, and metabolisms are generally composed by favorable and unfavorable

reactions influencing each other. In the extreme case where an irreversible reaction follows one or more reversible reactions, such as

$$A + B \rightleftharpoons C \rightleftharpoons D + E \rightarrow F$$

it is obvious that regardless of how unfavorable the first and second reactions may be, the end result is the total depletion of A and/or B and the formation of F. The same would happen in

$$A + B \rightleftharpoons C \rightleftharpoons D + E \begin{array}{l} \nearrow F \\ \searrow G \end{array}$$

but not in

$$A + B \rightleftharpoons C \rightleftharpoons D + E \begin{array}{l} \nearrow F \\ \searrow G \end{array}$$

in which the end products would be both F and G. Although both the formation of F and G are irreversible, they are competing reactions. Although they both occur to the total depletion of at least one of the reagents, the product of the fastest reaction dominates. One of the reactions may be much faster than the other, in which case only one product forms in practice.

In a branched chain of reactions with an irreversible conversion such as, for instance,

$$A + B \rightleftharpoons C \rightleftharpoons D + E \begin{array}{l} \nearrow F \rightleftharpoons J + K \rightleftharpoons L \\ \searrow G \rightleftharpoons H \rightleftharpoons I \end{array}$$

the final outcome critically depends on the irreversible steps and the branching points. Thus, the result may be complex.

Given the importance of the velocity of reactions to the course of chain reactions, we shall now address kinetics in more detail as ΔG^0 only accounts for how far the reaction goes in the degree of conversion of reactants into products, not kinetics.

4.2 Consecutive Reactions With Enzymes

The chain of reactions above is dominated by the irreversibility of reactions $D + E \rightarrow F$ and $D + E \rightarrow G$ ($\Delta G^0 \ll 0$ for both). If the latter, for instance, is much faster than the first, the metabolites G, H, and L will dominate. In the opposite case, F, J, K, and I will dominate.

Imagine now that (i) E is a key reactant of high reactivity that turns most reactions it enters irreversible ($\Delta G^0 \ll 0$ —in nature ATP fulfills this role, which makes metabolism critically dependent on this molecule, Box 4.2), and (ii) both reactions are catalyzed by enzymes. The combination of these two factors has a high impact in the variation of the concentrations of the metabolites over time (see an example in Fig. 4.3). Importantly, the concentration of E and the regulation of the activity of the enzymes are ways to control the course of reactions. The following typical situations are possible:

1. Low "level" (concentration) of E results in "accumulation" (increased concentration) of D.
2. A highly active catalysis of $D + E \rightarrow F$ with simultaneous absence of an enzyme to catalyze $D + E \rightarrow G$, or the presence of an enzyme that is inhibited, results in the production of F at a high rate. If $F \rightleftharpoons J + K$ is not catalyzed, a transient accumulation of F occurs.

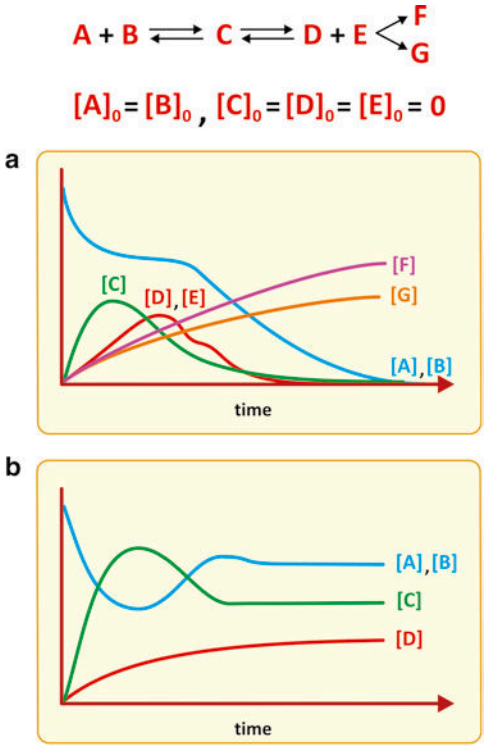


Fig. 4.3 Evolution of the concentration of metabolites in a hypothetical reaction. Depending on the reversibility of reactions and their velocity, the concentration of the metabolites following the addition of A and B at equal concentrations changes dramatically over time, in case reactions $D+E \rightarrow F$ and $D+E \rightarrow G$ are occurring (a) or not (panel b). In panel a the velocity of the formation of F is assumed to surpass the velocity of the formation of G. In panel b, [E] equals [D]

In most metabolic sequence of reactions ("pathways"), all reactions are catalyzed by enzymes, which generates a situation in which the concentration of metabolites may change very rapidly in high amplitude intervals, depending on the thermodynamics of the reactions involved, the enzymes present, and the eventual

degree to which their activity is affected by other molecules or physical conditions such as temperature.

4.2.1 *The Basis of Enzymatic Catalysis and Its Impact in Metabolism*

As addressed in previous sections and Box 4.2, ATP or related nucleotide triphosphate is a quasi-universal driver of metabolism for its hydrolytic dephosphorylation with $\Delta G^o \ll 0$. Coupling this reaction to others in a metabolic pathway grants the ability to perform reactions that would otherwise occur to a very low level. As long as ATP is available in the cell, the process is assured. This simplicity contrasts with the apparent complexity of the control of enzyme activity in the cellular environment. We say apparent because this complexity is founded on simple basic

Box 4.2: ATP—The Quasi-Universal Driver of Metabolism
ATP is a fascinating molecule for its ubiquity. ATP hydrolysis into $ADP + PO_4^{3-}$ or $AMP + PO_3^{2-} - O - PO_3^{2-}$ has $\Delta G^o \ll 0$, and these reactions are present in several metabolisms, coupled to unfavorable reactions. Any chemical bond splitting involves expenditure of energy, and ATP is no exception. The idea that ATP has "high-energy bonds" between phosphoryl groups whose energy is liberated and used to force the occurrence of unfavorable reactions is misleading albeit widely disseminated. The complete reaction of ATP hydrolysis is:

$$ATP + 3H_2O \rightarrow ADP + PO_4^{3-} + 2H_3O^+$$

but the intervention of water is frequently overlooked. The net balance of the bonds disrupted and created in this reaction is such that energy is liberated, yet the chemical bonds themselves are not energy depots that release energy when broken. It is the balance of bond dynamics involved the reaction that matters. Part of this misunderstanding comes from the fact that one tends to oversimplify the writing of chemical reactions. When written like

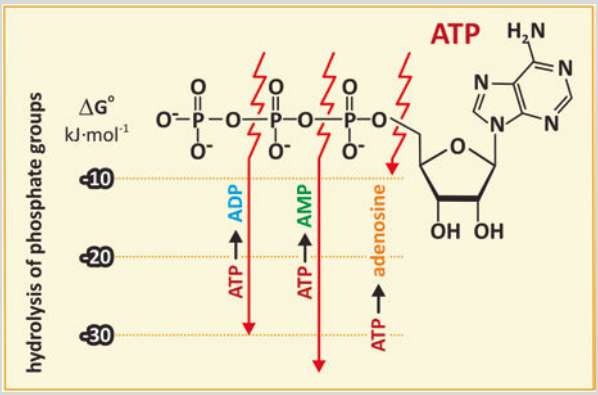
$$ATP \rightarrow ADP + P$$

the involvement of water is implicit but frequently overlooked. Only one bond fission is shown, leaving the idea that the released energy is contained in the chemical bond itself, which is illusory.

Figure 3.23 shows the structure of ATP. At first glance it may seem intriguing why the hydrolysis of ATP into ADP or AMP involves different ΔG^0.

(continued)

Box 4.2 (continued)
This happens because the phosphoryl groups are not equivalent. The electronic distribution in the molecule, for instance, is different, as shown in the following figure.



Having a common highly endergonic hydrolysis reaction to couple to other reactions is a big advantage for cells as only one molecule needs to be synthesized to drive metabolism, and the complexity and diversity of enzymes involved are very much decreased compared to a situation where many and diverse endergonic reactions would be used. To power metabolism, the cells use almost exclusively ATP and enzymes that catalyze its hydrolysis, coupling it to a simultaneous unfavorable reaction. Assuring that ATP is always present and never completely depleted is vital for the cell.

Specific processes that need to be driven thermodynamically by the use of endergonic reactions but should not be dependent on ATP make use of similar molecules such as GTP or UTP.

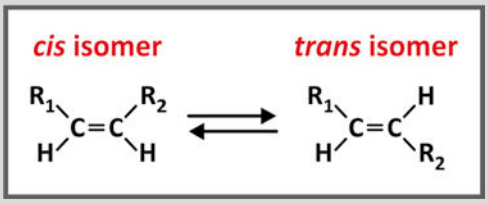
It is interesting that hydrolysis of $\mathrm{ATP} \rightarrow \mathrm{ADP}$, $\mathrm{ATP} \rightarrow \mathrm{AMP}$, and $\mathrm{P_2O_7} \rightarrow 2\mathrm{PO_4^{3-}}$ involve $\Delta G^o \ll 0$ in any case. Nevertheless, the frequency of $\mathrm{ATP} \rightarrow \mathrm{ADP}$ reaction over the others in metabolic processes is much higher. The eventual advantage may have been that, in parallel to the dedicated mechanisms cells have to convert ADP back to ATP using energy from nutrients, ATP can be readily obtained from $\mathrm{ADP} + \mathrm{ADP} \rightarrow \mathrm{ATP} + \mathrm{AMP}$ (see Sect. 8.4). The appearance of AMP may signal a state of ATP depletion that triggers regulatory events in the cell that favor ATP synthesis. This matter is still controversial and will be readdressed in Chap. 8 in connection to the use of ATP for muscle contraction.

principles and is not difficult to understand. The factors affecting enzyme activity are basically the ones affecting protein structure, as the essence of enzyme catalysis is the conformational dynamics of the protein with the bound substrate. Environmental factors affecting catalysis, such as pH or temperature, are not very important in regulating the activity of enzymes because most organelles in cell have buffered pH and constant temperature. The activity of enzymes changes depending on these conditions, but it is not feasible for the cells to change pH or temperature to influence the course of metabolic reactions. So, enzymes have a structure that fits optimal activity at specific temperature and pH ranges (recall the pH of different human tissues in Fig. 2.8), and their activity depends most on ligands, called effectors, that can improve activity ("activators") or decrease it ("inhibitors"). The exact mechanism these ligands use to act as activators or inhibitors of enzymes depends a lot on the structure of the enzyme and how catalysis takes places. The thermodynamics and performance of enzymes as catalysts can be studied under simple but rather abstract principles that describe generally how the velocity of catalysis is independent from the extent of reaction and how it changes with the concentration of substrate and effectors, but do not provide information on how a specific enzyme binds the substrate and distorts its electronic structure to facilitate catalysis (see Sect. 3.3.4).

The simplest thermodynamic description of catalysis is explained in Box 4.3. It illustrates that the velocity of reactions is independent of ΔG^{o}. The simplest

Box 4.3: The Thermodynamics of a Simple Catalyzed Reaction

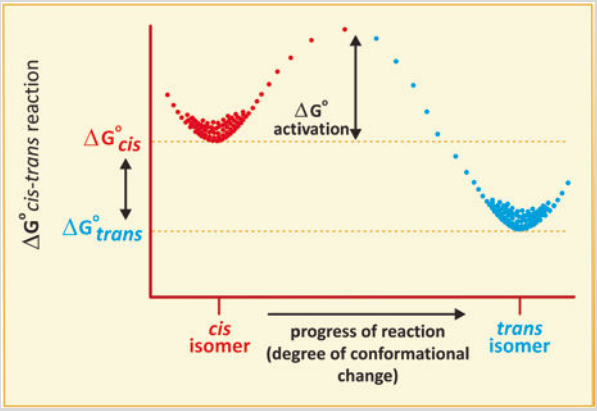
Isomerization is a simple process that we will use to illustrate the thermodynamics associated to the kinetics of reactions and the effect of enzymes on both. *Cis–trans* isomerization, for instance, can be represented by a simplified scheme:



in which R_1 and R_2 are any group of the molecule different from H. Depending on the temperature, solvent, and exact chemical nature of R_1 and R_2, the conformational dynamics of these molecules varies, but there is always a certain degree of flexibility that makes some molecules more distorted than others at any given instant. Some of these molecules acquire a structure that is intermediate between the *cis* and the *trans*-isomer, R_1, R_2, and

(continued)

Box 4.3 (continued)
the H being located in unstable positions that make the molecule more energetic. Higher energy states associated to intermediary conformations are very unstable, and few molecules acquire these conformations, although it may happen occasionally. Plotting a point for each molecule in an energy (of the total molecule) vs. degree of structural conversion plot, one would obtain at a given instant, in equilibrium, the distribution depicted in the following figure. Many molecules are in the *cis* and *trans* states because they correspond to energy minima, but few molecules reach conformations in between these states because these conformations correspond to electronic distributions and nuclei localizations that are not optimal in the balance of charge distributions. The difference between the energy of the reactant (*cis* isomer in this case) and the energy associated to the molecule in the most unlikely state (energy maximum) is the so-called activation energy because evolution from this point to the products corresponds to loss of energy (decrease in ΔG), i.e., the molecule spontaneously progresses to the product (*trans*-isomer) after this point.

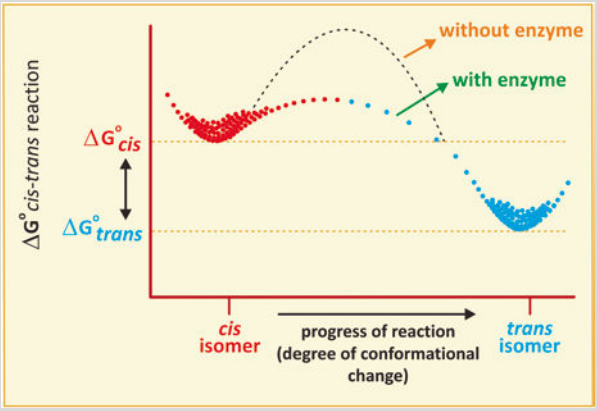


Energy of each molecule according to its degree of conversion between *cis* and *trans* conformations. Each point represents a molecule. This is a hypothetical distribution of a population of molecules in equilibrium at a certain given instant in time. Most molecules are in the cis or trans isomers conformation, which correspond to local minima of energy. The intermediate conformations correspond to molecules in high-energy states, therefore less populated. In practice this implies that very few molecules spontaneously convert between conformations

(continued)

Box 4.3 (continued)

When the activation energy is high, it is very rare that a molecule acquires the energy necessary to adopt the intermediate state that enables spontaneous conversion to products. In other words, the reaction progresses very slowly. Enzymes bind to substrates and distort the structure of the molecules in a way that the electronic distribution and nuclei interactions are more favorable. The energy of activation assigned to the reactant is decreased, and the consequence is that more molecules reach the maxima of energy and progress to products. This means that the velocity of the reaction increases (see figure). ΔG_{cis}° and ΔG_{trans}° remain unchanged, which means that the catalyzed reactions reach a state of equilibrium faster, but the extent of reaction (fraction of reactants converted to products) is not altered by the action of the enzyme.



Enzymes distort the molecular structure of substrates turning the intermediary conformations not so energetic, therefore more likely. Because more molecules reach the intermediary conformations, a higher velocity of product formation is achieved

formulation for an enzymatic reaction was devised mainly by Leonor Michaelis and Maud Menten (Box 4.4). In their abstract model of catalysis, the generic enzyme E_z would bind the generic substrate, S, to form a complex of undetermined nature from which the generic product, P, was formed irreversibly:

$$E_z + S \rightleftharpoons E_zS \rightarrow E_z + P$$

The mathematical deduction in Box 4.4 shows that in this case the velocity of the reaction (i.e., the variation in [P] per time unit) is:

$$v = \frac{V_{max}[S]}{K_m + [S]}$$

Box 4.4: Fundamentals of Enzyme Kinetics of Simple Reactions
Enzymes (E_z) associate to substrates (S) through specific amino acid residues that distort the substrate and make the conversion to products (P) faster. The enzyme interacts actively with the S molecule in the course of catalysis, but the end of reaction results in the release of P with E_z being in the original state as before binding S. In abstract, this situation can be represented by:

$$E_z + S \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} E_zS \underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} E_z + P \quad (1)$$

E_zS is the transitory complex in which E_z and S are in contact, S being distorted. k_x are the kinetic rate constants of each conversion ($x = 1, 2, -1, -2$), which are proportionality factors, between velocity and concentration of reactants. In broad terms, it may be said that k_x is the intrinsic propensity of step x to occur (e.g., k_{-1} is the intrinsic propensity for the fast unbinding of E_z and S after E_zS is formed, while k_2 is the intrinsic propensity for the fast release of P after E_zS is formed; once E_zS is formed, the processes to which k_{-1} and k_2 are associated compete with each other). The velocities of each of the four events of the reaction are:

$$v_1 = k_1[E_z][S], \quad v_{-1} = k_{-1}[E_zS], \quad v_2 = k_2[E_zS], \quad v_{-z} = k_{-2}[E_z][S] \quad (2)$$

Assuming that the reaction proceeds in a steady-state condition, the concentration of E_zS does not change with time, i.e., the velocity of creation of E_zS equals the velocity of consumption of E_zS:

$$v_1 + v_{-2} = v_2 + v_{-1} \quad (3)$$

$$\left(k_1[S] + k_{-2}[P]\right)[E_z] = \left(k_{-1} + k_2\right)[E_zS] \quad (4)$$

$[E_z]$ and $[E_zS]$ are not practical variables to work with as is the concentration of total enzyme ($[E_z]_o = [E_zS] + [E_z]$) that is known or more readily measurable in an experiment. At the same time, the velocity of the reaction is the velocity at which P is produced, v:

$$v = k_2[E_zS] - k_{-2}[E_z][P] \quad (5)$$

Combining all equations, it can be deduced that the velocity of product creation is:

$$v = [E_z]_0 \frac{k_2k_1[S] - k_{-2}k_{-1}[P]}{k_{-1} + k_2 + k_1[S] + k_{-2}[P]} \quad (6)$$

(continued)

Box 4.4 (continued)
This is an interesting equation that describes how the velocity of a catalyzed reaction varies with the total concentration of the enzyme, the concentration of the substrate, and the concentration of the product. It also describes how the velocity changes depending on the intrinsic kinetic properties of each step (k_1, k_2, k_{-1}, k_{-2}).

Regardless of the particular [S], [P], or kinetic constants, the equation shows that v is always proportional to $[E_z]_o$. The total concentration of enzyme has a direct impact on the velocity of the reaction. In fact, in cells, increasing or diminishing the expression of enzymes is a way to directly interfere with the rate of critical steps of metabolism.

It is important to further explore the equation in particular situations that may be of significance:

1. $[P] \approx 0$ and/or $k_{-2} \approx 0$ or any other conditions in which $k_{-2}[E_z][P] \ll k_2[E_zS]$ (Michaelis–Menten condition)

   In this case the reaction simplifies to

$$E_z + S \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} E_zS \xrightarrow{k_2} E_z + P \tag{7}$$

   and v simplifies to ($[P] \approx 0$ and/or $k_{-2} \approx 0$ in Eq. (6) of this box):

$$v = \frac{k_2 \left[E_2\right]_0 \left[S\right]}{\frac{k_{-1} + k_2}{k_1} + \left[S\right]} \tag{8}$$

$$v = \frac{V_{max} \left[S\right]}{K_M + \left[S\right]} \tag{9}$$

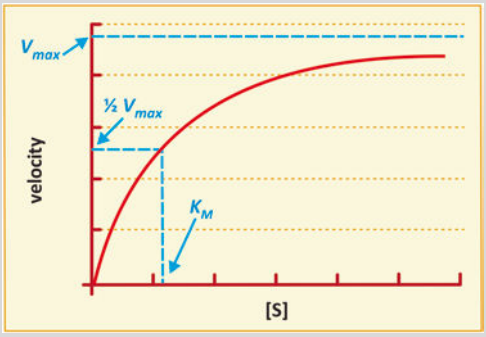
   $k_2[E_z]_o$ corresponds to the velocity of reaction when all the enzyme molecules are bound to S, which is the maximal possible velocity, V_{max}; $(k_{-1} + k_2)/k_1$ corresponds to the equilibrium constant of the dissociation of E_zS and is named Michaelis constant. As K_M^{-1} relates to the extent of the association between E_z and S in equilibrium, it is frequently taken as a measure of the enzyme–substrate affinity.

   Michaelis–Menten conditions lead to a dependence of v on [S] that is hyperbolic (see figure). Although corresponding to a very particular condition of a very simple reaction scheme, the Michaelis–Menten equation [Eq. (9)] has great historical importance, and most enzymes having

(continued)

Box 4.4 (continued)

hyperbolic-like kinetics are described by apparent K_M and V_{max}, even though the reaction scheme they follow is not always exactly the one in Eq. (7).



Dependence of v on [S], as predicted by Eq. (9) (Michaelis–Menten equation). This dependence is a particular case of a rectangular hyperbole (i.e., having asymptotes perpendicular to x and y axes; rectangular hyperbola may be expressed as $(x-h)(y-t)=m$, in which h, t, and m are constants that can be rearranged to the format of a Michaelis–Menten equation for particular cases of h, t, and m

2. $[S] \approx 0$ and/or $k_1 \approx 0$ or any other condition in which $k_1[E_z][S] \ll k_{-1}[E_zS]$

This is a situation similar to the one of Michaelis–Menten but in the reversed sense:

$$E_z + S \underset{k_{-1}}{\overset{}{\leftarrow}} E_zS \underset{k_2}{\overset{k_{-2}}{\rightleftharpoons}} E_z + P \qquad (10)$$

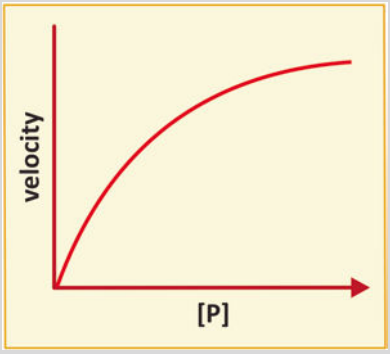
and the velocity dependence on [P] is similar ($[S]=0$ or $k_1=0$ in Eq. (6)):

$$v = \frac{V_{-\max}[P]}{K_{-M} + [P]} \qquad (11)$$

(The subscripts "–" are used to refer the inverse sense of the reaction, i.e., negative velocities.)

(continued)

Box 4.4 (continued)



3. Intermediate values of [S] and [P]
   Because we are working with an example in which the conversion between S and P follows a 1:1 stoichiometry, one may consider a situation where the reaction starts with S being at concentration $[S]_o$ in the absence of P:

$$[S] = [S]_o - [P] \tag{12}$$

   Combining Eqs. (12) and (6):

$$v = [E_z]_0 \frac{k_2 k_1 - (k_2 k_1 + k_{-2} k_{-1}) \frac{[P]}{[S]_0}}{\frac{k_2 + k_{-1}}{[S]_0} + k_1 + (k_{-2} - k_1) \frac{[P]}{[S]_0}} \tag{13}$$

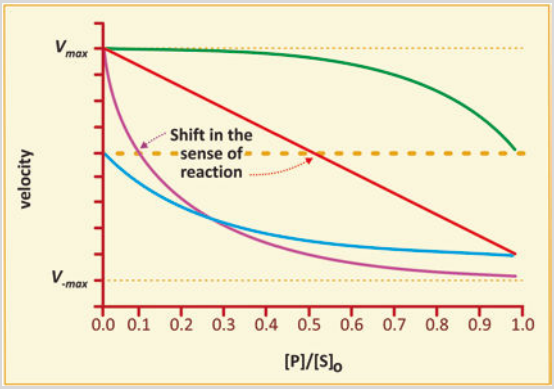
   For a given initial concentration of S, the velocity of reaction changes as P is being produced, as depicted in the figure below. The exact curves depend on the specific values of k_1, k_2, k_{-1}, and k_{-2} for the reactions, but the pictorial examples in the figure show that when [S] dominates over [P] ($[P]/[S]_o \approx 0$), the reaction proceeds with net transformation of S into P with a velocity proportional to [S]. When [P] dominates over [S] ($[P]/[S]_o \approx 1$), the reaction proceeds with net conversion of P to S with a velocity that is proportional to [P]. The negative values of v in the graph reflect the net conversion of P into S (i.e., an overall course of reaction in the reverse direction). The $[P]/[S]_o$ point at which $v = 0$ (i.e., the velocity of formation of P and consumption of P is equal) is obtained setting $v = 0$ in Eq. (13):

$$\left(\frac{[P]}{[S]_0} \right)_{v=0} = \frac{k_2 k_1}{k_2 k_1 + k_{-2} k_{-1}} \tag{14}$$

(continued)

Box 4.4 (continued)

This shows that the shifting point at which the enzyme action changes the sense of the reaction is in fact a balance between the conjugated parameters of the forward and reverse sense steps.



The velocity of a reversible reaction catalyzed by a single enzyme in both senses shifts from positive (net consumption of substrate and production of P) to negative (net consumption of P and production of the substrate) depending on the balance of the kinetic constants involved and the concentration of the substrate and product. The shifting point ($v=0$) is attained when [P] relative to $[S]_0$ is $\frac{[P]}{[S]_0} = \frac{k_2 k_1}{k_2 k_1 + k_{-2} k_{-1}}$.

The *green line* corresponds to $k_{-2}=0$ (Michaelis–Menten condition), $k_1=k_{-1}=k_2$; the *blue line* corresponds to $k_1=0$, $k_{-1}=k_{-2}=k_2$; the *red line* corresponds to $k_1=k_{-1}=k_{-2}=k_2$; the *magenta line* corresponds to $k_1=k_{-1}=k_2=k_{-2}/10$

The practical implication of Eq. (13) is immense. It shows that enzymes catalyzing the simple reaction described by equation 1 are able to increase the velocity of the reaction in both senses depending whether S or P is accumulating, always favoring the consumption of the metabolite (S or P) that is accumulating. This is vital to understand metabolic regulation.

in which V_{max} is the maximal possible velocity for the reaction at a given concentration of enzyme and K_m is the dissociation constant of E_zS (if the first reaction reaches equilibrium). K_m is named Michaelis constant and K_m^{-1} reflects the extent to which E_z binds S, which is usually referred to as the "affinity" of E_z toward S, an intuitive but rather ambiguous concept.

The irreversible step makes the model simpler, but in many cases the second step is reversible, and the same enzyme can catalyze a reaction both ways, from S to P and vice versa. In the excess of S, the dominant reaction is $S \rightarrow P$, and when P accumulates, $P \rightarrow S$ dominates.

Box 4.4 shows that in a reversible reaction, the velocity of reaction is dependent on the relative concentrations of S and P. When the concentration of P reaches cer-

tain critical points, the velocity of the conversion of P to S surpasses the velocity of the opposite process, and the net result is the consumption of P and production of S (Fig. 4.4). This is very important in metabolism as most enzymes catalyze reactions in both directions and the dominant direction depends on the relative concentrations of the metabolites.

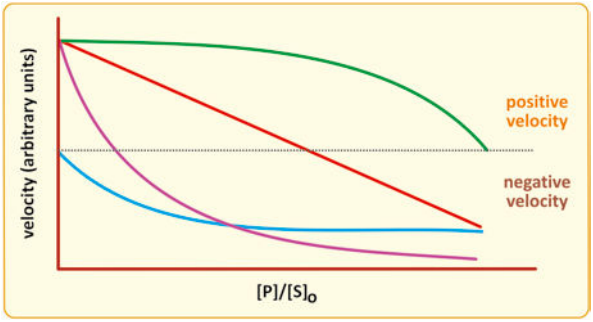
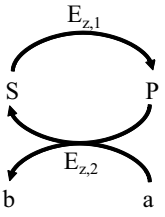


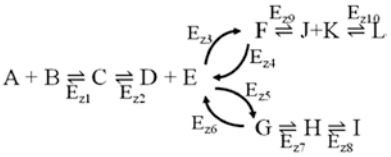
Fig. 4.4 Velocity of the reversible reaction $S \rightleftharpoons P$ catalyzed by an enzyme. $[S]_o$ is the initial concentration of the substrate; [P] equals $[S]_o$ ($[P]/[S]_o = 1$) when all S was converted to P. Positive values of velocity correspond to a net conversion of S to P; negative values correspond to net conversion of P to S. The exact $[P]/[S]_o$ at which $v = 0$ (no net production of P or S) depends on the specific enzymes and substrates to be considered, but the transition from positive to negative values of velocity is a generic trend among enzymes that catalyze reversible reactions. The *red line* corresponds to the variation in velocity for an enzyme that catalyzes the conversion of S to P and P to S equally. In this case, the turning point of velocity corresponds to half conversion of substrates ($[P]/[S]_o = 0.5$). The *magenta line* corresponds to an enzyme that is more efficient in converting P into S: a small concentration of P is enough to drive the net reaction in the direction of converting P into S. The *green line* corresponds to a Michaelis–Menten enzyme: catalysis only converts S into P (always positive velocities) with maximal velocity for maximal concentration of [S] ($[P] = 0$). The *blue line* corresponds to a Michaelis–Menten enzyme that only converts P into S: the velocity is always negative and maximal for maximal concentrations of P ($[S] = 0$)

It is important to stress that a certain enzyme may catalyze the conversion of S to P irreversibly, and a second enzyme may catalyze the inverse reaction also irreversibly. In this way S and P are interconvertible, but two enzymes are involved. Moreover, third molecules may be involved in one of the directions only. This is the case of



where two different enzymes are involved, and the conversion of P to S may also imply conversion of a to b, which may have $\Delta G^o \ll 0$ to make this particular reaction more favorable. It should be noted that with this reaction scheme, the conversion of S to P may be blocked (absent or inactive E_{z1}) without interfering in the conversion of P to S, or even while E_{z2} is activated. When the same enzyme catalyzes $S \rightleftharpoons P$, this possibility does not exist; this enzyme may be activated or inhibited, although both directions will be affected. In the reaction above catalyzed by $E_{z,1}$ and $E_{z,2}$, a fine-tuning of the direction of reaction is possible, but in $S \rightleftharpoons P$ is not.

Returning to the last metabolic scheme in Sect. 4.1, now modified to include enzyme-catalyzed reactions:



It is clear that E_{z3}, E_{z4}, E_{z5}, and E_{z6} are the ones that determine the direction of the flux of the metabolism. There are mechanisms that prevent cells from having E_{z5} and E_{z6}, and E_{z3} and E_{z4} simultaneously activated, so that depending exclusively on which of these key enzymes are active or inactive, this metabolic pathway may be producing L or I, or both, at the expense of consuming A and B, or it can be producing A and B at the expense of consuming I or L, or both. This is the key concept of metabolic regulation. The mechanisms used in cells to activate or inhibit enzymes are then an issue of critical importance and will be addressed in Chap. 5.

An additional factor improves the metabolic processes further. The velocities of reactions catalyzed by key enzymes such as E_{z3}, E_{z4}, E_{z5}, or E_{z6} usually do not follow equation. Whereas enzymes following Michaelis–Menten-like kinetics (Sect. 4.2.1 increase the velocity of reactions proportional to the substrate concentration when [S] is low, these other enzymes change the velocity of reaction abruptly around critical concentrations of the substrate. Velocity varies sigmoidly in response to [S] (Fig. 4.5), in the same way oxygen fixation efficiency by hemoglobin depends on oxygen concentration. This means that some reactions are triggered at high velocity only when the substrate accumulates to a certain level.

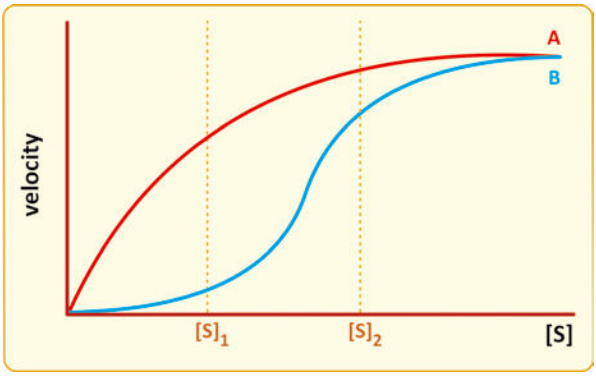


Fig. 4.5 Two types of dependencies of the velocities of reactions catalyzed by enzymes on the concentration of substrate. Enzymes following Michaelis–Menten kinetics (A) have a hyperbolic-like dependence of velocity on [S]. The initial trend (up to $[S]_1$) is linear. In this range, accumulation of the substrate is counteracted by a proportional increase in the velocity of its conversion to product. Enzymes with sigmoidal kinetics (B) have a lag initial regime in which the velocity is low until [S] reaches a critical value, $[S]_1$, after which the velocity increases abruptly. In both cases (A and B), high substrate concentrations ($[S] > [S]_2$) correspond to almost maximal velocity of the catalyzed reaction. In this situation, the enzymes do not modulate velocity in response to substrate concentration because they are already working at maximal possible capacity

Box 4.5: The Importance of K_M, the Michaelis Constant

In strict terms, K_M is only valid for an enzyme that catalyzes a reaction described by

$$E_z + S \underset{k_1}{\overset{k_{-1}}{\rightleftharpoons}} E_zS \xrightarrow{k_2} E_z + P \qquad (1)$$

as explained in Box 4.4. In practice, most reactions cannot be described this way. For instance, reactions involving more than one substrate do not fit in this scheme. Yet K_M is a combination of all kinetic constants ($(k_2 + k_{-1})/k_1$) that reflects the extent of dissociation of E_zS when equilibrium conditions are reached ($k_2 \ll k_{-1}$). Thus K_M^{-1} reflects the "affinity" of S for E_z and is an extremely valuable tool to compare the "preference" of the same enzyme for different substrates. For this reason, K_M acquired great importance in the study of enzymes from the early days of enzymology, the discipline that is

(continued)

Box 4.5 (continued)
devoted to the study of the enzymes. Even enzymes that did not follow reaction scheme 1 but had hyperbolic-like dependences of catalytic velocity vs. [S] had apparent K_M^{-1} assigned. Other enzymes were studied in conditions in which the velocity would vary in a hyperbolic-like manner with [S] and apparent K_M^{-1} calculated. When more than one substrate is used, for instance, if all substrate concentrations are kept high and only one substrate concentration varies, the velocity of the reaction depends on the concentration of that substrate. The dependence is usually hyperbolic-like and an apparent K_M is estimated, which is valid for that specific substrate. Somewhat mistakenly, K_M became the key parameter to describe the kinetic properties of enzymes, and the quest for methods on how to calculate K_M for specific E_z–S pairs became an import part of biochemistry for many years.

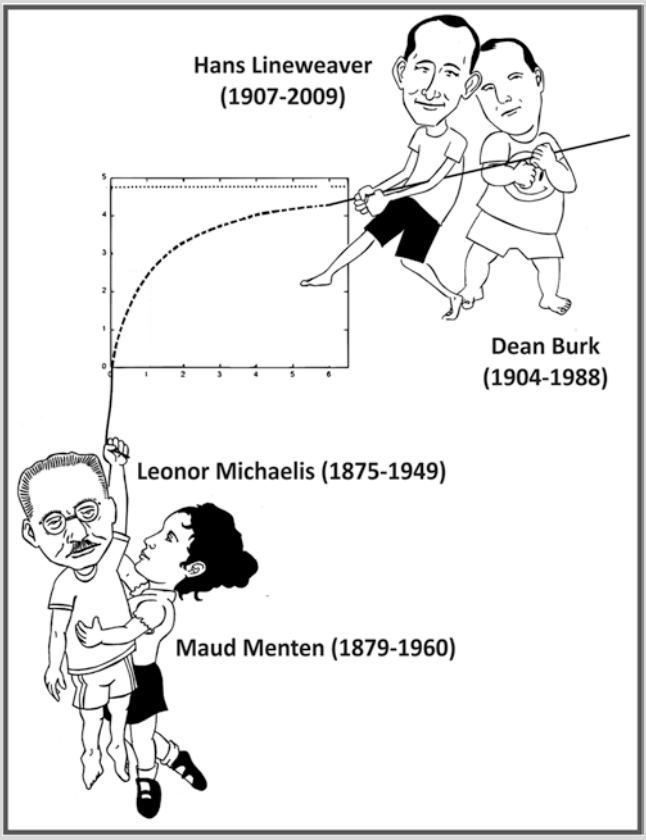
It is obvious from the Michaelis–Menten equation:

$$v = \frac{V_{\max[S]}}{K_M + [S]} \tag{2}$$

that when [S] equals K_M, $v = V_{max}/2$. The graphical interpretation is immediate, as depicted in the figure below. V_{max} is the asymptotic limit of v when [S] tends to infinity; K_M is the [S] in which v is half V_{max}. This is the reason why K_M is so popular among biochemists studying metabolism. When the substrate concentration drops below K_M, the velocity of the reaction is considerably below V_{max} and the process being catalyzed loses efficacy. So K_M is the reference value to estimate the metabolic impact of drops in the concentration of metabolites.

Box 4.5 (continued)
Calculation of K_M is thus of the uppermost importance. However, in an experimental plot of v vs. [S] with discrete data, V_{max} is not easy to identify as experimental data at very high [S] are difficult to attain (see figure below). Nowadays, a computational nonlinear regression fit of Eq. (2) to the experimental data selects the best statistical K_m and V_{max} easily, fastly, and accurately, but in the early twentieth century, when Leonor Michaelis and Maud Menten derived Eq. (1), this was not an option. In the 1930s Dean Burk and Hans Lineweaver overcame the limitation of V_{max} determination by linearizing the Michaelis–Menten equation. When rewritten in the form $1/v$ vs. $1/[S]$, Eq. (1) becomes:

$$\frac{1}{v} = \frac{K_M}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}} \tag{3}$$



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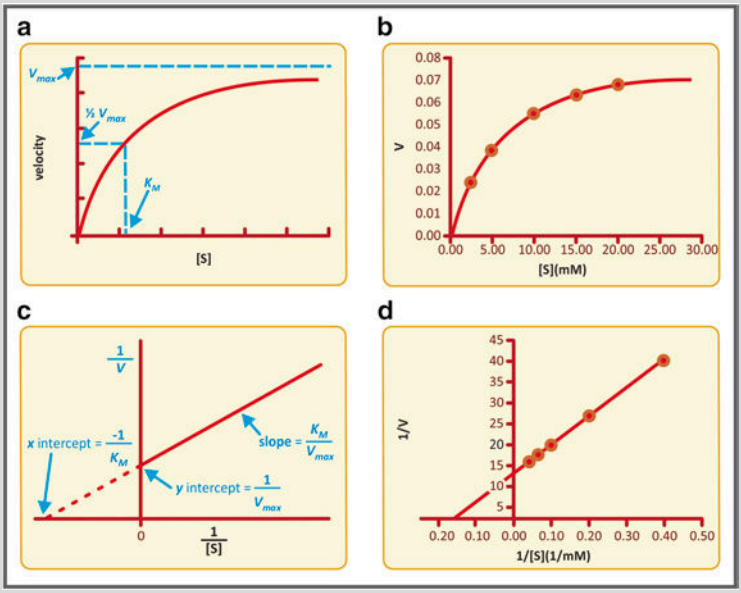
Box 4.5 (continued)
which describes a straight line from which both K_M and V_{max} are readily calculated (see panels c and d below).



Graphical interpretation of the Michaelis–Menten rectangular hyperbole (**a**). K_M is the substrate concentration at which the reaction velocity is half of the maximal velocity, V_{max}, which is the asymptotic limit of the velocity for infinite [S]. In practice, with experimental data (**b**), V_{max} is very difficult to identify as [S] cannot be extended indefinitely due to solubility or cost limitations. Historically, the most common option to obtain K_M and V_{max} was to perform the linearization of the Michaelis–Menten plot to estimate K_M and V_{max} graphically (**c**): the y intercept is $1/V_{max}$ and the slope is K_M/V_{max}. K_M can also be estimated from the x intercept, which is $-1/K_m$. Panel (**d**) shows the linearization of the data in panel (**b**). For the data presented in the figure, $K_M = 6.39$ mM and $V_{max} = 0.085$ Ms^{-1}. The data were extracted from a 1951 study on the hydrolysis of carbobenzoxyglycyl-L-tryptophan using pancreatic carboxypeptidase (R. Lumry, E. L. Smith, and R. R. Glantz, 1951, J. Am. Chem. Soc. 73, 4330). The linearization of the Michaelis–Menten equation is owed to Hans Lineweaver and Dean Burk, who used the method for the first time in 1934. With modern computational techniques and statistical methods, linearization is not mandatory as V_{max} and K_M can be estimated by nonlinear regression methods that fit the Michaelis–Menten equation directly to experimental data [*red line* in panel (**b**)]

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Chapter 5
The Regulation of Metabolisms

In a cell, the fluxes of matter and energy are highly controlled so that cells can maintain their organization and multiply when needed. We have discussed in the previous chapter that consecutive reactions can be driven through coupling favorable reactions to unfavorable reactions, many of those benefiting from the ΔG^{o} of ATP hydrolysis. Synthesizing ATP involves using energy associated to the chemical processing of nutrients or molecules stored for this purpose (catabolic metabolism). When in excess, the nutrients tend to engage a series of reactions whose end products are the storage molecules (anabolic metabolism) for later use. This shift implies a complex network of metabolisms that must be inhibited or activated. Inhibition and activation occur selectively at specific reactions which in turn occur in specific locations and precise timings inside the cells. In addition, the shift requires that different cells in the same tissue or in different tissues operate coordinately. Liver, adipose tissue, muscle, and brain, for instance, need to be coordinated so that when the brain and muscle require specific nutrients to operate, this process does not conflict with processes in other organs and no failure of the body function as a whole occurs.

Only specific selected sets of reactions take place at a given time in each organelle of a cell. Chemical entities (hormones) circulate in the body and are captured by cell receptors that trigger short chemical reaction sequences, generically named "signal transduction pathways" (see Box 5.1 and Fig. 5.1) upon binding. In the end, these short sequences of chemical reactions modify enzymes stimulating or inhibiting the catalysis of metabolic reactions (Fig. 5.1). The same hormone may be sensed by different cells in different organs. The metabolic events triggered in different organs are not necessarily the same but are coordinated. For instance, during prolonged aerobic exercise, muscles are consumers of fatty acids, which must be mobilized from the adipose tissues; in this situation both the muscle and the adipose tissue are not activating the same metabolic pathways, but they are certainly coordinated. A drop in glycemia (glucose concentration in blood) leads to the release of glucagon, a peptide hormone synthesized in the pancreas that binds to receptors in hepatocytes (liver cells) and triggers events that activate reactions leading to the

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production of glucose (see Chap. 9). Glucose is then released in the blood through the optimized mesh of capillaries throughout the whole liver. This matter will be revisited later in Box 8.1.

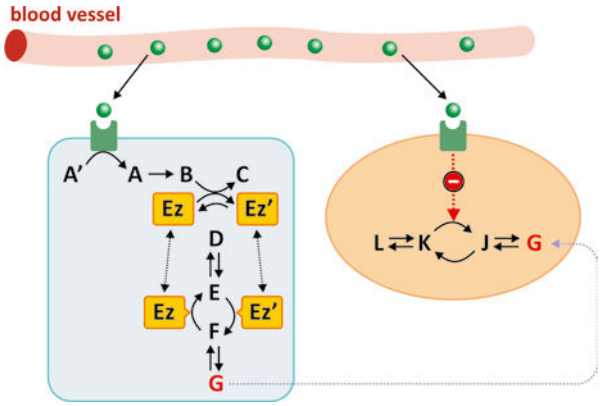


Fig. 5.1 When the hormone (green sphere) binds to its receptor, which may be located on the membrane or may be intracellular, this translates into a short series of chemical reactions (exemplified as compounds A′ to C) named "signal transduction." One of the consequences may be that one enzyme is modified (Ez to Ez′ in the picture). This transformation may be phosphorylation, for instance (Ez′ being in this case Ez with a covalently bound phosphate group). In this hypothetical situation in which Ez and Ez′ catalyze opposing reactions irreversibly, the practical effect of the hormone is to dictate the sense of metabolic reactions. In the case depicted in the figure, the presence of the hormone in blood generates metabolite G into the cell on the left. The same hormone may be acting at the same time in different cells (colored light blue and salmon) in different organs triggering different metabolic pathways. The hormone may stimulate G-consuming pathways in cells from other organs (e.g., leading to inhibition of the enzyme that converts K in J)

Box 5.1: Biosignaling, the Communication Among Cells and Inside Cells

In a complex organism, such as the human body, having specialized systems, with specialized organs, specialized tissues, and specialized cells, coordination requires fine-tuning and reliability. The homeostasis of the human body requires that the action of different organs is not conflicting. Imagine a situation such as prolonged starvation. The liver synthesizes glucose and releases glucose in blood to keep the glycemia within safe levels for the brain to operate. What would happen if other organs such as striated muscle were subtracting glucose from the blood to synthesize glycogen, for instance? This conflict between liver action and striated muscle action would be fatal or, at least, result in a paramount waste of energy and matter. There are mechanisms that prevent conflicts of this kind. These mechanisms coordinate the action of cells that may be in contact with each other in the same tissue or in very remote

(continued)

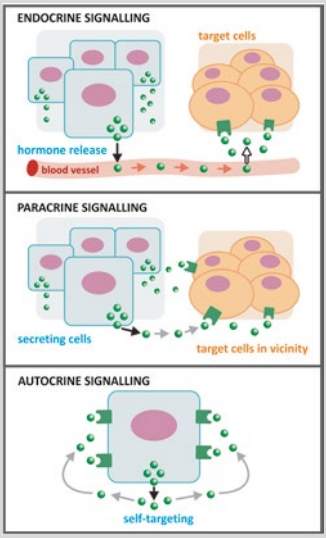
Box 5.1 (continued)
locations relative to each other, such as different organs. There are molecules that serve as "signals" that are released and trigger synchronized and compatible events in different cells. This is known as cellular communication or biosignaling.

Naturally, biosignaling, such as any communication process, requires that there is a source for the signal (e.g., hormone), the means for the signal to disseminate, and a receptor, or receptors. The signal is a molecule that is synthesized, so the steps of biosignaling are (1) synthesis of the molecule that will serve as signal; (2) release of the signal molecule; (3) transport/dissemination to the target cells, i.e., cells with the receptors that bind and are responsive to the signal molecule; (4) interaction with the receptor, usually a protein, in the target cell; (5) triggering intracellular chemical or physical events that result directly from signal–receptor interaction ("signal transduction"); and (6) generation of other events, frequently a series of events ("cascade" or "signaling pathway"), which constitutes the final response of the cell to the signal.

Hormones, neurotransmitters, prostaglandins, growth factors, and cytokines, such as interleukins and interferons, serve as biochemical communication signals. These signals may act at short range or long range (see figure). Short-range communication involves diffusion of signals in the extracellular medium in the immediate vicinity of the cell that secretes the signal molecule. This is named paracrine signaling. Synaptic signaling is an example of paracrine signaling: neuronal termini release neurotransmitters that bind to receptors in the postsynaptic cells. Long-range signaling is known as endocrine signaling. In this case, specialized cells in specialized organs synthesize hormones, which are usually released into the bloodstream and distributed throughout the body. Hydrophilic hormones are soluble in blood and are easily distributed; hydrophobic hormones might need transporters and/or have severe limitations in the concentrations they can achieve in blood. Steroid hormones, for instance, are hydrophobic hormones derived from cholesterol, and they need to be associated to carrier proteins to be distributed in the body through the blood.

(continued)

Box 5.1: (continued)



A third form of signaling exists in addition to endocrine and paracrine: autocrine. However, this signaling is mainly found in pathological conditions such as cancers. Tumor cells release growth factors that bind to receptors in the surface of the same cell that releases them, stimulating cell growth and cell division. These events become unbalanced and the tumors grow uncontrolled.

Most molecular signals have very high selectivity and affinity for their receptors. High selectivity means that the receptor is responsive only for a very precise structure of the ligand, i.e., the molecule that serves as signal. High affinity means that the binding equilibrium of the ligand signal to the receptor is very extensively shifted toward the bound ligand–receptor complex, which in practice means that very low concentrations of the ligand generate an integrated high response from the receptor. Nanomolar (10^{-9} M) concentration of hormones usually suffices to trigger physiological responses because the high affinity for the receptor compensates the low concentration of the ligand.

Depending on whether the hormones may translocate through the plasma membrane or not, the receptors may be located on the surface of cells, or inside the cells, in the cytoplasm or nucleus. Adrenalin, insulin, and glucagon, for instance, have surface receptors; in contrast, testosterone and progesterone, for instance, bind to receptors intracellularly.

(continued)

Box 5.1: (continued)
Upon binding of the ligand to receptor, the signal transduction process may be of three main different types depending on the functionality of the receptor:

1. When the receptor is an ionic channel, the ligand may activate or inhibit the flux of ions through the channel. This is the case of the receptor of acetylcholine in the neuromuscular junction.
2. When the receptor is coupled to G protein (guanosine nucleotide-binding proteins), binding of the ligand causes conformational changes in the receptor that indirectly activate the G protein, which detaches it from the receptor and binds to adenylate cyclase (also known as adenylyl cyclase) or a phospholipase or another enzyme that catalyzes the formation of the molecules so-called second messengers. Second messengers then initiate series of reactions that will interfere with metabolic processes. Cyclic AMP, cyclic GMP, inositol triphosphate, and diacylglycerol are examples of second messengers, and the receptors of adrenaline and serotonin are examples of G protein-coupled receptors.
3. When the receptor has catalytic tyrosine kinase activity, binding of the ligand causes conformational changes that make the enzyme active and thus able to phosphorylate proteins in Tyr residues using ATP as a source of phosphate. The insulin receptor is of this kind.

The intracellular signaling pathways that follow the binding of the signal molecule to the receptor are diverse. The main signaling pathways associated to the regulation of metabolism are addressed individually in the main text.

Glycemia is a key factor in energetic metabolism as the brain uses exclusively glucose as a source of ATP production (the only known exception occurs in long-term starvation; see Sect. 9.3.4), and there is no mechanism to keep a higher concentration of glucose in the central nervous system than in blood because glucose transporters across the blood–brain barrier do not operate against a concentration gradient. Therefore, the coordination of metabolisms occurring in different organs simultaneously is such that glucose concentration in the blood is kept higher than a specific threshold. Values under this threshold produce loss of conscience, coma and death may occur (Fig. 5.2). Given the importance of glycemia control and the investment represented by regulatory pathways it is not surprising that biochemists tend to overemphasize the importance of glucose as a nutrient compared to amino acids and lipids. Yet, lipids and proteins are the most important energetic reserves in the human body (see Table 9.1). Energetic metabolisms are often but erroneously associated to carbohydrate metabolism only. In reality, fatty acid, amino acids, and glucose metabolisms are interconnected. But despite the connections, the metabolites pertaining to each pathway are not necessarily interconvertible. Lipids are typically storage molecules so they cannot be converted to glucose or proteins; it can only be converted in metabolites that in principle will follow reaction routes leading to ATP synthesis (Fig. 5.2).

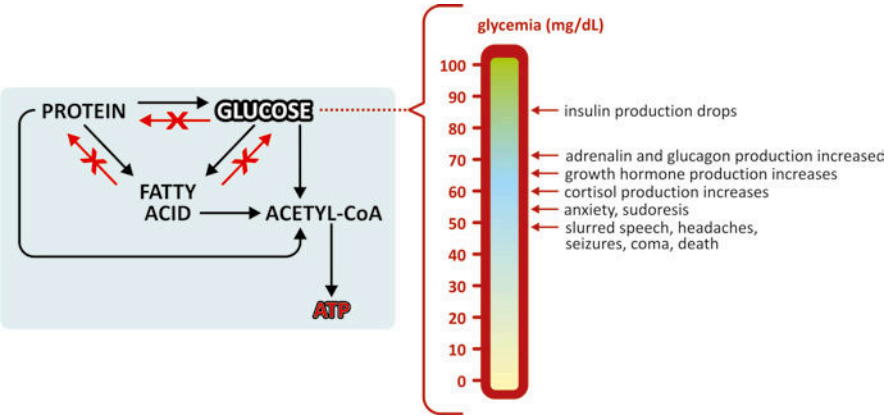


Fig. 5.2 Allowed and forbidden conversion routes in human metabolism. Glucose and fatty acids cannot be converted to proteins. Fatty acids cannot be converted to proteins or glucose. This implies that all excess nutrients from food intake end in lipids, stored in adipose tissue. Glycemia (the concentration of glucose in blood) needs to be kept above a certain threshold, even in the absence of food intake. In this case, synthesis of glucose is possible using amino acids from muscle protein degradation

In summary, metabolisms need regulation both inside the cells and in different tissues, frequently in different organs. Therefore, different levels of regulation exist. They are associated to mechanisms with different efficacies, time scales, and areas of impact.

5.1 Levels of Regulation: Impact and Time Scale

Think about your simplest daily routines, like eating, moving, and sleeping. They seem extremely banal and simple, almost unnoticeable in our lives, but they are demanding challenges from the metabolic point of view. They involve relatively fast alternation between states of nutrient abundance (meals) and nutrient absence (fasting), rest (e.g., sleeping), moderate exercise (e.g., walking), exercise bursts (e.g., short run to catch a bus) or intense enduring exercise (e.g., athletic running or swimming), and all possible combinations of feeding state with exercise. At the same time, there are processes that constantly consume energy such as brain activity (any basal activity, not only mental work), heartbeat, or keeping body temperature. Metabolic adaptation to fluctuating conditions on top of basal permanent activities requires mechanisms of metabolic regulation that are (1) fast, (2) efficient, and (3) robust. In fact these factors are not independent: robustness comes from redundancy of mechanisms—having more than one mechanism to assure the same effect decreases the chances of failure; redundancy is a cooperative combination of similar mechanisms—a fast one, albeit not so efficient, and a very efficient one, albeit not very fast. Usually fast regulation mechanisms consists in controlling the availability of substrates and/or the activity of enzymes, while very efficient regulation

mechanisms consist in controlling the presence of enzymes, i.e., their genetic expression, which is naturally a slower process. Genetic expression is usually dependent on hormones, which guarantee coordination among the metabolism in different organs, all under the influence of the same circulating hormones. This interplay between mechanisms is illustrated in Fig. 5.3.

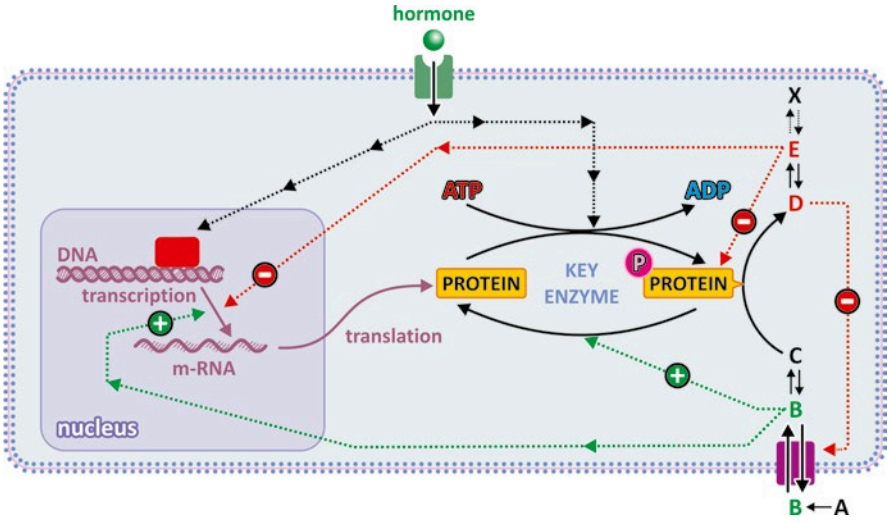


Fig. 5.3 The different levels of regulation are (1) substrate availability through control of transport across membranes (metabolite B); (2) enzyme activation by upstream metabolites (B) in the metabolic pathway and/or enzyme inhibition by the product of downstream metabolites (E) in the metabolic pathway; (3) activation or inactivation of enzymes by covalent attachment of a phosphate group (P), which is usually the end effect of a cascade of events caused by the binding of a hormone to its receptor (signal transduction); and (4) regulatory enzymes' expression/translation controlled by hormones through signal transduction inside cells. Hormone-dependent mechanisms are slower; the direct effect of metabolites on enzymes (activation or inhibition) is faster because enzymes coexist with regulatory metabolites (named effectors) in the same cell compartment

5.2 Inhibition and Activation of Enzymes by Ligands

As mentioned in the previous section, controlling the activity of enzymes (i.e., changing their kinetic characteristics such as K_M or V_{max}) is a fast way to influence the rate and course of metabolic pathways. Physical factors such as temperature and pH have a direct effect on the structure of proteins because they alter the intramolecular forces that stabilize protein folding. Therefore, they affect the activity of enzymes. Enzymes have optimal temperature and pH ranges to operate (Fig. 5.4). Below and above the optimal ranges, the reaction velocity decreases. Human enzymes are adapted to body temperature and have optimal activity around 37 °C. They are also adapted to the pH of their micro-environments. Pepsin and other stomach enzymes, for instance, have optimal activity at acidic pH.

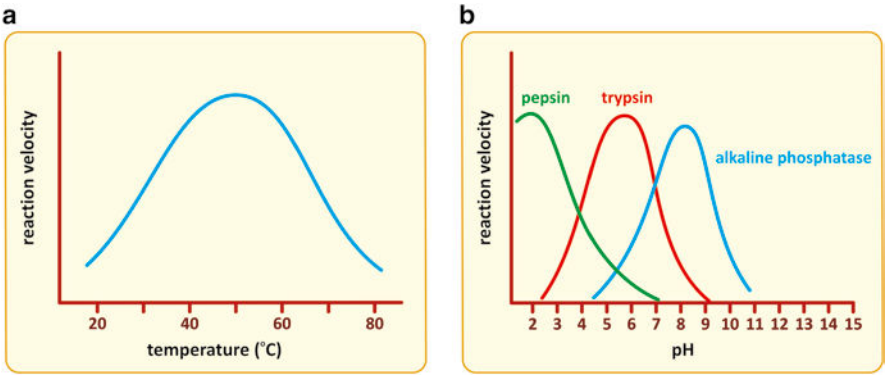


Fig. 5.4 Enzymes suffer structural alteration in their folding when physical factors such as temperature (**a**) or pH (**b**) change, which in turn cause alterations in the kinetics of catalysis. The reaction velocity is maximal in a limited interval of temperature or pH, decreasing for higher or lower values. Enzymes are adapted to the local pH of the different micro-environments of the human body from very acidic (such as pepsin) to alkaline (such as alkaline phosphatase), as illustrated in panel (**b**)

There are microorganisms living in extreme environments such as hot springs, at low pH and high temperatures. They are called extremophiles for this reason. Nevertheless, even in these cases, there is adaptation of the enzymes to the local pH and temperature. These molecules are very appealing to the biotechnological industry because they can be used in industrial processes that combine extreme pHs with high temperature; however, they are not in the realm of human biochemistry and will not be further discussed here.

In principle enzymatic activity could be modulated by changing pH or temperature, but this is not an option as these factors do not affect specifically a single enzyme in a specific metabolic pathway. The inhibition or activation of an enzyme has to be very selective. Shutting down a metabolic pathway demands inhibition and/or activation of very specific enzymes (recall Sect. 4.2.1), which is not compatible with the manipulation of pH or temperature. Instead, having small molecules that bind specifically to unique binding sites of enzymes, affect their conformation, and influence their kinetics is a much better way to specifically modulate the activities of selected enzymes. These small molecules are called ligands, and they can slow (inhibitors) or accelerate (activators) the catalytic process.

In the case of an enzyme obeying Michaelis–Menten kinetics, ligands may in principle affect V_{max}, K_M, or both. The molecular mechanisms behind the influence of ligands on V_{max} or K_M may be very diverse, even if the end result is the same. Two different inhibitors may impact on V_{max}, for instance, binding to different sites of the same enzyme and producing different effects on enzyme structure. In other words, the alterations ligands cause on kinetic parameters tell nothing about *how* ligands and enzymes interact. To relate kinetics with mechanism of interaction, one has to resort to models, i.e., hypothetical arrangements and postulated events, and deduce on the final in kinetics.

This means that the examples in Box 4.4 can be extended to the case in which an additional molecule, the inhibitor or the activator, interacts with the enzyme E_z, in

addition to S (the substrate) and P (the product). Let's work on a simple example. Postulating that there is a ligand that binds to E_z and prevents S from binding to E_z (in this case the ligand acts as an inhibitor and will be represented by I), the reaction scheme is composed of

$$E_z + S \rightleftharpoons E_zS \rightarrow E_z + P \quad \left(\text{The unperturbed catalysis itself}\right) \tag{5.1}$$

$$E_z + I \rightleftharpoons E_zI \quad \left(\text{The action of the inhibitor}\right) \tag{5.2}$$

The action of I is to prevent part of E_z to take part in the reaction. The effective concentration of E_z available to interact with S is decreased, and naturally, K_M appears to be increased. Having less extensive binding of E_z to S appears to be a decreased affinity of E_z to S. In reality the intrinsic binding of E_z to S is not affected in its nature; it is the "sequestration" of E_z by I that causes the alteration in K_M. For very high S concentration, S is so abundant relative to I that, in practice, the influence of I on binding of E_z to S is not relevant and so V_{max} remains constant. V_{max} is $k_2.[E_z]_0$ (Box 4.4) and I do not change k_2 or $[E_z]_0$. The reaction velocity vs. [S] plot shows a decreased slope at small [S] but the same asymptotic value (V_{max}) in the presence of I when compared to the plot in the absence of I (Fig. 5.5).

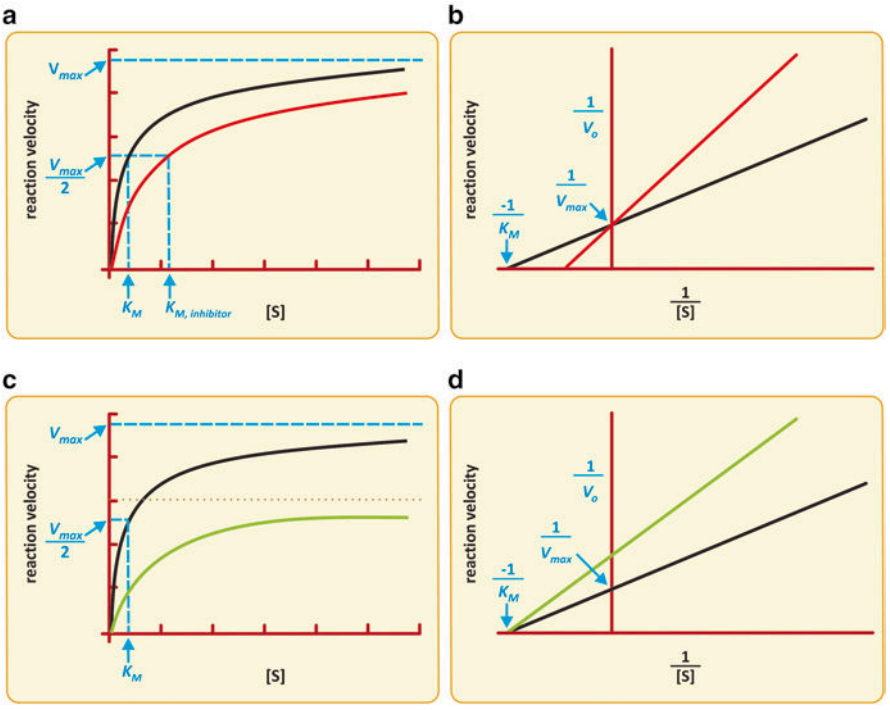


Fig. 5.5 Two possible effects of an inhibitor on the Michaelis–Menten kinetics of an enzyme. (**a**, **b**) K_M is increased with no alteration in V_{max}. (**c**, **d**) V_{max} is decreased with no alteration of K_M. Simultaneous alterations on V_{max} and K_m are also possible (not shown), which are the most frequent situations in practice

If I binds to E_z irreversibly $\left(E_z + I \rightarrow E_z I\right)$, part of enzyme population is permanently blocked by I, so the effective concentration of E_z free to interact with S is decreased even at high concentrations of S. In this case, V_{max} is decreased (recall again $V_{max} = k_2 \cdot [E_z]_0$—Box 4.4).

In the reaction scheme (5.1) and (5.2) shown above, E_z was assumed to bind to S or I but not to both simultaneously. This is expected to occur in cases in which S and I compete for the active catalytic site of the enzyme. This mechanism is thus called "competitive." There are other reaction schemes that consider simultaneous binding of I and S to E_z (which implies that they are not competing for the same binding site—"non-competitive" inhibition):

$$E_z + S \rightleftharpoons E_z S \rightarrow E_z + P \quad \left(\text{The unperturbed catalysis itself}\right) \tag{5.3}$$

$$E_z + I \rightleftharpoons E_z I \quad \left(\text{The action of the inhibitor}\right) \tag{5.4}$$

$$E_z I + S \rightleftharpoons E_z SI \quad \left(\text{Enzyme binding S after binding I}\right) \tag{5.5}$$

$$E_z S + I \rightleftharpoons E_z SI \quad \left(\text{Enzyme binding I after binding S}\right) \tag{5.6}$$

In this case, binding of I to E_z hinders the catalytic process but not the binding of S to the catalytic site itself, so V_{max} is decreased but K_M is not altered (Fig. 5.5). Even at high [S], a fraction of E_z is bound to I. The total amount of E_z available for catalysis is decreased and V_{max} is also decreased (again recall that $V_{max} = k_2 \cdot [E_z]_o$—Box 4.4). In practice, it is not frequent to find inhibitors that perturb the structures of proteins with a selective effect on V_{max} leaving K_M unchanged but non-competitive inhibition retains didactic interest.

It is interesting to note that V_{max} is also decreased in cases in which k_2 is decreased. This may happen when E_zSI in reactions 3 and 4 above retains catalytic activity $\left(E_z SI \rightleftarrows E_z S + P\right)$ but at slower rate when compared to free E_z. The catalytic rate constant (k_2) is decreased in the fraction of enzyme associated to I (E_zI) and V_{max} decreases.

It may also happen that E_z bind ligands that have the opposite effect of inhibitors on E_z: to increase k_2, thus increasing the velocity of the catalytic process. In this case the ligand is called an "activator," as opposed to "inhibitor." There are also activators that decrease K_M. The binding of S to E_z may be affected when the structure of the catalytic site of E_z is altered by the conformational changes caused by the binding of the activator to its specific binding site in the protein.

Depending on the characteristics of the reaction schemes, namely, stoichiometries of interactions, diversity of interacting molecules, reversibility of the reactions, etc., the expected kinetics may vary greatly. Figure 5.6 has simple illustrative examples based on Michaelis–Menten kinetics.

When performing work on enzymology, one has to bear in mind that there is no direct unequivocal correspondence between the experimental kinetics, reaction schemes, and molecular mechanisms of action. One may conceive mechanisms,

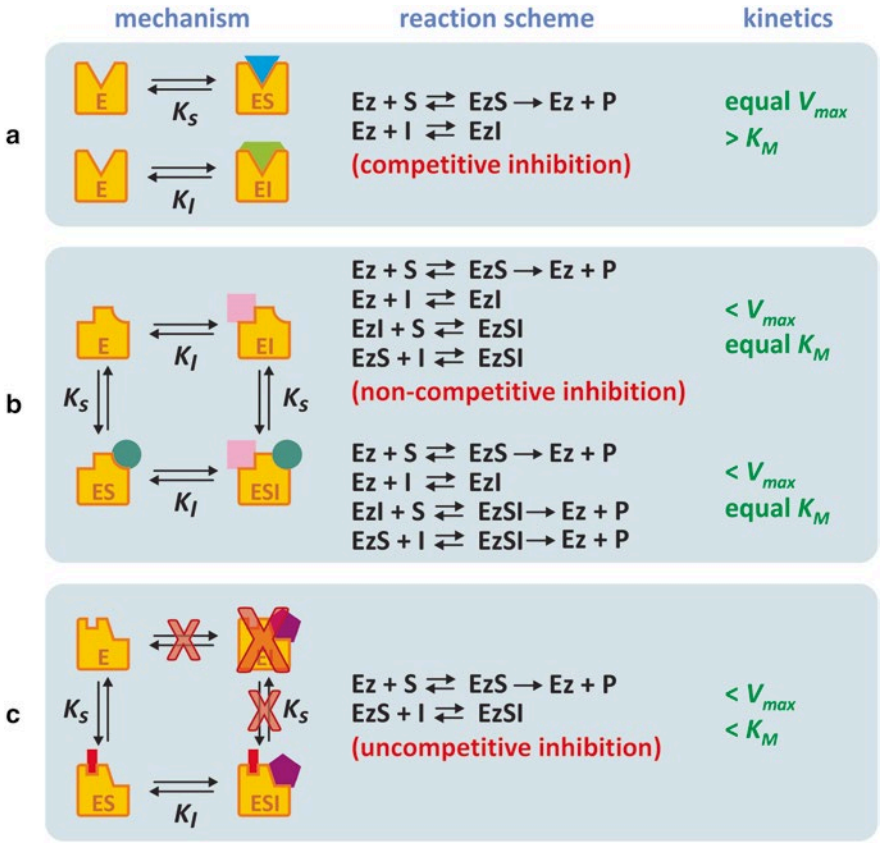


Fig. 5.6 Four examples of reaction mechanisms and associated kinetic alteration in Michaelis–Menten or Michaelis–Menten-like enzymes. Depending on the mechanisms and respective reaction scheme, the resulting apparent V_{max} and K_M may differ or not from V_{max} and K_M in the absence of the inhibitor, I. The first mechanism is named "competitive inhibition," but the other mechanisms bear names that are not always consensual. The second and third mechanisms are usually referred to as "non-competitive" and the fourth as "uncompetitive"

infer the corresponding underlying reaction scheme, deduce mathematically the associated kinetics, and study the match of the deduced kinetics to the experimental data, but the inverse is not possible. It is not possible to deduce unambiguously mechanism from experimental kinetic curves. In this way, it is premature to deduce a competitive mechanism in a situation in which experimental data show higher K_M and equal V_{max} as other mechanisms may result in similar kinetics. However, for most practical purposes in the health sciences, the exact mechanisms of action are not all that relevant, and this reasoning of enzymology research practice will not be further developed.

It is worth stressing that all that has been mentioned about enzymes having a Michaelis–Menten (or a Michaelis–Menten-like) kinetics is valid with adaptations for enzymes having more complex kinetics such as sigmoidal (Fig. 5.7). Thus, the

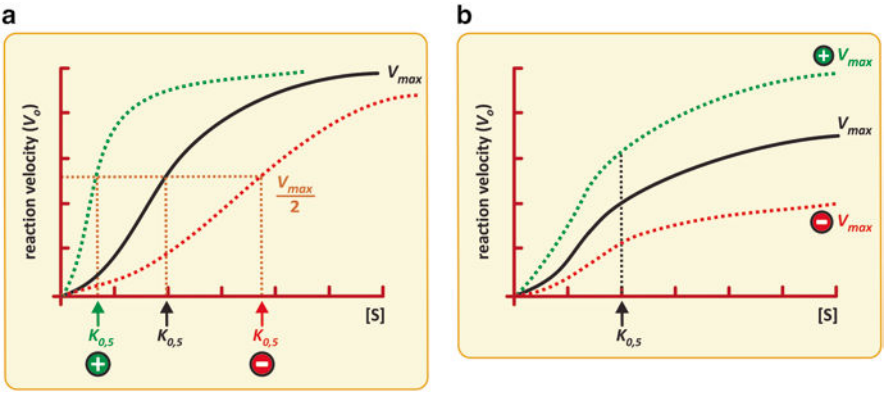


Fig. 5.7 Effect of activation and inhibition in sigmoidal kinetics of catalysis. The impact may be on the [S] needed to achieve 50 % of the maximal velocity ($K_{0.5}$) (**a**) or the maximal attained velocity, V_{max} (**b**)

general concept of inhibiting or activating enzymes using small molecules as ligands may potentially apply to all enzymes in metabolism.

The importance of enzyme inhibitors in drug discovery and development is huge. Many of the drugs in current medicines target enzymes (Table 5.1) and were conceived to block processes in which those enzymes are essential. Inhibiting

Table 5.1 Examples of drugs, targeted enzymes, and field of therapy

Drug	Target enzyme	Field of therapy
Aspirin	Cyclooxygenase	Anti-inflammatory
Captopril and enalapril	Angiotensin-converting enzyme (ACE)	Antihypertension
Simvastatin	HMG-CoA reductase	Lowering of cholesterol levels
Desipramine	Monoamine oxidase	Antidepression
Clorgyline	Morpramine oxidase A	Antidepression
Selegiline	Morpramine oxidase B	Treatment of Parkinson's disease
Methotrexate	Dihydrofolate reductase	Anticancer
5-Fluorouracil	Thymidylate synthase	Anticancer
Viagra	Phosphodiesterase enzyme	Treatment of male erectile dysfunction
Allopurinol	Xanthine oxidase	Treatment of gout
U75875	HIV protease	AIDS therapy
Ro41-0960	Catechol-O-methyltransferase	Treatment of Parkinson's disease
Omeprazole	H^+/K^+ ATPase proton pump	Ulcer therapy
Organophosphates	Acetylcholinesterase	Treatment of myasthenia gravis, glaucoma, and Alzheimer's disease
Acetazolamide	Carbonic anhydrase	Diuretic
Zileuton	5-Lipoxygenase	Anti-asthmatic

HMG-CoA reductase, for instance, results in the inhibition of cholesterol synthesis. One of the most common strategies to create inhibitors is to synthesize molecules that are similar enough to the substrate to bind the catalytic site but incapable of suffering catalysis. Figure 5.8 shows several examples of natural substrates and similar molecules that act as inhibitors of the enzymes. In some cases binding of the inhibitor to the enzymes is reversible, but in other cases, inhibitors react covalently with the enzyme and the binding is thus irreversible in practice.

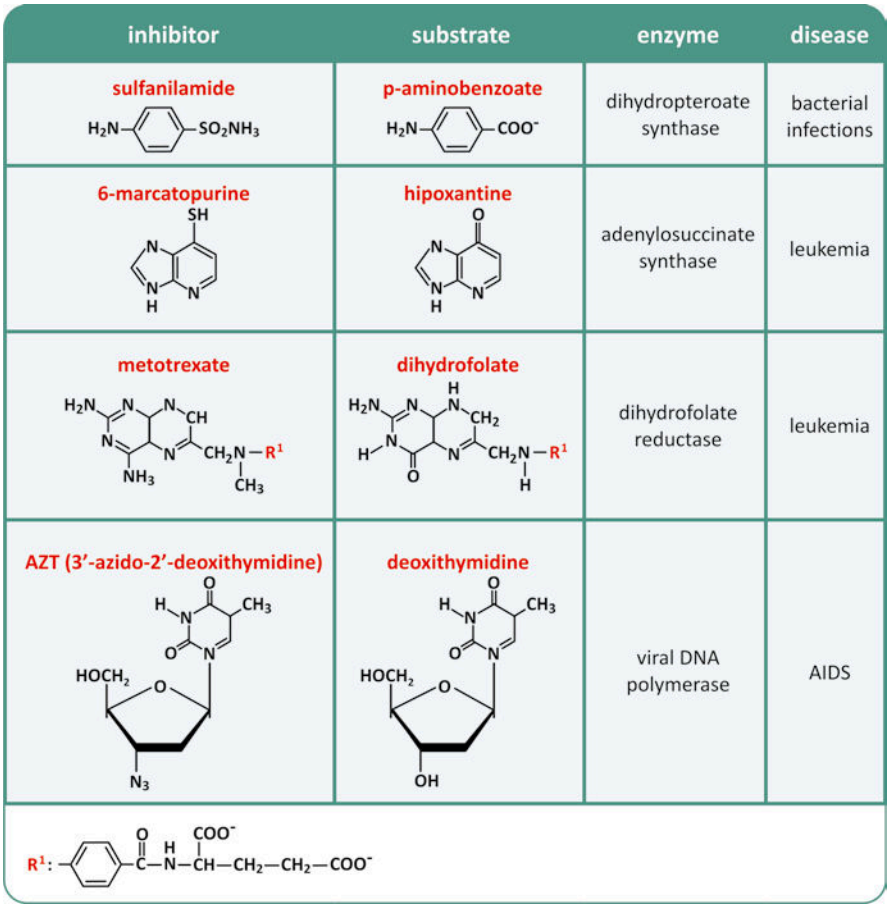


Fig. 5.8 Examples of enzyme inhibitors that are very similar in chemical structure to the natural substrates

5.2.1 Nomenclature of Ligands

Ligands other than the substrates that have a significant effect on the velocity of catalysis are generally called effectors. They can be activators (speed catalysis up) or inhibitors (slow down catalysis). In principle, effectors bind and dissociate from

enzymes without undergoing chemical modification, in contrast to substrates. For historical reasons, some ubiquitous substrates, such as NADH or NADPH, are often named separately as coenzymes. These so-called coenzymes are small molecules that transfer or accept groups from another substrate. This should not be confused with prosthetic groups. Prosthetic groups are chemical entities other than amino acid residues covalently attached to enzymes, such as heme groups of iron–sulfur centers (Fig. 5.9). They may have a direct role in catalysis, but they are not considered substrates as they are part of the enzyme itself in chemical terms.

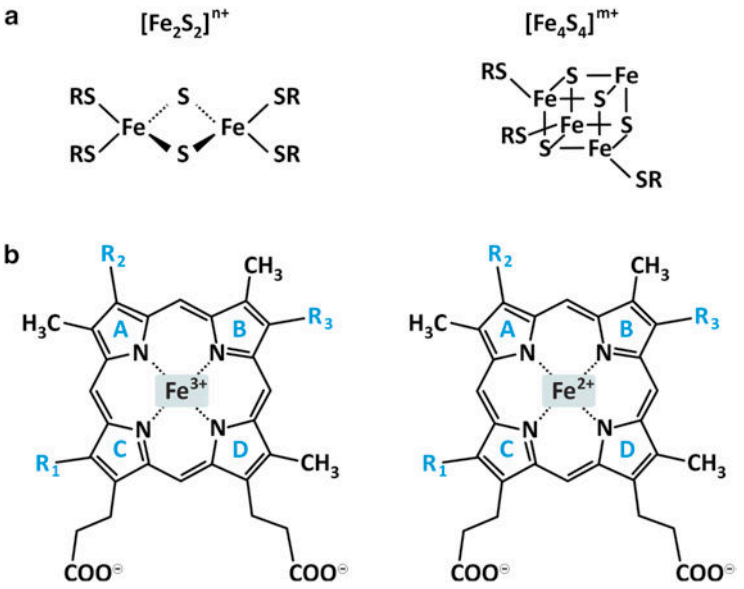


Fig. 5.9 Iron–sulfur centers (**a**) and heme groups (**b**) are examples of prosthetic groups as they are not amino acid residues and bind covalently to enzymes. The A–D multiring structure (tetrapyrrole) in panel (**b**) is the basic structure of a family of molecules named porphyrins. The central metal ion varies among porphyrins. Porphyrins conjugating iron ions are hemes. Both iron oxidation states (+3, *left*, or +2, *right*) are accommodated by the tetrapyrrole, meaning the central iron ion can engage redox reactions while inserted in the heme. The organic groups R_1–R_3 vary among hemes from different sources

The rules of nomenclature are not well defined. ATP for instance is often referred to as a coenzyme, but this is not a general rule. The same happens with CoA. NADH and NADPH are more consensually classified as coenzymes. FADH is often referred to as coenzyme, but it occurs in nature covalently bound to proteins so it is in reality a prosthetic group. While it is important to have precise nomenclature for the purpose of efficient and unbiased communication, one should not overemphasize name over action and one shall not further discuss semantics.

5.3 The Availability of Primary Precursors in a Metabolic Pathway

In a metabolic pathway such as the one depicted in Fig. 5.1, the velocity of reactions is not only controlled by the kinetic characteristics of the enzymes involved but also by the accumulation of G in the cell where it appears as the end product and availability of G in the cell where it is the primary precursor. To leave one cell and enter another, metabolite G needs to cross the membranes of both cells. Assuming that G is polar, it will not diffuse across the lipid bilayer of the membrane. It will need to be carried by specialized molecules or molecular assemblies. Take glucose as an example. It may be produced by hepatocytes in a metabolic pathway called gluconeogenesis and be consumed in neurons. Glucose is a polar molecule, soluble in plasma. To leave the hepatocyte and enter the neurons, glucose uses transmembrane proteins that assist in the process of translocating glucose across membranes.

Given the importance of transport across membranes to metabolisms, we shall address this issue in some detail in the next section.

5.3.1 Transport of Metabolites and Effectors Across Membranes

Figure 5.10 represents the six typical alternative routes molecules use to cross membranes. Small relatively hydrophobic molecules such as ethanol may not need transporters as they are able to simply diffuse across the lipid bilayer. Small ions have hindered diffusion across the bilayer due to their polarity, but they are able to cross

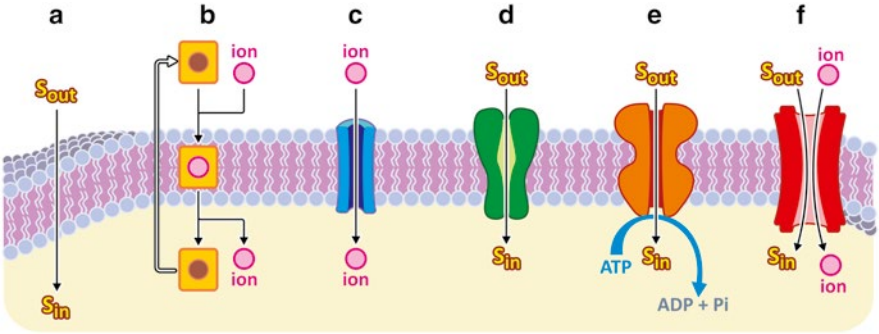


Fig. 5.10 The six different routes that molecules and ions may use to translocate lipid membranes: simple diffusion (**a**), ionophore mediated (**b**), ion channels (**c**), facilitated diffusion (**d**), primary active transport (**e**), and secondary active transport (**f**). Routes (**a**)–(**c**) are driven by electrochemical potential gradient (difference of concentration and charge distribution between both sides of the membrane)—passive transport. Routes (**e**)–(**f**) use ATP hydrolysis or the dissipation of an ion gradient, respectively, as sources of energy—active transport

membranes if encapsulated by chelating agents that are soluble in lipids. Ions can also cross membranes through ion channels, proteins that connect both sides of the membranes and are selectively permeable to specific ions. Similar proteins constitute channels for small molecules, which have thus a facilitated diffusion across the membrane. All these processes, from simple diffusion to facilitated diffusion, operate with net translocation of molecules in one direction, from the higher electrochemical gradient to lower electrochemical gradient. If the concentration and charge distribution is the same on both sides of the membrane, there is no net mass movement across the membrane, as the velocity of transfer on one sense equals the velocity of the opposite sense.

Figure 5.11 focuses on the molecular structure of a K^+-specific channel and aquaporin, a water channel across membranes.

Transporting molecules against electrochemical gradients is not energetically favorable. Thus, to transport molecules against an electrochemical gradient, an external source of energy has to be used. Some use ATP (primary active transport—Fig. 5.10d); others couple the transport of the solute to the transport of an ion along its electrochemical gradient (secondary active transport—Fig. 5.10e). The coupling may be such that both ion and solute are co-transported in the same direction (symport) or opposed directions (antiport). Primary and secondary transport mechanisms are coordinated when they transport common ions or solutes (Fig. 5.12). An example of simple diffusion and antiport is shown in Fig. 5.13. Glucose transport in intestinal epithelial cells is depicted in Fig. 5.14 as example of transport assembly that can be found in human epithelia.

Channels and transporters operate under the same thermodynamic principles as enzymes. Diffusion through membranes mediated by transporters has a decreased activation energy (Fig. 5.15). Moreover, transporters are proteins whose actions shares similarities with enzymes. Assuming that the transport of a solute, S, from the outer to the inner side of a membrane by the transporter, T, is described by

$$S_{out} + T \rightleftharpoons ST \rightarrow S_{in} + T$$

the associated kinetics of transport is hyperbolic, like a Michaelis–Menten process (Fig. 5.16). Likewise, there are inhibitors and activators of transporters. Thus, transporters may also be key points of regulation of metabolism and therefore drug targets.

Another way to control the action of transporters is to control their availability on the surface of cells. Transporters are embedded in the bilayer matrix so that they can be removed from the surface of the cell by vesiculation. This happens with glucose transporters such as glucose transport 4 (GLUT4). GLUT4 is responsible for insulin-stimulated glucose uptake in muscle and adipose tissue. Binding of insulin to its receptor on the surface of membranes leads to the fusion of intracellular vesicles having membranes loaded with GLUT4 with the cell

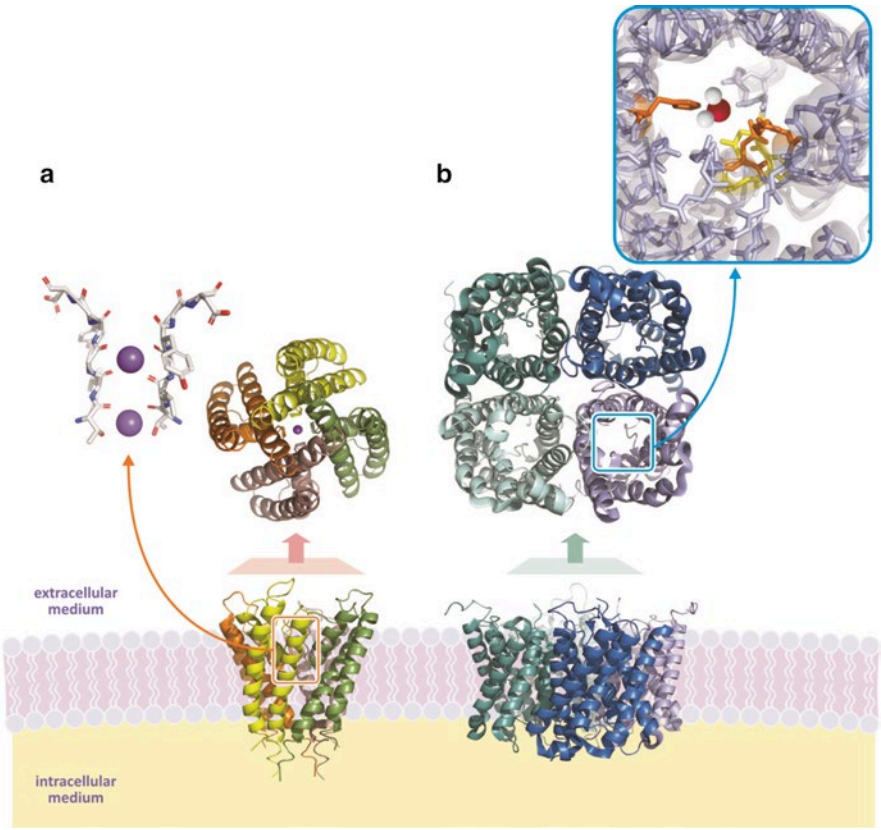


Fig. 5.11 (**a**) Molecular structure of the K^+ channel of *Streptomyces lividans* (PDB 1BL8) in longitudinal (*bottom*) and perpendicular (*top*) views in relation to the membrane. K^+ channels are the most widely distributed ion channel in living organisms. Four identical subunits, containing two transmembrane helices each, arranged in a conic structure in which the central backbone carbonyl oxygens form K^+-selective pores that fit the ion precisely (see detail in the *left*, PDB 1J95), removing the hydration shell from the ion when it enters the channel. The channel accommodates four K^+ sites that are occupied alternately. (**b**) Structure of the spinach aquaporin (PDB 2B5F) in longitudinal (*bottom*) and perpendicular (*top*) views in relation to the membrane. Aquaporin selectively transports water molecules in and out of the cells. The protein is a tetramer of identical subunits, each of them containing a transmembrane pore (see detail in the *right*). All aquaporins contain a conserved sequence of Asn-Pro-Ala (highlighted in *yellow*) as part of the water channel. Water channel also contains a conserved His that narrows pore diameter limiting the passage of molecules larger than water and a conserved Arg that repels cations, including H_3O^+ (highlighted in *orange*). The positioning of the water molecule is tentative for illustrative purposes only

membrane, exposing the transporters (see Sect. 8.4.3). Glucose transport may thus occur, and this molecule becomes available inside the cells (Fig. 5.17). From that moment on, metabolic pathways having glucose as precursor, such as glycolysis, may initiate.

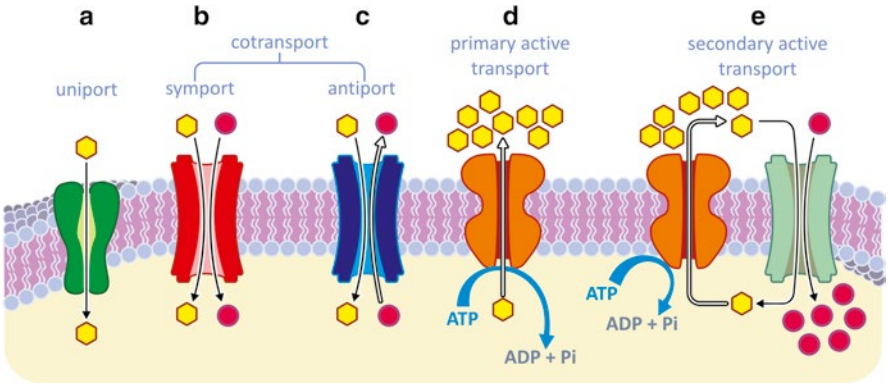


Fig. 5.12 Unidirectional transport of a single solute is called uniport (**a**). Co-transport implies that two solutes (or a solute and an ion) are transported simultaneously, either in the same direction (symport—**b**) or in opposite directions (antiport—**c**). Primary active transport (**d**) is sometimes coupled to a co-transport (**e**) by the creation of an ionic or molecular gradient (*yellow molecule*) that is used to transport the red solute independent of its gradient

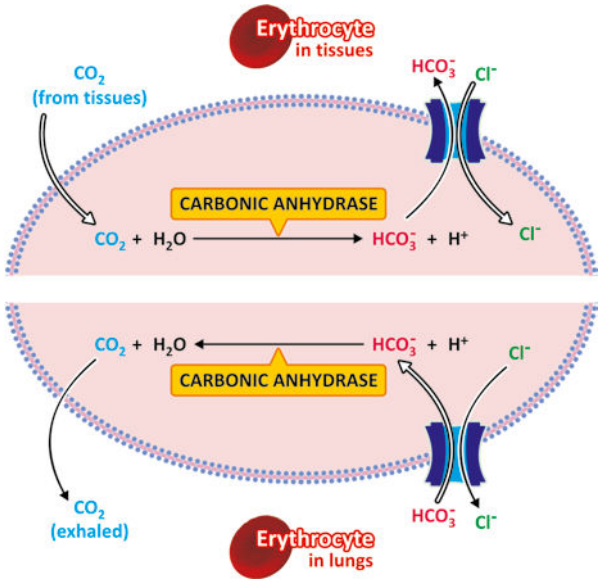


Fig. 5.13 Transport of ions and solutes across the erythrocyte membrane. CO_2 is a small molecule able to diffuse directly across the membrane according to its concentration gradient (in lungs: in → out; in respiring tissues: out → in). Hydrogen carbonate (bicarbonate) is co-transported with chloride. The direction of transport depends on CO_2 concentration because CO_2 equilibrates with HCO^-_3

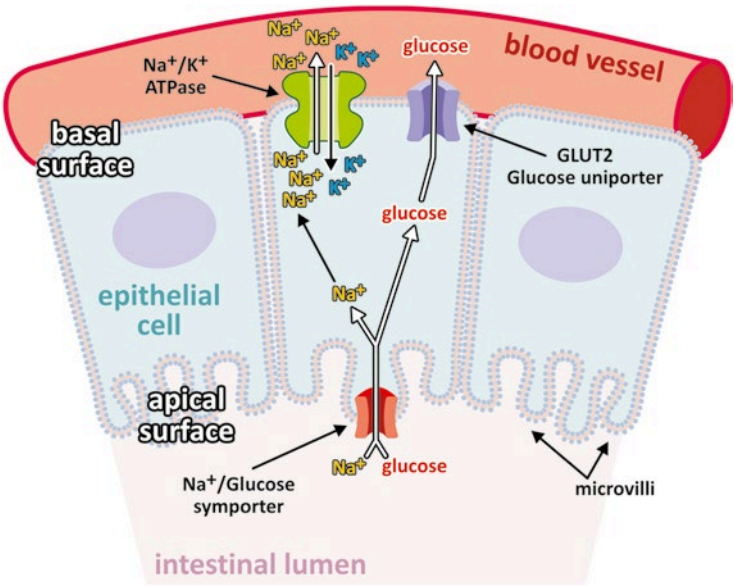


Fig. 5.14 The system of glucose transport in intestinal epithelial cells is complex and includes transport from the intestinal lumen to the cytoplasm and from the cytoplasm to blood. Na^+ has a central role by coupling both steps

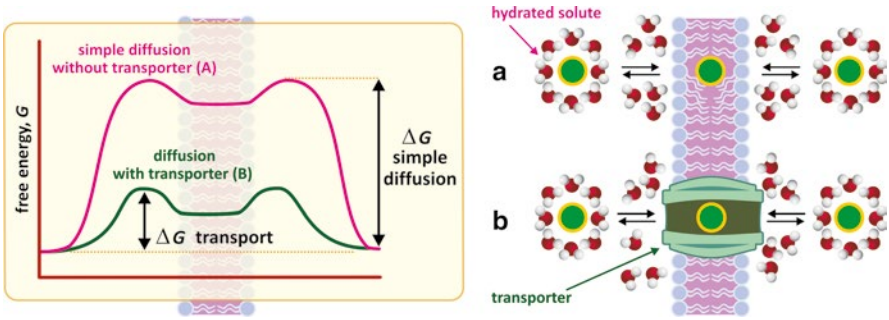


Fig. 5.15 Thermodynamics of the passage of a polar molecule or ion (*green circle*) across a lipid membrane. Simplified diffusion (**a**) implies a high energetic cost to remove the hydration shell around the solute. The action of a transporter (**b**) leads to the reduction of the activation energy (the ΔG represented in the *left panel*) of the membrane translocation by the molecule or ion. The transporter replaces water by forming hydrogen bonds with the solute

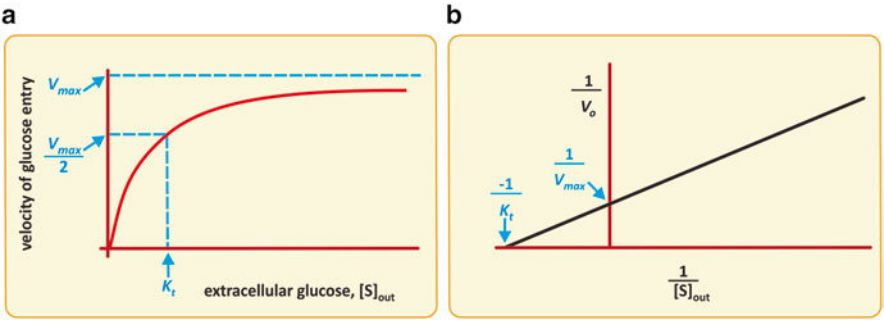


Fig. 5.16 Michaelis–Menten-like kinetics in solute transport across membranes. K_t is equivalent to K_M, except that it refers to transport, not catalysis

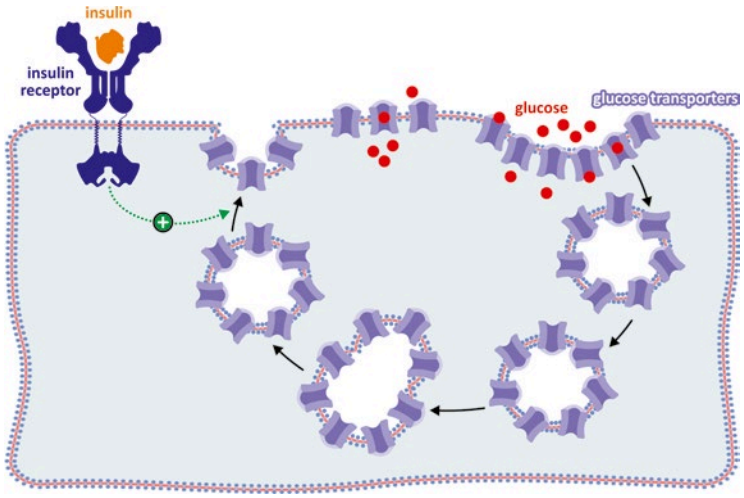


Fig. 5.17 Insulin-stimulated exposure of GLUT4 on the surface of muscle and adipose cells. Vesicles bearing GLUT4 are recruited and fused with the plasma membrane. GLUT4 is automatically exposed. In the absence of insulin, the process is reversed

5.4 Slow (But Efficient!) Mechanisms of Controlling Enzyme Action

Enzyme response to binding of effectors is very fast. Concomitant to the effector's accumulation or depletion, the binding or dissociation, respectively, of the effector is nearly immediate. Yet, the efficacy of the process of inhibition or activation is concentration dependent. At extreme concentrations, the effect is also extreme, but at intermediate concentrations of the effector, the inhibition or activation is only partial. In contrast to non-covalent binding of effectors, there are two mechanisms

of enzyme action that are highly efficient, although not immediate. These are the covalent modification of enzymes using phosphates, which may take up to minutes, and the synthesis or degradation of the enzymes themselves, which may take up to hours or days.

Phosphorylation of enzymes occurs at –OH groups; thus, it is a process that occurs in exposed Ser, Thr, or Tyr residues. This process frequently involves ATP and is catalyzed by a second enzyme, a kinase. The inverse process, dephosphorylation, is also catalyzed by enzymes, called phosphatases (Fig. 5.18). Phosphate is the ubiquitous group used for covalent modification of enzymes in nature, but there is no rule on whether the active forms of the enzymes are the ones phosphorylated or not. Table 5.2 shows examples of enzymes that are active or inactive when phosphorylated.

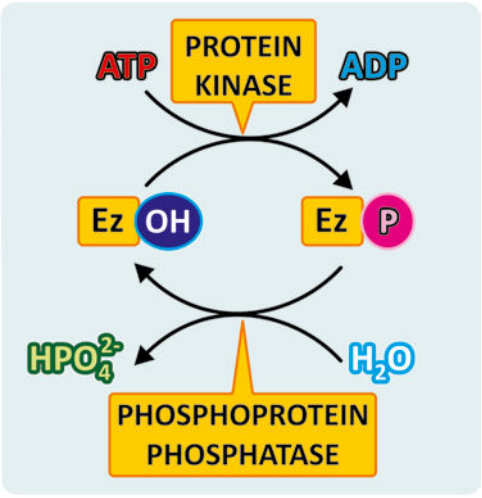


Fig. 5.18 Typical reactions for phosphorylation or dephosphorylation of enzymes. The process involves the action of the two other enzymes: a kinase and a phosphatase

The presence or absence of a phosphate group on the protein may have a high impact on the domain where the reaction takes place. Local changes in structure may propagate through the 3D architecture of the protein and trigger or block enzyme activity. The reason why phosphates are the only choice of nature to perform this task is intriguing. This is believed to be related to the abundance of phosphates and its chemical nature. Phosphates bind to organic molecules, forming esters, keeping their anionic charge, which protects them from hydrolysis. Nucleophiles, such as the hydroxide ion, are repelled by negative charges and therefore react less rapidly with anions than with neutral substrates. Furthermore, the same is generally true with respect to electrically neutral nucleophiles such as water.

The rate constant for the attack of hydroxide to the dimethyl phosphate anion is less than that for the attack of hydroxide ion to the trimethyl phosphate by a factor of more than 10^5 (Table 5.3).

Table 5.2 Some enzymes are active when phosphorylated; others are inactive. There is no general rule in this domain

Metabolic pathway	Enzyme	Phosphorylated form	
		Active	Inactive
Glycogenolysis	Glycogen phosphorylase kinase	✓	
	Glycogen phosphorylase	✓	
Glycogenogenesis	Glycogen synthase		✓
Glycolysis and gluconeogenesis	6-phosphofructo-2-kinase		✓
	Fructose 2,6-bisphosphatase	✓	
	Pyruvate kinase		✓
Lipolysis (conversion of triacylglycerols to glycerol and fatty acids)	Lipase	✓	
Lipogenesis	Citrate lyase		✓
	Acetyl-CoA carboxylase		✓
	3-Hidroxy-3-methylglutaryl-CoA reductase		✓

Table 5.3 Rates of reaction of esters with OH^- at 35 °C

Ester	k (M^{-1} s^{-1})	Fold increase in k[a]
$(CH_3O)_2PO^-{}_2$	2×10^{-9}	1.0
$(CH_3O)_3P{=}O$	3.4×10^{-4}	1.7×10^{5}
$CH_3CO^3{}_2C_2H_5$	1.0×10^{-2}	5.0×10^{6}

[a]Relative to $(CH_3O)_2PO^-{}_2$

The phosphorylation/dephosphorylation switch to turn on or turn off enzymes is found for key enzymes of metabolisms. The primary triggering of the events that result in phosphorylation or dephosphorylation of enzymes is the interaction of a hormone with its receptor, as illustrated before in Fig. 5.1. The figure shows that binding of H to its receptor triggers a series or transformations whose end result is the phosphorylation/dephosphorylation of the key enzyme. These intermediary steps are generally referred to as signal transduction and the chemical species involved as second messengers. We shall not address the theme of signal transduction in detail because in this section, we intend to remain focused on the basics of metabolic regulation (signal transduction triggered by specific hormones will be discussed in detail in the chapters that explore the regulation of metabolism in different physiological situations—Chaps. 8–11). Nonetheless, it is worth mentioning that there are two main classes of receptors that trigger signal transduction: G protein-coupled receptor and kinase-linked receptors. Binding of the ligand (usually named "agonist" in pharmacology) to the receptors of the first kind causes conversion of GDP into GTP and the release of a complex of proteins associated to the receptor that will associate to other molecular targets, such as adenylyl cyclase, which converts ATP into cyclic AMP (cAMP). The increase in cAMP concentration may in turn stimulate other chemical reactions in a cascade-like manner. Besides adenylyl cyclase-coupled receptors (Fig. 5.19), there are other cases, such as phospholipase C-coupled receptors. In this

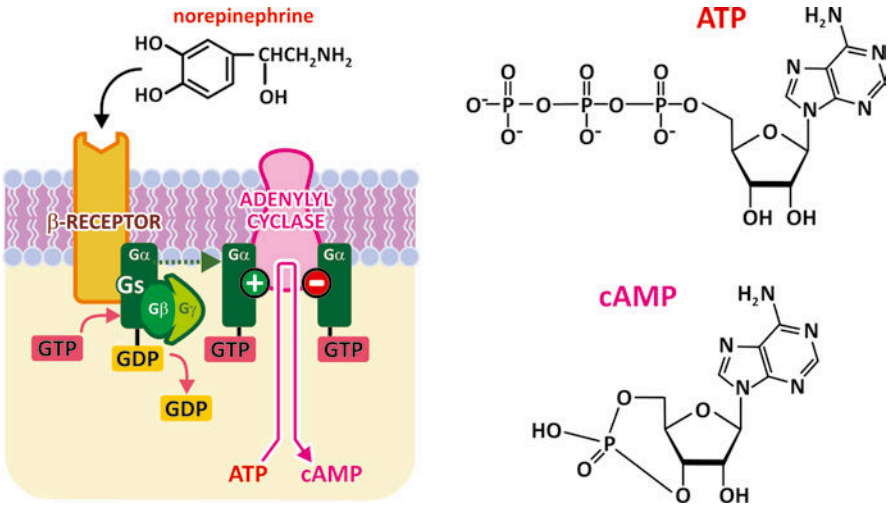


Fig. 5.19 Generic representation of a G protein-coupled receptor. Upon binding of the agonist ligand to the receptor (e.g., norepinephrine), adenylyl cyclase is activated and cAMP is produced

case the GTP-associated released complex of proteins associate to phospholipase C and leads to hydrolysis of the phosphatidylinositol bisphosphate in the membranes into diacylglycerol and inositol triphosphate, the latter serving as second messenger.

Kinase-linked receptors are transmembrane proteins with extracellular domains that bind agonist ligands, including hormones, and intracellular domains having amino acid residues that undergo phosphorylation. A cascade of events is initiated ("kinase cascade").

The most important signal transduction systems for metabolic dynamics and regulation will be shown throughout the following chapters, together with the metabolic pathway for which they are relevant. However, it is important to highlight that the end effect of some hormones that bind to cell surface receptors may take place in the nucleus, like the recruitment of transcription factors (Fig. 5.20). This enables hormones to control the activity of enzymes at a higher level: enzyme biosynthesis. Not only hormones may activate/inhibit enzymes by phosphorylation or dephosphorylation, but they can also control the availability of the enzymes through the control of their biosynthesis. This redundancy assures high efficacy in a wide time range as covalent modification of enzymes is operational in the minute to minute time scale and the control of synthesis is operational in hours to days. Cholesterol synthesis is shown as example in Fig. 5.21.

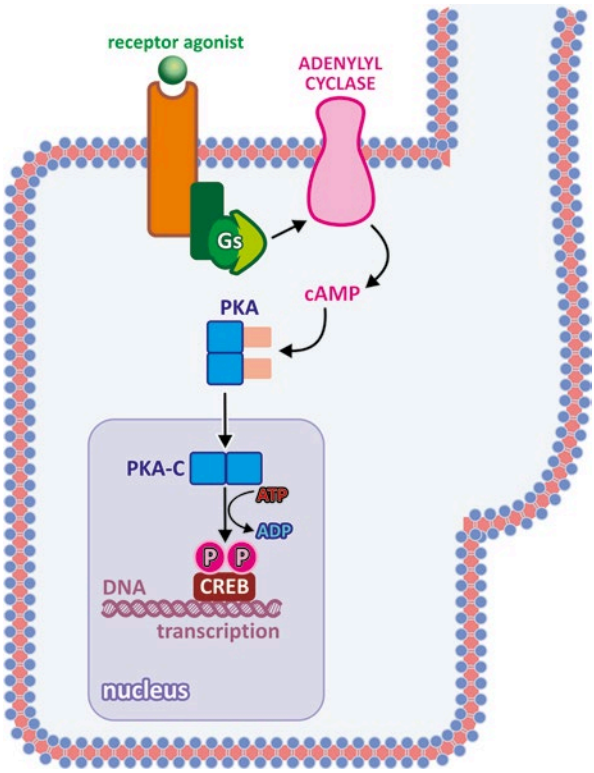


Fig. 5.20 Impact of a receptor agonist on transcription via adenylyl cyclase. Hormones may interfere in enzyme biosynthesis using similar mechanisms. Gs - stimulative regulative G-protein, PKA - Protein kinase A, PKA-C - PKA catalytic subunit, CREB - cAMP response element-binding protein

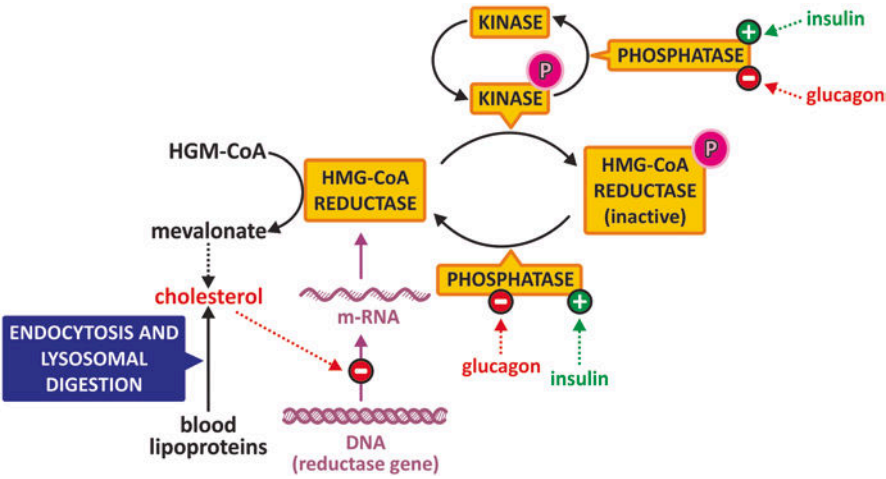


Fig. 5.21 HMG-CoA reductase is the key enzyme in the biosynthesis of cholesterol. It is regulated by slow mechanisms: covalent modification (inactivation by phosphorylation) and transcription inhibition by the end product, cholesterol. The hormones insulin and glucagon have opposite effects: insulin stimulates cholesterol synthesis and glucagon stimulates the inhibition of HMG-CoA reductase, shutting down the synthetic pathway

5.5 Key Molecules in Energy Metabolism

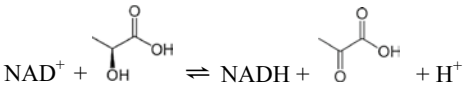
In the same way ATP is frequently used in metabolism because its favorable hydrolysis coupled to unfavorable reaction makes the whole set of chemical reactions favorable, NADH (see NADH chemical structure in Fig. 3.23) is also a molecule frequently used to drive redox processes. The process can be represented by

$$NAD^+ + 2H \rightleftharpoons NADH + H^+$$

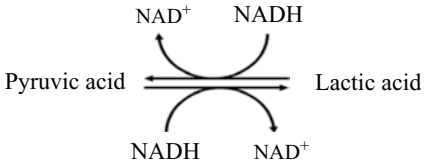
but this is not elucidative for its role in redox reactions. A better representation is

$$NAD^+ + RH_2 \rightleftharpoons NADH + RH^+ \left(\text{or } NADH + R + H^+\right)$$

in which RH_2 is a generic molecule. In this reaction the reduction of NAD^+ is coupled to the oxidation of RH_2. This is the case of



In biochemical literature, the reaction stoichiometries and the involvement of H_2O of H^+ (or H_3O^+) are not always represented for the sake of simplicity, and this process can simply be written as



but one must not forget the chemical details implied albeit not explicit.

NADH is so ubiquitous in cells that it can be considered as a "universal" electron carrier. NADH is used in cells due to its strong reducing (electron-donating) power; NAD^+ is converted back to NADH by oxidation of other molecules. Most reactions involving NADH/NAD^+ in metabolism are catalyzed. The enzymes that catalyze the oxidation of a substrate with the transfer of one or more hydrides (H^-) to an electron acceptor such as NAD^+ (or equivalent molecules such as $NADP^+$, or FAD, or FMN; see Fig. 3.23) are named dehydrogenases.

There are other examples of ubiquitous molecules in metabolism, although not as striking as NADH or ATP. Biotin, for instance, is a non-proteic "prosthetic" (not an amino acid residue) group that exists in carboxylases and participates in the addition of CO_2 groups from HCO_3^- to organic molecules, as exemplified in Fig. 5.22 (in which E_z is the enzyme):

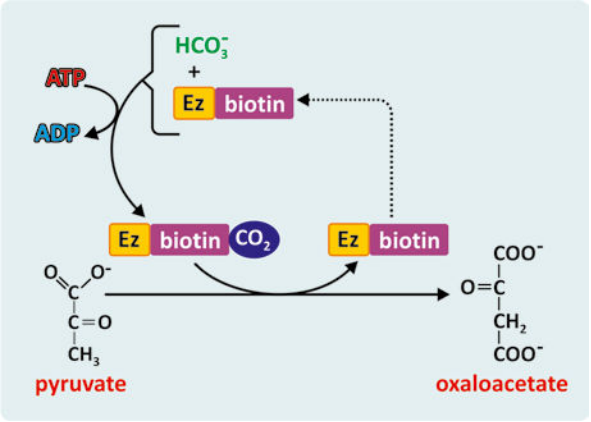
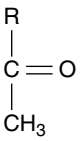


Fig. 5.22 Biotin occurs in carboxylases (E_z-biotin) and participates in the addition of CO_2 groups from HCO_3^- to organic molecules such as pyruvate

Acetyl-CoA (acetyl bound to coenzyme A, CoA—Fig. 5.24) is an amazing molecule as it is common metabolite. Acetyl-CoA is important in glucose metabolism, fatty acid degradation, amino acids metabolism, and cholesterol synthesis. Its ubiquity is not as simple to explain as the universality of ATP (hydrolyses with $\Delta G^o \ll 0$), NADH (strong reducing power; $\Delta G^o \ll 0$ in reduction), or biotin (specific functionality in carboxylation processes). Acetyl groups seem to be the universal "carbon transfer unit" and CoA their carrier for this purpose. Acetyl (or ethanoyl) is a two-carbon group of the kind:



In this formula R represents the generic molecule to which acetyl group is attached to (i.e., the acetylated molecule). In acetyl-CoA, R is CoA. Degradation of glucose, fatty acids or amino acids results in acetyl groups being attached to CoA. The acetyl groups can then be used to generate NADH from NAD^+, which in turn will help in transforming ADP to ATP. Yet, under different circumstances, acetyl groups in acetyl-CoA may also be used to synthesize molecules, serving as a carbon supplier for the growth of the carbon backbones. CoA concurs with biotin-containing enzymes for this purpose.

A generic scheme of the metabolisms built around the "universal molecules" ATP, NADH, and acetyl-CoA is presented in Fig. 5.23).

It is very curious that the fraction of the structure of the ATP, NADH, or acetyl-CoA molecules involved in chemical reactivity itself is very small when compared to the whole molecule (Fig. 5.24). Moreover, at first glance it may seem puzzling that in some reactions in metabolism, ATP is replaced by GTP or UTP, and NADH is replaced by $FADH_2$ or NADPH (Fig. 5.24). GTP or UTP do not differ from ATP in the phosphodiester bond broken to form ADP (which is analogous to GDP or UDP). Phosphorylation of NAD to form NADPH occurs at a site that does not affect the electron donor group. What is then the advantage of having relatively large molecules to perform simple chemical reactions that involve only small groups in their structures? What is the advantage in having analogous molecules with same reactivity that differ only in the "inert" part of these molecules? The answers are simple: having molecules of a significant dimension facilitates recognition and specificity of the binding sites of enzymes, and small variations in the chemical structure of these molecules determine that other enzymes should be used instead. In reactions in which NADPH intervenes, the associated enzyme is specific for NADPH, not NADH. The occurence of these reactions is an advantage in the sense of allowing the donation of electrons without affecting NADH pools in the cell. The reaction only occurs if NADPH is present. Therefore, the use of NADPH in addition to NADH enables that

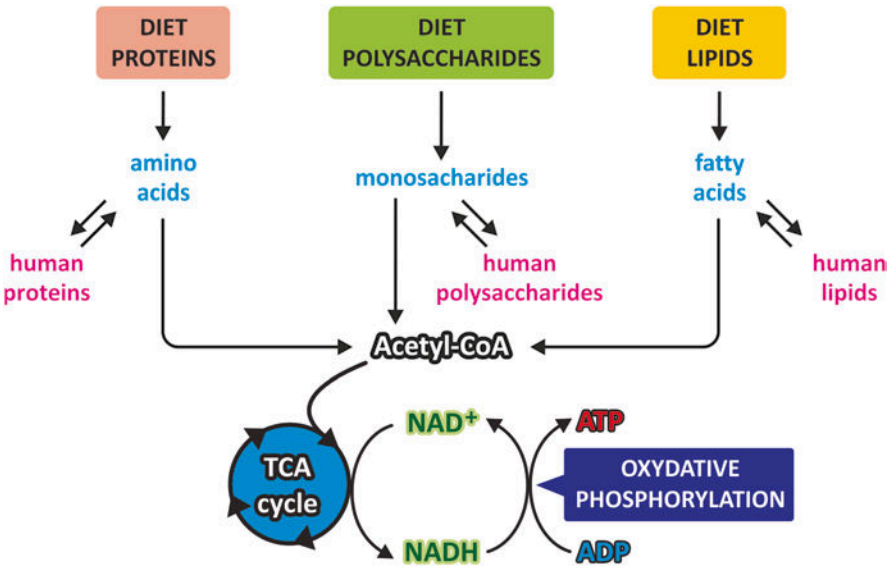


Fig. 5.23 Very broad and abbreviated view of human energy metabolism. Intermediary metabolites are not shown for the sake of simplicity. The main interplay of NADH, acetyl-CoA, and ATP is highlighted. TCA - Tricarboxylic acids cycle (also known as citrate cycle or Krebs cycle)

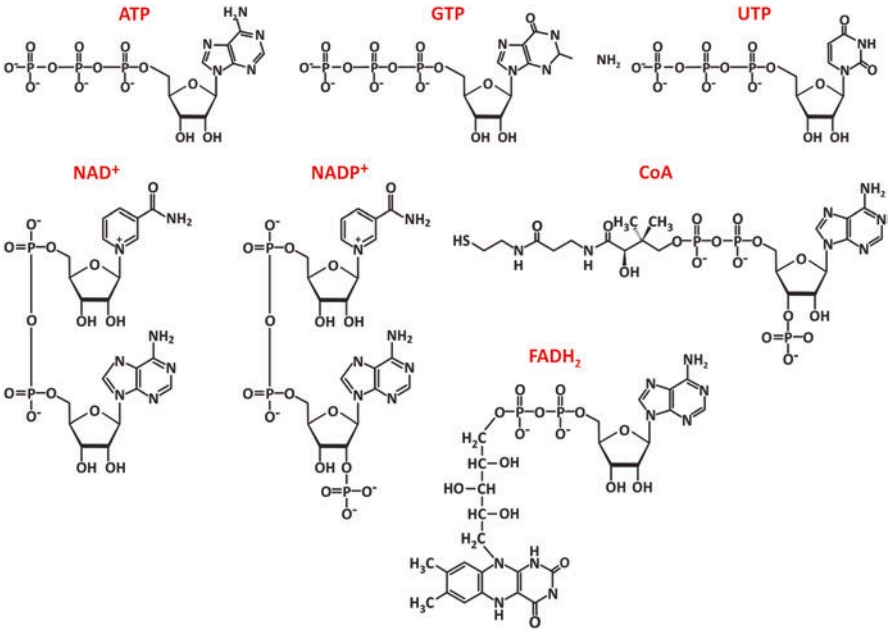


Fig. 5.24 The reactive groups of ATP, NAD^+, or CoA are relatively small compared to the whole molecule. Analogous molecules to ATP or NAD^+ differ in the "non-reactive" part of the molecule. Although not involved directly in reactivity, these parts of the molecules are important to grant specificity to different enzymes

specific redox reactions in the cell may be regulated regardless of the metabolic state of cell at the moment, i.e., regardless of NADH levels. Protective mechanisms against reactive oxygen species (ROS), for instance, are very much dependent on the reductive action of NADPH. The capacity to repair the chemical damage imposed by ROS is thus mantained regardless of low concentrations of NADH (see Sect. 6.2.5). Likewise, the use of GTP or UTP in some reactions instead of ATP may have the same advantage.

In the following chapters we will devote our attention to the synthesis of ATP, which in turn is dependent of the NADH production, which in turn is dependent on acetyl-CoA that is obtained from nutrients: fatty acids, monosaccharides, and amino acids.

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Chapter 6
Energy Conservation in Metabolism: The Mechanisms of ATP Synthesis

In living organisms, different types of energy are always interconverting into one another within the cell to allow the distinct cellular functions to be performed. This can be illustrated by several examples, such as the conversion of the energy of light into chemical energy in photosynthetic organisms or the chemical energy into mechanical energy in muscle contraction. Also, virtually all the cells need to use chemical energy to transport ions and other compounds across a membrane, generating a concentration gradient and thus converting chemical into osmotic energy. Finally, chemical energy is continuously converted in other forms of chemical energy during the biosynthesis of new molecules in cellular metabolism.

In the beginning of the twentieth century, a set of experiments carried out by Arthur Harden and William J. Young, in which they showed that phosphate is essential for yeast alcoholic fermentation, started a new era for the understanding on how energy is obtained from the environment and stored within the cells for later use. This discovery was the first association between phosphate and energy transformations in living cells, paving the way for the subsequent identification of ATP, more specifically its phosphoanhydride bond, as the main cellular energy carrier.

In this chapter, we will discuss the principles and the most relevant steps of the main processes of ATP synthesis in heterotrophic cells.

Heterotrophic organisms conserve the energy of nutrient molecules by coupling the breaking of their chemical bonds to the synthesis of ATP, which occurs through two distinct mechanisms.

The first mechanism of ATP synthesis to be identified is known as substrate-level phosphorylation. It does not depend on oxygen and thus may occur in anaerobiosis. The general principle involved in ATP synthesis through this mechanism is the formation of a phosphorylated molecule that presents a high-energy phosphate bond or, in a more precise term coined by Fritz Lipmann, a high potential of transferring its phosphoryl group, which is used to phosphorylate ADP, generating ATP.

The second mechanism of ATP synthesis is known as oxidative phosphorylation. It depends on organized membranes and, in eukaryotic cells, takes place in mitochondria. The unraveling of this mechanism was possible due to someone who

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could see beyond the energy of the chemical bonds. This man was Peter Mitchell, who postulated the revolutionary chemiosmotic theory, which states that the synthesis of ATP from ADP and inorganic phosphate (Pi) is driven by the pH gradient across the mitochondria inner membrane and the consequent formation of a transmembrane electrical potential.

6.1 Fermentation: The Anaerobic Pathway for ATP Synthesis

As the first forms of life appeared on Earth when the atmosphere contained no oxygen yet, the anaerobic use of glucose may be considered the most ancient metabolic pathway of energy conservation.

In humans, this pathway is still very important since there are cell types that lack the oxidative apparatus, specially the mitochondria, organelles in which the oxidative phosphorylation takes place. These cells include the mature erythrocytes, cells from the crystalline, and some cells of the retina. There are also many situations in which oxygen availability is limited, and thus the ATP synthesis through fermentation becomes essential. This is the case of muscle cells in intense contraction activity, working in low oxygen conditions due to the adrenaline-induced contraction of peripheral blood vessels (see Chap. 10). There are also pathological situations in which fermentation overcomes oxidative metabolism even in the presence of oxygen. This is the case of cancer cells, in what is called the "Warburg effect" (see Box 6.4).

It is also important to note that only sugars can be used as energy source in anaerobic conditions. The degradation of the other nutrient molecules, the lipids and the proteins, depends on metabolic pathways that occur in mitochondria and

culminate with ATP synthesis through oxidative phosphorylation, which requires oxygen as the final substrate (see details in Sect. 6.2).

6.1.1 A Historical Perspective of the Discovery of the Fermentation Process

Since ancient times, man has dealt with processes involving fermentation, although only recently fermentation has been understood as a metabolic pathway associated to the reactions of energy transformation essential for life.

Fermentation is the basis of the production of bread, beer, and wine, activities that accompanied humans since the early civilizations. The term fermentation arose from these practices, coming from the Roman word *fermentare*, which is related to the formation of bubbles. Thus, fermentation was firstly associated to a process in which a gas was produced. Although now we know that depending on the organism fermentation leads to different end products, which do not always include a gas, in the alcoholic fermentation, which occurs during the production of bread, beer, and wine, sugars from fruits or cereals are converted into ethanol and CO_2, which is the gas released.

Despite several advances in the scientific studies on fermentation during the nineteenth century, Louis Pasteur was the first to connect living organisms to fermentation, which was the basis for its understanding as a biological process (see Box 6.1).

Box 6.1: The Impact of Pasteur's Ideas About Fermentation

The concept that fermentation was performed by living organisms was not readily accepted when proposed by Pasteur, especially because it seemed to reinforce the theory of vitalism, which assumed that organic substances in living organisms could be formed only under the influence of a mysterious "vital force." Jakob Berzelius, Justus von Liebig, and Friedrich Wohler, important chemists from that time, were strongly against the vitalistic ideas, and although they were right in their concepts on the chemical processes, their convictions made them very reluctant to acknowledge the valuable contributions of Pasteur's observations. This can be illustrated by the ironic text published by Woehler and von Liebig in 1839 in the Annals of Chemistry regarding the participation of yeast in the transformation of sugars in ethanol and CO_2 during beer production: "Beer yeast, when dispersed in water, breaks down into an infinite number of small spheres. If these spheres are transferred into an aqueous solution of sugar, they develop into small animals. They are endowed with a sort of suction trunk with which they gulp the sugar from the solution. Digestion is immediate and clearly recognizable because of the discharge of excrements. These animals evacuate ethyl alcohol from their bowels and carbon dioxide from their urinary organs. Thus one can observe how a specifically lighter fluid is extruded from the anus and rises vertically, whereas a stream of carbon dioxide is ejected at very short intervals from their enormously large genitals."

Pasteur observed microscopic globules in a wine sample (Fig. 6.1a) and sustained that these globules were living microorganisms responsible for the sugar transformation into ethanol and CO_2. He also demonstrated that each type of fermentation is linked to a specific microorganism or, as he named, a specific "ferment." Interestingly, similar globules have been described in a sample of beer very earlier, in 1680, by Anton van Leeuwenhoek, the pioneer of the use of the microscope (Fig. 6.1b), although there is no evidence that van Leeuwenhoek had associated these globules to living organisms.

Besides associating fermentation to the presence of living organisms, Pasteur also carried out crucial experiments showing that it occurred in the absence of oxygen. These experiments discredit the current view of fermentation as a chemical process resulted from the reaction between oxygen and sugars, reinforcing the idea that fermentation was a biological process. Pasteur's findings also allowed the formulation of other two important biological concepts derived from his experiments. The first one was that some forms of life can exist without oxygen, in what he referred in one of his most famous articles, published in 1861, as *la vie sans l´air* (the life without air). The second concept came from an intriguing observation that he made in those experiments: more CO_2 was produced by yeast in the absence than in the presence of oxygen, a phenomenon known as the "Pasteur effect" (Fig. 6.1c), which is now explained by the fact that the number of ATP molecules synthesized per glucose molecule in fermentation is much lower than that in oxidative phosphorylation (see in Chap. 4 the stoichiometry of ATP synthesis in the oxidative metabolism of different nutrients).

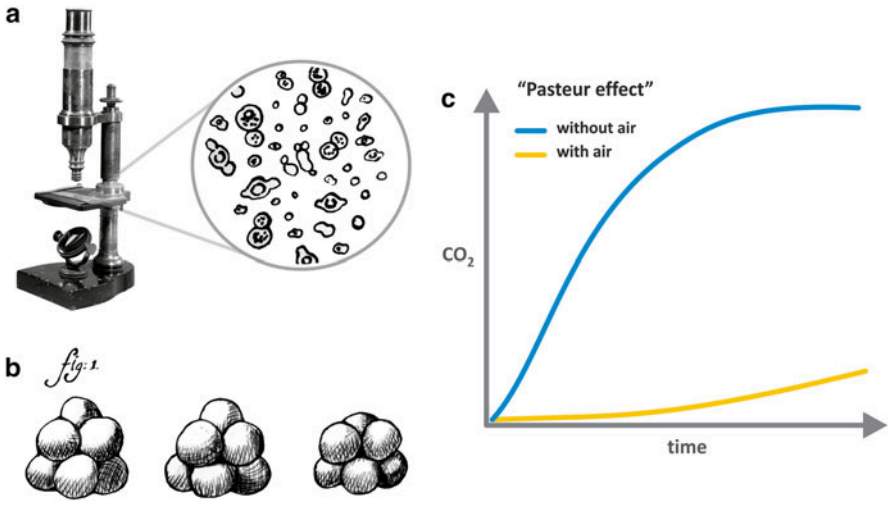


Fig. 6.1 (**a**) The microscope used by Pasteur to observe the wine samples and his drawing representing the "globules" he had observed in the sample (Adapted from Pasteur's book "Études sur la bière," 1876). (**b**) Drawing by van Leeuwenhoek, reporting in 1680 the observation of a beer sample in the microscope that he developed. (**c**) Representation of the "Pasteur effect," in which the production of CO_2 by yeast is much higher in the absence than in the presence of oxygen

The next significant advance in the understanding of fermentation came from an unexpected observation. Eduard Buchner, working with his brother Hans in the preparation of a yeast extract to treat patients with tuberculosis, discovered that even when all the yeast cells were completely disrupted, fermentation continued to proceed normally. This finding marks the end of the vitalism theory by showing that the presence of living cells was not necessary for fermentation and can be regarded as the dawn of biochemistry, introducing the concept that biological reactions are catalyzed by molecules, which Buchner called zymases, the term firstly used to refer to what we now call enzymes. Due to the great impact of his discoveries, Eduard Buchner was the first scientist to be awarded the Nobel Prize in Chemistry, in 1907.

Finally, we must comment the experiment by Harden and Young, mentioned in the beginning of this chapter. Using the cell-free system developed by the Buchners, they observed that the fermentation rate, which decreased with time, could be restored with the addition of salts of phosphoric acid (Fig. 6.2). This was the first evidence that phosphate was involved in cell metabolism, opening the way in the subsequent years to the recognition of the ubiquitous and the crucially important biological phosphorylations in life.

Few years later, Otto Meyerhof, studying muscle contraction in different organisms, showed that contraction occurred in the absence of oxygen, a similar phenomenon to that observed by Pasteur for yeast alcoholic fermentation (Fig. 6.3a). However, in the case of muscle cell fermentation, the end product was lactate. The molecular similarity of the end products of alcoholic and lactic fermentations suggested that both processes could be equivalent (Fig. 6.3b).

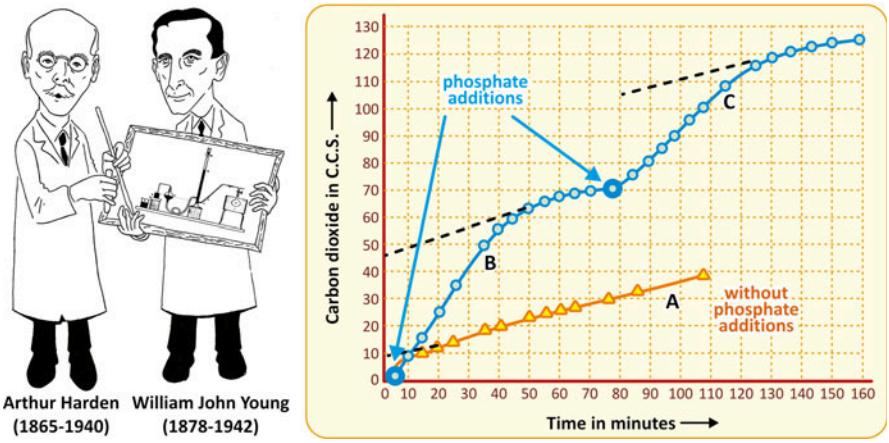


Fig. 6.2 Original figure from the article by Harden and Young published in 1906 (Adapted from Harden & Young. Proc. Royal Soc. Lond. B 77:405–520, 1906), showing the production of CO_2 by yeast fermenting glucose in the absence of any addition (curve A) and when salts of phosphoric acid were added at the indicated times (curves B and C)

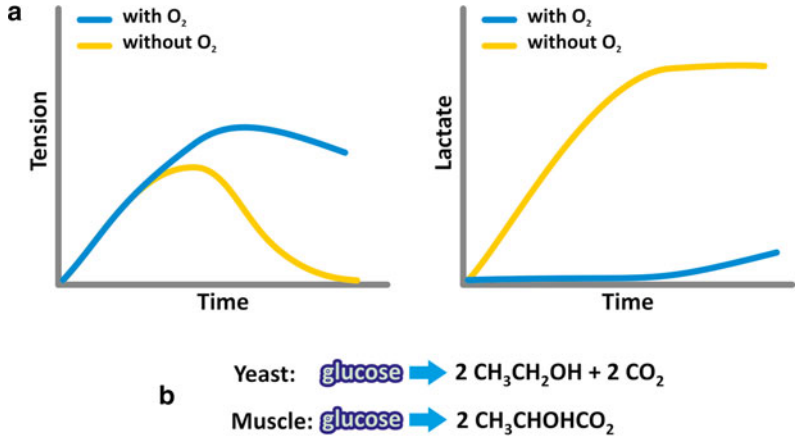


Fig. 6.3 (**a**) Schematic representation of the results obtained by Otto Meyerhof when a muscle fiber was incubated with glucose; tension due to contraction and lactate production were measured as a function of time. (**b**) The analysis of the end products of both, alcoholic fermentation performed by yeast and lactic fermentation performed by the muscular fibers, shows great similarity

Additionally, the same requirement of phosphate was observed for fermentation in the muscle cells. Now we know that indeed the processes are identical, except for the last reaction (see next section).

6.1.2 An Overview of the ATP Synthesis by Substrate-Level Phosphorylation During Fermentation

The general mechanism of ATP synthesis that occurs during fermentation consists in a series of reactions that rearranges the molecular structure of an initially phosphorylated monosaccharide, which is a hexose phosphate, in such a way as to form phosphorylated compounds with high potential of transferring their phosphoryl group (see Box 6.2). These high-energy intermediates are triose-phosphate molecules originated from a cleavage step and whose phosphoryl group is transferred to ADP, generating ATP (Fig. 6.4a). This process of ATP synthesis is named substrate-level phosphorylation, since the phosphoryl group of an intermediate of the pathway is directly used to phosphorylate ADP.

The initial substrate for fermentation is usually glucose, the most abundant monosaccharide derived from a regular human diet. The two initial steps of phosphorylation use ATP as the phosphoryl group donor (Fig. 6.4a). This may seem nonsensical at a first glance, if we think that this is a metabolic pathway for the synthesis of ATP. However, as it will become clear through the analysis of the stoichiometry of the entire pathway, although two ATP molecules are used for each

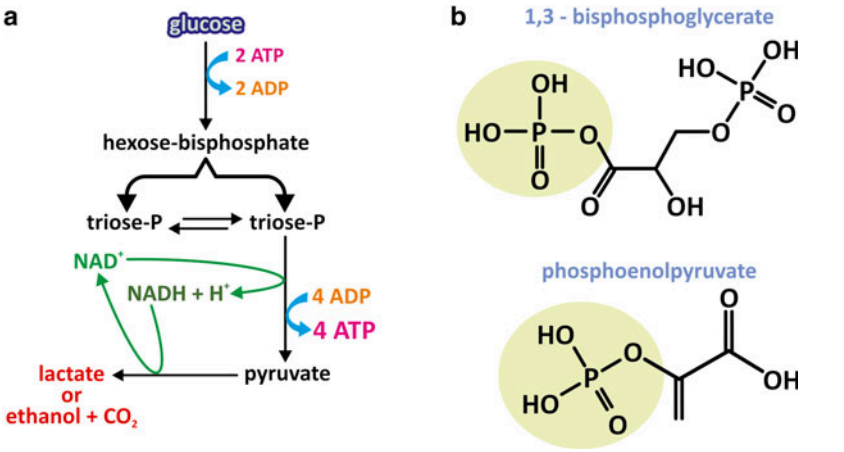


Fig. 6.4 General overview of the essentials of the fermentation process. (**a**) The pathway initiates with a hexose (usually glucose). After two steps of phosphorylation with ATP as the phosphate donor, the resultant hexose bisphosphate is cleaved into two triose phosphates, which may interconvert. One of them follows the pathway, which includes one NAD^+-dependent oxidation reaction and two transfers of the phosphate group of a high-energy triose phosphate to ADP, generating ATP and, at the second of those reactions, pyruvate. Pyruvate must be reduced to allow NADH reoxidation. The main end products of fermentation are lactate or ethanol and CO_2, depending on the organism. (**b**) Chemical structures of the high-energy triose-phosphate molecules 1,3-bisphosphoglycerate and phosphoenolpyruvate

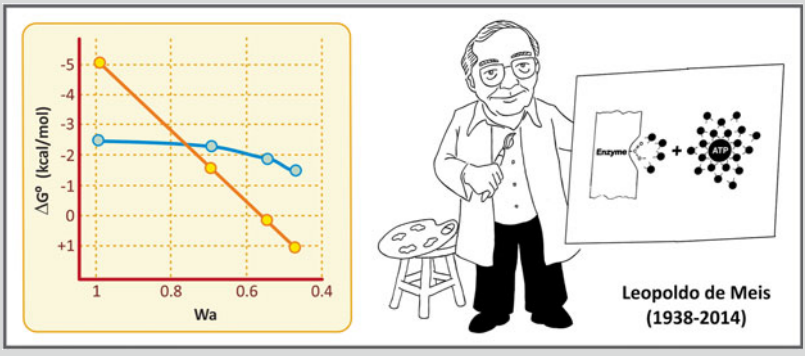
hexose that enters the pathway in its initials steps, four ATP molecules are synthesized at the end, yielding a positive balance of two ATP molecules for each hexose molecule that is metabolized. Moreover, consumption of ATP is mandatory to "force" the first steps to occur in terms of thermodynamics (see Box 4.2).

The triose phosphates with high potential of transferring their phosphoryl group are 1,3-bisphosphoglycerate and phosphoenolpyruvate (Fig. 6.4b). In the case of 1,3-bisphosphoglycerate, an anhydride bond links the carboxyl group to the phosphate, forming what is called an acyl phosphate. In the case of phosphoenolpyruvate, the double bond between carbons 2 and 3 favors the phosphoryl group transfer.

Additionally, the pathway includes an oxidative step in which the oxidation of a three-carbon intermediate is coupled to the reduction of the coenzyme nicotinamide adenine dinucleotide (NAD^+), generating NADH. Since the typical amount of NAD^+ in the cytoplasm is much lower than the amount of glucose metabolized, fermentation should end with a step leading to the reoxidation of the NADH molecule. The reaction that does this is the one that synthesizes the end product of the pathway, which in human cells is lactate (Fig. 6.4a).

Box 6.2: The Concepts of Energy-Rich Phosphate Compounds

In his classical article from 1941, Fritz Lipmann classified the phosphate compounds as "energy rich" and "energy poor." This classification was based on the conception that the energy released by the hydrolysis of these compounds was essentially determined by the nature of the chemical bond that links the phosphate to the molecule. Phosphoanhydride linkages generate "energy-rich" compounds, while phosphoester linkages generate "energy-poor" compounds. According to this concept, the free energy of hydrolysis of a phosphate compound would be determined essentially by the contribution of enthalpy. Due to the experimental approaches used at that time, the contribution of the environment in which the reaction takes place could not be taken into consideration, but new data obtained after 1970 introduced the idea that the energy of the hydrolysis of phosphate compounds would be also determined by the differences in their solvation energy and thus would vary greatly depending on the medium. This was found valid for compounds containing phosphoanhydride bonds, such as ATP, pyrophosphate, or acyl phosphates, but not for compounds in which the phosphate group is linked to the molecule through a phosphoester bond (see figure comparing the energy of hydrolysis of the phosphoanhydride bond of pyrophosphate with the phosphoester bond of glucose-6-phosphate as a function of water activity). Based on these observations, a new conception was formulated by the Brazilian scientist Leopoldo de Meis, according to which molecules containing phosphoanhydride bonds are susceptible to changes in the entropic energy (dependent on the solvation energy and thus on the environment), which contributes to the free energy involved in the reaction.



Effect of water activity (Wa) on the energy of hydrolysis of pyrophosphate (*yellow circles*) and glucose-6-phosphate (*blue circle*). Graph reproduced from de Meis in Calcium and Cellular Metabolism: Transport and Regulation, chapter 8. Plenum Press, NY, 1997

With this in mind, it is possible to understand the mechanism of ATP synthesis by ATP synthase presented in Sect. 6.2.4, in which the energy required in the reaction is not needed for the condensation of ADP and Pi, but for the ATP release from the catalytic site of the enzyme.

6.1.3 Glucose Fermentation Reactions

The first ten reactions of the fermentation process, starting with glucose and ending with the formation of two pyruvate molecules, are the same in many organisms that synthesize ATP anaerobically. The last step, which involves NADH reoxidation, is different depending on the organism, resulting in either lactate or ethanol and CO_2 as the end products. Furthermore, the reactions from glucose to pyruvate are also the same that occur when carbohydrates are used aerobically (see Sect. 7.4).

The pathway from glucose to pyruvate is named glycolysis and may be divided in two parts.

In the first part, two phosphorylation steps using ATP generate a hexose with two phosphoryl groups linked, the fructose-1,6-bisphosphate, which was the compound discovered by Harden and Young (see Sect. 6.1.1), and the first intermediate of fermentation to be identified, known as the Harden–Young ester. In the first phosphorylation step, the enzyme hexokinase catalyzes the conversion of glucose in glucose-6-phosphate, which is then isomerized to fructose-6-phosphate by the phosphohexose isomerase. The second phosphorylation step is catalyzed by the phosphofructokinase and occurs at the hydroxyl group on carbon 1 of fructose-6-phosphate, generating fructose-1,6-bisphosphate. Fructose-1,6-bisphosphate is then cleaved by the aldolase into two triose-phosphate molecules, glyceraldehyde-3-phosphate and dihydroxyacetone phosphate. These triose phosphates may interconvert in a reaction catalyzed by the triose-phosphate isomerase, with dihydroxyacetone phosphate forming glyceraldehyde-3-phosphate, the next intermediate of the pathway. Thus, in the end of this first phase, one glucose molecule is converted into two glyceraldehyde-3-phosphate molecules (Fig. 6.5). In the light of the points discussed in the previous section, it is important to note that all the phosphorylated compounds of this first part of glycolysis are phosphoesters.

In the second part of the pathway, each glyceraldehyde-3-phosphate molecule is oxidized in a NAD^+-dependent reaction followed by a phosphorylation step, in a reaction catalyzed by the glyceraldehyde-3-phosphate dehydrogenase, which forms 1,3-bisphosphoglycerate. Note that this phosphorylation step uses inorganic phosphate directly and not ATP as the phosphate donor. 1,3-bisphosphoglycerate is the first high-energy phosphorylated compound generated in the pathway. The oxidation of the aldehyde group of glyceraldehyde coupled to the phosphorylation reaction forms, instead of a free carboxyl group, an acyl phosphate, from which the phosphoryl group is transferred to ADP, generating ATP and 3-phosphoglycerate, in a reaction catalyzed by the phosphoglycerate kinase. Phosphoglycerate mutase converts 3-phosphoglycerate into 2-phosphoglycerate, which is then dehydrated by the enolase, forming the second compound in the pathway with high potential of transferring the phosphoryl group, the phosphoenolpyruvate (PEP). Then, another substrate-level phosphorylation step occurs, with the phosphoryl group of PEP transferred to ADP, generating ATP and pyruvate, a reaction catalyzed by the pyruvate kinase (Fig. 6.5).

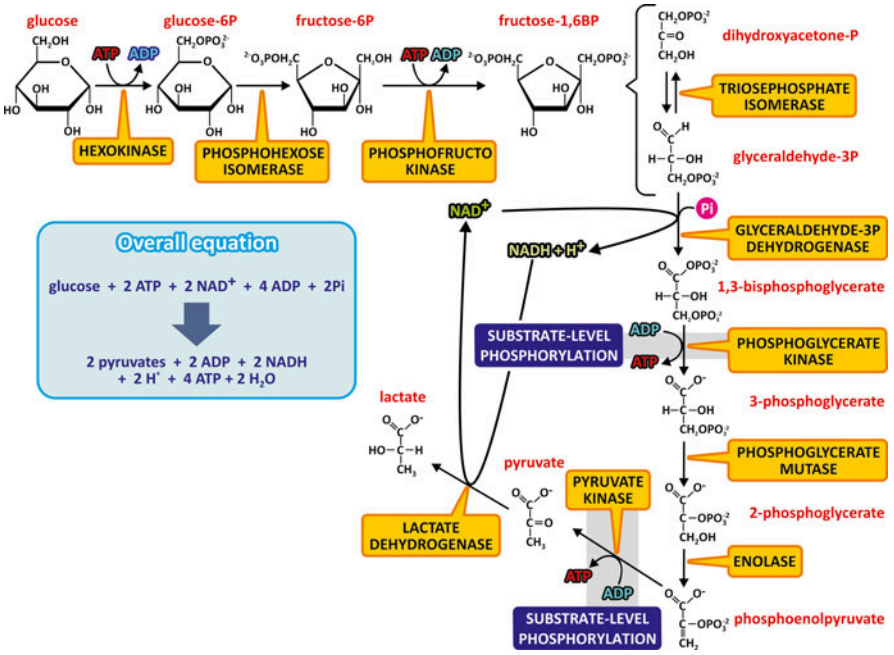


Fig. 6.5 Reactions of the fermentation of glucose to lactate. The first ten reactions consist in the metabolic pathway named glycolysis, which is also the pathway for the aerobic metabolization of carbohydrates. The last step occurs when pyruvate is not oxidized through the aerobic metabolism and NADH must be reoxidized. It should be noticed that each fructose-1,6-bisphosphate molecule leads to two glyceraldehyde-3-phosphate molecules, as dihydroxyacetone phosphate will be converted to glyceraldehyde-3-phosphate. The names of the enzymes are highlighted in *yellow boxes*. The steps that involve substrate-level phosphorylation are also indicated

The last step in the fermentation of glucose in human cells is the reduction of pyruvate to lactate, catalyzed by the lactate dehydrogenase, allowing the reoxidation of NADH (Fig. 6.5).

When glycolysis is the means of carbohydrate utilization with concomitant use of oxygen, instead of pyruvate being reduced in the last step of fermentation, it is completely oxidized to CO_2 in mitochondria (see Sect. 7.4).

6.2 Oxidative Phosphorylation: The Main Mechanism of ATP Synthesis in Most Human Cells

Oxidative phosphorylation accounts for 95 % of ATP synthesis in the human organism. This metabolic pathway is compartmentalized in mitochondria, the organelles that comprise most of the bioenergetic functions within the eukaryotic cell (see Box 6.3).

The mitochondria are unique organelles composed of two lipid membranes (Fig. 6.6). The outer mitochondrial membrane contains several porins, proteins that

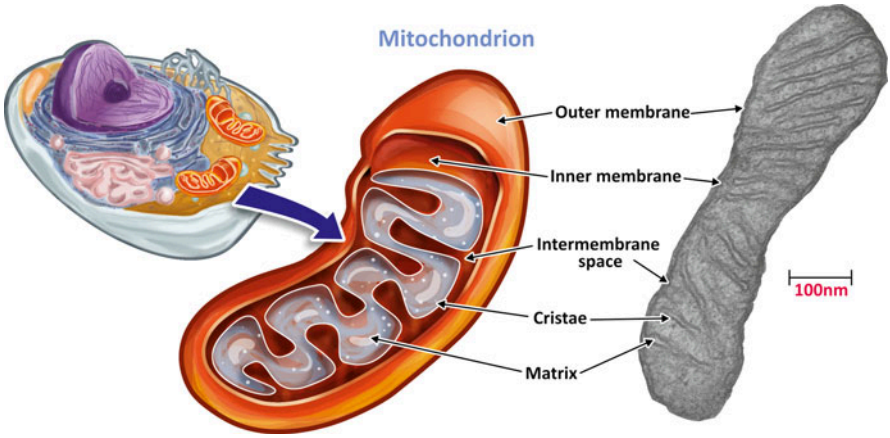


Fig. 6.6 The structure of a mitochondrion. Mitochondria are organelles formed by a permeable outer membrane and a very impermeable inner membrane with several convolution named cristae. The intramitochondrial medium forms the mitochondrial matrix. Mitochondrion transmission electron micrograph: courtesy from Prof. Marlene Benchimol

make membrane permeable to ions and small molecules (with molecular mass lower than 5 kDa). The inner membrane has a very high protein–lipid ratio and is rich in an unusual phospholipid, cardiolipin. It is very impermeable, with the transport of molecules and ions, including H^+, occurring only through specific proteins. This membrane has a very large surface area provided by several convolutions called mitochondrial cristae (Fig. 6.6). The inner membrane encloses the mitochondrial matrix, which comprises most of the enzymes of the oxidative metabolism, the mitochondrial ribosomes, tRNA, and several copies of the mitochondrial DNA (Fig. 6.6).

Box 6.3: The Endosymbiotic Theory for the Origin of Mitochondria

The incorporation of the mitochondria was an important event in the evolution of the eukaryotic cells. It is believed that it occurred more than 1.5 billion years ago through the invasion of a heterotrophic anaerobic cell by an aerobic bacterium. This is known as the endosymbiotic theory, which was postulated in the beginning of the twentieth century but was revived and better argued by Lynn Margulis, in the 1960s. Several genetic evidences suggest that the ancestral symbiont was an aerobic α-proteobacterium that consumed oxygen through a respiratory chain. The role of the anaerobic host in the symbiosis would be to make pyruvate accessible to the endosymbiont metabolism.

(continued)

Box 6.3 (continued)

On the other hand, evidence indicate that the role of the ancestral symbiont in the initial phase of the evolution of the mitochondria was to protect the anaerobic cell components from the toxic effects of oxygen through the activity of the last respiratory chain component, the enzyme cytochrome oxidase, which converts oxygen to water (see next sections of this chapter). In fact, the sharp increase in the oxygen tension around two billion years ago introduced a great threat for the anaerobic ancient cells, which did not have the detoxifying enzymes peroxidases, catalases, or superoxide dismutases that protect modern cells against the toxic effects of reactive oxygen species. Thus, the aerobic symbiont would function as an oxygen scavenger inside the host cell. With time, evolution of host genomes probably contributed with new functions to the symbiont, including the ATP/ADP transporter, transforming it into an organelle with an ATP-exporting function.

6.2.1 A Historical Perspective of the Understanding of Cellular Respiration

Since Lavoisier's experiments in the eighteenth century, which correlated respiration to the combustion of organic matter, both processes involving oxygen consumption and CO_2 release together with heat production, the concept that aerobic metabolism proceeds with the direct reaction between oxygen and the organic compounds in the body was established. However, the connection between metabolic oxidative reactions and oxygen consumption in respiration remained illusive for a long time.

In the first decades of the twentieth century, there was a serious controversy regarding the mechanisms of biological oxidations in aerobic metabolism (Fig. 6.7).

On one side, Heinrich Wieland postulated, together with Torsten Thumberg, that in the biological oxidations, the reactions catalyzed by the dehydrogenases "activated" some hydrogen atoms of the metabolic intermediates, making them labile to be transferred to a hydrogen acceptor. It is important to mention that their hypothesis, although incomplete by not taking into account the role of oxygen, correctly supported the concept that free oxygen does not directly combine with carbon to form CO_2.

On the opposite side was Otto Warburg (Box 6.4), who defended the oxygen-activating hypothesis, in which he argued that the dehydrogenase concept was unnecessary. In this hypothesis, Warburg postulated that the oxidation of all metabolites was catalyzed by an iron-containing enzyme, which he named *Atmungsferment* (meaning oxygen-transferring enzyme or respiratory enzyme) and in which the iron atom was oxidized to its ferric state (Fe^{3+}) by oxygen and reduced back to its ferrous form (Fe^{2+}) after reaction with organic substances.

The key to solve the Wieland–Warburg controversy was provided by David Keilin, who working as entomologist and parasitologist changed biochemistry with his findings (Fig. 6.7).

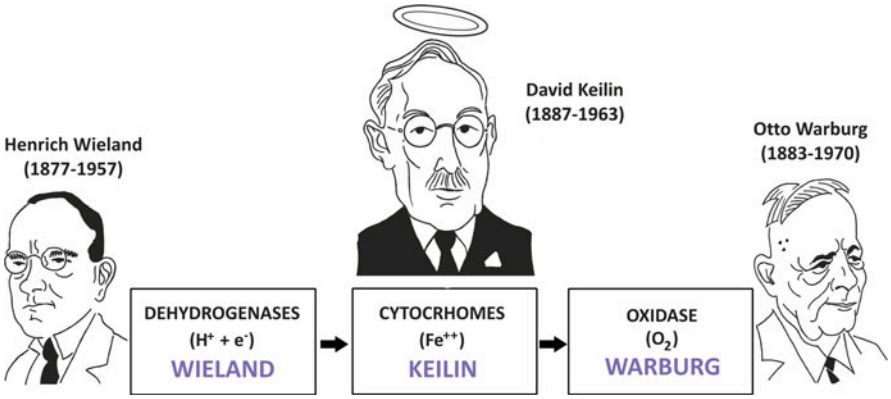
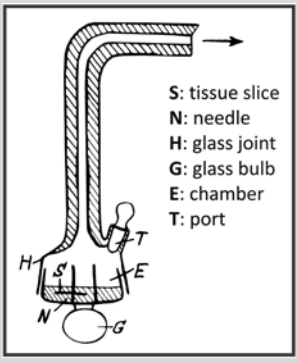


Fig. 6.7 Solution of Wieland–Warburg controversy with the discovery of the cytochromes by Keilin. Wieland postulated that the reactions catalyzed by the dehydrogenases "activated" some hydrogen atoms of the metabolic intermediates, making them labile to be transferred to a hydrogen acceptor while Warburg defended the oxygen-activating hypothesis, in which biological oxidations were catalyzed by an iron-containing enzyme. Keilin proposed that the cytochromes connected the dehydrogenases and the oxidase, being alternately reduced by the dehydrogenases and oxidized by oxygen through the Warburg enzyme

Box 6.4: The Diversity of Otto Warburg's Contributions for Science

Otto Warburg was a very active and interdisciplinary scientist, giving outstanding contributions to different fields in science, including respiration, photosynthesis, and cancer cell metabolism.

Warburg strongly defended the use of quantitative methods and worked on the improvement of the instruments to get reliable measurements. Using manometric techniques, he developed an apparatus, the Warburg respirometer (see figure below), to quantify O_2 uptake by thin slices of a tissue, through the changes in the chamber pressure, measured by the connection of a manometer

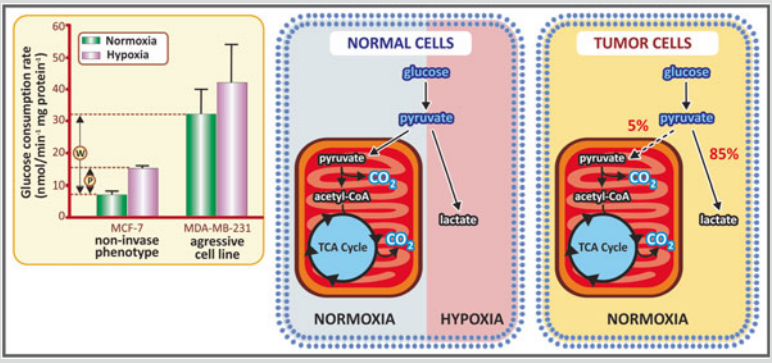


(continued)

Box 6.4 (continued)

to the end of the glass joint (H). The apparatus also allowed the measurements of CO_2 emission by adding, for example, potassium hydroxide in the chamber (E) to precipitate CO_2. Due to his pioneering studies on cellular respiration in the beginning of the twentieth century, in which he proposed the existence of an iron-containing respiratory enzyme (the Warburg *Atmungsferment*), he was awarded the Nobel Prize in Physiology or Medicine in 1931.

Warburg also dedicated much time of his life investigating cancer cell metabolism. Studying different types of cancer cells in the decade of 1920, Warburg made a very intriguing observation: he found a behavior that was the opposite of the Pasteur effect (the inhibition of fermentation by O_2; see Sect. 6.1.1). Warburg showed that cancer cells produced lactic acid from glucose even under aerobic conditions, which is known as the Warburg effect (see figure below). This was firstly interpreted as a consequence of a damaged respiration in cancer cells, but now we know that the Warburg effect occurs due to alterations in the regulation of glycolysis in tumorigenesis.



Pasteur (P) and Warburg (W) effects. Graph in the left reproduced by permission from Macmillan Publishers Ltd: Gatenby & Gillies. Nat. Rev. Cancer 4:891–899, 2004

Studying the physiology of insects, Keilin rediscovered a substance that has been described more than a century before by Charles A. MacMunn, but was forgotten after it has been considered a hemoglobin contaminant present in MacMunn's preparations (see Box 6.5). Keilin showed that it was not hemoglobin and named this substance "cytochrome" (meaning cellular pigment) because he had found it in cells of many different organisms, such as insects, worms, yeast, and plants, and due to

its characteristic absorption spectrum containing four distinct bands, which he named *a*, *b*, *c*, and *d*. Keilin proposed that this pigment acted as the link between the Wieland dehydrogenases and the Warburg oxidase, being alternately reduced by the dehydrogenases and oxidized by oxygen through the Warburg enzyme.

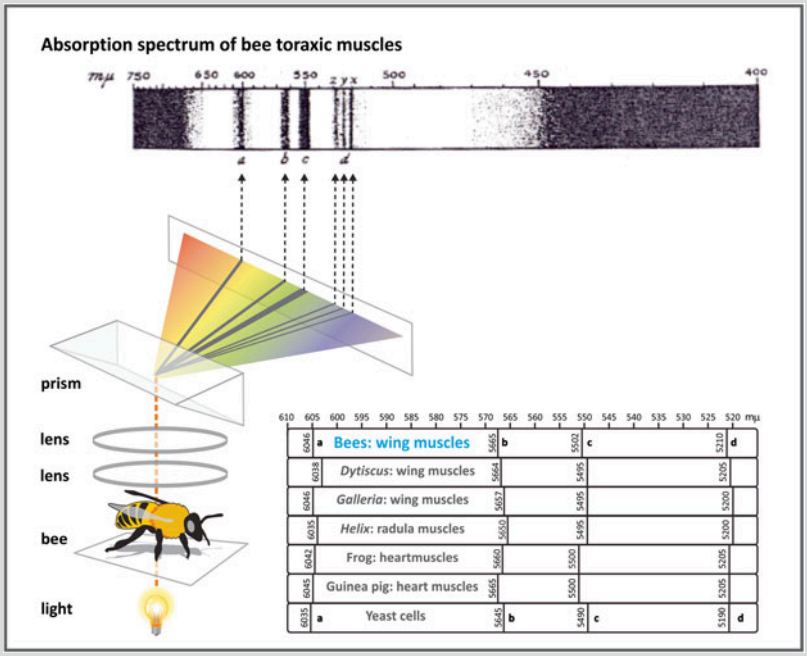
Keilin's experiments provided a great advance in the comprehension of the energy transformations in aerobic organisms, establishing the concept of what we now know as the respiratory chain, a series of membrane-associated redox carriers that transfer the electrons from the metabolic substrates to molecular oxygen. However, the process by which this sequence of redox reactions is coupled to energy storing into the cells was still unknown at that time.

Box 6.5: The Discovery of the Cytochromes by David Keilin

The basic features of the respiratory chain were established between the 1920s and 1930s by the entomologist and parasitologist David Keilin. In 1925, he published his first paper in this field, entitled "*On cytochrome, a respiratory pigment, common to animals, yeast, and higher plants*" (Keilin. Proc. R. Soc. Lond. B 98:312–339, 1925), marking a new phase in the studies of the biological oxidations. It is interesting to revisit the reflexions of E. F. Hartree, who worked with Keilin on this subject, published in the periodic Biochemical Education, in 1973, in which he comments the history of the discovery of the cytochromes: "In these days, when the world of science is under pressure to organize for the pursuit of practical ends, when the scale of scientific endeavour is making the lone furrow an anachronism, it is salutary to recall the simple-handed achievements of men of science stimulated solely by an urge to understand the living world. The discovery of cytochromes is a notable example of such achievements, not only because the disarming simplicity of the experimental approach by the discoverer, David Keilin, but also because the discovery came at a critical moment. It resolved a serious dilemma that was impeding the evolution of biochemistry from an untidy and rather primitive branch of chemistry into a major scientific discipline: more succinctly the transformation of Bio-Chemistry into Biochemistry." At that time, Keilin was interested in studying the adaptations of a fly larva that parasitizes the stomach of horses. Using a microspectroscope to compare the absorption spectra of the larvae and the adult thoracic muscle, he found the presence of four sharp absorption bands in the spectrum of the adult muscle, which were very different from the bands seen for hemoglobin and oxyhemoglobin (see in the figure the results published in the 1925's paper with a schematic representation of the experiment). Intrigued by this absorption pattern, he examined many other organisms, including different insects, yeast and other microorganisms, plants, and animal tissues, finding always the same absorption spectra with the four bands.

(continued)

Box 6.5 (continued)



A more intriguing observation was that when a yeast suspension was submitted to vigorous shaking, the bands disappeared, appearing again after a period of rest. Keilin's interpretation of this phenomenon was that the pigment, which he named cytochrome, became oxidized during the shaking in the air, leading to the disappearance of the bands. After interruption of the shaking, yeast respiratory activity led to reduction of the pigment, which in the reduced form displayed the four-banded absorption spectrum. Using insects, Keilin observed strikingly similar results: the absorption band pattern observed in the thoracic muscle was strong when the wings were stimulated to vibrate but disappeared after the movement stopped. Thus, Keilin concluded that the cytochrome was acting as a respiratory carrier linking oxygen and the oxidizable metabolic intermediates.

One of the landmarks in the discovery of this missing link was the article published by Belitser and Tsybakova in 1939, showing a direct correlation between oxygen consumption in cellular respiration and phosphate esterification, as it can be seen in Fig. 6.8, a reproduction of the original figure of this article.

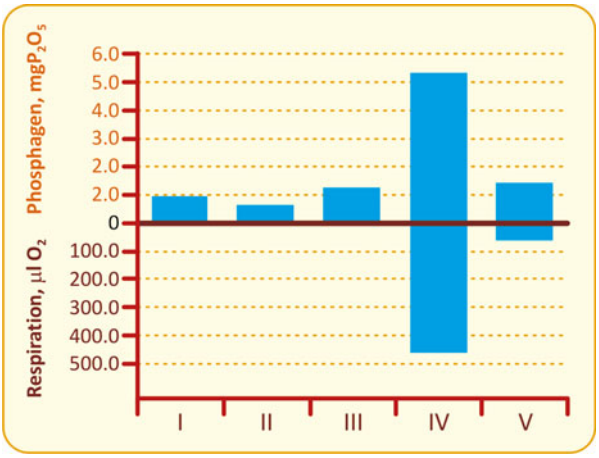


Fig. 6.8 Correlation between phosphate incorporation (*top*) and O_2 consumption (*bottom*) in a pigeon muscle preparation in the following conditions: (I) before any incubation; (II) in the absence of O_2 (in a N_2 atmosphere) without addition of a respiratory substrate; (III) in the presence of pyruvate but in the absence of O_2; (IV) in the presence of pyruvate and O_2; and (V) in the presence of O_2 but in the absence pyruvate. Figure adapted from Belitser & Tsybakova. Biokhimia 4:516–535, 1939

After this, years and years were spent searching a high-energy intermediate, which, in analogy to the substrate-level phosphorylation process already known to be the mechanism of ATP synthesis in fermentation (see Sect. 6.1.2), would couple the redox process to ATP synthesis. This intermediate had never been found, and how oxidative phosphorylation occurred remained one of the most challenging questions of Biochemistry for a long time. The basic principles behind this process could only be understood after the proposal of the chemiosmotic hypothesis by Peter Mitchell (see next section).

6.2.2 An Overview of Oxidative Phosphorylation Process

In the oxidative reactions of catabolism, the electrons removed from the metabolic intermediates are transferred to two major electron carrier coenzymes, nicotinamide adenine dinucleotide (NAD^+) and flavin adenine dinucleotide (FAD), which are converted to their reduced forms, NADH and $FADH_2$ (see Chap. 7). These reactions are catalyzed by dehydrogenases, as firstly postulated by Wieland and Thumberg (see Sect. 6.2.1).

Oxidative phosphorylation depends on the electron transport from NADH or $FADH_2$ to O_2, which is reduced to H_2O. Electron transport occurs through a number of protein complexes associated to the inner mitochondrial membrane. Some of these protein complexes contain cytochromes (the Keilin's pigment) as part of their structures. These cytochromes are in fact proteins that contain a heme prosthetic

group. Heme is a complex ring structure named protoporphyrin that binds an iron atom. As detected by Keilin, there are different classes of cytochromes that contain different types of heme (Fig. 6.9), distinguishable by their characteristic absorption spectra. Heme oxidation leads to a decrease in light absorption, explaining the observations made by Keilin in his experiments (see Box 6.5). The heme groups in the cytochromes *a* and *b* are tightly but not covalently bound to the protein, while in cytochrome *c* the heme is covalently linked to specific Cys residues of the protein.

The structures of respiratory protein complexes are now known with detail, as it will be explained in the next section, but one can safely say that the concept and the principles of their role in electron transport were established by Keilin after the discovery and the study of the cytochromes, which he defined as "oxidation–reduction catalysts" (see Sect. 6.2.1).

The mechanism by which the electron transport is coupled to ATP synthesis was proposed by Peter Mitchell, in 1961, in his revolutionary chemiosmotic theory. Initially it was difficult to be accepted, since most of the scientists in the field believed that a high-energy intermediate would link oxidation to phosphorylation reactions (as occurs in the substrate-level phosphorylation process; see Sect. 6.1.2). However, the chemiosmotic hypothesis was proved to be correct, and Mitchell was awarded the Nobel Prize in Chemistry in 1978.

The chemiosmotic theory postulates that the electron transfer through the respiratory protein complexes is coupled to proton (H^+) pumping across the proton-impermeable mitochondrial inner membrane, from the mitochondrial matrix to intermembrane space. H^+ pumping generates what Mitchell called the proton-motive force, the simultaneous effect of the pH gradient across the membrane and transmembrane electrical potential that drives the ATP synthesis from ADP and Pi (Fig. 6.10).

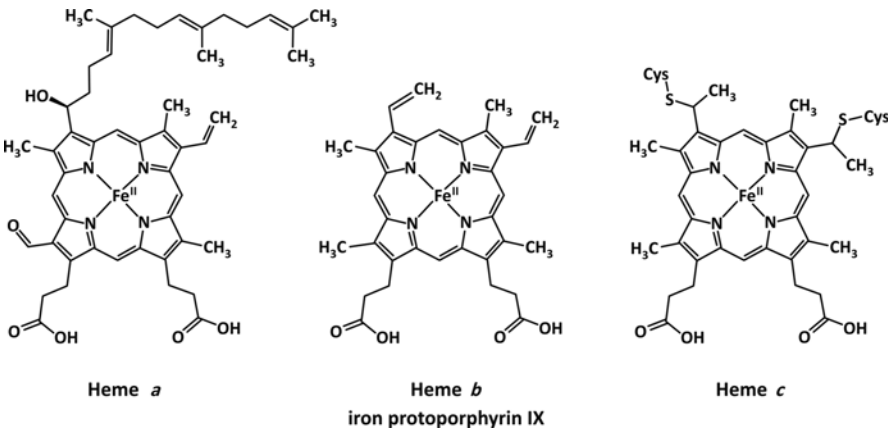


Fig. 6.9 The distinct types of heme prosthetic groups of the cytochromes: heme *a*, which has a long isoprenoid tail attached to the porphyrin ring; heme *b*, which is a protoporphyrin IX; and heme *c*, which is covalently bound to Cys residues in the polyprotein chain of the cytochrome *c*

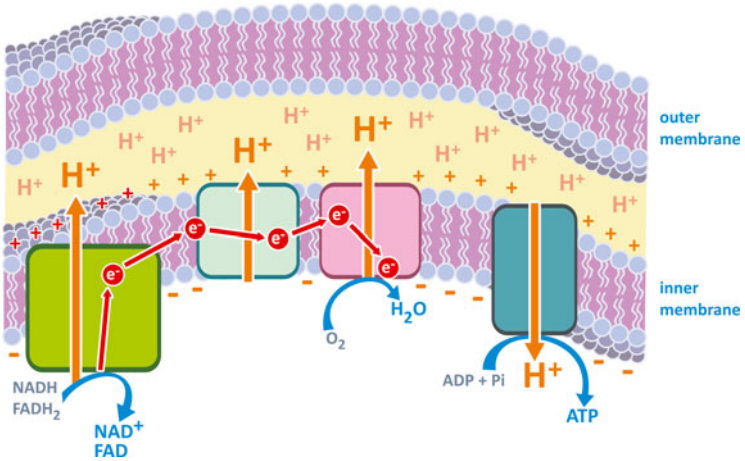


Fig. 6.10 Schematic representation of the general mechanism of ATP synthesis in oxidative phosphorylation. Electrons are transferred from NADH or $FADH_2$ through a number of protein complexes associated to the inner mitochondrial membrane to the final acceptor oxygen, which is reduced to H_2O. Electron transport is coupled to H^+ pumping across mitochondrial inner membrane, generating a pH gradient and a transmembrane electrical potential, which is the driving force for ATP synthesis by ATP synthase. Note that this is a simplified scheme in which the NAD- or FAD-associated complexes are represented as one entity, although they are different protein complexes. However, it is important to point out that electron transport through FAD-associated complex is not coupled to H^+ pumping across mitochondrial membrane, as detailed in the next section

The H^+ pumping occurs through specific protein segments that are part of some of the respiratory complexes. The ATP synthesis is catalyzed by another protein complex in the mitochondrial membrane, the ATP synthase, through which the H^+ ions return to the matrix (Fig. 6.10).

6.2.3 The Electron Transport System

The electron transport complexes are integral membrane proteins that contain attached chemical groups (flavins, iron–sulfur groups, heme or cooper ions) capable of accepting and donating electrons (see detailed structures in the next section).

6.2.3.1 The Sequence of Electron Transfer Between the Electron Carrier Groups

A form to deduce the sequence in which the electrons are transferred between the carriers is to compare the reduction potential of each individual electron carrier group (Table 6.1). The reduction potential is a measure of how "easy" is for a molecule to accept an electron.

Table 6.1 Reduction potential of respiratory electron carriers

Reaction	Reduction potential (V)
$2H^+ + 2e^- \rightarrow H_2$	–0.414
$NAD + H^+ + 2e^- \rightarrow NADH$	–0.320
NADH dehydrogenase (FNM) $+ 2H^+ + 2e^- \rightarrow$ NADH dehydrogenase ($FNMH_2$)	–0.300
Ubiquinone $+ 2H^+ + 2e^-$ → ubiquinol	0.045
Cytochrome *b* (Fe^{3+}) $+ e^-$ → cytochrome *b* (Fe^{2+})	0.077
Cytochrome c_1 (Fe^{3+}) $+ e^-$ → cytochrome c_1 (Fe^{2+})	0.220
Cytochrome *c* (Fe^{3+}) $+ e^-$ → cytochrome *c* (Fe^{2+})	0.254
Cytochrome *a* (Fe^{3+}) $+ e^-$ → cytochrome *a* (Fe^{2+})	0.290
Cytochrome a_3 (Fe^{3+}) $+ e^-$ → cytochrome a_3 (Fe^{2+})	0.350
½ $O_2 + 2H^+ + 2e^- \rightarrow H_2O$	0.817

The sequence deduced by the reduction potential was confirmed experimentally. Keilin in his first experiments on the cytochromes (see Box 6.5) observed that the bands do not appear nor disappear simultaneously. From this observation, he deduced that what he firstly named simply cytochrome was a mixture of pigments containing three components, designated cytochromes *a*, *b*, and *c*. Afterward, it was possible to distinguish two components in what was considered as cytochrome *a*, whose absorption bands were superimposed. One of them, the cytochrome *a*3, is the last component in the chain, being directly oxidized by oxygen. Furthermore, the use of specific inhibitors of the electron transport allowed the complete sequence to be determined, confirming that the sequence follows the order of increasing reduction potential, as expected.

6.2.3.2 The Organization of the Respiratory Complexes in the Inner Mitochondrial Membrane

Although Keilin's experiments allowed the determination of the sequence through which the electrons flow in the respiratory chain, nothing was known at that time about how the electron-transferring groups were organized. Keilin always worked with a grinded heart muscle preparation that later was identified as submitochondrial particles, which are actually vesicles of the inner mitochondrial membrane. At the end of the 1940s, the group of David Green was able to fractionate four components of the respiratory chain, which they named Complexes I, II, III, and IV. These components corresponded to four integral membrane protein complexes, the NADH/ubiquinone oxidoreductase (or NADH dehydrogenase), the succinate/ubiquinone oxidoreductase (or succinate dehydrogenase), the ubiquinone/cytochrome *c* oxidoreductase (or cytochrome *bc*1 complex), and the cytochrome *c* oxidase, respectively (Fig. 6.11).

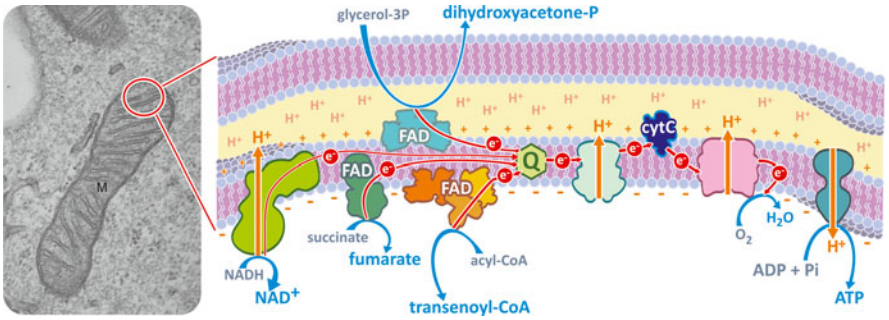


Fig. 6.11 Schematic representation of the electron transfer complexes in the inner mitochondrial membrane. The electrons flow from NADH to O_2 through three protein complexes: NADH/ubiquinone oxidoreductase (*light green*), ubiquinone/cytochrome *c* oxidoreductase (*light blue*), and cytochrome *c* oxidase (*pink*). The electron transference from NADH/ubiquinone oxidoreductase to ubiquinone/cytochrome *c* oxidoreductase is mediated by the membrane-soluble molecule ubiquinone (*green hexagon* labeled with a Q), and the transference from ubiquinone/cytochrome *c* oxidoreductase to cytochrome *c* oxidase is mediated by the small protein cytochrome *c* (*dark blue* labeled with cytC). O_2 is also reduced by electrons coming from $FADH_2$, which is linked to FAD-dependent dehydrogenases, such as succinate/ubiquinone oxidoreductase (*dark green*), the acyl-CoA dehydrogenase (*orange*), and the glycerol-phosphate dehydrogenase (*blue*). The electrons from $FADH_2$ are transferred to ubiquinone and then flow to O_2 through ubiquinone/cytochrome *c* oxidoreductase and cytochrome *c* oxidase, as described for the electrons transferred from NADH. The electron transferring through NADH/ubiquinone oxidoreductase, ubiquinone/cytochrome *c* oxidoreductase, and cytochrome *c* oxidase is coupled to H^+ pumping to the intermembrane space. The H^+ returns to the matrix through the enzyme ATP synthase, driving ATP synthesis. The transmission electron micrograph in the *left* shows a cell thin section with a mitochondrion (courtesy from Prof. Marlene Benchimol)

Now we know that in addition to these four protein complexes, there are two other proteins that participate in the transfer of electrons from metabolic substrates to the respiratory chain, the acyl-CoA dehydrogenase and the glycerol-phosphate dehydrogenase (Fig. 6.11).

Electron transport between the respiratory complexes occurs through two more mobile electron carriers, the ubiquinone (also called coenzyme Q), a very hydrophobic molecule with high mobility in the mitochondrial membrane (independently on its protonation state), and cytochrome *c*, a small protein associated to the outer face of the inner mitochondrial membrane (Fig. 6.11).

NADH formed in metabolic NAD^+-dependent oxidative reactions is a water-soluble electron carrier that reversibly associates to specific dehydrogenases. The electrons are transferred from NADH to O_2 through three protein complexes: NADH/ubiquinone oxidoreductase, ubiquinone/cytochrome *c* oxidoreductase, and cytochrome *c* oxidase.

The electron carrier FAD is usually covalently attached to a FAD-dependent dehydrogenase. This type of enzyme includes the succinate dehydrogenase, the acyl-CoA dehydrogenase, and the glycerol-phosphate dehydrogenase. In the reac-

tions catalyzed by these three FAD-dependent enzymes, FAD is reduced to $FADH_2$, whose electrons are then transferred to O_2 through ubiquinone/cytochrome *c* oxidoreductase and cytochrome *c* oxidase, as described for NADH/ubiquinone oxidoreductase electrons (Fig. 6.11).

The numbering of the four firstly identified electron transport complexes from I to IV and the concept that they form a chain, as inferred by the usually used name of "electron transport chain" or "respiratory chain," give rise to a not entirely correct idea that the electron transport complexes are arranged sequentially and that the electron transport occurs through a linear pathway.

A more appropriated terminology emerges from the concept of a convergent "electron transport system," as proposed by Erich Gnaiger, in which electrons either from NADH via Complex I or from $FADH_2$ through three different FAD-associated complexes, the Complex II, the electron-transferring flavoprotein (ETF), or the glycerol-phosphate dehydrogenase (GpDH), converge to ubiquinone, in what he named the "Q-junction" (Fig. 6.12). After this point of convergence, the electrons flow to oxygen through a "linear" pathway composed of Complex III, cytochrome *c*, and Complex IV.

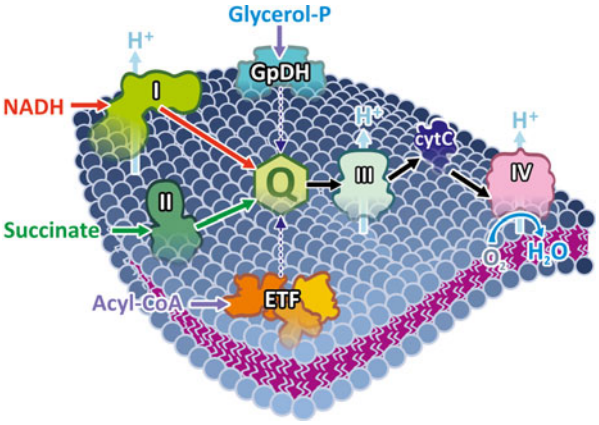


Fig. 6.12 Convergent electron transport system. Ubiquinone receives the electrons from four different protein complexes: NADH/ubiquinone oxidoreductase (or Complex I), succinate/ubiquinone oxidoreductase (or Complex II), ETF/Q oxidoreductase (ETF); and glyceraldehyde dehydrogenase (GpDH). NADH comes from NAD-dependent dehydrogenases, mainly glyceraldehyde-3-phosphate dehydrogenase, pyruvate dehydrogenase, isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, and malate dehydrogenase. $FADH_2$ is linked to succinate/ubiquinone oxidoreductase, GpDH, or acyl-CoA dehydrogenase, which in turn is associated to ETF. Then, electrons are transported to O_2 through a "linear" pathway composed of ubiquinone/cytochrome *c* oxidoreductase (or Complex III) and cytochrome *c* and cytochrome *c* oxidase (or Complex IV)

6.2.3.3 The Structure of the Electron-Transferring Components

A detailed description of each electron-transferring component of the respiratory chain is presented below as these are paradigmatic cases of structure/function correlation.

NADH/ubiquinone oxidoreductase (or Complex I) is composed of 45 polypeptide chains associated to several electron-transferring groups: a flavin nucleotide (FMN) and many iron–sulfur (Fe–S) centers. The complete crystal structure of NADH/ubiquinone oxidoreductase is only available for the simpler prokaryotic enzyme (Fig. 6.13a), but the high degree of sequence conservation suggests that the bacterial

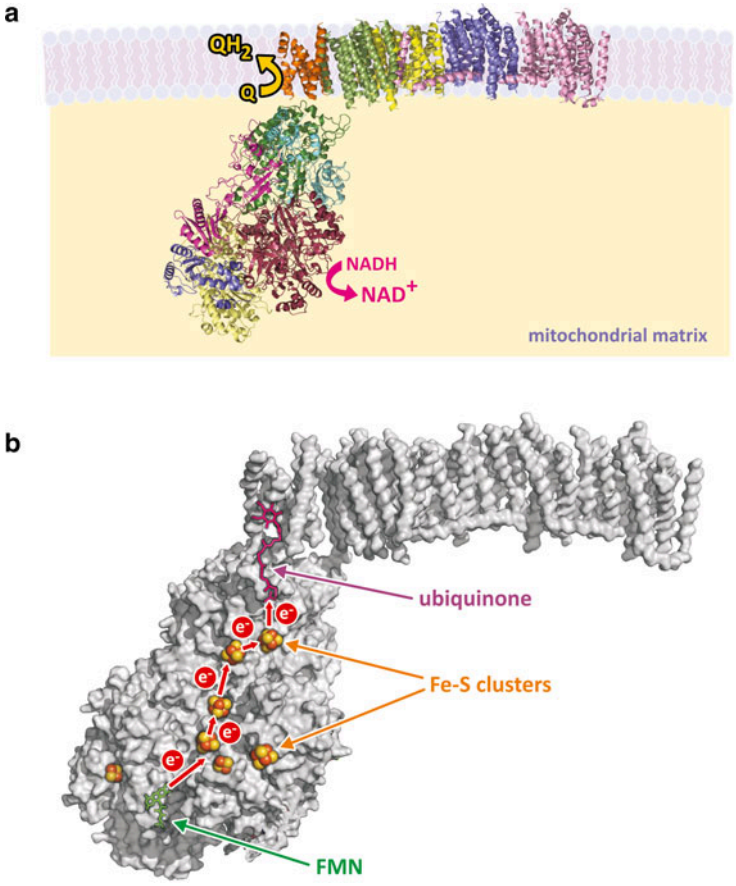


Fig. 6.13 (**a**) Structure NADH/ubiquinone oxidoreductase complex from *Thermus thermophilus* (PDB 3M9S), with each subunit colored differently. This complex catalyzes the oxidation of NADH to NAD^+, with the reduction of ubiquinone (Q) to ubiquinol (QH_2). (**b**) The transfer of electrons (e^-) from NADH to ubiquinone (shown in *magenta sticks*), flowing through FMN (shown in *green sticks*) and iron–sulfur clusters (*orange* and *yellow spheres*), is represented by the *red arrows* over the protein surface map. Electron transfer is coupled to the translocation of four protons

enzyme represents a minimal model of human enzyme. The electrons removed from NADH flow through FMN and then to the Fe–S groups to finally reduce ubiquinone to ubiquinol (Fig. 6.13b). The electron transfer through the enzyme components drives the transport of four H^+ from the matrix to the intermembrane space.

Succinate/ubiquinone oxidoreductase (or Complex II) is an FAD-dependent mitochondrial membrane enzyme that catalyzes the oxidation of succinate to fumarate, a reaction of the tricarboxylic acid (TCA) cycle, the pathway that accounts for the complete oxidation of acetyl-CoA, which, in turn, is the convergent product of the degradation pathways of sugars, lipids, and some amino acids (see Sect. 7.2). This enzyme is usually referred as succinate dehydrogenase, but since the oxidation of succinate to fumarate is coupled to electron transference to ubiquinone, succinate/ubiquinone oxidoreductase is a more precise denomination. The enzyme contains four polypeptide chains, a catalytic heterodimer composed of subunit A, containing a covalently bound FAD, and subunit B, containing three iron–sulfur clusters, and two transmembrane polypeptides that anchor the enzyme in the mitochondrial membrane and where a heme *b* group is bound (Fig. 6.14).

Acyl-CoA dehydrogenase (ACAD) catalyzes the first step of mitochondrial fatty acid oxidation: the conversion of an acyl-CoA to trans-2,3-enoil-CoA with the

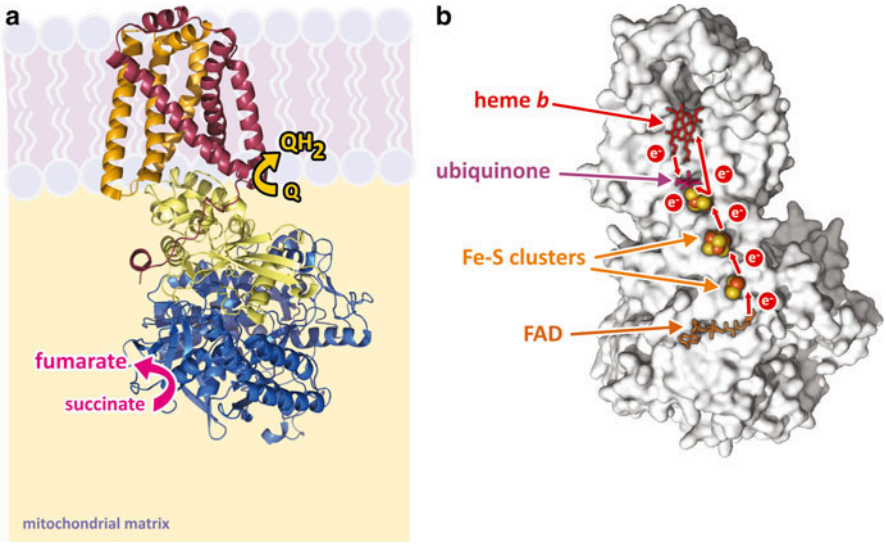


Fig. 6.14 (**a**) Structure of succinate/ubiquinone oxidoreductase complex from porcine heart (PDB 1ZOY). The FAD binding protein, or subunit A, is shown in *blue*; the iron–sulfur protein, or subunit B, is shown in *light yellow*; and the transmembrane proteins are shown in *pink* and *orange*. The enzyme catalyzes an FAD-dependent oxidation of succinate to fumarate, with the concomitant reduction of ubiquinone (Q) to ubiquinol (QH_2). (**b**) The electron (e^-) transfer pathway from FAD (shown in *orange sticks*) to heme *b* (shown in *red sticks*), flowing through the iron–sulfur groups (*orange* and *yellow spheres*) to finally reduce the ubiquinone (shown in *magenta sticks*), is represented by the *red arrows* over the protein surface map

reduction of the enzyme-bound FAD coenzyme (see Sect. 7.4.4). ACADs associate to the electron-transferring flavoprotein (ETF), which reoxidizes ACAD-bound $FADH_2$. ETF then transfers the electrons to the ETF/Q oxidoreductase that in turn reduces ubiquinone after electron transport through the Fe–S centers (Fig. 6.15). There are five isoforms of this enzyme showing distinct specificity for the fatty acyl chain length. The very-long-chain acyl-CoA dehydrogenase (VLCAD) forms homodimers of 67 kDa subunits bound to the inner mitochondrial membrane through the 180 last residues of the C-terminal. These residues are lacking in the long-, medium-, and short-chain acyl-CoA dehydrogenases (LCAD, MCAD, and SCAD, respectively), isoforms that are soluble homotetramers with 45 kDa subunits.

Glycerol-phosphate dehydrogenase (GpDH) is a dimeric enzyme associated to the outer face of the inner mitochondrial membrane that oxidizes glycerol-3-phosphate to dihydroxyacetone phosphate (DHAP) with reduction of the enzyme-bound FAD that mediates the transfer of the electrons to ubiquinone (Fig. 6.16).

Ubiquinone is a lipid-soluble benzoquinone with an isoprenoid tail, which in mammals is composed of 10 isoprenyl units, making the molecule very hydrophobic and thus soluble in membranes (Fig. 6.17). It is reduced to ubiquinol after accepting two electrons and two H^+ from NADH/ubiquinone oxidoreductase, succinate/ubiquinone oxidoreductase, ETF/Q oxidoreductase, or GpDH and diffuses in the membrane, reaching ubiquinone/cytochrome *c* oxidoreductase to which the electrons are transferred.

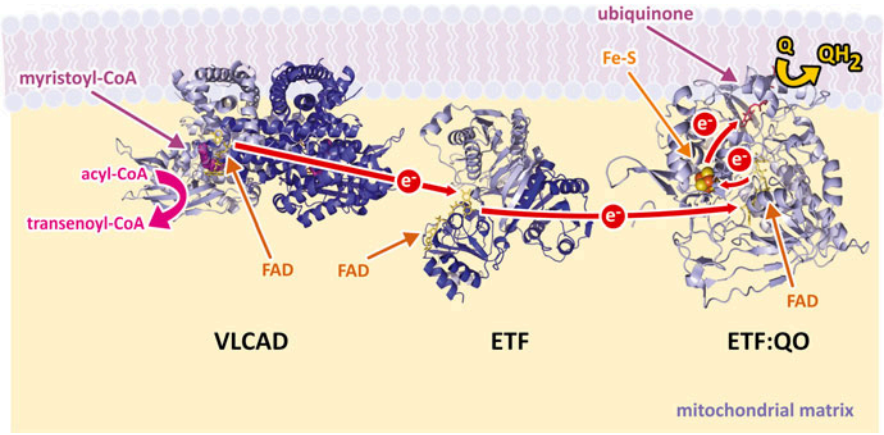


Fig. 6.15 Representation of the multistep electron transfer from an acyl-CoA (oxidized to transenoyl-CoA) to ubiquinone (Q, reduced to ubiquinol, QH_2). The known structures used as examples are the human VLCAD dimer (PDB 3B96) complexed with the substrate myristoyl-CoA (shown in *pink spheres*); the human ETF (PDB 1EFV); and the ETF/QO from pig liver (PDB 2GMH) complexed with ubiquinone (shown in *magenta sticks*). The electrons (e^-) are transferred through each enzyme-bound FAD (shown in *orange sticks*) to the iron–sulfur center (shown in *orange* and *yellow spheres*) in ETF/QO to finally reduce the ubiquinone

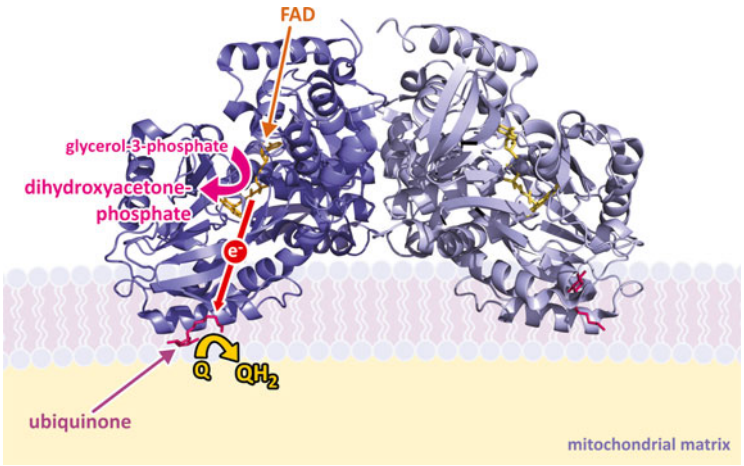


Fig. 6.16 Structure of the GpDH dimer from *E. coli* (PDB 2QCU), showing the enzyme-bound FAD (in *orange sticks*), which transfers electrons (e^-) to ubiquinone (Q, reduced to ubiquinol, QH_2; molecular structure shown in *magenta sticks*)

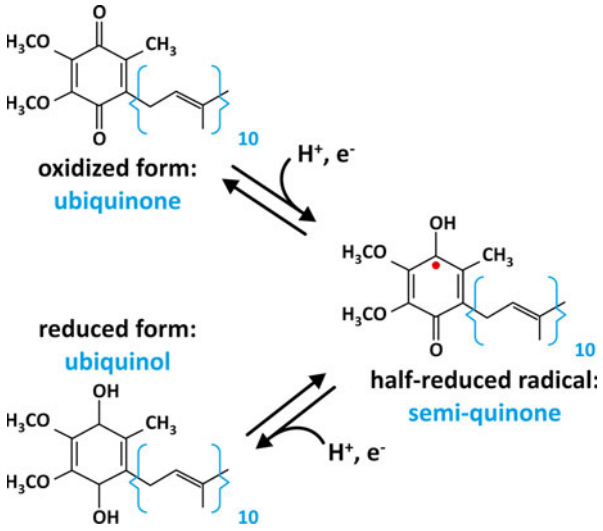


Fig. 6.17 Structures of the redox forms of coenzyme Q: ubiquinone is the oxidized form that is completely reduced to ubiquinol by accepting two electrons and two H^+ in the reactions catalyzed by NADH/ubiquinone oxidoreductase, succinate/ubiquinone oxidoreductase, ETF/Q oxidoreductase, or GpDH. Ubiquinol is reoxidized by the ubiquinone/cytochrome *c* oxidoreductase. The reduction of ubiquinone and the oxidation of ubiquinol involves an intermediate step in which a half-reduced semiquinone radical ($^{\bullet}Q^-$) is formed. The implications of this will be discussed in Sect. 6.2.5

Ubiquinone/cytochrome *c* oxidoreductase (or Complex III) transfers the electrons from ubiquinol to cytochrome *c* with the coupled transport of four H^+ from the matrix to the intermembrane space. This protein complex is a dimeric structure with each monomer being a complex assembly of 11 polypeptide chains. The electrons are transported through three functional groups associated to each monomer: cytochrome *b* containing two *b*-type heme groups, an Fe–S center, and cytochrome *c*1 containing one *c*-type heme group (Fig. 6.18).

Cytochrome *c* is a monomeric protein containing one heme group (Fig. 6.19). This protein is located in the intermembrane space in close association to the inner mitochondrial membrane. It is reduced by ubiquinone/cytochrome *c* oxidoreductase and reoxidized by cytochrome *c* oxidase.

Cytochrome *c* oxidase (or Complex IV) is a dimeric complex of 13 subunit monomers that transfers electrons from cytochrome *c* to O_2, forming H_2O. It contains two Cu ions associated to the SH groups of two Cys of the subunit that receives the electrons from cytochrome *c* and transfers them to an *a*-type heme group that in turn transfers the electrons to another heme *a* group, heme a_3, this one associated to another Cu ion, which finally transfers the electrons to O_2 (Fig. 6.20).

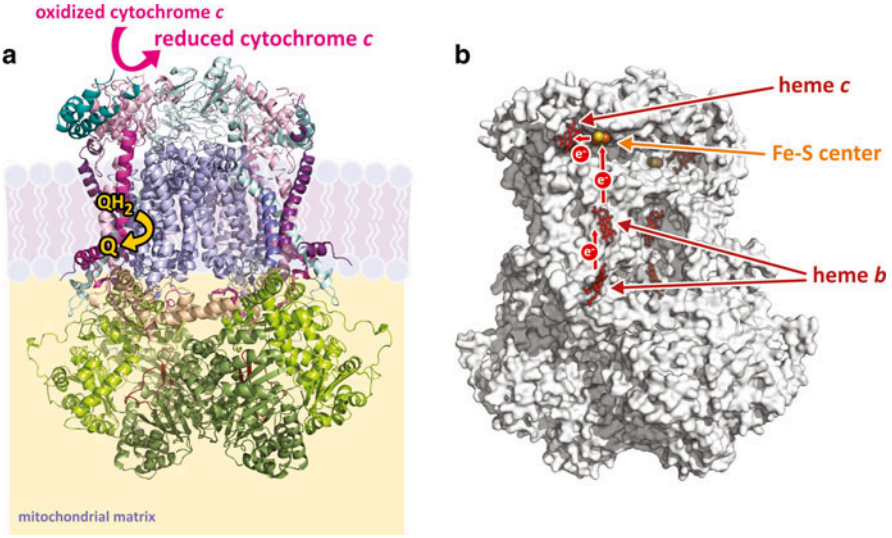


Fig. 6.18 (**a**) The complete dimeric structure of the bovine ubiquinone/cytochrome *c* oxidoreductase (PDB 1BE3) with the 11 subunits colored differently. (**b**) The electron (e^-) transfer pathway from ubiquinol to cytochrome *c*, flowing through the heme *b* groups (shown in *red sticks*) of the cytochrome *b* subunit (shown in *light purple* in A), the iron–sulfur center (shown in *orange* and *yellow spheres*), and the heme *c* group (shown in *red sticks*) of the cytochrome *c*1 subunit (shown in *pink* in A), to finally reduce cytochrome *c*, is represented by the *red arrows* over the protein surface map

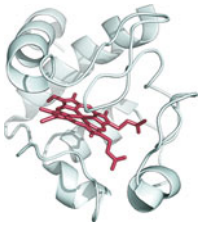


Fig. 6.19 Structure of human cytochrome *c* (PBD 1HCR), showing its heme group in *red sticks*

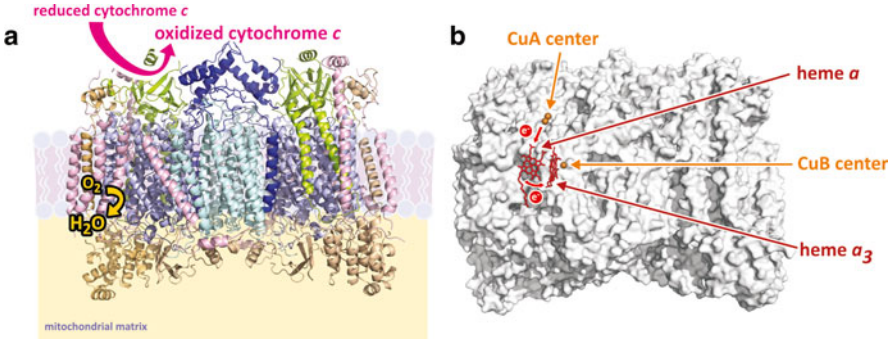


Fig. 6.20 (**a**) Dimeric structure of the bovine cytochrome *c* oxidase (PDB 1OCC), with the 13 subunits of each monomer colored differently. The enzyme catalyzes the oxidation of cytochrome *c* with the reduction of O_2 to H_2O. (**b**) The electrons (e^-) are sequentially transferred through the CuA center (shown in *brown spheres*), to heme *a* (shown in *red sticks*), and heme *a3* (shown in *red sticks*)-CuB center (shown in *brown spheres*) to finally reduce the O_2 to H_2O, as represented by the *red arrows* over the protein surface map

6.2.4 The ATP Synthesis Through Oxidative Phosphorylation

The synthesis of ATP is catalyzed by a large protein complex, the ATP synthase, located, as the respiratory complexes, in the inner mitochondrial membrane. The enzyme uses as substrates ADP and Pi in a reaction dependent on the flow of H^+ from the intermembrane space to the mitochondrial matrix.

In electron microscopy imaging, ATP synthase is seen as characteristic head connected to the membrane by a long stalk. Through studies using the technique of electron cryotomography, it was possible to observe that the ATP synthase dimers are arranged in long rows along the highly curved mitochondrial cristae edges, an organization similar in mitochondria from mammals, fungi, or plants (Fig. 6.21).

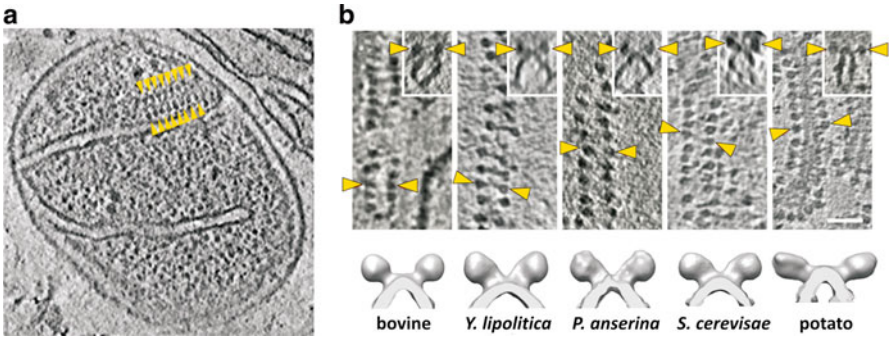


Fig. 6.21 (**a**) Tomographic slices showing the arrays of F_1–F_o ATP synthase dimers (*yellow arrows*) in whole mitochondria of *Podospora anserina*. (**b**) Rows of F_1–F_o ATP synthase in mitochondrial membranes from bovine heart, *Yarrowia lipolytica*, *Podospora anserina*, *Saccharomyces cerevisiae*, and potato. A side view of each array with the dimers in relation to the membrane is shown in the inset. *Yellow arrowheads* indicate F1 heads of one dimer. Scale bar, 50 nm. The surface representation of each dimer is shown in the *bottom*. Figures reproduced with permission from Davies et al. Proc. Natl. Acad. Sci. USA 108:14121–14126, 2011

6.2.4.1 The Structure of ATP Synthase

The overall structure of this large enzyme may be separated in two components: the F_1 portion, a peripheral membrane protein clearly seen in electron microscopy images as projections in the inner mitochondrial membrane (see Fig. 6.21), and the F_o portion (whose denomination comes from its sensitivity to oligomycin), an integral mitochondrial membrane protein, through which H^+ flow from the intermembrane space to the mitochondrial matrix (Fig. 6.22).

The F_1 portion of the ATP synthase was isolated and purified by Efraim Racker, whose studies were decisive for the comprehension of the mechanism of ATP synthesis reaction. The crystallographic structure of F_1, determined by John E. Walker (Fig. 6.22a), revealed that it has nine subunits of five different types, three α-subunits, three β-subunits, and one of each γ-, δ-, and ε-subunits (Fig. 6.22). F_1 δ- and ε-subunits interact with the membrane-embedded transmembrane helices of F_o portion of the enzyme. The catalytic sites of ATP synthesis are located in each of the β-subunits, whose conformations in the enzyme structure are different from each other due to differences in their interactions with the other subunits of the enzyme. This is essential for the mechanism of ATP synthesis, as it will be detailed in the next section.

The F_o complex is composed of three types of subunits: one a subunits; two b subunits, which associate to F_1 α- and β-subunits; and 10–12 small c subunits, which are hydrophobic polypeptides consisting of two transmembrane helices that form a membrane-embedded cylinder that interacts with the δ- and ε-subunits of F_1 complex (see schematic representation in Fig. 6.22b).

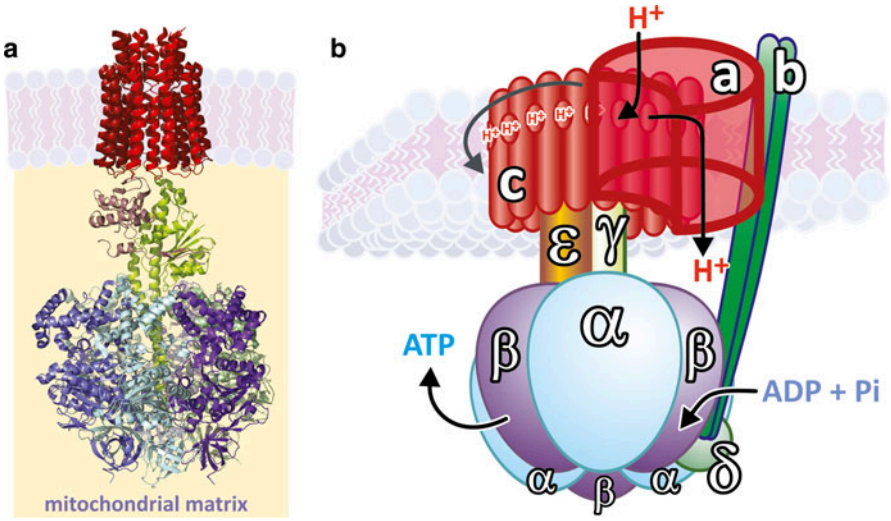


Fig. 6.22 (**A**) Structure of the F_1 and part of the F_o portions of ATP synthase from *Saccharomyces cerevisiae* (PDB 2XOK). The F_1 α-, β-, γ-, and ε-subunits are represented in *cyan*, *purple*, *light green*, and *pink*, respectively. The c subunits of the F_o portions are shown in *red*. (**b**) Schematic representation of the entire F_1F_o-ATP synthase, showing the δ-subunit of F_1 portion as well as the a- and b-subunits of F_o portion, which were not yet determined and thus are not represented in the crystallographic structure shown in (**a**)

6.2.4.2 The Mechanism of ATP Synthesis by the ATP Synthase

The synthesis of ATP from ADP and Pi is a very endergonic reaction in aqueous solution. However, one important point to understand the mechanism of ATP synthesis by ATP synthase is that when occurring in F_1 environment, it is readily reversible, with a free-energy change close to zero (see Box 6.2). For the reaction catalyzed by F_1, the energy barrier consists in the step of ATP release from the enzyme. This energy barrier is overcome by the energy input from the H^+ gradient, since flow through F_o promotes conformational changes in the β-subunit, leading to the loss of its affinity to ATP.

This view of ATP synthesis was formulated by Paul D. Boyer. From his kinetic studies, two main new concepts emerged. The first was that the three catalytic sites of the ATP synthase participate sequentially and cooperatively in the catalytic cycle; the second was that the catalytic mechanism would be seen as "a rotational catalysis" mechanism, as Boyer named, in which the three catalytic sites alternate the reaction catalysis (see Box 6.6). One clue for this proposal was given by the crystallographic structure of F_1, which revealed that the three β-subunits were differentially occupied during the catalytic cycle, one having ADP bound, other having ATP bound, and the third being empty (see Fig. 6.23a).

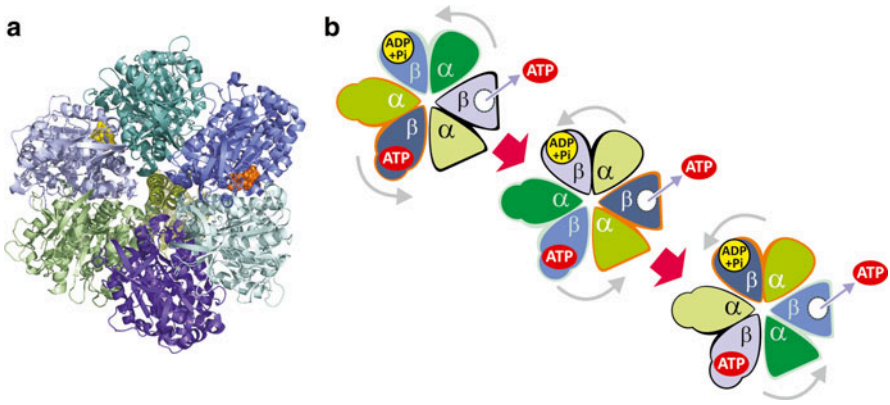


Fig. 6.23 (**a**) Structure of mitochondrial bovine F_1 showing ADP bound to one of the β-subunits, in *yellow*, and a non-hydrolyzable ATP analogue (phosphoaminophosphonic acid-adenylate ester) bound to another β-subunit, in *orange* (PDB 1BMF). (**b**) Schematic representation of the different conformations assumed by F_1 subunits: ADP and Pi bind to the catalytic β-subunit, which is in the β-ADP conformation. The enzyme rotation driven by H^+ flow through F_o portion promotes a conformation change in the β-subunit that acquires the β-ATP conformation, which stabilizes ATP in the active site. Then, another F_1 rotation leads the subunit to its empty conformation, which loses the affinity to ATP, releasing it to the medium

The rotational catalysis mechanism may be summarized in the model shown in Fig. 6.23b. ADP and Pi from the medium bind to the β-subunit catalytic site that is in the β-ADP conformation. The conformation of this β-subunit changes to the β-ATP conformation due to enzyme rotation driven by H^+ flow through F_o portion. In this conformation, the β-subunit stabilizes ATP, which is in equilibrium in the active site with ADP and Pi. Then, another F_1 rotation occurs, leading this β-subunit to change conformation again, now to the empty conformation, which loses the affinity to ATP, releasing it to the medium. Another round starts when another F_1 rotation leads the β-subunit again to the β-ADP conformation. This rotational movement frequently justifies the label of "molecular machine" to ATP synthase.

Box 6.6: The Confirmation of Boyer's Model by Real-Time Microscopy

F_1 rotation could be directly seen in an ingenious experiment performed by the research groups of Masasuke Yoshida and Kazuhiko Kinosita (published in Nature 386:299–302, 1977), in which they attached to the γ-subunit of F_o a long fluorescent actin filament and observed its movement as ATP was hydrolyzed, in real time in a microscope, in relation to the $\alpha_3\beta_3$ core immobilized in the microscope slide. They also observed that the rotation occurred in three discrete steps of 120°, completely confirming Boyer's model.

Due to his great contribution to the understanding of the mechanism of ATP synthesis, Boyer shared the Nobel Prize in Chemistry, in 1997, with John Walker, who determined the crystallographic structure of the F_1 portion of the enzyme, an essential step for the comprehension of the catalytic mechanism.

6.2.5 *Regulation of Oxidative Phosphorylation*

Oxidative phosphorylation is generally limited by the availability of ADP, so that the major control of ATP synthesis by oxidative phosphorylation is the cellular ATP requirement.

When respiratory substrates are freely available, ATP is synthesized so that the ratio ATP/ADP increases. If the levels of ADP become very low (when ATP synthesis overcomes its utilization in cellular metabolism), but the substrates are still available, the H^+ gradient reaches the maximum level. This prevents electron transport and hence respiration, since the gradient cannot be dissipated through ATP synthase, which cannot work due to the absence of its substrate, ADP. This situation is called state 4 respiration (Fig. 6.24) and illustrates the low permeability of the inner mitochondrial membrane to H^+ and the coupling of electron transport to ATP synthesis.

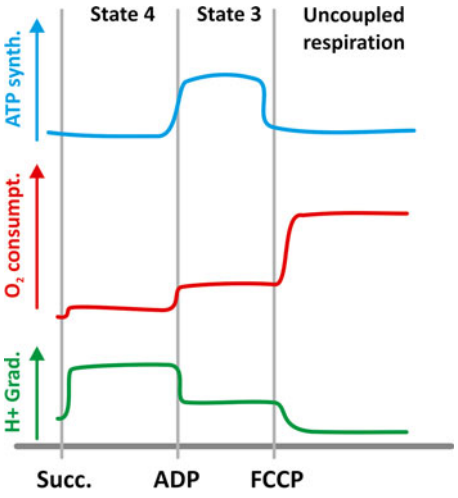


Fig. 6.24 Representation of the extent of the proton gradient, oxygen consumption, and ATP synthesis when a respiratory substrate (e.g., succinate), ADP, and an uncoupler (e.g., *p*-trifluoromethoxycarbonyl cyanide phenylhydrazone, FCCP) are added to intact mitochondria in vitro

When ADP becomes available, the ATP synthase phosphorylates it to ATP, and H^+ gradient is reduced to such an extent that respiration is allowed to proceed, while ADP is available. In this situation, respiration occurs at the same rate of ATP synthesis, in what is called state 3 respiration (Fig. 6.24).

However, even in state 4, or when ADP phosphorylation is inhibited (e.g., using oligomycin), some oxygen consumption is observed, demonstrating that the coupling of respiration to ATP synthesis is imperfect, and part of the energy will be normally dissipated as heat. This occurs to some extent due to H^+ leaking through the inner mitochondrial membrane. This phenomenon can be reproduced by the use of substances called uncouplers (e.g., *p*-trifluoromethoxycarbonylcyanide phenylhydrazone—FCCP), whose effect on O_2 consumption, H^+ gradient, and ATP synthesis is shown in Fig. 6.24. In the presence of uncouplers, H^+ readily move back into the matrix, bypassing the ATP synthase and collapsing the gradient, which causes respiration to be accelerated but uncoupled from ADP phosphorylation.

6.2.5.1 Uncoupling Proteins: The Physiological Uncouplers

Physiologically, uncoupling of electron transport to ADP phosphorylation is provided by a family of uncoupling proteins (UCP). The best studied of them, and the first to be identified, UCP1, is expressed exclusively in a specialized tissue called brown adipose tissue (BAT). In contrast to white adipocytes, brown adipocytes contain several lipid droplets and a much higher number of mitochondria (Fig. 6.25a), which confer the brown color to the tissue.

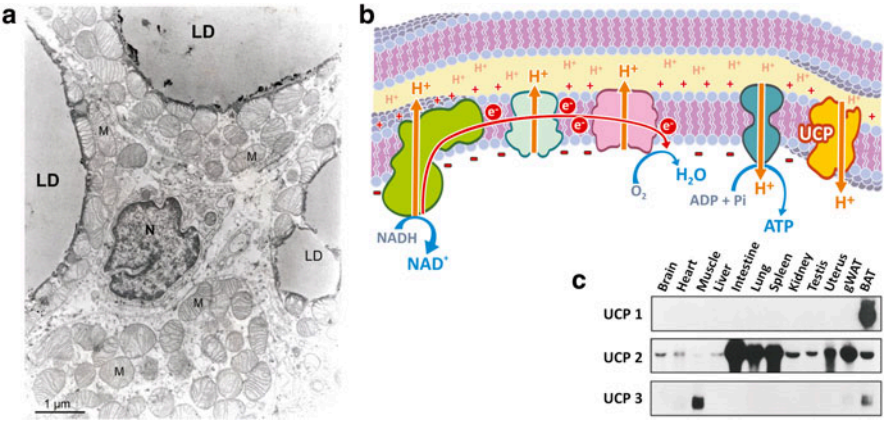


Fig. 6.25 (**a**) Transmission electron micrograph of a BAT adipocyte thin section showing lipid droplets (LD) and a high number of mitochondria (M); N: nucleus. (Courtesy from Prof. Marlene Benchimol). (**b**) The UCPs are integral inner mitochondrial membrane proteins that allow H^+ flow bypassing ATP synthase, dissipating the gradient and accelerating respiration. (**c**) Western blotting showing the expression pattern of the three UCP isoforms in mouse tissues (Reproduced with permission of Portland Press from Ricquier & Bouillaud. Biochem J. 345:161–179, 2000)

In humans, BAT is mostly found in the newborn, regulating thermogenesis through the expression of the UCP1. This protein occurs in abundance in the inner mitochondrial membrane and provides an alternative route, bypassing ATP synthase, for H^+ to return to the mitochondrial matrix, dissipating the gradient and accelerating respiration, resulting in heat production in a regulated manner (Fig. 6.25b). Until very recently, this tissue has been considered to have no physiological relevance to adult humans, but some recent findings suggest that reminiscent BAT cells may proliferate in response to cold exposure (Box 6.7). This effect seems to be more pronounced in lean subjects, suggesting that regulated uncoupled respiration may also be a way to control energy expenditure (see Chap. 11).

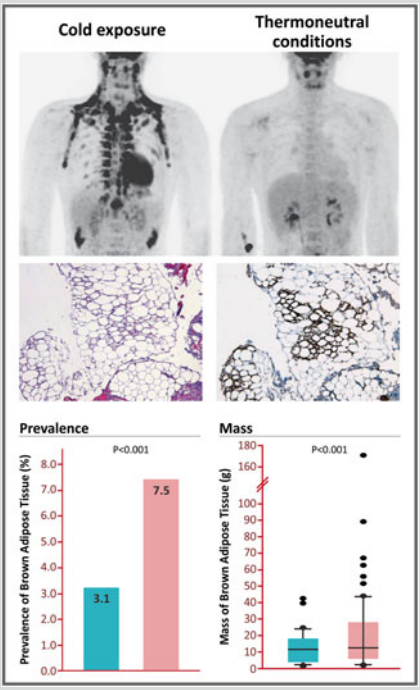
Two other UCP isoforms have been characterized, UCP2 and UCP3. They show a more ubiquitous tissue distribution (Fig. 6.25c) and probably play a role in protecting cells against reactive oxygen species, which may be produced in excess in mitochondria in some situations, such as when an excessive amount of substrates is supplied to the cells (see next section).

Box 6.7: BAT in Adult Humans

Positron-emission tomography combined with computed tomographic (PET–CT) scans, with ^{18}F-fluorodeoxyglucose (^{18}F-FDG) as a tracer, is generally used to diagnose neoplasms and their metastases, since tumor cells present a much higher glucose uptake when compared to other cells (see Box 6.4 about the Warburg effect). In these tests, a high glucose uptake in the supraclavicular tissue is usually observed, sometimes confusing the diagnosis. It was speculated that these highly glycolytic cells in this region would be brown adipose tissue (BAT). Recently, a set of studies was performed to investigate this issue. In some of these studies, healthy volunteers were exposed to 16 °C for 2 h before the tests. Comparative PET–CT scans revealed a great increase in ^{18}F-FDG uptake in the supraclavicular region upon cold exposure (see figure). Furthermore, tissue biopsies were used for immunostaining with UCP1-specific antiserum, confirming the presence of substantial amounts of metabolically active BAT in adult humans. A systematic examination of the presence and distribution of BAT in lean and obese men during exposure to cold temperature showed that BAT activity is inversely correlated to body mass index (BMI). Additionally, analysis of 3640 consecutive PET–CT scans performed for various diagnostic reasons in 1972 patients showed substantial BAT depots in regions extending from the anterior neck to the thorax for 76 of 1013 women (7.5 %) and 30 of 959 men (3.1 %), with also a larger mass in women.

(continued)

Box 6.7 (continued)



Presence of BAT in humans detected by PET-CT scans after exposure to cold (*top left*) and under thermoneutral conditions (*top right*), and histologic images of biopsy specimens showing UCP1 staining (*middle right*), and the quantification of prevalence (*bottom left*) and amount (*bottom right*) of BAT in men (*blue*) and women (*pink*). (Reproduced with permission from Lichtenbelt et al. New Engl. J. Med. 360:1500–1508, 2009, and Cypess et al. New Engl. J. Med. 360:1509–1517, 2009)

6.2.5.2 Production of Reactive Oxygen Species in Mitochondria

In physiological or pathological situations in which the input of electrons into the respiratory chain overcomes their transfer to oxygen, such as in hypoxia, the formation of reactive species of oxygen (ROS) is increased. This occurs because the passage of electrons from complex I to ubiquinone and from ubiquinone to complex III involves the formation of a partially reduced ubiquinone radical ($^{\bullet}Q^{-}$) as an intermediate. When the electron flow through the respiratory chain is impaired, the probability of this radical to react with cellular components before being completely reduced or oxidized greatly increases. $^{\bullet}Q^{-}$ can react with oxygen, generating the superoxide radical ($^{\bullet}O_2^{-}$). This radical is very reactive and its formation may lead to the production of an even more reactive radical, the hydroxyl radical ($^{\bullet}OH$). These

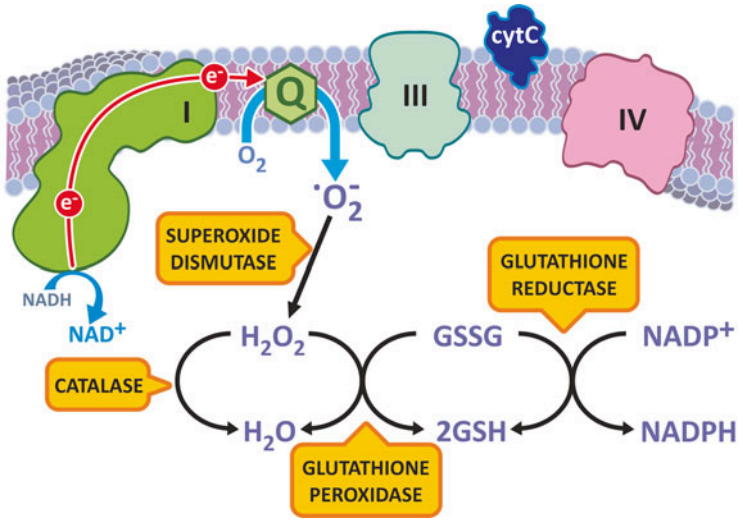


Fig. 6.26 A partially reduced ubiquinone radical ($^{\bullet}Q^{-}$) is formed as an intermediate in the reduction of ubiquinone to ubiquinol by complex I or the oxidation of ubiquinol to ubiquinone by complex III. If $^{\bullet}Q^{-}$ accumulates, it may react with O_2, forming $^{\bullet}O_2^{-}$ and $^{\bullet}OH$. Cells have different enzymatic systems to prevent oxidative damage caused by ROS, including the enzymes superoxide dismutase, glutathione peroxidase and catalase

ROS can react and damage enzymes, lipids, and nucleic acids. They can also alter cellular gene expression, leading to several modifications in cellular functions.

Cells have different enzymatic systems to prevent oxidative damage caused by ROS. This includes the enzyme superoxide dismutase that converts $^{\bullet}O_2^{-}$ in hydrogen peroxide (H_2O_2), which in turn may be used by glutathione peroxidase to reduce glutathione or by catalase to form H_2O (Fig. 6.26).

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Chapter 7
Catabolism of the Major Biomolecules

Catabolism is the group of metabolic pathways in which the chemical energy contained in the nutrient molecules is transferred to other compounds, especially ATP, which in turn provides the energy for different cellular processes such as the synthesis of new biomolecules, ion transport across cellular membranes, and muscle contraction.

In Sect. 6.2, we discussed in detail the mechanism of ATP synthesis through oxidative phosphorylation, starting with the electron transfer from the reduced coenzymes NADH and $FADH_2$ going to the components of the respiratory system. In this chapter we will present the metabolic pathways along which the oxidation of the nutrient molecules results in the reduction of NAD^+ and FAD, generating the reduced coenzymes that feed the electron transport system.

7.1 An Overview of Catabolism

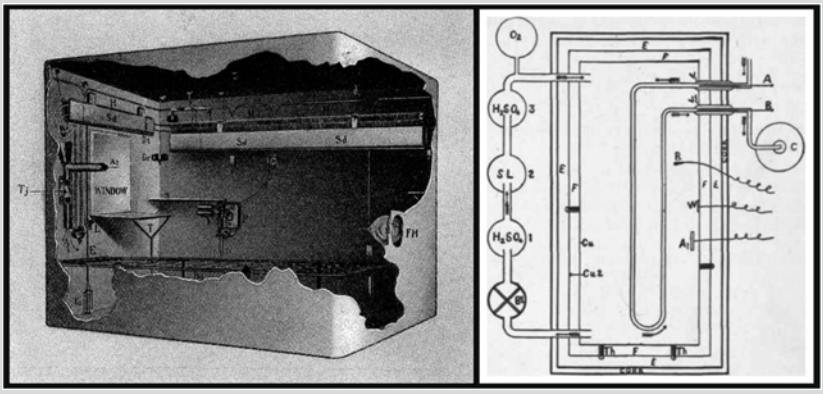
Carbohydrates, lipids, and proteins are the major constituents of food and serve as fuel molecules for the human body. The net energy yielded by the metabolism of each of these nutrients was determined for humans in the end of the nineteenth century, by the pioneer work of Wilbur O. Atwater, who developed a human respiration calorimeter (see Box 7.1) and obtained the values of 4.0, 4.0, and 8.9 kcal/g for carbohydrate, protein, and fat, respectively, values that are still considered valid today.

The main products of carbohydrates, lipids, and protein digestion (monosaccharides, fatty acids, and amino acids, respectively) reach the bloodstream and enter the cells, where they are firstly metabolized through a specific catabolic route that converges to a central pathway in the metabolism, the tricarboxylic acid (TCA) cycle (Fig. 7.1), also referred to as the Krebs cycle (see Sect. 7.2.3).

A.T. Da Poian, M.A.R.B. Castanho, *Integrative Human Biochemistry*,
DOI 10.1007/978-1-4939-3058-6_7

Box 7.1: Wilbur O. Atwater and the Studies on Human Nutrition

Atwater started his scientific career in agricultural chemistry, studying fertilizers, but his major contributions for the advancement of science came when he changed his scientific interest to human nutrition after he spent a period in Carl von Voit's laboratory, in Germany. Atwater developed systematic studies on the composition and the digestibility of foods as well as on the kind of foods people from different ethnic groups in different areas were eating. At that time, part of his interest was focused on finding out how poor people could make more economical choices of food maintaining its nutritional value. In 1893, Atwater's group developed a respiration calorimeter for studies of human metabolism, known as the Atwater–Rosa–Benedict's human calorimeter (see figure). This system allowed them to measure the metabolizable energy contributed by each of the nutrient molecules.



Inside view of the Atwater–Rosa–Benedict's human respiration calorimeter (image from http://vlp.mpiwg-berlin.mpg.de/references?id=lit15620&page=p0213) and the diagrammatic representation of its components (reproduced from the book *The Elements of the Science of Human Nutrition*, by Lusk G, 1917). The studied subject entered a copper chamber with a capacity of 1123 l and lay on a bed, and the chamber was hermetically closed. The air within the chamber passes through three bottles previously weighed containing (1) sulfuric acid, which removes the water; (2) moist soda lime, which removes the CO_2; (3) and sulfuric acid again, which absorbs the moisture taken from the soda lime. The gain in weight of bottle 1 represents water absorbed, and the gain in weight of bottles 2 and 3 equals the CO_2 absorbed. O_2 was automatically fed into the system by cylinder, and O_2 consumption was calculated by weighting the cylinder before and at the end of the experiment

The formation of a molecule of acetyl-coenzyme A (acetyl-CoA) is the convergence point of the degradation pathways of monosaccharides, fatty acids, and some of the amino acids (Fig. 7.1). Then, acetyl-CoA is completely oxidized to CO_2 in the TCA cycle, to which the products of the degradation of other amino acid also converge. In the oxidative reactions of the TCA cycle, electrons are transferred from the TCA cycle intermediates to NAD^+ or FAD, generating NADH and $FADH_2$ that

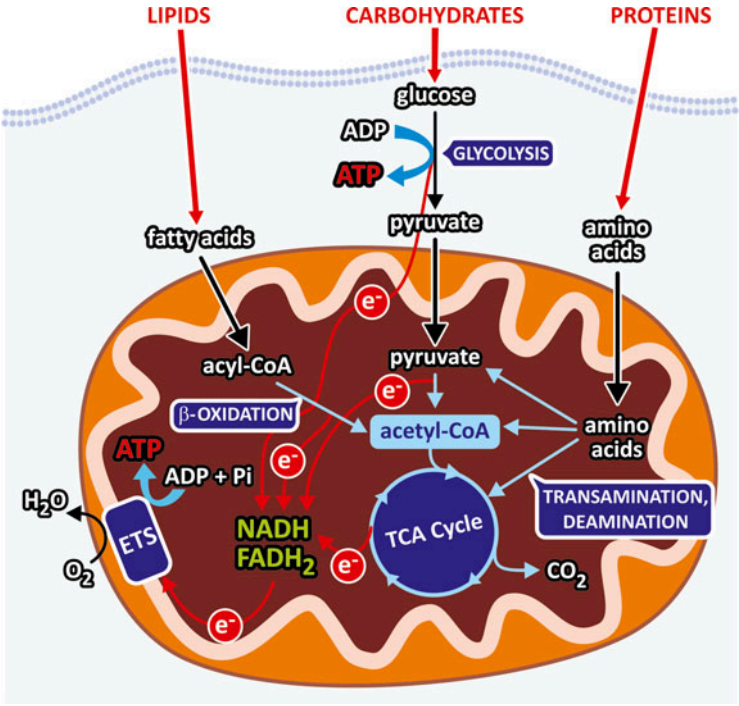


Fig. 7.1 Schematic representation of a generic cell showing the catabolic pathways. Carbohydrate digestion generates monosaccharides, mainly glucose, which is degraded through glycolysis into two pyruvate molecules. In this pathway, an oxidative step is coupled to NAD^+ reduction, generating NADH that feeds the respiratory chain (electron transport system, ETS) with electrons (e^-) that ultimately reduce O_2 to H_2O. Pyruvate is converted to acetyl-CoA in the mitochondrial matrix in a reaction involving an oxidative decarboxylation with concomitant NAD^+ reduction to NADH. Degradation of fatty acids occurs through the pathway known as β-oxidation, which sequentially removes from the fatty acid chain a two-carbon fragment, in the form of acetyl-CoA, with concomitant reduction of FAD and NAD^+. Amino acids, the products of protein hydrolysis, undergo transamination or deamination reactions that remove their amino group and generate intermediates of the central metabolic pathway. The circles in the electron representation are used to indicate that they are not free in the cellular medium but are transferred by the compounds of each pathway

in turn will transfer the electrons to O_2 through the respiratory system (see Sect. 6.2.2). Thus, in the overall process, O_2 is consumed and CO_2 is produced. This process is called cellular respiration, in analogy to the physiological term respiration, which is attributed to the gas exchanges that occur in the lungs.

The specific pathways of fatty acids and glucose degradation, the β-oxidation and glycolysis, respectively, also have oxidative steps in which FAD and/or NAD^+ are reduced (Fig. 7.1). On the other hand, the reactions of amino acid metabolism that generates the intermediates of the central metabolic pathway consist basically in transamination or deamination reactions that remove the amino group from the amino acid molecules, followed in some cases by carbon skeleton rearrangements.

In this chapter we will first focus on the central pathway of acetyl-CoA oxidation, the TCA cycle, to which all the nutrient degradation products ultimately converge to feed the electron transport system. Then, we will describe each specific pathway for the degradation of monosaccharides, fatty acids, and amino acids.

It is important to bear in mind that the use of each nutrient molecule as a source of energy for ATP synthesis depends on the metabolic status (Fig. 7.2). For example,

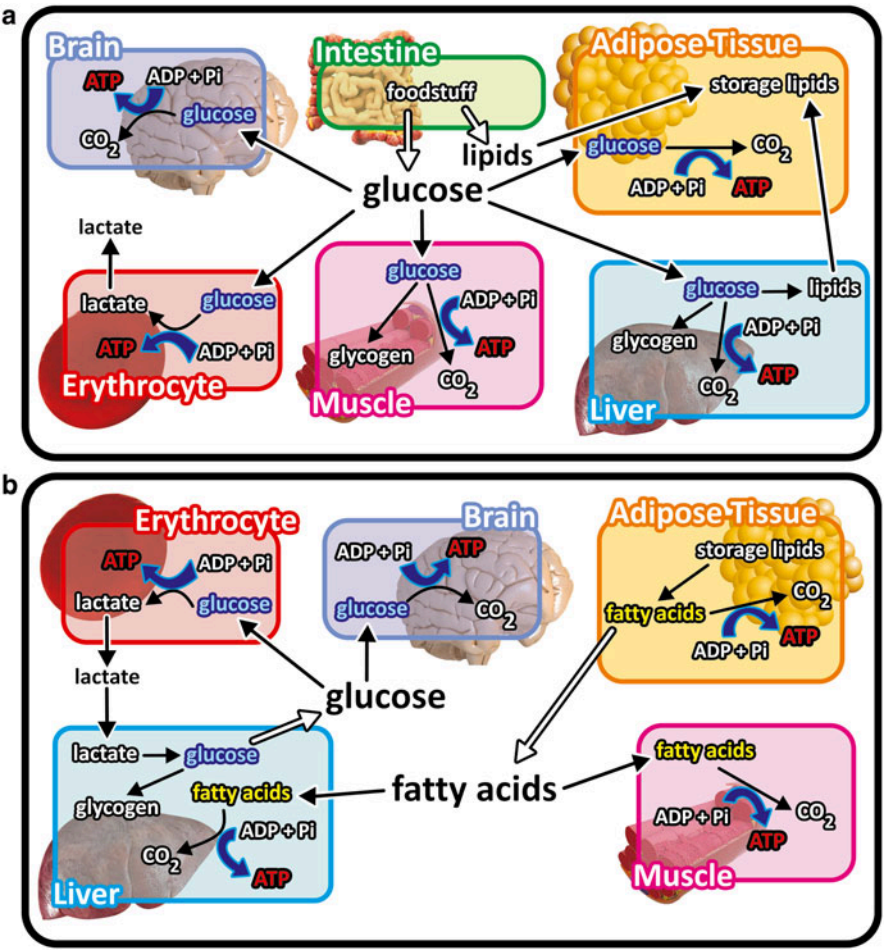


Fig. 7.2 Overview of the integration of metabolism in different metabolic situations emphasizing the catabolism of the different nutrient molecules. (**a**) After a balanced meal, carbohydrate catabolism is the main pathway for ATP synthesis in virtually all the cells in the body. Ingested lipids are stored in the adipose tissue. The excess of glucose is stored as glycogen in muscle and liver cells or converted into lipids in the liver and in the adipose tissue. (**b**) When glucose concentration decreases in the blood, fatty acids are mobilized from the adipose tissue, becoming the main energy source for most of the tissues. Cells lacking mitochondria, as erythrocytes, or isolated from systemic circulation, as those of the central nervous system, are exceptions and maintain glucose catabolism in this situation

after a balanced meal, the increase in blood glucose concentration triggers a series of regulatory events that select the carbohydrates as the main class of nutrients to be used as energy source, while fats are stored in the adipose tissue (see Chap. 8). In contrast, during the intervals between the meals or during fasting or low-carbohydrate diets, fatty acids are mobilized from the adipose tissue and become the major source of energy for most of the tissues in the body (see Chap. 9).

7.2 Tricarboxylic Acid Cycle: The Central Pathway for the Oxidation of the Three Classes of Nutrient Molecules

In aerobic metabolism, the products of nutrients' degradation converge to the TCA cycle, a central pathway consisting in eight reactions (Fig. 7.3). In this pathway, the acetyl group of acetyl-CoA resulting from the catabolism of monosaccharides, fatty acids, or amino acids, is completely oxidized to CO_2 with concomitant reduction of the electron-transporting coenzymes (NADH and $FADH_2$).

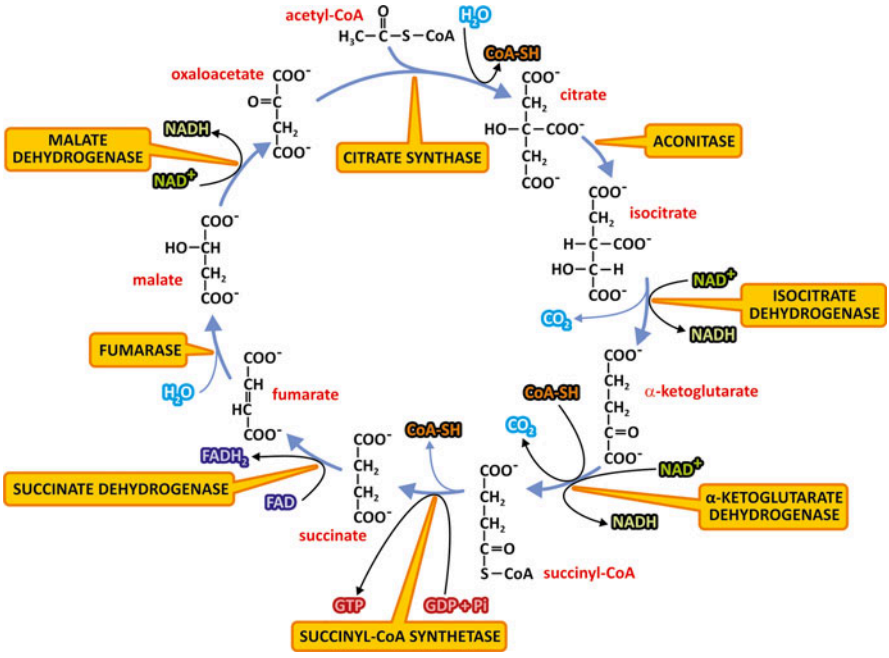


Fig. 7.3 Reactions of the TCA cycle. The NAD^+ or FAD molecules reduced to NADH or $FADH_2$ are indicated at the respective oxidation reactions, as well as the CO_2 released at the decarboxylation reactions. The GTP molecule synthesized by substrate-level phosphorylation is also shown. The names of the enzymes are highlighted in *yellow boxes*. Remind that succinate dehydrogenase is the Complex II of the electron transport system

7.2.1 TCA Cycle Reactions

We start the description of TCA cycle reactions with the condensation of acetyl-CoA and oxaloacetate, which generates citrate (Fig. 7.3). The next seven steps, which regenerate oxaloacetate, include four oxidation reactions in which NAD^+ or FAD are reduced to NADH or $FADH_2$, whose electrons will be then transferred to O_2 in the respiratory chain. Additionally, a GTP or an ATP molecule is directly formed by the mechanism of substrate-level phosphorylation (see Sect. 6.1).

The condensation of the two-carbon acetyl group of acetyl-CoA with the four-carbon molecule oxaloacetate, forming citrate, a six-carbon molecule, is catalyzed by the enzyme citrate synthase. A thioester intermediate, citroyl-CoA, is formed in the active site of the enzyme, and its hydrolysis makes the reaction highly exergonic. The coenzyme A released in this reaction may be recycled in the conversion of pyruvate to acetyl-CoA by pyruvate dehydrogenase complex (see Sect. 7.3).

Citrate is then transformed to isocitrate, in a reaction catalyzed by the enzyme aconitase.

The next reaction is an oxidative decarboxylation catalyzed by isocitrate dehydrogenase, which converts the six-carbon compound isocitrate in the five-carbon molecule α-ketoglutarate, with the release of CO_2 and reduction of an NAD^+ molecule.

The next step is also an oxidative decarboxylation, the conversion of α-ketoglutarate in the four-carbon molecule succinyl-CoA, catalyzed by the enzyme complex α-ketoglutarate dehydrogenase. In this reaction, CO_2 is released, and NAD^+ works as the electron acceptor, with the subsequent binding of coenzyme A through a thioester bond forming succinyl-CoA. It is interesting to notice that α-ketoglutarate dehydrogenase is structurally similar to the pyruvate dehydrogenase complex, the enzyme complex that catalyzes the conversion of pyruvate into acetyl-CoA (see Sect. 7.3.1).

Succinyl-CoA is then converted to succinate by succinyl-CoA synthetase. In this reaction, the hydrolysis of the thioester bond of succinyl-CoA with concomitant enzyme phosphorylation is coupled to the transfer of a phosphate group bound to the enzyme to GDP or ADP through the mechanism of substrate-level phosphorylation.

Succinate dehydrogenase oxidizes succinate to fumarate. This enzyme is the Complex II of the respiratory system (see Sect. 6.2), a FAD-linked enzyme through which the electrons from succinate enter in the respiratory chain.

Then, fumarate is hydrated to form malate by the enzyme fumarase.

Finally, oxaloacetate is regenerated by the oxidation of malate catalyzed by malate dehydrogenase, with concomitant reduction of NAD^+.

It is important to point out that although the O_2 does not participate directly in the TCA cycle, the cycle only operates in aerobic conditions since the oxidized NAD^+ and FAD are regenerated in the respiratory chain.

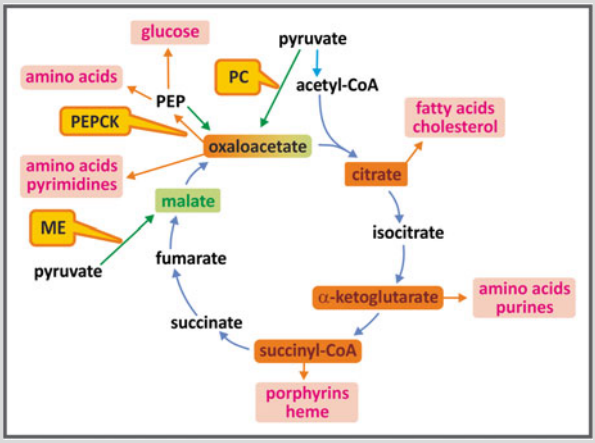
7.2.2 TCA Cycle as a Dynamic Pathway

The TCA cycle is not a pathway that is only fed by acetyl-CoA. Likewise, CO_2 is not its unique end product. Different four- and five-carbon metabolic intermediates

Box 7.2: The Anabolic Role of the TCA Cycle

Many TCA cycle intermediates may be used as precursors for biosynthetic pathways (see figure). Citrate leaves mitochondria yielding acetyl-CoA for the synthesis of fatty acids and other lipids (see Sect. 8.3). α-ketoglutarate and oxaloacetate are the precursors of several amino acids. The synthesis of porphyrins starts with succinyl-CoA. Oxaloacetate generates glucose through the pathway of gluconeogenesis (see Sect. 9.3.1) and is also the precursor of purines and pyrimidines that form the nucleotides. It is important to mention that when the TCA cycle intermediates are withdrawn for biosynthetic processes, a replacement mechanism should operate to avoid impairment of the oxidative metabolism. This occurs through replenishing reactions, known as anaplerotic reactions, in which pyruvate or phosphoenolpyruvate (PEP) are carboxylated, generating oxaloacetate or malate (see figure).

The most important of these reactions is the one catalyzed by pyruvate carboxylase (see Sect. 9.3.1). This reaction is highly regulated by acetyl-CoA, so that when this molecule accumulates, indicating that cycle intermediates are not sufficient to perform its complete oxidation, pyruvate is shifted toward the formation of oxaloacetate, increasing the capacity of the TCA cycle to oxidize acetyl-CoA. Pyruvate may also be converted to malate by the malic enzyme. The other anaplerotic reaction is that catalyzed by PEP carboxykinase, which generates oxaloacetate from PEP with the transfer of the phosphate from PEP to GDP, forming also a GTP molecule. This reaction occurs in the opposite direction in the gluconeogenesis pathway (see Sect. 9.3.1).



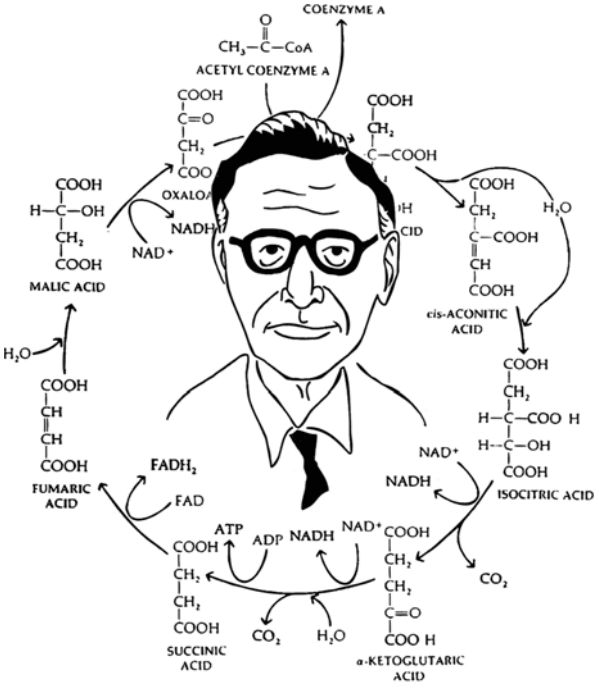
TCA cycle intermediates shown in orange boxes may be used as precursors for the synthesis of fatty acids, cholesterol and steroids, amino acids, purines and pyrimidines, porphyrins, and heme. Anaplerotic reactions (shown by green arrows), catalyzed by the enzymes pyruvate carboxylase (PC), phosphoenolpyruvate carboxykinase (PEPCK), or malic enzyme (ME), replenish the cycle, maintaining its oxidative capacity

may enter the cycle at different points to be oxidized, as occurs, for instance, with the amino acid glutamate after being converted to α-ketoglutarate by a transamination reaction (for details, see Sect. 7.5). The same occurs with the other amino acids, which are also converted in TCA cycle intermediates.

Besides receiving metabolic intermediates to be oxidized, the TCA cycle has also an anabolic role in metabolism, since many of its intermediates may be withdrawn from the cycle to be used as the precursors of biosynthetic processes (see Box 7.2). Due to its role in both catabolism and anabolism, the TCA cycle is considered an amphibolic pathway.

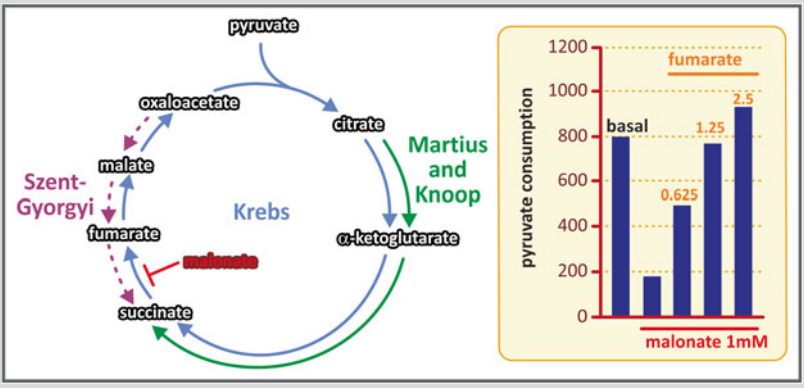
7.2.3 A Historical Overview of the TCA Cycle Discovery

The TCA cycle is also known as the "Krebs cycle" in recognition of the contribution of Sir Hans Krebs to its discovery (see Box 7.3). As stated in Krebs obituary, written by J. R. Quayle, "his life's work, published in over 360 publications, spanned a whole era of biochemistry, namely, the elucidation of the major part of central intermediary metabolism and its regulation … Perhaps his greatest contribution to biological sciences as a whole was his discovery of the concept of metabolic cycles, how to recognize them, how to establish them unequivocally, and how to delineate and to assess their physiological function."



Box 7.3: Sir Hans Krebs and the Discovery of the TCA Cycle

Sir Hans Krebs was awarded the Nobel Prize in Physiology or Medicine in 1953, for the discovery of the TCA cycle. The prize was shared with Fritz Lipmann, who discovered the coenzyme A and its importance for the intermediary metabolism (see Box 7.4). Krebs began the studies that led him to the discovery of the TCA cycle working with Otto Warburg (see Box 6.4), who introduced to him the technique of using tissue slices for metabolic studies. This experimental procedure allowed working with intact cells in controlled media with free gas exchange between cells and the medium, an approach only possible before for microbiological studies. Using this technique, Krebs could propose for the first time a cycled pathway in the intermediate metabolism, the urea cycle (see Sect. 7.5). In the case of the TCA cycle, Krebs based his proposal on several observations carried out during the decade of 1930. In 1935, Albert Szent-Gyorgyi discovered the sequence of reactions from succinate to fumarate to malate to oxaloacetate (see figure), showing that these dicarboxylic acids present in animal tissues stimulate the O_2 consumption. Two years later, Carl Martius and Franz Knoop found the sequence from citrate to α-ketoglutarate to succinate. Krebs integrated these findings adding his own observations about the effect of the addition of tricarboxylic acids to slices of pigeon breast muscle. He found that these acids even in very low concentrations promoted the oxidation of a much higher amount of pyruvate in the muscle slices, suggesting a catalytic effect of these compounds. He also observed that malonate, an inhibitor of succinate dehydrogenase, inhibited the oxidation of pyruvate and that the addition of fumarate to the medium in this condition restored pyruvate consumption (see figure). Assembling these information, Krebs elegantly showed the cyclic nature of the pathway.



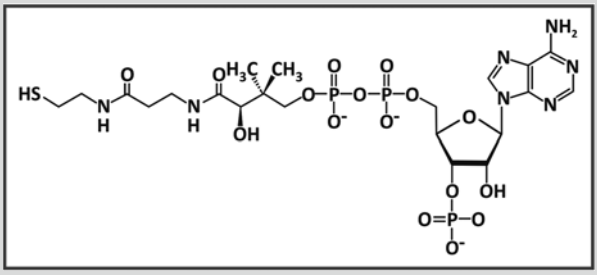
Sequence of reactions described by Szent-Gyorgyi (*purple*), Martius and Knoop (*green*), and Krebs (*blue*). The graph in the *right* shows the results of the crucial experiment done by Krebs that proved the cycled nature of the reactions for pyruvate oxidation: the inhibition of pyruvate consumption by the addition of malonate (an inhibitor of the enzyme succinate dehydrogenase) was abolished by fumarate addition to the system

When Krebs proposed the TCA cycle, in 1937, he thought that citrate was synthesized from oxaloacetate and pyruvate (see Box 7.3). Thus, the TCA cycle was seen as a pathway that serves only for carbohydrate oxidation, since pyruvate was known to be the product of carbohydrate metabolization (see Chap. 6). The importance of the TCA cycle in the oxidation of other compounds, such as the fatty acids, was only realized after three important discoveries in the middle of the twentieth century: (a) the isolation of coenzyme A (CoA), by Fritz Lipmann, in 1945 (see Box 7.4); (b) the isolation of acetyl-CoA by Feodor Lynen, in 1951; and (c) the subsequent work by Joseph Stern, Severo Ochoa, and Lynen, showing that the reaction between acetyl-CoA and oxaloacetate forming citrate was accompanied by the generation of stoichiometric amounts of sulfhydryl groups, indicating that acetyl-CoA was the molecule that donated the acetyl group to oxaloacetate.

Box 7.4: Fritz Lipmann and the Discovery of Coenzyme A

The discovery of coenzyme A led Fritz Lipmann to be awarded the Nobel Prize in Physiology or Medicine in 1953. Lipmann dedicated many years of his scientific career working on the process of ATP synthesis. In 1941, he published a landmark paper in which he introduced the term "energy-rich phosphate bond" and established the basis for the understanding of the substrate-level phosphorylation mechanism of ATP synthesis (see Chap. 6). As a follow-up to these studies, Lipmann got interested in the capacity of some molecules to transfer acetyl groups, which he thought would be an important step in some biosynthetic processes. In the search for this "activated acetate," Lipmann found a dialyzable, heat-stable factor present in all tissues he studied, which could not be replaced by any other known cofactor.

He purified and identified this new coenzyme and named it coenzyme A, in which "A" is related to the activation of acetate. Coenzyme A is composed of a phosphoadenosine diphosphate linked through a phosphoester bond to a pantothenic acid, which is in turn bound through an amide linkage to a β-mercaptoethylamine (see figure).



Since acetyl-CoA is the product of the oxidation of pyruvate (and carbohydrates) (see Sect. 7.3) and fatty acids (see Sect. 7.4), as well as being it generated in the metabolization of some amino acids (see Sect. 7.5), one can recognize acetyl-CoA as the convergence point of the oxidative metabolism (see Fig. 7.1).

7.2.4 Regulation of the TCA Cycle

The rate of the TCA cycle is basically regulated by the flux of the dehydrogenases—isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinate dehydrogenase, and malate dehydrogenase. The activities of the dehydrogenases, in turn, are determined by the mitochondrial ratios $NADH/NAD^+$ and $FADH_2/FAD$, which depend on the ATP/ADP ratio. Therefore, when the concentration of ATP increases, the electron flow through the electron transport chain slows and the $NADH/NAD^+$ and $FADH_2/FAD$ ratios increase, leading to a decrease in TCA cycle rate. On the other hand, when ATP is used, the increase in ADP levels allows the electron transport, increasing NADH and $FADH_2$ oxidation and then enhancing TCA cycle rate.

7.3 Catabolism of Carbohydrates

Human diet contains different carbohydrates, including polysaccharides, such as starch and glycogen; disaccharides, such as sucrose, lactose, and maltose; and monosaccharides, such as glucose and fructose. Polysaccharides and disaccharides are broken in the digestive tract to their respective monosaccharides, which together with the free monosaccharides present in the diet reach the bloodstream and enter the cells. Inside the cells, monosaccharides are converted to a phosphorylated derivative that is degraded in two pyruvate molecules through a metabolic pathway named glycolysis (Fig. 7.4).

It is important to mention that besides being the pathway for carbohydrate degradation in aerobiosis, glycolysis also affords ATP synthesis in the absence of oxygen (see Chap. 6).

7.3.1 Carbohydrate Oxidation Reactions

Glycolysis itself is composed of ten reactions, which are presented in detail in Sect. 6.1.3. Thus, in this section we will focus our attention in the conversion of pyruvate to acetyl-CoA, the connecting point between glycolysis and the TCA cycle.

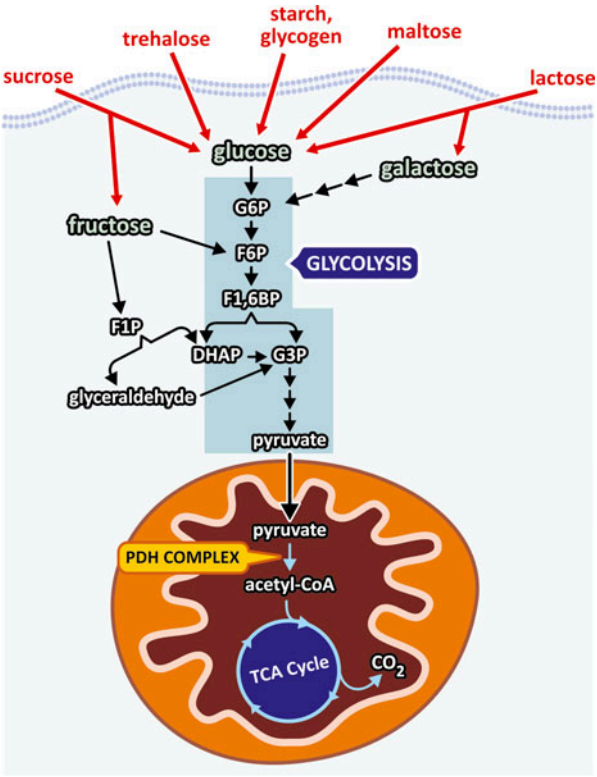


Fig. 7.4 Dietary carbohydrates are digested and reach the bloodstream mainly as monosaccharides that enter the cells where they are phosphorylated and degraded to pyruvate through the pathway named glycolysis. Pyruvate is transported into the mitochondria where it is converted to acetyl-CoA, which in turn is completely oxidized in the TCA cycle. G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; F1,6BP, fructose-1,6-bisphosphate; F1P, fructose-1-phosphate; G3P, glyceraldehyde-3-phosphate; DHAP dihydroxyacetone phosphate

Pyruvate molecules, generated in the cytosol as the product of either glycolysis or the degradation of some amino acids, need to be transported to the mitochondrial matrix where it is converted to acetyl-CoA (Fig. 7.4).

The oxidative decarboxylation of pyruvate to form acetyl-CoA is catalyzed by one of the largest cellular multienzyme complexes known, the pyruvate dehydrogenase complex (PDH), with about 50 nm in diameter and a molecular mass of almost 10 MDa (Fig. 7.5a). Eukaryotic PDH is composed of multiple copies of four distinct proteins, three of them presenting enzymatic activity, E1 (pyruvate decarboxylase), E2 (dihydrolipoyl acetyltransferase), and E3 (dihydrolipoyl dehydrogenase) and the fourth, E3-binding protein (E3BP), which mediates E3

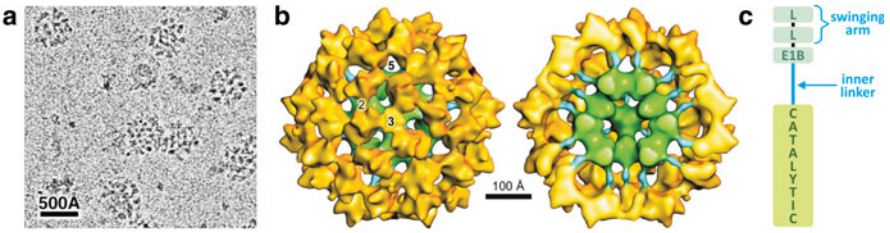


Fig. 7.5 (**a**) Cryoelectron microscopy of bovine PDH showing the multienzyme complex with ~500 Å. (**b**) Reconstructed structure of the reconstituted complex and its half section (*right*) showing the dodecahedral core formed by E2 catalytic domains (*green*) and E2 inner linker (*blue*) that connects the core to the E1–E3 outer shell (*yellow*). (**c**) Diagrammatic representation of E2 four structural domains connected by linkers: the catalytic domain, the E1-binding domain (E1B), and the two lypoil domains (L) that form the "swinging arm." (**a**) and (**b**): reproduced with permission from Zhou et al. Proc. Natl. Acad. Sci. USA 98:14802–14807, 2001

integration in the complex. Additionally, regulatory kinases and phosphatases are also associated to PDH complex.

The core of the PDH complex is formed by 60 E2 subunits arranged as a pentagonal dodecahedron of 20 E2 trimers, which acts as a scaffold for the complex organization (Fig. 7.5b). Human E2 contains four distinct domains connected by linkers (Fig. 7.5c). The C-terminal half comprises the catalytic domain, which self-associates forming the organized core. The N-terminal half is extended, generating an annular gap between the core and the E2E3-outer shell. It contains, besides the E1-binding domain, a "swinging arm" in which two highly flexible lipoyl domains allow an efficient transfer of the reaction intermediates between the active sites. Human E3BP structural organization is very similar to that of E2, and it is suggested that its addition to the core structure replaces part of the E2 subunits generating a final core structure containing up to 48 E2 and about 12 E3BP subunits. To this core, it is estimated that 20–30 $\alpha_2\beta_2$ E1 tetramers and 6–12 E3 homodimers are tightly bound.

The reaction catalyzed by PDH complex consists of five steps in which the intermediates remain bound to the enzyme components (Fig. 7.6). Firstly E1 catalyzes the thiamine diphosphate (TPP)-dependent decarboxylation of pyruvate with release of CO_2 and formation of a hydroxyethyl derivative bound to TPP. This is followed by the transfer of two electrons and the remaining acetyl group to the oxidized lipoyl group linked to the lipoyl domain of E2, a reaction also catalyzed by E1. The flexible linker that connects the lipoyl domain in E2 allows the movement of the acetyl group to the catalytic site of E2, where its transfer to CoA is catalyzed, generating acetyl-CoA that is released from the enzyme. Then, the lipoyl group, which is reduced at this point, moves to the E3 catalytic site where it is oxidized with the reduction of the enzyme-bound FAD. Finally, the electrons are transferred from E3-bound $FADH_2$ to NAD^+, forming NADH. The flexible lipoyl domains allow the reaction intermediates to be channeled through the catalytic sites during the catalytic cycle, in what is called "swinging-arm mechanism" (Fig. 7.6).

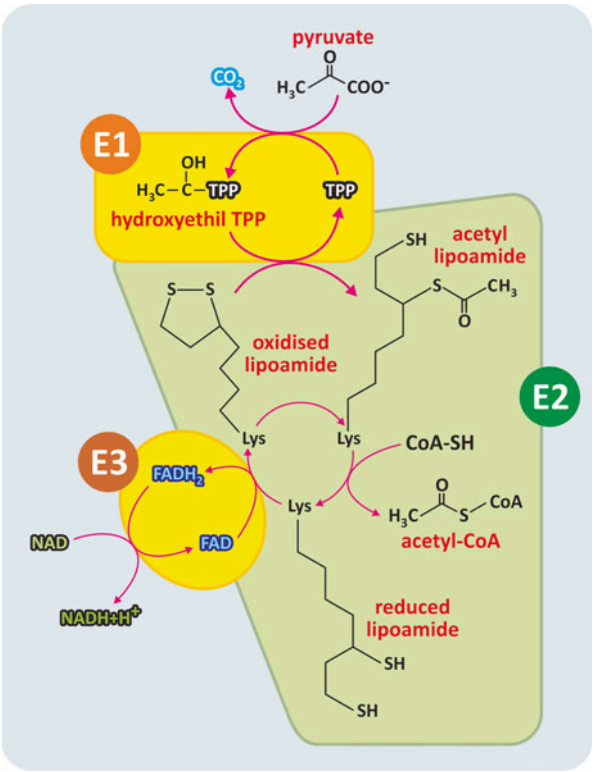


Fig. 7.6 Schematic representation of the catalytic cycle of PDH complex. Step (1) consists of the TPP-dependent decarboxylation of pyruvate, catalyzed by E1, in which CO_2 is released and a hydroxyethyl derivative remains bound to TPP. In the step (2), also catalyzed by E1, two electrons reduce the lipoyl group bound to E2, and the remaining acetyl group is transferred to one of the reduced sulfhydryl groups. In step (3) the acetyl group is transferred to the coenzyme A, forming acetyl-CoA. In step (4) the reduced sulfhydryl transferred the electrons to E3-bound FAD. In step (5) the electrons are transferred from $FADH_2$ to NAD^+, generating NADH

7.3.2 Regulation of Pyruvate Conversion to Acetyl-CoA

The PDH complex is mainly regulated by phosphorylation and dephosphorylation, catalyzed by pyruvate dehydrogenase kinase (PDK) and pyruvate dehydrogenase phosphatase (PDP), respectively. Both enzymes are bound to the lipoyl domain of E2 in PDH complex.

Three phosphorylation sites were identified in the human enzyme, all of them located in E1: site 1 in Ser264, site 2 in Ser271, and site 3 in Ser203. Phosphorylation leads to enzyme inactivation, being the phosphorylation of site 1 the one that causes the major effect.

Four PDK isoforms with tissue-specific expression have been identified. PDK1 is mostly expressed in heart, PDK2 in most tissues, PDK3 in testis, and PDK4 in heart and skeletal muscles. Two PDP isoforms, PDP1 and PDP2, both requiring Mg^{2+} for their activity, have been identified.

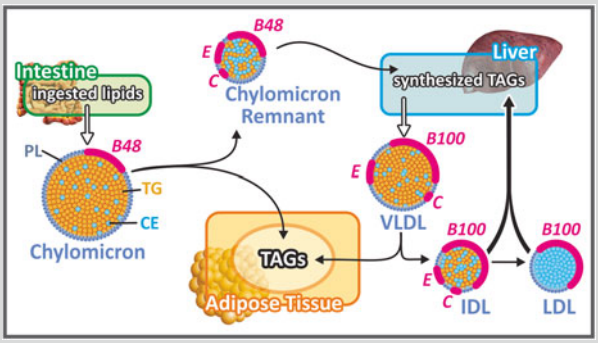
7.4 Catabolism of Lipids

Fatty acids are the main source of energy for most of human cells. They are stored in the adipose tissue as triacylglycerol (TAG), a molecule formed by one glycerol esterified to three fatty acid molecules. TAGs may be obtained from the diet or can be synthesized from carbohydrates and accumulate in adipocytes after being transported from the intestine or from the liver by specialized lipoproteins (see Box 7.5).

Box 7.5: Lipoproteins and Lipid Transport in the Body

The transport of lipids in the body fluids needs special requirements due to their chemical properties (see Chap. 3). Most lipids are insoluble in aqueous media and would form drops when circulating, causing fat embolisms. Other lipids have amphipathic properties that would affect cellular membrane integrity. These problems can be overcome because lipids circulate associated to lipoprotein complexes.

The lipoproteins differ in their size and composition according to their origin (for details on the different types of lipoproteins, see Sect. 3.1.2). Lipids obtained from the diet are incorporated in lipoproteins named chylomicrons, which are released in the lymphatic system to reach the blood. The chylomicrons transport TAGs mainly to the adipose tissue, where they are stored in lipid droplets (see figure). The excess of carbohydrates is converted to lipids in the liver and in the adipose tissue itself (see Chap. 8). TAGs synthesized in the liver are transported to the adipose tissue associated to the very-low-density lipoprotein (VLDL).



Transport of lipids in the body fluids through different lipoproteins. Lipoproteins contain a core of neutral lipids, mainly triacylglycerols and cholesterol esters (TG and CE, represented as yellow and blue circles, respectively), surrounded by a phospholipid (PL) monolayer to which different proteins are associated (represented as the pink structures—apolipoproteins are named with letters, sometimes followed by numbers that correspond to their molecular mass)

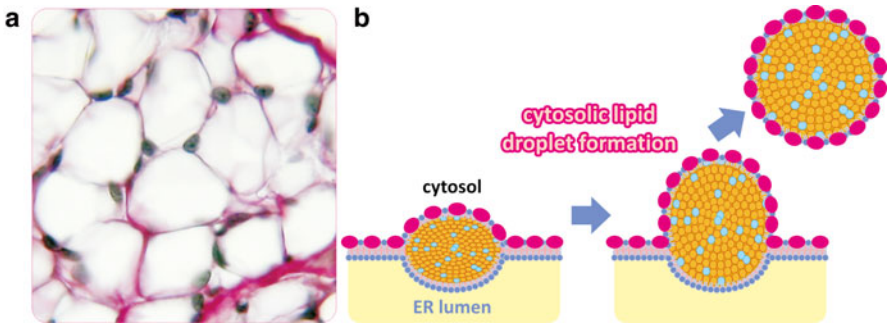


Fig. 7.7 (**a**) Section of white adipose tissue showing the adipocytes with a large lipid droplet inside (Reproduced with permission of Instituto de Histologia e Biologia do Desenvolvimento, Faculdade de Medicina, Universidade de Lisboa, FMUL). (**b**) Model of lipid droplet formation. Synthesized neutral lipids accumulate between the leaflets of the endoplasmic reticulum membrane until the lipid droplet buds as an independent organelle limited by a phospholipid monolayer with associated proteins among which the most abundant are the members of the PAT (perilipin, ADRP, and TIP47) proteins

In the adipocytes, TAGs accumulate in a large lipid droplet that occupies most of the cell volume (Fig. 7.7a). This organelle buds from the endoplasmic reticulum (ER) after accumulation of lipids in between ER membrane leaflets. Lipid droplets consist in a hydrophobic core containing mainly neutral lipids, as TAGs and cholesterol esters, surrounded by a phospholipid monolayer with associated proteins (Fig. 7.7b). The most abundant protein in the lipid droplets from adipocytes is named perilipin, which, besides its structural function, has a central role in the activation of lipolysis (see next section).

7.4.1 TAG Mobilization and Fatty Acid Transport in the Bloodstream

Hormones, mainly catecholamines, and probably also glucagon, secreted in response of hypoglycemia or other stress situations, trigger a signaling pathway in the adipocytes that culminates with the activation of the protein kinase A (PKA) (see Chaps. 9 and 10).

PKA phosphorylates two adipocyte proteins essential for TAG mobilization, perilipin, the major protein on the surface of adipocyte lipid droplets, and the enzyme hormone-sensitive lipase (HSL), which catalyzes the hydrolysis of the ester linkages of the TAG molecules. Phosphorylated perilipin recruits HSL to the surface of the lipid droplet, coordinating the access of HSL to TAG molecules. HSL in turn becomes active upon phosphorylation, converting TAGs to glycerol and fatty acids, which are released in the bloodstream (Fig. 7.8). Fatty acids circulate associated to serum albumin, reaching different tissues in the body where they are used as energy source.

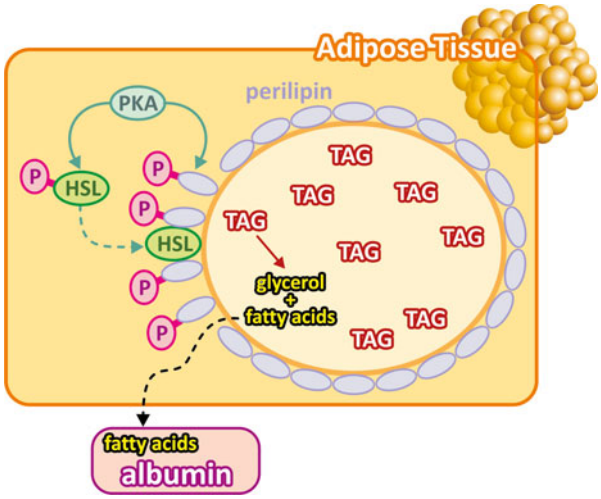


Fig. 7.8 TAG mobilization in adipocyte. PKA, activated by hormone signaling, phosphorylates the enzyme HSL and the LD surface protein perilipin. Phosphorylation activates HSL and induces a conformational rearrangement in perilipin that allows access of HSL to TAG molecules inside the LDs. HSL catalyzes the hydrolysis of TAG in glycerol and fatty acids, which are released to the bloodstream. Fatty acids circulate associated to serum albumin. The "P" symbol in a *pink circle* indicates the phosphate group

7.4.2 Activation of Fatty Acids

The oxidation of fatty acids occurs inside the mitochondria. To be transported to the mitochondrial matrix, long-chain fatty acids should be activated in an ATP-dependent reaction in which their carboxyl group is linked to the thiol group of the coenzyme A through a thioester bond forming acyl-CoA, in a reaction catalyzed by the acyl-CoA synthetases. In this reaction, ATP is cleaved forming AMP and pyrophosphate. Pyrophosphate is in turn promptly hydrolyzed by the action of the inorganic pyrophosphatase, yielding two phosphate molecules. The AMP is temporally linked to the fatty acyl group, which is then transferred to coenzyme A (Fig. 7.9).

Acyl-CoA synthetases consist in a family of isozymes that differ in their subcellular localization and specificity for fatty acids with different chain length. The long-chain acyl-CoA synthetase is associated to the outer mitochondrial membrane, to peroxisomes, and to ER membrane. It acts efficiently for 10- to 20-carbon saturated fatty acids or 16- to 20-carbon unsaturated fatty acids, which are the most common fatty acids used as energy source by the cells. Fatty acids with less than ten carbon atoms cross freely the mitochondrial membrane and become activated in the mitochondrial matrix by the short- or medium-chain acyl-CoA synthetases.

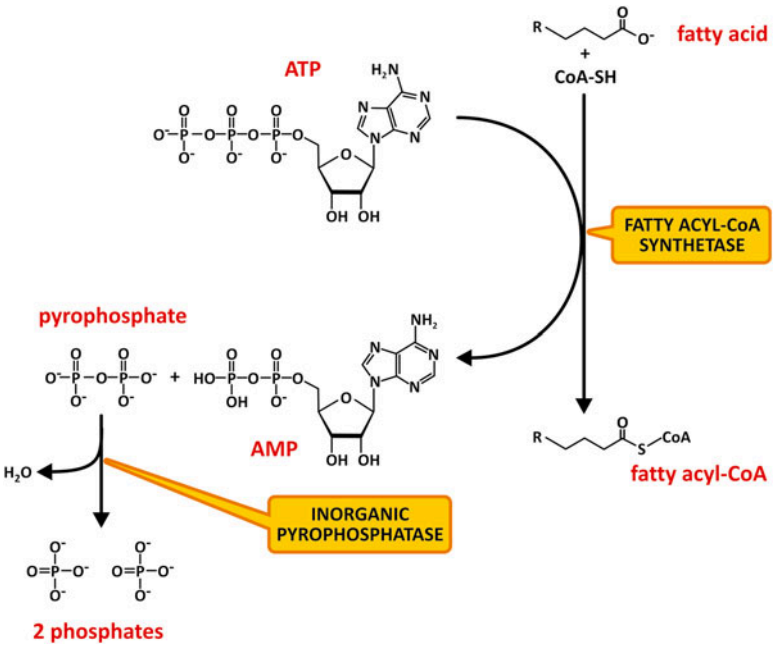


Fig. 7.9 Activation of fatty acids through the formation of acyl-CoA. Acyl-CoA synthetase links the carboxyl group of the fatty acid molecule to the thiol group of the coenzyme A, in a reaction dependent on ATP hydrolysis in AMP and PPi with an intermediate step in which the fatty acyl group in bound to AMP. PPi is hydrolyzed by the enzyme inorganic pyrophosphatase, driving the entire process toward fatty acyl-CoA formation

7.4.3 Fatty Acid Transport into Mitochondria

The fatty acyl-CoA formed in the cytosol cannot be directly transported into the mitochondrial matrix since the inner mitochondrial membrane is impermeable to coenzyme A. Thus, to be transported, the fatty acyl group is firstly transferred from coenzyme A to carnitine (Fig. 7.10a). This reaction is catalyzed by an enzyme associated to outer face of the outer mitochondrial membrane named carnitine/palmitoyl transferase I (CPT-I), which generates acyl-carnitine and free coenzyme A in the cytosol (Fig. 7.10b).

The acyl-carnitine crosses the inner mitochondrial membrane through the carnitine-acyl-carnitine transporter, a transmembrane protein that exchanges acyl-carnitine for carnitine (Fig. 7.10b).

Finally, the acyl group of the acyl-carnitine is transferred to a mitochondrial coenzyme A, in a reaction catalyzed by an enzyme located in the inner side of the inner mitochondrial membrane, carnitine/palmitoyl transferase II (CPT-II) (Fig. 7.10b).

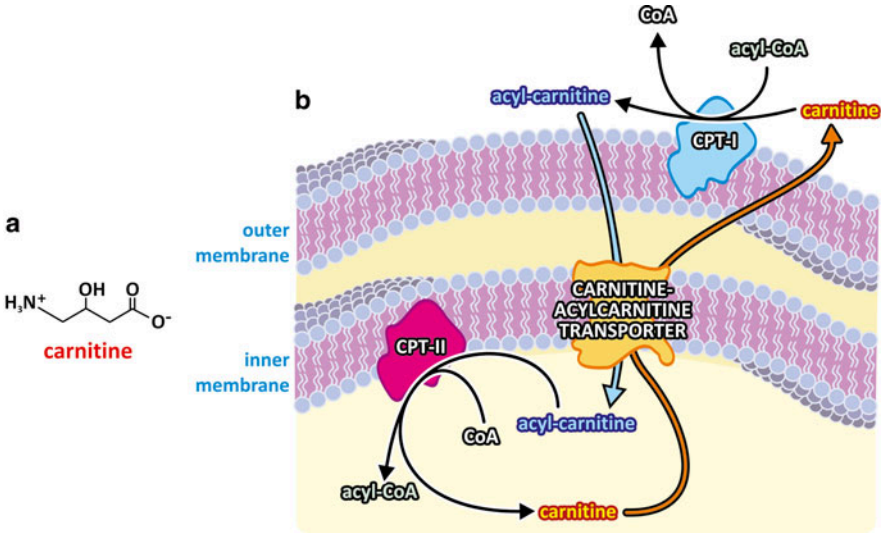


Fig. 7.10 (**a**) Carnitine molecular structure. (**b**) Schematic representation of the fatty acyl-CoA transport across the mitochondrial membranes. CPT-I catalyzes the transfer of the acyl group from acyl-CoA to carnitine, generating acyl-carnitine and free coenzyme A in the cytosol. The carnitine-acyl-carnitine transporter in the inner mitochondrial membrane exchanges the acyl-carnitine with free carnitine, generated in the mitochondrial matrix by the action of CPT-II, which catalyzes the transfer of the acyl group of the acyl-carnitine to coenzyme A, regenerating the acyl-CoA in the matrix

7.4.4 β-Oxidation: The Pathway for Fatty Acid Degradation

The main pathway of fatty acid oxidation is known as β-oxidation, a process in which two-carbon units are progressively removed from the carboxyl end of the fatty acid molecule. For the oxidation of saturated fatty acids, the process consists in four reactions that generate acetyl-CoA and the acyl-CoA molecule shortened by two carbons, with the concomitant reduction of FAD and NAD^+. These reactions are continuously repeated until the acyl-CoA is entirely oxidized to acetyl-CoA.

The experiments carried out by Franz Knoop in the beginning of the 1900s were decisive to the elucidation of the pathway for fatty acid oxidation (Box 7.6). These

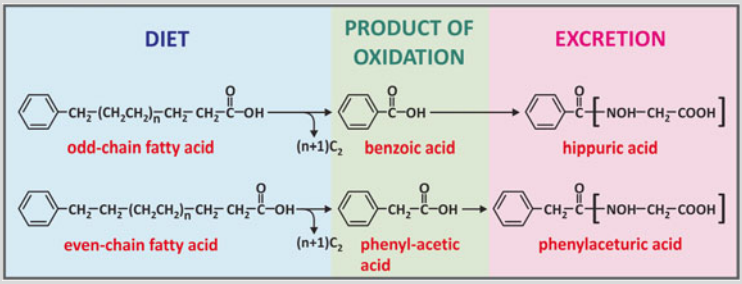
Box 7.6: Franz Knoop and the Discovery of the *β*-Oxidation of Fatty Acids

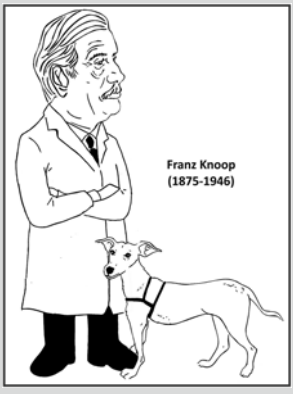
In 1904, Franz Knoop proposed the basis for the fatty acid oxidation through the conception of a very bright experiment that probably represents the first time in which a tracer was used to follow a compound along its metabolic pathway. Knoop fed dogs with fatty acid molecules of different chain length in which the terminal methyl group has been replaced by a phenyl ring.

(continued)

Box 7.6 (continued)
Then, he observed the phenyl-containing products in the urine of these dogs. He found that when the ingested fatty acid had an even number of carbon atoms, the final product was always phenylacetate, excreted as phenylaceturic acid, a glycine conjugate (see figure).

On the other hand, when the fatty acid molecules contained an odd number of carbon atoms, they yielded benzoate, excreted as hippuric acid, also a glycine conjugate (see figure). From these results, Knoop concluded that the fatty acids were degraded by the oxidation of the β-carbon followed by the cleavage of the chemical bond between carbons α and β, in a sequential process that generated molecules of two carbons, which Knoop assumed to be acetate. Now we know that the two-carbon product of fatty acid oxidation is acetyl-CoA.





experiments revealed that the β-carbon atom of the fatty acid molecule is oxidized, leading to the cleavage of the bond between carbons β and α. This made this pathway to be known as β-oxidation. Nowadays the lipid carbons nomenclature has changed (see Sect. 3.1) but the pathway designation remains.

7.4.4.1 Oxidation of Saturated Fatty Acids with Even Number of Carbons Atoms

The four reactions of the β-oxidation of saturated fatty acids are repeated in successive cycles. Each cycle consists in a sequence of an FAD-dependent oxidation, a hydration, an NAD-dependent oxidation, and a thiolysis.

The first reaction is the oxidation of the acyl-CoA to *trans*-enoyl-CoA with the concomitant reduction of an enzyme-bound FAD molecule (Fig. 7.11). This reaction is catalyzed by four types of acyl-CoA dehydrogenases (ACADs). ACADs present distinct but overlapping chain length specificities, being named as very-long-, long-, medium-, and small-chain acyl-CoA dehydrogenases (VLCAD, LCAD, MCAD, and SCAD), which show optimum activities for fatty acids of 16, 14, 8, and 4 carbons atoms, respectively. VLCAD is a homodimer bound to the inner mitochondrial membrane, whereas the other three ACADs are homotetramers located in the mitochondrial matrix.

The resulting $FADH_2$ bound to the ACADs is reoxidized by the electron-transferring flavoprotein (ETF), which in turn transfers the electrons to another flavoprotein, the ETF/ubiquinone oxidoreductase. Finally, the latter enzyme transfers the electrons to the ubiquinone in the electron transport chain (see Sect. 6.2.3).

The three other reactions of β-oxidation are also catalyzed by families of enzymes with chain-length specificities. The reactions are the hydration of the *trans*-enoyl-

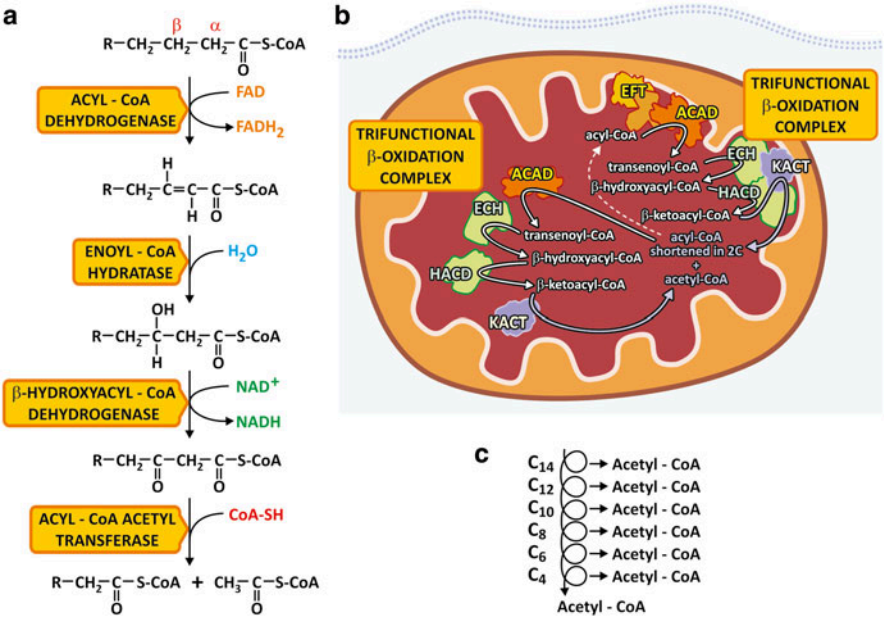


Fig. 7.11 (**a**) Reactions of β-oxidation. (**b**) Schematic representation of the β-oxidation enzymes, with those with long-chain specificity associated to the inner mitochondrial membrane, and those with short-chain specificity, localized in the matrix. Long-chain fatty acyl-CoA are oxidized by the membrane-associated enzymes until being shortened to about 12 to 8 carbons atoms, when they become substrates to the matrix enzymes. (**c**) Scheme representing the sequential β-oxidation reactions resulting in the conversion of a fatty acid of n atoms of carbons in n/2 acetyl-CoA molecules

CoA to β-hydroxyacyl-CoA, catalyzed by enoyl-CoA hydratases; the oxidation of the β-hydroxyacyl-CoA to β-ketoacyl-CoA, with concurrent NAD reduction, catalyzed by β-hydroxyacyl-CoA dehydrogenases; and the cleavage of the bond between carbons α and β of the β-ketoacyl-CoA with the transfer of the resultant acyl residue shortened in two carbons to coenzyme A, catalyzed by thiolases (Fig. 7.11a).

The enzymes with long-chain specificity form a complex associated to the inner mitochondrial membrane, the trifunctional β-oxidation complex (Fig. 7.11b). This complex channels the substrates from one enzyme to the other in a way that impairs the isolation of the intermediates from mitochondria. The enzymes with preference for medium-chain and short-chain acyl-CoA are soluble mitochondrial matrix proteins. Thus, a model for the entire process of β-oxidation would include an initial stage in which the long-chain intermediates were processed by the membrane-associated enzymes (VLCAD and the trifunctional β-oxidation complex). When the acyl-CoA molecules reach about 12 to 8 carbons, the membrane-associated enzymes lose their affinity for them. These medium-chain intermediates become substrates for the soluble enzymes that act on them until the acyl-CoA molecules are entirely oxidized to acetyl-CoA, which may finally enter the TCA cycle.

The oxidation of a fatty acid of 16 carbon atoms results in eight acetyl-CoA, seven $FADH_2$, and seven NADH molecules, after seven cycles of the four β-oxidation reactions. It is important to point out that β-oxidation reactions themselves reduce the electron carriers FAD and NAD, which in turn transfer the electrons to the respiratory chain components. $FADH_2$ associated to ACADs transfers the electrons to ubiquinone via ETF, and NADH is oxidized by the NADH dehydrogenase complex or Complex I of the respiratory chain (see Sect. 6.2.3). Thus, even if acetyl-CoA does not enter the TCA cycle, the β-oxidation pathway itself results in ATP synthesis through oxidative phosphorylation.

It is important to mention that β-oxidation of some fatty acids may occur in another organelle, the peroxisome. In this case the pathway is not directly associated to ATP synthesis, but to the formation of hydrogen peroxide (H_2O_2) (see Box 7.7).

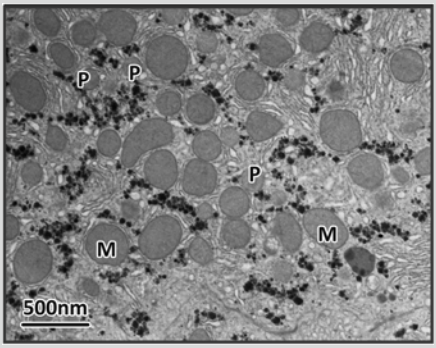
Box 7.7: *β*-Oxidation in Peroxisomes

Peroxisomes are cellular organelles (see figure), whose major function is to breakdown very-long-chain fatty acids through β-oxidation. Peroxisomes contain acyl-CoA oxidases instead of ACADs. The acyl-CoA oxidase is also a FAD-associated enzyme, but the electrons transferred to FAD via the oxidation of the acyl-CoA molecule directly reduce O_2, generating H_2O_2. Thus, O_2 is consumed but ATP is not produced in these organelles. Since catalase is present in the peroxisomes, H_2O_2 is readily converted to H_2O. The other steps of peroxisomal β-oxidation are the same as those of mitochondrial β-oxidation, but the peroxisomal enzymes are specific for long-chain and branched-chain acyl-CoAs. Thus, the products of β-oxidation in peroxisomes are short-chain acyl-CoAs, mainly octanoyl-CoA, acetyl-CoA, and NADH.

The short-chain acyl-CoA and the acetyl-CoA molecules are then sent to mitochondria through a carnitine transport system also present in peroxisomes.

(continued)

Box 7.7 (continued)



Transmission electron micrograph of a liver thin section showing the peroxisomes (P) and the mitochondria (M) (courtesy from Prof. Marlene Benchimol)

The fate of acetyl-CoA molecules generated in β-oxidation depends on the metabolic situation or on the cellular type: they may be completely oxidized in the TCA cycle, may be used in the synthesis of the ketone bodies (see Sect. 7.4.6), or may act as substrate for the synthesis of some amino acids.

7.4.4.2 Oxidation of Odd-Chain Fatty Acids

Human diet is mainly composed of fatty acids with an even number of carbon atoms, but fatty acids with odd number of carbons also occur, especially in diets containing vegetables and marine organisms. Odd-chain fatty acids are also oxidized through the β-oxidation pathway. The difference from the oxidation of the even-chain fatty acids is that the last cycle of reactions generates one acetyl-CoA and one propionyl-CoA (a three carbon molecule), instead of two acetyl-CoA molecules. Propionyl-CoA is then converted to succinyl-CoA, a TCA cycle intermediate, through a sequence of three reactions that occur in the mitochondrial matrix (Fig. 7.12).

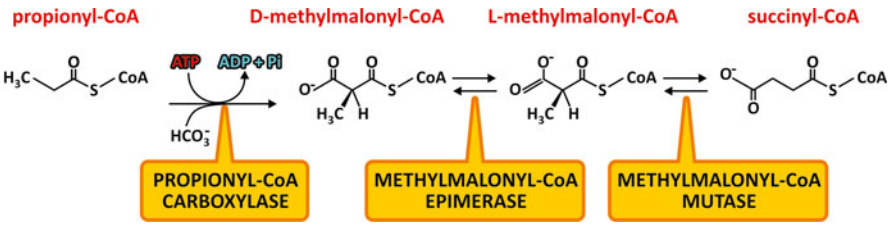


Fig. 7.12 The propionyl-CoA formed in the last cycle of the β-oxidation of the odd-chain fatty acids is converted to succinyl-CoA by an ATP-dependent carboxylation reaction followed by structural rearrangements

7.4.4.3 Oxidation of Unsaturated Fatty Acids

Human diet also contains monounsaturated fatty acids, such as oleic acid, or polyunsaturated fatty acids, such as linoleic acid and linolenic acid. These molecules are oxidized through β-oxidation pathway until reaching the double bond. At this point, if the double bond is in *trans* configuration and occurs between carbons α and β, this intermediate will enter β-oxidation in the enoyl-CoA hydratase reaction. However, in the naturally occurring unsaturated fatty acids, the double bonds are generally in the *cis* configuration. Thus, an additional enzyme is required: an enoyl-CoA isomerase that converts the *cis*-double bond in a *trans*-double bond. The product, a *trans*-enoyl-CoA, can then proceed in the β-oxidation pathway. Additionally, if the double bond is not at the right position, a reductase is also required.

7.4.5 Regulation of Fatty Acid Oxidation

The major regulation point of fatty acid oxidation is the reaction catalyzed by CPT-I, which is inhibited by malonyl-CoA. Malonyl-CoA is the product of the carboxylation of acetyl-CoA, a reaction catalyzed by the enzyme acetyl-CoA carboxylase (ACC). ACC reaction is generally activated when blood glucose concentration is high (see Sect. 8.3.1). Thus, when glucose is highly available as energy source, fatty acid oxidation is inhibited, whereas when blood glucose concentration decreases, the intracellular levels of malonyl-CoA diminishes allowing fatty acyl-CoA transport into mitochondrial matrix where they are oxidized.

7.4.6 Fatty Acid Conversion to Ketone Bodies

The acetyl-CoA molecules produced in β-oxidation does not always enter the TCA cycle; their fate may differ depending on the metabolic situation. As mentioned in the beginning of this chapter (see Sect. 7.1), fatty acids, mobilized from the adipose tissue, become the major source of energy for most of the tissues in the body when the glucose concentration in the blood starts to decrease during the periods in between the meals or in fasting situations. In these situations, the liver plays an essential role in the maintenance of glycemia by synthesizing glucose through a pathway known as gluconeogenesis (see Chap. 9).

In gluconeogenesis, TCA cycle intermediates act as substrates for the synthesis of glucose, and thus, these molecules, especially oxaloacetate, are removed from the cycle (Fig. 7.13a). The low levels of oxaloacetate decrease the capacity of the TCA cycle to oxidize acetyl-CoA, which accumulates in the mitochondrial matrix. Acetyl-CoA, even when in excess, continues to be produced to ensure ATP synthesis in the liver (remember that the oxidation steps of β-oxidation generate $FADH_2$ and NADH, allowing ATP synthesis through oxidative phosphorylation without

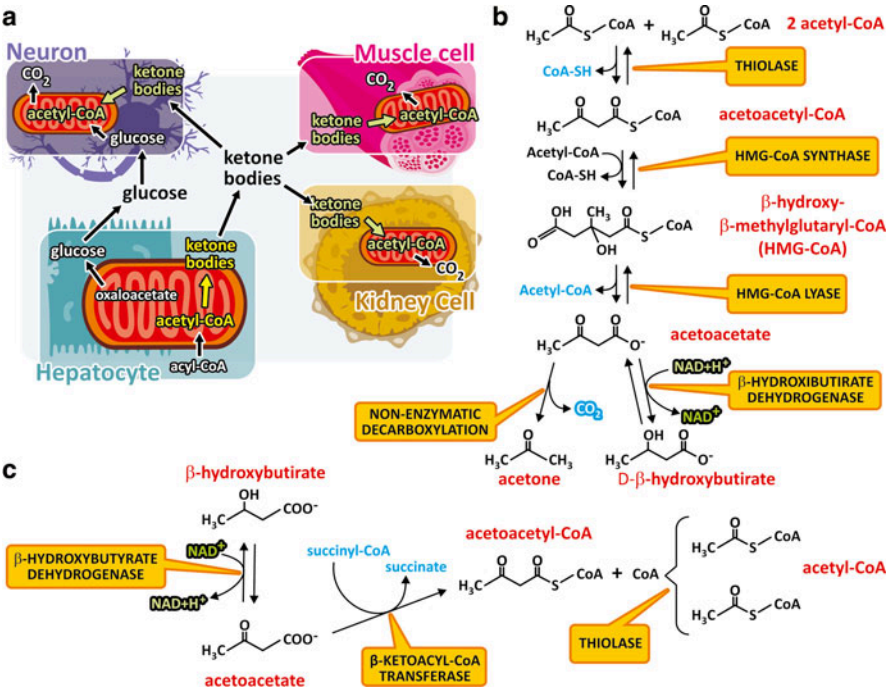


Fig. 7.13 (**a**) Schematic representation of a hypoglycemia situation in which acetyl-CoA in the liver is converted to ketone bodies, which are released in the bloodstream and used as energy source by several tissues. The *yellow arrow* in the hepatocyte highlights the pathway of ketone bodies synthesis, occurring only in the liver; the *green arrows* highlight the pathway of degradation of ketone bodies, which occurs in different extrahepatic tissues. (**b**) Reactions for the synthesis of the ketone bodies, a pathway restricted to the liver. (**c**) Reactions for the degradation of the ketone bodies, which occur in several extrahepatic tissues, including the skeletal and cardiac muscles, kidneys, and brain. The names of the enzymes are highlighted in *yellow boxes*

requirement of the TCA cycle). Thus, to recycle mitochondrial coenzyme A pool, acetyl-CoA is converted to acetoacetate, β-hydroxybutyrate, and acetone (Fig. 7.13b), molecules known as the ketone bodies, a nomenclature rather inappropriate since these molecules are not insoluble as the word "bodies" would suggest, neither the β-hydroxybutyrate has a ketone function in its molecular structure. In fact, two of the "ketone bodies" are carboxylic acids, making this functional group as important as ketone group in "ketone bodies."

The ketone bodies are released in the bloodstream, and several extrahepatic tissues, especially the brain, kidney, heart, and skeletal muscle, may use these molecules as energy source (Fig. 7.13a). In these tissues, β-hydroxybutyrate and acetoacetate are converted to acetyl-CoA (Fig. 7.13c), which enters the TCA cycle being completely oxidized. The use of ketone bodies by the brain has special importance during long periods of fasting, as it will be deeply discussed in detail in Chap. 9.

7.5 Catabolism of Amino Acids

The catabolism of the amino acids varies greatly depending on the metabolic situation, but it is calculated that about 15 % of the resting energy expenditure comes from amino acid oxidation in different tissues, in particular the liver, the main site of amino acid metabolization, and muscle, especially in the case of the branched-chain amino acids.

It is important to bear in mind that amino acids cannot be stored in the body, using the word storage in the strict sense of its meaning. Proteins in the body play a number of functions, and even the proteins that occur in very high amounts, such as the muscle contractile proteins, cannot rigorously be seen as an energy storage. However, proteins are constantly being synthesized and degraded, both as a result of the normal process of protein turnover as well as due to a controlled degradation of a specific protein, and it is believed that between 200 and 300 g of protein are formed and destroyed each day in an adult of average size.

Additionally, the protein content in the diet is also a determining factor for their use as energy sources. Thus, the balance between availability of amino acids and their requirement for protein synthesis may result in an excess of free amino acids, which then become available for oxidative degradation.

7.5.1 An Overview of the Amino Acid Catabolism

As occurs in carbohydrate and fatty acid catabolism, the products of amino acid degradation converge to the TCA cycle (see Fig. 7.1). However, the fact that there are at least 20 different amino acids as well as the presence of nitrogen in their molecular structure make the catabolism of amino acids more complex than those of glucose and fatty acids.

Before the metabolic use of amino acids, nitrogen should be separated from the amino acid carbon skeleton to be excreted or recycled for the biosynthesis of nitrogenous compounds, such as the nucleotides and other amino acids (Fig. 7.14). Nitrogen is removed from the amino acid molecules in the form of ammonia (NH_3) that in solution, at physiological pH, exists as the ammonium ion (NH_4^+). This occurs through deamination reactions, which must be precisely regulated since ammonia is very toxic. The liver is the main site of amino acid metabolization, where NH_4^+ is converted to urea, the nitrogenous compound that is excreted in humans.

Distinct degradation pathways to each carbon skeleton generated from different amino acids are necessary, but all amino acids are ultimately converted to pyruvate, acetyl-CoA, or some of the intermediates of the TCA cycle, which can be completely oxidized. In the liver, the products of amino acid degradation may also be converted in glucose or ketone bodies that are released in the bloodstream and used by other tissues (Fig. 7.14).

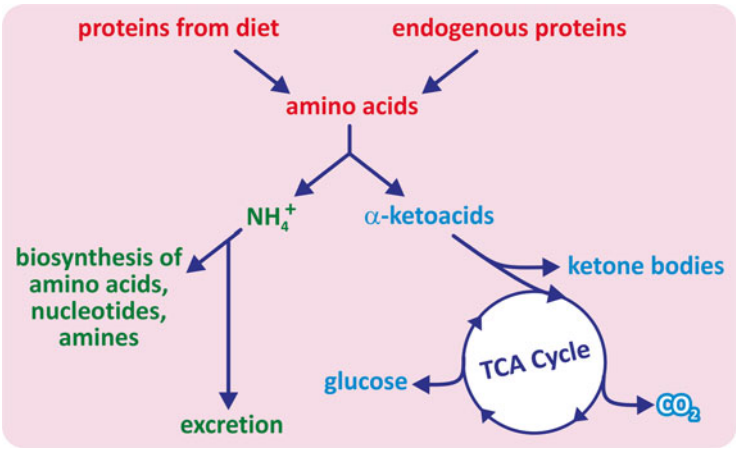


Fig. 7.14 Overview of amino acid degradation in the liver

7.5.2 Amino Acid Metabolism in the Liver

The liver is the major site of amino acid metabolization. The pathway that converts NH_4^+ in urea, the nitrogenous compound that is excreted in humans, occurs only in the liver, as well as the main metabolic strategy used to separate the nitrogen from the carbon skeleton of the amino acids, which consists in the combination of a transamination reaction followed by a deamination reaction, a process known as transdeamination.

7.5.2.1 Oxidative Deamination of the Amino Acids

The evolutionary strategy to cope with amino acid diversity relied on the conversion of all the amino acids into a specific one, Glu, followed by its highly specific and efficient deamination. The oxidative deamination of Glu is, thus, of central importance for the amino acid metabolism and consists in the main deamination reaction in the liver. This reaction generates α-ketoglutarate and NH_4^+ and is catalyzed by Glu dehydrogenase (GDH), a hexameric enzyme restricted to liver mitochondria that displays an unusual ability to use both NAD^+ and $NADP^+$ as the electron acceptor (Fig. 7.15). The fact that the first steps of the conversion of NH_4^+

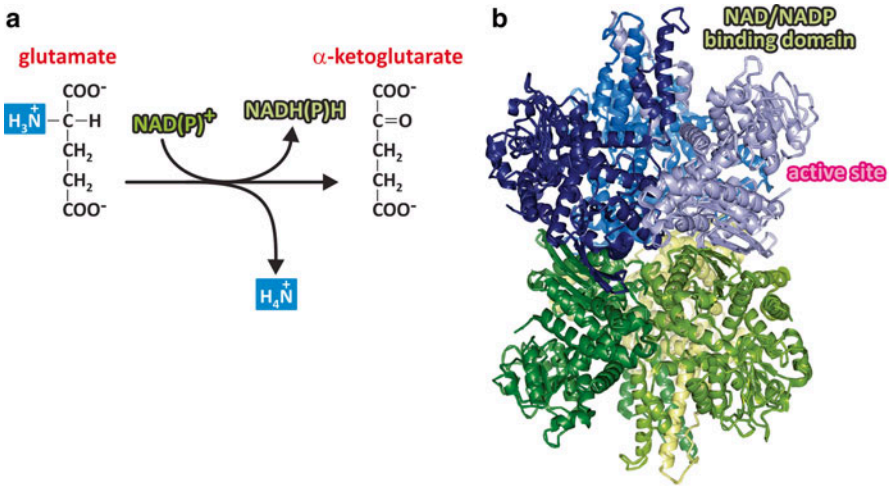


Fig. 7.15 (**a**) Reaction catalyzed by GDH. (**b**) Structure of the hexameric human GDH with each subunit represented in a *different color* (PDB 1L1F), showing the NAD/NADP-binding domain and the active site

in urea also occur in the mitochondria of the hepatocytes prevents ammonia dissemination in the body, thus avoiding its toxic effects.

Since the GDH uses only Glu, a mechanism for the conversion of all other amino acids into Glu is required. This occurs through transamination reactions.

7.5.2.2 Amino Acid Interconversion: The Transamination Reactions

The enzymes transaminases or aminotransferases convert amino acids in their respective α-ketoacids by transferring the amino group of one amino acid to an α-ketoacid, in a reaction dependent on the cofactor pyridoxal phosphate, or vitamin B6, which is covalently bound to the enzyme. This allows the amino acids to be interconverted (Fig. 7.16).

The main α-ketoacid that acts as the amino group acceptor is the α-ketoglutarate, which is the product of the GDH reaction and whose transamination generates Glu. Thus, the transamination reactions ultimately convert all the amino acids in Glu, which in turn is deaminated by GDH (Fig. 7.17). Through this strategy, the amino groups of all the amino acids are separated from their carbon skeleton.

Transaminases are present in both the cytosol and mitochondria, but their high activities in the cytosol imply that most of the formed Glu is transported to the mitochondrial matrix where GDH is located.

It is important to point out that the transaminases are also widely distributed in other tissues besides the liver, where their main function is amino acid interconversion instead of Glu formation, since GDH and urea synthesis are restricted to the liver.

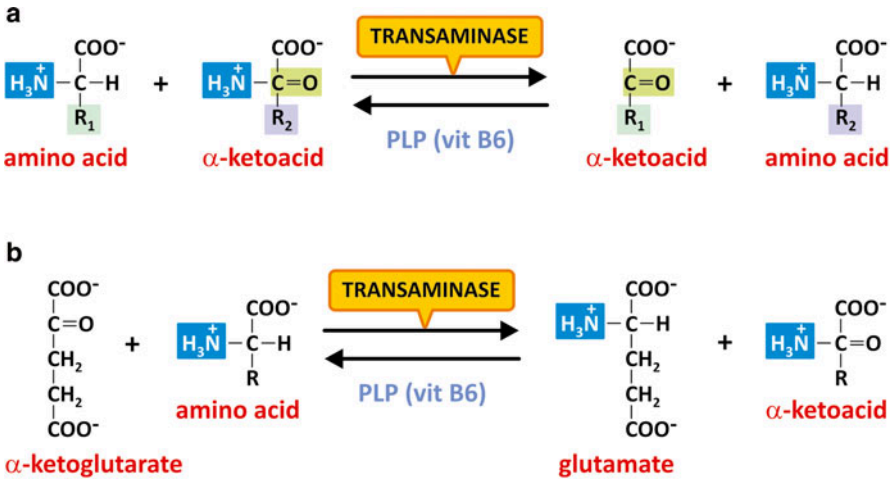


Fig. 7.16 (**a**) A general scheme of the transamination reaction. Radical groups R_1 and R_2 are swopped between an amino acid and an α-ketoacid. (**b**) Transamination reaction that makes all the amino acids to be converted into Glu, which uses α-ketoglutarate as the α-ketoacid that accepts the amino group of the amino acid, generating Glu and the α-ketoacid correspondent to the amino acid

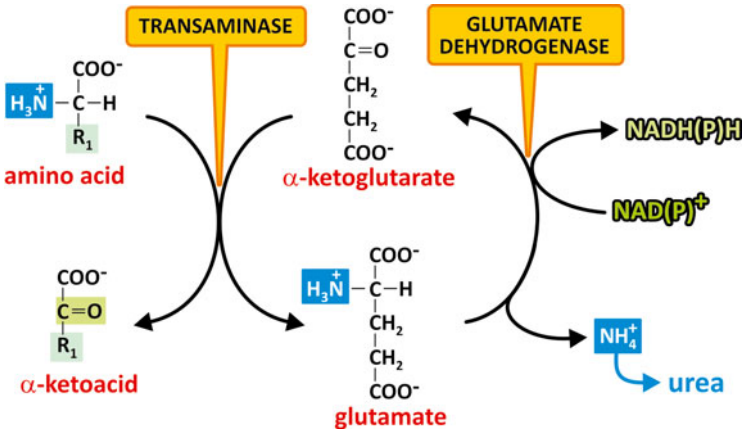


Fig. 7.17 Coupling of transamination and oxidative deamination reactions in the liver. Transamination of each amino acid with α-ketoglutarate generates Glu, which in turn is the substrate of GDH that removes the amino group of Glu in the form of NH_4^+, regenerating the α-ketoglutarate

7.5.2.3 Pathways for the Metabolism of the Amino Acid Carbon Skeletons

As mentioned in the beginning of this section, the existence of at least 20 different amino acids implies a number of distinct pathways for their metabolization. Here we will give only a general overview of this process. The detailed pathways for the

metabolization of each amino acid may be found in more specialized literature.

For some amino acids, a single transamination step directly generates an intermediate of the central metabolic pathway. This is the case of the transamination of Ala, Asp, or Glu that forms directly pyruvate, oxaloacetate, or α-ketoglutarate, respectively. For other amino acids, a complex set of reactions is required. Additionally, some amino acids are converted to other amino acids before removal of the amino group, such as Phe that is converted to Tyr and Gln, His, Pro, and Arg that are converted to Glu.

But, regardless whether the metabolization pathway is very simple or complex, the end product is one of the following six intermediates of the central metabolic pathway: pyruvate, acetyl-CoA or one of the four intermediates of the TCA cycle, α-ketoglutarate, succinyl-CoA, fumarate, or oxaloacetate (Fig. 7.18).

Depending on the situation, the carbon skeleton generated by transdeamination in the liver, instead of being directly oxidized in hepatocytes, may be converted to glucose or ketone bodies that are released into the bloodstream to be used as energy source by other tissues (see Chap. 9).

It is also important to point out that complete oxidation is not the only fate for all the carbon skeletons generated by amino acid degradation. When pyruvate and/or acetyl-CoA are generated, the complete oxidation can proceed directly. On the other hand, entering into the TCA cycle in positions other than acetyl-CoA increases the amount of TCA cycle intermediates, meaning that they increase the oxidative capacity of the cycle, but this does not result in the actual oxidation of the entering compound to CO_2. In this case, the intermediates may be converted to oxaloacetate, which can be converted into phosphoenolpyruvate (PEP) by the enzyme PEP carboxykinase (see Sect. 9.3.2). PEP is then converted to pyruvate by the glycolytic

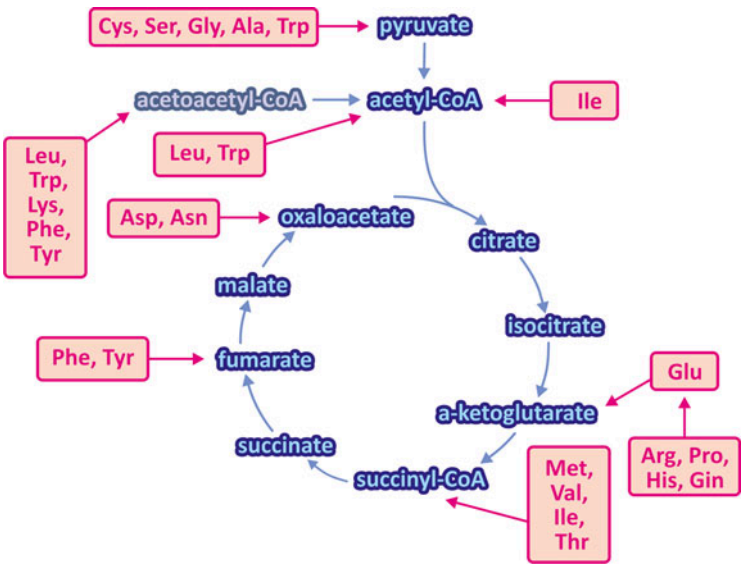


Fig. 7.18 Entry of deaminated amino acids into the TCA cycle

enzyme pyruvate kinase (see Sect. 6.1.3) and then to acetyl-CoA, which can now be completely oxidized through TCA cycle reactions.

Glucose is synthesized from the amino acids that generate TCA cycle intermediates or pyruvate, which are converted in oxaloacetate that in turn follows the pathway known as gluconeogenesis (see Chap. 9). These amino acids are classified as gluconeogenic amino acids. Amino acids that generate only acetyl-CoA (Leu and Lys) cannot be converted to glucose.

Ketone bodies are synthesized from the amino acid whose metabolism generates acetyl-CoA (see Sect. 7.4.6). These amino acids are classified as ketogenic amino acids.

7.5.2.4 Synthesis of Urea for Nitrogen Excretion

As commented in Box 7.3, in the beginning of this chapter, the pathway that converts NH_4^+ to urea was the first metabolic cycle to be described. This landmark in Biochemistry was discovered by Sir Hans Krebs. This took place in 1932, but remains an illustrative timeless plan of work in Biochemistry (see Box 7.8). Indeed, Krebs may be seen nowadays as one of the most, if not the most, prominent biochemist ever.

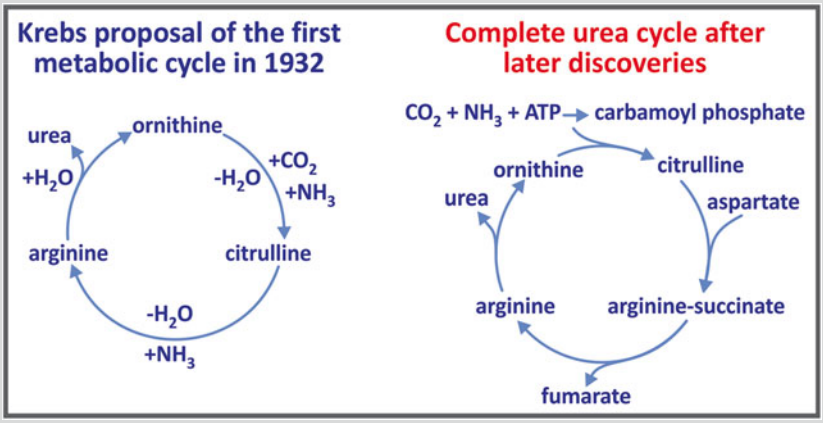
Box 7.8: Sir Hans Krebs and the Discovery of the Urea Cycle

The motivation that led Krebs to study the synthesis of urea came from the experience of working with tissue slices in the period he was at Otto Warburg laboratory. Krebs was impressed by the new possibilities that the use of tissue slices technique opened, allowing approaches that before were only feasible using microorganisms. Since Warburg used this technique to study only degradative metabolic pathways (glycolysis and respiration), Krebs decided to evaluate whether it would also be possible to explore biosynthetic processes, at that time restricted to the use of entire organisms and perfused organs. He chose urea synthesis because it seemed to occur through a simple pathway at a very high rate. Firstly Krebs developed a simple and reproducible method to measure urea and created a new reaction medium that simulated blood plasma. With this system, Krebs and Kurt Henseleit, a medical student whose M.D. thesis was supervised by Krebs on this subject, designed a working plan that included the quantification of urea synthesis using as substrates the combination of ammonia with different amino acids. These experiments resulted in two main observations with nonobvious connection: (a) the unexpected finding that ornithine together with ammonia yields exceptionally high rates of urea synthesis and (b) the presence of high levels of the enzyme arginase in the liver, an enzyme that converts the amino acid Arg into ornithine and urea. The connection between these findings came with the subsequent observation that for each ornithine added to the medium, 20 urea molecules were formed and that the total urea nitrogen was equivalent to the ammonia disappearance.

(continued)

Box 7.8 (continued)
This led to the crucial proposal that ornithine would act as a catalyst: it was combined to ammonia generating an intermediate in the production of Arg, whose hydrolysis recovered ornithine levels in the system, yielding urea as the final product.

The next steps were the search for the intermediates between ornithine and Arg. At the same time, two biochemist independently identified citrulline as a molecule that would fulfill this gap. Indeed, when Krebs tested the effect of citrulline on the urea synthesis from ammonia, it worked in the same way as ornithine. So, Krebs proposed the first metabolic cycle as the pathway of urea synthesis. The finding of additional intermediates later completed the cycle as we know it today (see figure).



Urea is synthesized almost exclusively in the liver, from which it is released in the bloodstream, reaching the kidneys for excretion in the urine. The molecule of urea contains two nitrogen atoms, one coming from the NH_4^+ produced by the reactions catalyzed by GDH or glutaminase within the mitochondria and the other coming from the amino group of the amino acid Asp, generated in the hepatocyte cytosol by transamination of any amino acid with oxaloacetate. In a series of reactions, these two amino groups ultimately become part of an Arg molecule, which after hydrolysis forms urea and ornithine (see figure in Box 7.8). The detailed sequence of reactions that comprise the pathway is represented in Fig. 7.19 and described below.

Firstly the NH_4^+ reacts with HCO_3^- and two molecules of ATP in the mitochondrial matrix, in a reaction catalyzed by the enzyme carbamoyl-phosphate synthase I, yielding two ADPs and one Pi and the intermediate carbamoyl phosphate, which enters the cycle by transferring its carbamoyl group to ornithine, forming citrulline and Pi, in a reaction catalyzed by ornithine transcarbamoylase. Citrulline leaves mitochondria and reacts with Asp in an ATP-dependent reaction catalyzed by the enzyme argininosuccinate synthase, which generates argininosuccinate, AMP, and PPi. The enzyme

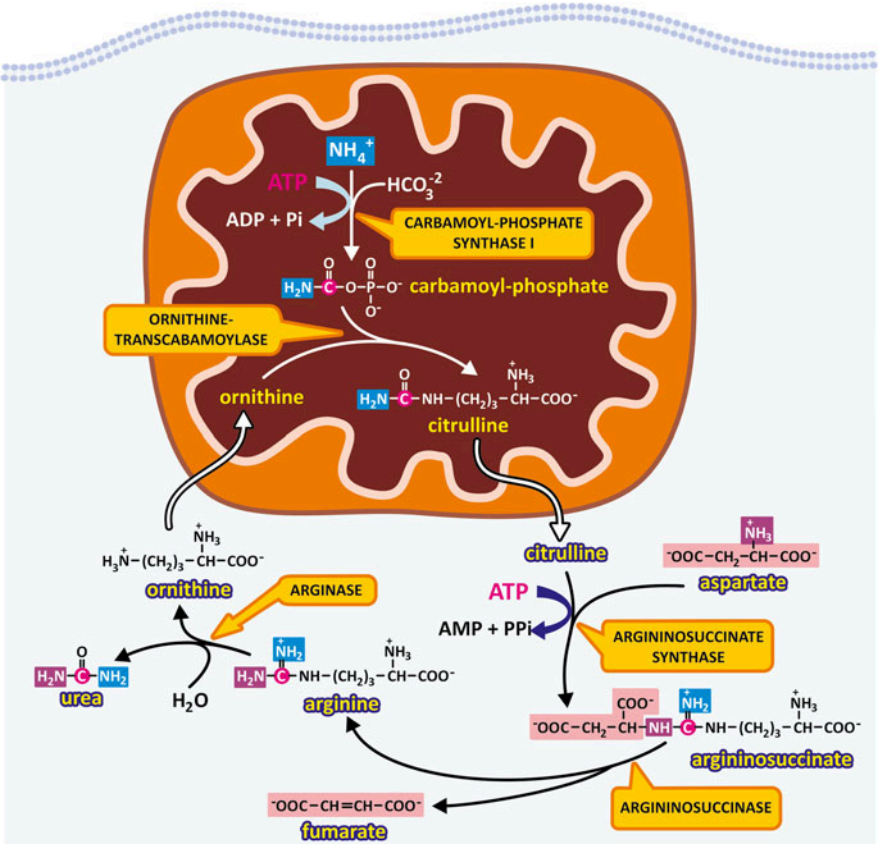


Fig. 7.19 The urea cycle. Urea is synthesized in the liver through a cyclic pathway with part of the reactions occurring in the mitochondria and part in the cytoplasm. One of the nitrogen atoms of urea molecule comes from NH_4^+ produced by the GDH or glutaminase reactions (highlighted with a *blue box*) within the mitochondria. The other comes from the amino group of Asp (highlighted with a *purple box*). The names of the enzymes are highlighted in *yellow boxes*

argininosuccinase cleaves the argininosuccinate into fumarate and arginine, which finally is hydrolyzed to urea and ornithine by the enzyme arginase (Fig. 7.19).

It is important to point out that the oxaloacetate molecules removed from the TCA cycle to be transaminated, generating Asp, are replenished since the reaction of argininosuccinase releases fumarate, which enters the TCA cycle to regenerate oxaloacetate.

7.5.3 Amino Acid Metabolism in Other Tissues

Although much more information is available about amino acid metabolism in the liver, other tissues also oxidize amino acids, with a special emphasis on the use of branched-chain amino acids by muscle.

Amino acid deamination reactions in different tissues form NH_4^+ that is generally combined to Glu yielding Gln, in a reaction catalyzed by the enzyme glutamine synthetase (Fig. 7.20). Gln is then released in the bloodstream, being the main transporter of amino groups in blood. This explains why Gln concentration in blood is much higher than that of other amino acids. Gln is taken up mainly by the liver and the kidney, where the enzyme glutaminase removes the amido group from Gln, generating Glu and NH_4^+. In the liver, NH_4^+ enters the urea cycle, and Glu undergoes oxidative deamination by GDH (see previous section). In kidney, NH_4^+ is directly excreted (Fig. 7.20).

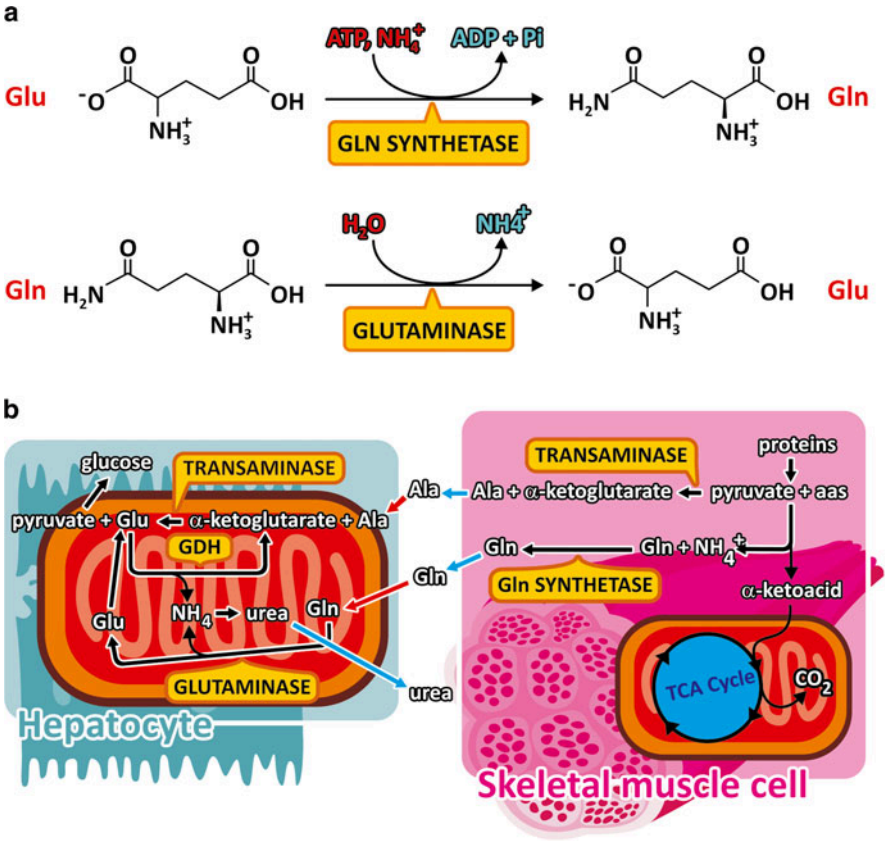


Fig. 7.20 (**a**) Reactions catalyzed by the enzyme glutamine synthetase and by the enzyme glutaminase. (**b**) Integration of amino acid metabolism. In extrahepatic tissues (specially skeletal muscle), acids from diet, intracellular protein turnover, or degradation undergo transamination with pyruvate generating Ala or deamination forming NH_4^+ that is combined to Glu yielding Gln. Gln and Ala are released in the bloodstream reaching the liver, where Ala is transaminated with α-ketoglutarate, forming pyruvate and Glu. Glu undergoes oxidative deamination, generating NH_4^+ that enters the urea cycle. Pyruvate is converted in glucose or is completely oxidized. Gln is deaminated yielding NH_4^+ that enters the urea cycle and Glu that undergoes oxidative deamination. Urea is released in the bloodstream and excreted by the kidneys. The names of the enzymes are highlighted in *yellow boxes*

Amino acids in muscle also undergo transamination with pyruvate, generating Ala. This is especially important in fasting, when the amino acids released from the degradation of contractile proteins are converted to Ala, which in turn is released in the bloodstream reaching the liver, where pyruvate is regenerated (and converted to glucose) and NH_4^+ is converted to urea (Fig. 7.20) see also Sect. 9.3.4. Indeed, in fasting, the proportions of amino acids released from the muscle do not reflect the muscle protein composition. Ala and Gln correspond to about 60 % of the amino acid released from muscle cells in the bloodstream.

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Chapter 8
Metabolic Responses to Hyperglycemia: Regulation and Integration of Metabolism in the Absorptive State

Carbohydrates, lipids, and proteins are the main components of foods and serve as "fuel" molecules that provide energy for the organism. A regular meal is generally composed of 45–65 % carbohydrates, 20–35 % lipids, and 10–30 % protein. After ingestion, these nutrients are broken down into smaller molecules, which are subsequently absorbed and metabolized (see Chap. 7).

The end products of carbohydrate and protein digestion are monosaccharides (mainly glucose) and small peptides and amino acids, respectively, which reach the portal vein after absorption by intestinal cells (Fig. 8.1). Before reaching the systemic circulation, these metabolites pass through the liver, which absorbs and stores from ½ to ¾ of the nutrients coming from the digestive tract (see Box 8.1).

Monoacylglycerol and long-chain fatty acids which originated from lipid digestion are not soluble in aqueous medium and need specific transport mechanisms. They are reconverted to triacylglycerols in the intestinal mucosal cells and then are released into the lymph in the form of lipoproteins named chylomicrons (Fig. 8.1).

This chapter will focus on the metabolic adaptations that take place after the ingestion of nutrients, which allow the components of the diet to be used as energy sources or to be stored as reserves that will be mobilized in the postabsorptive or fasting states. This main topic will be introduced with a discussion on how the cells can sense the variations in blood glucose concentration, how they respond to this, and how this sensing mechanism controls the secretion of insulin. Then, we will discuss in detail the metabolic pathways involved in the biosynthesis of the main energy storage molecules, glycogen and triacylglycerol. Additionally, we will present the mechanism of action of insulin and its effects on different target tissues, giving an overview of the fate of the main metabolites absorbed after a meal in different cell types.

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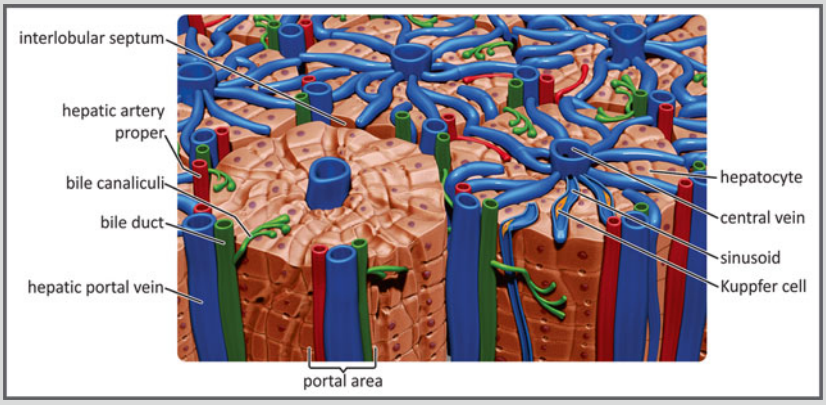
Box 8.1: The Hepatic Portal System

Blood is supplied to the liver by the hepatic portal vein and hepatic arteries. About 75 % of the blood entering the liver is venous blood drained from the intestine, pancreas, and spleen, which converges to the portal vein. Thus, everything absorbed by the digestive tract, including nutrients and toxins, as well as the pancreatic secretions and the blood cells and their degradation products released from the spleen, passes through the liver before reaching the systemic circulation. The remaining 25 % of the blood supply to the liver is arterial blood coming from the hepatic arteries.

The functional units of the liver are the hepatic lobules, a polygonal arrangement of plates of hepatocytes radiating outward a central vein (see figure). The hepatocytes make contact with the blood in the sinusoids, vascular channels that receive the blood coming from the terminal branches of both the portal vein and hepatic arteries. The sinusoids are lined with highly fenestrated endothelial cells, allowing a considerable amount of plasma to be filtered into the space between the endothelium and hepatocytes. Blood flows through the sinusoids and empties into the central vein of each lobule that coalesces into hepatic veins, which leave the liver. Of special importance in the context of this chapter is the fact that the liver is responsible for removing part of glucose from the blood, converting it into glycogen or lipids, before it reaches the systemic circulation.



Schematic representation of the hepatic lobules. Portal vein branches and the sinusoids, carrying venous blood, are shown in *blue*, and the hepatic artery branches are shown in *red*. The bile ducts that transport the bile from the hepatocytes to the gallbladder or the duodenum are shown in *green*

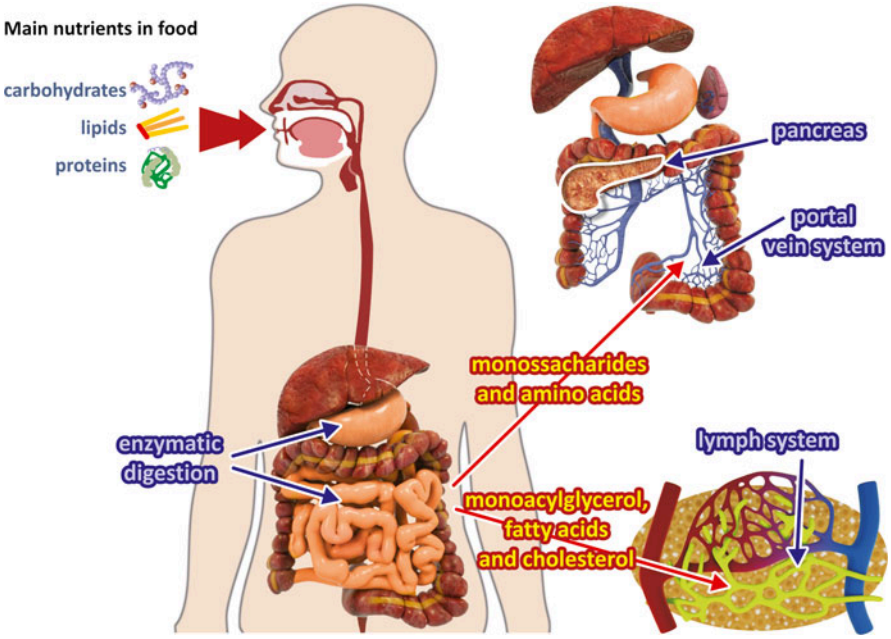


Fig. 8.1 Absorption of the main nutrients. Digestion of carbohydrates and proteins generates monosaccharides and amino acids that are absorbed and travel through the portal vein system to the liver, entering the general circulation by the way of the hepatic vein. Digestion of lipids generates fatty acids and monoacylglycerol, which are released into the lymph

8.1 Glucose Sensing by Cells

The increase in blood glucose concentration is the main signal that transmits the information that food was ingested to most cells in human body, directly or indirectly by regulating the secretion of different hormones, which in turn control the energy metabolism. After a carbohydrate-rich meal, the increase in blood glucose concentration triggers the secretion of the hormone insulin (Fig. 8.2a). The action of this hormone on its target cells as well as the increase in glycemia itself produce a rapid glucose utilization by different tissues, both as energy source and as the precursor for the synthesis of storage molecules.

Glucose metabolism homeostasis depends on rapid responses to changes in glycemia. These responses occur when blood glucose concentration decreases (see Chap. 9), but also when glycemia rises. In both cases, the metabolic adaptations operate until the basal level of blood glucose concentration, which is approximately 5 mM, is reestablished (Fig. 8.2a). This set point is maintained due to the opposite effects of glucose on the rate of pancreatic secretion of the hormones glucagon and insulin, which shows a crossover point of 5 mM glucose (Fig. 8.2b), concentration below which glucagon action predominates, whereas above it insulin effects prevail.

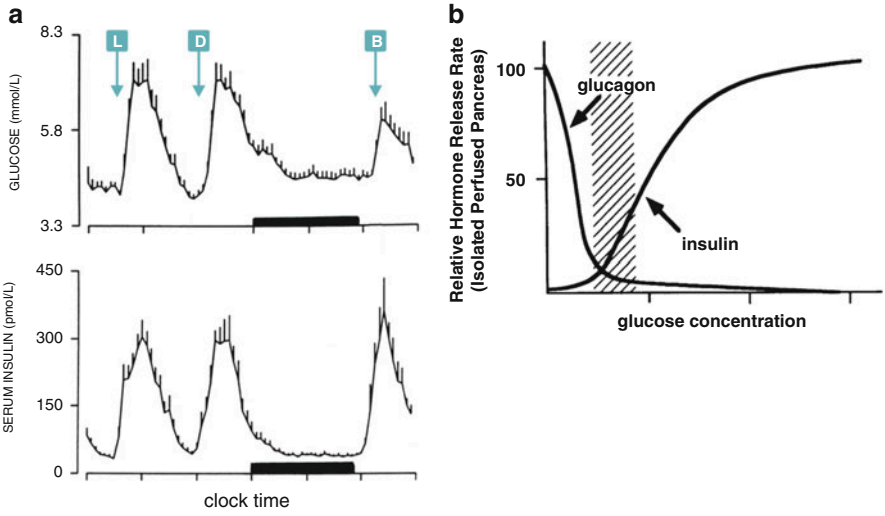


Fig. 8.2 (**a**) Day profile of plasma glucose and serum insulin of nine human subjects (mean + SE). L, D, and B indicate lunch, dinner, and breakfast time, respectively. The *black bar* represents the sleep period (Reproduced with permission from Biston et al. Hypertension 28:863–871, 1996). (**b**) Hormone secretion profile in isolated perfused pancreas as a function of glucose concentration. The *dashed region* corresponds to the physiological blood glucose range (Reproduced with permission of the American Society for Clinical Investigation, from Matschinsky et al. J. Clin. Invest. 92:2092–2098, 1993)

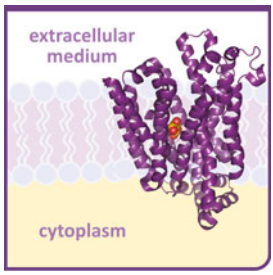
Insulin is produced and secreted by the β-cells of pancreatic Langerhans islets. The mechanism involved in secretion induction will be discussed in Sect. 8.4, but a question that may be posed at this point is: How can the β-cells sense the increase in blood glucose concentration and promptly respond by increasing insulin secretion?

To answer this question, we will first analyze two important steps that precede and determine glucose utilization in cellular metabolism: (a) the transport of glucose across the plasma membrane and (b) its phosphorylation to glucose-6-phosphate.

Glucose enters the cells by facilitated diffusion mediated by membrane transport proteins, the glucose transporters (GLUTs). Fourteen GLUT isoforms are expressed in human cells, which are characterized by distinct kinetic and/or regulatory properties, substrate specificity, cellular location, as well as tissue-specific expression. The best studied GLUTs are the isoforms 1–4 (Table 8.1), which play a central role in glucose homeostasis by conferring specific properties to glucose uptake depending on the cell type. The other isoforms seem to transport fructose, myoinositol and urate, in addition to glucose, and are probably also involved in the transport substrates yet to be identified.

In the liver and pancreatic β-cells, the uptake of glucose occurs predominantly through GLUT2, which is characterized by a very high K_M for glucose uptake (~17 mM) and by a very high expression level that gives these cells a high capacity for

Table 8.1 Properties of the four best studied GLUT isoforms

Transporter	K_M (mM)	Distribution	Features	Putative Structure[a]
GLUT-1	1–2	Ubiqitous, erythrocytes	Constitutive glucose transporter	
GLUT-2	20	Liver, β-cells, intestine, kidney	Low affinity, high capacity transporter	
GLUT-3	1	Neurons, Placenta	Low affinity transporter	
GLUT-4	5	Adipose tissue, skeletal muscle heart	Insulin-dependent transporter	

[a]The structures of mammalian GLUTs have not been determined yet, so the figure shows the structure of the *E. coli* homologue of GLUTs, the xylose-H^+ symporter (XylE), complexed with xylose (*orange*) (PDB 4GBY). It contains 12 segments that cross the plasma membrane, forming a "pore" through which the sugar is transported

transport. These properties ensure the fast equalization of extracellular and cytosolic concentrations of glucose after any change in the physiological glycemic levels.

Thus, although GLUT proteins are important for the regulation of the glucose uptake in other tissues (see Sect. 8.4.3 for glucose transport in the muscle and adipose tissue), in the case of β-cells GLUT2 properties are important for balancing the extra- and intracellular glucose concentrations.

To complement the answer to our question about how β-cells sense the changes in glycemia, we have also to take into account the next step of glucose utilization by cells. Glucose metabolization starts with its phosphorylation to glucose-6-phosphate, a reaction catalyzed by a family of enzymes named hexokinases (HK). Once glucose is phosphorylated, it cannot leave the cell unless the phosphate group is removed by the action of glucose-6-phosphatase. Since this enzyme is only expressed in liver and kidney cells (see Sect. 9.3.1), glucose phosphorylation in most cells, including β-cells, marks it for metabolization inside the cell.

There are four isoforms of HKs (HK 1–4). The isoform expressed in β-cells is the HK 4, also named glucokinase (GK) since glucose is its preferred substrate. GK is a monomer with low affinity for the glucose, with an $S_{0.5}$ of 8 mM, and cooperativeness with a Hill number of 1.7, giving to the curve that describes its activity as a function of glucose concentration an inflection point of approximately 4 mM glucose (Fig. 8.3). Additionally, unlike other HK isoforms, GK is not inhibited by the reaction product, glucose-6-phosphate. These kinetic properties make it possible that changes in GK activity occur exactly over the range of physiological blood glucose concentrations, as can easily be seen in Fig. 8.3, which compares the activity profile of HK isoforms as a function of glucose concentration. HK 1 activity is the same (and maximal) over all glucose concentrations in the physiological range, while a great increment in glucose phosphorylation by GK occurs as the glucose concentration increases from 2 to 12 mM. Reminding that extra- and intracellular concentrations of glucose are equalized due to the properties of GLUT2 in β-cells,

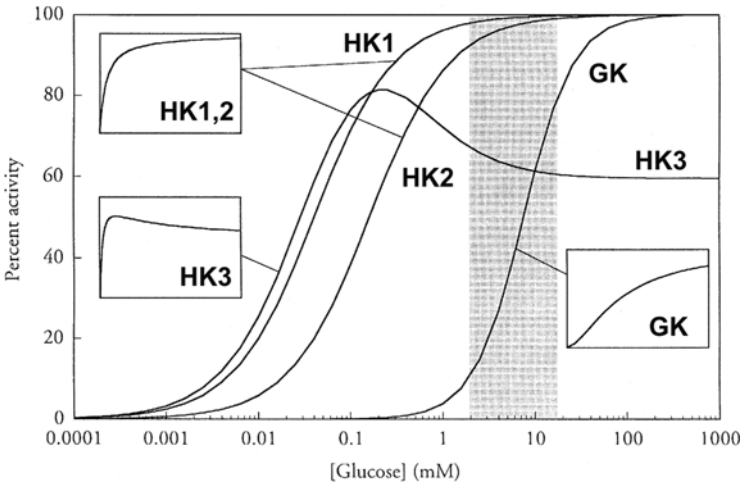


Fig. 8.3 Comparison of the kinetic properties of HK isoforms. The physiological range of blood glucose concentration is indicated by the *gray section*. The *insets* show the curves redrawn with a linear scale of glucose concentration to better illustrate the kinetic profile (Reproduced from Cardenas et al. Biochim. Biophys. Acta 1401:242–264, 1998, with permission from Elsevier)

the kinetic features of GK makes it possible that the rate of glucose metabolization in β-cells is determined by blood glucose concentration, making GK the intracellular sensor of glycemia. Nevertheless, mutations in the gene encoding GK cause different pathologies characterized by the impairment in glycemia control (Box 8.2).

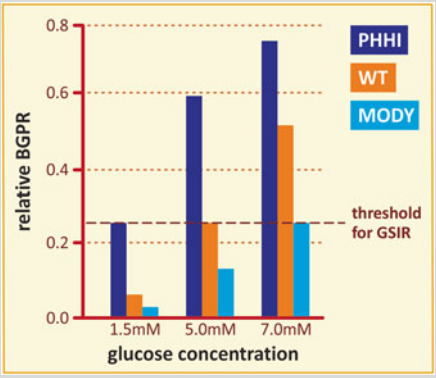
GK seems to play the role of glucose sensor in all GK-containing cells, which include, besides the β-cells, the hepatocytes, the specialized hypothalamic neurons, and the endocrine enterocytes. However, the responses to glucose sensing may be different in each of these cells. In β-cells, the increase in blood glucose concentration stimulates insulin secretion (see Sect. 8.4.2), whereas in hepatic cells, it induces the expression of glycolytic and lipogenic genes. Additionally, glucose sensing in hypothalamic cells participates in the regulation of food ingestion and energy expenditure, impacting on the control of body weight, as it will be discussed in Chap. 11.

Before describing the mechanisms involved in insulin secretion and the direct effects of this hormone on the regulation of metabolism of different target cells, we will present in detail the main metabolic pathways that are activated when glycemia rises.

Figure 8.2a shows how glucose is rapidly removed from the bloodstream in a period of 2 h after a carbohydrate-rich meal. In this period, the major fates of glucose are (a) its use as energy source by most, if not all, of the cells in the body (which does not imply that it is the only energy source of the human body); (b) its incorporation into glycogen molecules, in which glucose is stored by polymerization, therefore, without major changes in its molecular structure; and (c) its transformation into fatty acids, which are incorporated in the triacylglycerol molecules, the main energy storage molecules in the human body (Fig. 8.4).

Box 8.2: GK-Related Pathologies

The role of GK as the glucose sensor in β-cells is strongly supported by the consequences of several mutations in GK gene, which are manifested in at least three distinct pathologies: (a) persistent hyperinsulinemic hypoglycemia (PHHI), caused by mutations that activate the enzyme; (b) permanent neonatal diabetes mellitus (PNDM), which occurs in newborns carrying inactivating mutations in the two alleles of the gene; and (c) maturity-onset diabetes of the young (MODY), occurring due to one defective allele with a mutation that inactivates the enzyme. The β-cell threshold for glucose stimulation of insulin release (GSIR) may be as low as 1.5 mM in PHHI patients because of lowered glucose $S_{0.5}$ and/or increase in k_{cat}, while it may be increased to 7 mM due to a single inactivating mutation in a MODY patient (figure). Therefore, these syndromes can be explained on the basis that glucose concentration threshold for GSIR is determined by the catalytic capacity and/or in the substrate affinity of β-cell GK, supporting the concept that this enzyme plays a role in glucose sensing.



Comparison of the relative glucose phosphorylation rate as a function of glucose concentration for the wild type and two different mutants of GK. The *dashed line* indicates the threshold for glucose stimulation of insulin release, GSIR (Based on data from Matschinsky. Diabetes 51:S394–S404, 2002)

The use of glucose as energy source has already been discussed in Chaps. 6 and 7. In this chapter, we will detail the metabolic pathways that convert glucose in energy reserves, the synthesis of glycogen and the synthesis of lipids. We will also present the pentose-phosphate pathway, an additional pathway for glucose-6-phosphate oxidation that generates NADPH for the reductive biosynthesis and pentose phosphate for nucleotide synthesis.

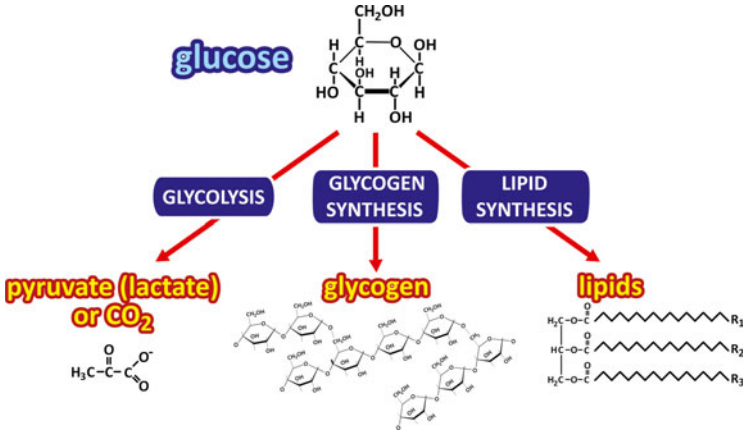


Fig. 8.4 Possible fates of glucose after its uptake by the cells. The metabolic pathways that may be followed by glucose are shown in the *blue boxes*

8.2 Biosynthesis of Glycogen

Glycogen is a branched polymer of glucose that forms granular structures corresponding to aggregates of glycogen itself associated to the enzymes involved in its synthesis and degradation as well as their regulatory machinery (Fig. 8.5).

In humans, glycogen is accumulated mainly in the liver and skeletal muscle in response to an increase in blood glucose concentration that generally occurs after a meal. It serves as an immediate source of glucose, which in the liver is released to maintain glycemia (see Sect. 9.2.1), while in muscle it serves as a fuel reserve to ensure ATP synthesis during intense contractile activity (see Sect. 10.4.2). The general pathways for storing and mobilizing glycogen are the same in both tissues, with slight differences in the enzymes, especially with respect to the mechanisms of their regulation.

In the beginning of the twentieth century, Carl and Gerty Cori discovered and studied glycogen phosphorylase (GP), the enzyme that catalyzes glycogen degradation (see Sect. 9.2.1). Since they demonstrated using different tissue preparations that the reaction of catalyzed by this enzyme was reversible in vitro, the synthesis of glycogen was firstly believed to occur as the reversal of glycogenolysis.

The existence of a different pathway for glycogen synthesis became more evident after the discovery of the cause of McArdle's disease, the most common disorder of glycogen metabolism, which is caused by a mutation in the muscle isoform of GP, resulting in a nonfunctioning enzyme and in a complete block of glycogen degradation in muscle. Patients with McArdle's disease have chronic high muscle glycogen levels, showing that glycogen synthesis occurs independently of GP activity.

Both in the liver and muscle, blood glucose is the major precursor for glycogen synthesis, which is mainly regulated by insulin action, as it will be discussed in Sect. 8.4. In the skeletal muscle, the transport of glucose into the cells is also insulin dependent (GLUT4 transporter).

The synthesis of glycogen can be divided in two distinct parts, the initiation and the elongation phases. The initiation phase is required when a new glycogen

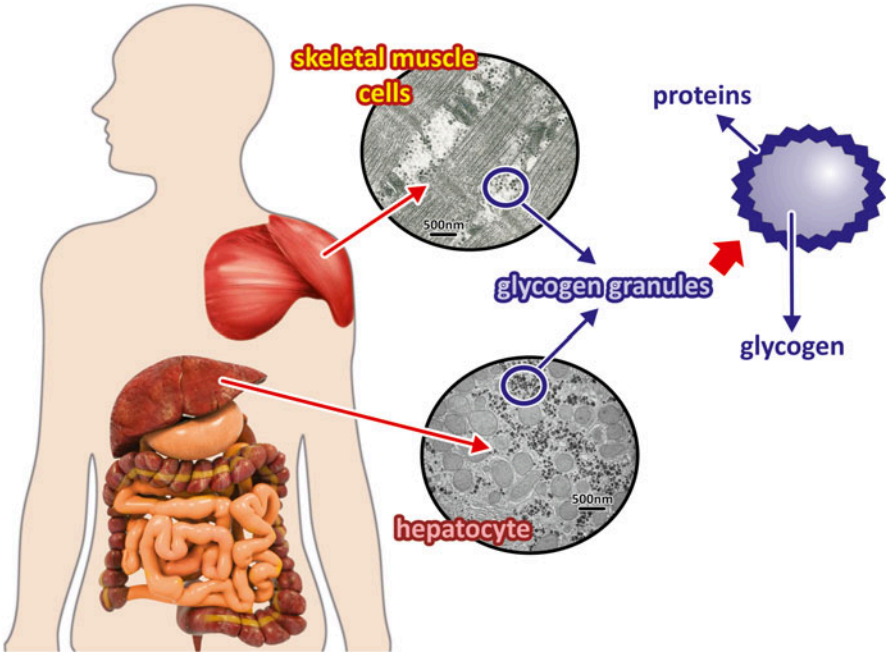
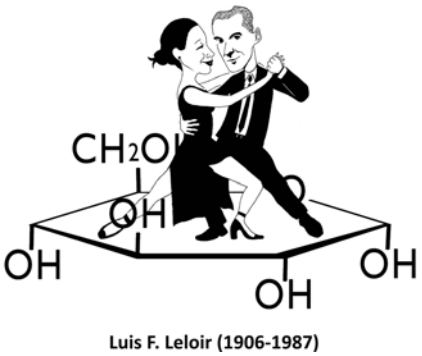


Fig. 8.5 Liver and muscle glycogen granules. Glycogen, a polymer of glucose molecules, is stored mainly in the liver and in muscles as granules with the glycogen molecule in the center surrounded by proteins and enzymes involved in its metabolism. hepatocyte The electron micrographs show sections of a skeletal muscle cell (*top*) and a hepatocyte (*bottom*) in which glycogen depots can be seen as electron dense. (Transmission electron micrograph images: skeletal muscle cell section, reprinted from the archives of Prof. David Ferreira, with the permission of Karin David Ferreira; hepatocyte section, courtesy from Prof. Marlene Benchimol)

molecule is synthesized and is mediated by glycogenin, a protein that, besides catalyzing the incorporation of the first glucose units in the nascent glycogen chain, remains covalently linked to the final glycogen molecule. The elongation phase depends on two different enzymes: (a) the glycogen synthase (GS), which catalyzes the successive addition of α-1,4-linked glucose units to a nonreducing end of the glycogen branch, and (b) the branching enzyme, which has a glucosyl(4 → 6)transferase activity that creates the α-1,6-glucosidic bond that starts a new branch.

For both initiation and elongation reactions, the donor of the glucose units to be incorporated in the glycogen molecule is UDP-glucose.

The role of UDP-glucose as a donor of glucose units for the synthesis of disaccharides and glycogen was discovered by Luis Leloir, an Argentine biochemist. After Leloir's findings, it became clear that the formation of a sugar nucleotide was a general activation step in the reactions of hexose polymerization, including the formation of disaccharides, glycogen, extracellular polysaccharides, and aminohexoses and deoxyhexoses found in some of these polysaccharides. Due to this great scientific contribution that opened the way for the understanding of carbohydrate biosynthesis, Leloir was awarded the Nobel Prize in Chemistry in 1970.



8.2.1 *Formation of UDP-Glucose*

The pathway from blood glucose to UDP-glucose differs in the liver and muscle in the beginning but converges to the same reactions in the end (Fig. 8.6a).

In the liver cells, glucose enters the cells through GLUT2 and is phosphorylated by GK. As already discussed for β-cells (see Sect. 8.1), this makes the synthesis of glucose-6-phosphate dependent on glucose concentration in the blood. Additionally, since the liver cells express the enzyme glucose-6-phosphatase, which removes the phosphate group from glucose-6-phosphate, when intracellular concentration of glucose-6-phosphate becomes high, some of the molecules may be dephosphorylated to glucose, which then leaves the cells. Thus, part of glucose molecules that

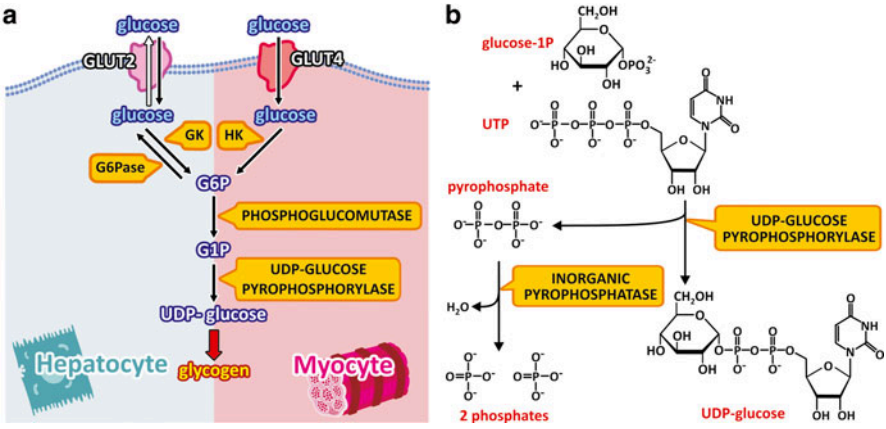


Fig. 8.6 (**a**) Pathway from blood glucose to UDP-glucose in the liver and muscle cells. Glucose enters the cell through GLUT2 or GLUT4 and is phosphorylated by GK or HK in the liver or muscle cells, respectively. Glucose-6-phosphate (G6P) is converted to glucose-1-phosphate (G1P), which is the substrate to the formation of UDP-glucose in both cell types. UDP-glucose is the substrate for glycogen synthesis. The names of the enzymes are highlighted in *yellow boxes*. (**b**) Reaction for UDP-glucose synthesis catalyzed by UDP-glucose pyrophosphorylase

enter the liver are metabolized in this organ, but the excess returns to the bloodstream to be used by other tissues.

In the muscle cells, the GLUT4 transports glucose into the cytoplasm. This transport is dependent on insulin action, as it will be discussed in detail in Sect. 8.4. Once inside the cell, glucose is phosphorylated to glucose-6-phosphate by HK, which has a high affinity for glucose.

Once glucose-6-phosphate is formed, in both the liver and muscle, the enzyme phosphoglucomutase converts glucose-6-phosphate to glucose-1-phosphate, which is the substrate to UDP-glucose synthesis. Glucose-1-phosphate reacts with UTP to form UDP-glucose, which is the immediate precursor for glycogen synthesis (Fig. 8.6b). This reaction is catalyzed by UDP-glucose pyrophosphorylase, with the formation of pyrophosphate besides UDP-glucose. Although the enzyme name, UDP-glucose pyrophosphorylase, refers to the reverse reaction, the synthesis of UDP-glucose is irreversible in physiological conditions. This occurs because pyrophosphate is readily hydrolyzed by the enzyme inorganic phosphatase, in a very exergonic reaction that pushes the glycogen synthesis forward.

8.2.2 Reactions for the Initiation of Glycogen Synthesis from UDP-Glucose

The initiation of glycogen synthesis depends on a very unusual protein, named glycogenin (Fig. 8.7). This protein occupies the core of glycogen molecule and is also the enzyme that catalyzes the initiation of glycogen synthesis.

Glycogenin is a member of glucosyltransferase superfamily. It is dimeric and shares with other glucosyltransferases the nucleotide-binding fold comprising a four-stranded αβ-domain that is responsible for the majority of the interactions with the UDP moiety of UDP-glucose (Fig. 8.7a). In mammals, there are two tissue-specific isoforms: glycogenin-1 predominantly expressed in muscle and glycogenin-2 mainly expressed in the liver.

The initial reaction catalyzed by glycogenin is unique, since the protein is the substrate, the catalyst, and the product of the reaction. First, glycogenin catalyzes the glucosylation of its Tyr195 by transferring a glucose unit from UDP-glucose to form a glucose-1-*O*-tyrosyl linkage (Fig. 8.7b). Then, glycogenin sequentially incorporates α-1,4-linked glucose residues to the nascent glycogen chain starting from the Tyr195-linked glucose and using UDP-glucose as substrate until the chain reaches 8 glucose residues, with the chain remaining attached to glycogenin (Fig. 8.7b).

8.2.3 Reactions for the Elongation of Glycogen Chain

Elongation of glycogen chain depends on two different enzymes, the GS and the branching enzyme.

GS catalyzes the formation of an α-1,4-glucosidic bond between a glucose unit from UDP-glucose and a nonreducing end of a glycogen chain (Fig. 8.8a).

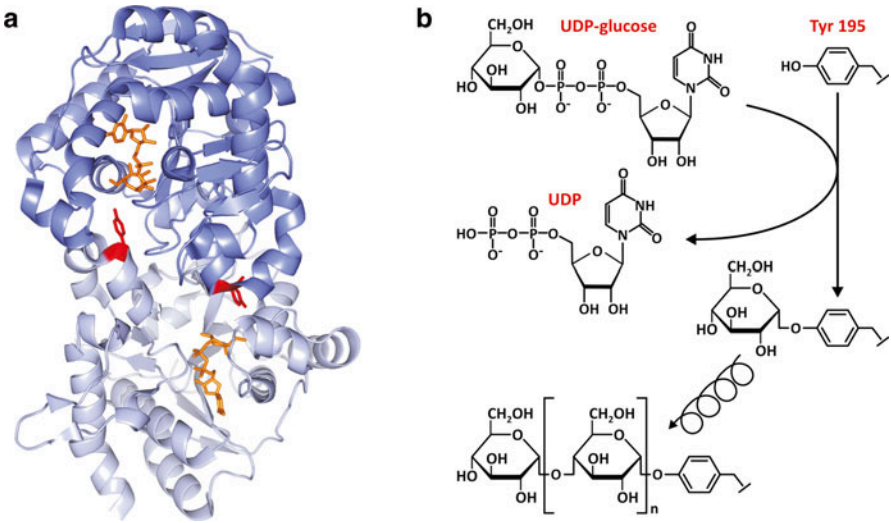


Fig. 8.7 (**a**) Structure of dimeric human glycogenin-1 (PDB 3T7O), highlighting Tyr195 (*red*), the residue that is glycosylated by the transfer of a glucose unit from UDP-glucose (*orange*), which is shown bound to the nucleotide-binding domain. (**b**) The two chemically distinct reactions that are catalyzed by glycogenin: the initial glucosylation of Tyr195 through the formation of a glucose-O-tyrosyl linkage (*top*) and the subsequent formation of the α-1,4-glucosidic linkages (*bottom*)

When the glycogen chain reaches at least 11 residues, it becomes a substrate for the branching enzyme, which catalyzes the transfer of a terminal segment of 7 glucose residues to a hydroxyl group on the position 6 of a glucose residue in the same or in a neighboring chain, creating an α-1,6-linked branch (Fig. 8.8b). Extra glucose residues may be added to the new branch or to the original chain by the action of GS.

Branching enzyme activity is important not only because the branches increase glycogen solubility inside the cell but also because it increases the number of nonreducing ends that are the sites of action of GS and GP, making glycogen molecule more readily metabolized.

Glycogen molecule usually grows until 12 tiers are formed, each of them with 12–14 glucose residues and two branches, resulting in approximately 55,000 glucose residues incorporated, which gives to the molecule an average molecular weight of 10^7 and a diameter of 21 nm.

8.2.4 Regulation of Glycogen Synthesis

GS is the key enzyme in the regulation of glycogen synthesis. GS activity is inhibited by phosphorylation, which can occur in multiple sites by at least 11 different protein kinases (Box 8.3).

Although phosphorylation causes GS inhibition, this effect can be completely overcome by the binding of glucose-6-phosphate, the major allosteric activator of GS (Fig. 8.9).

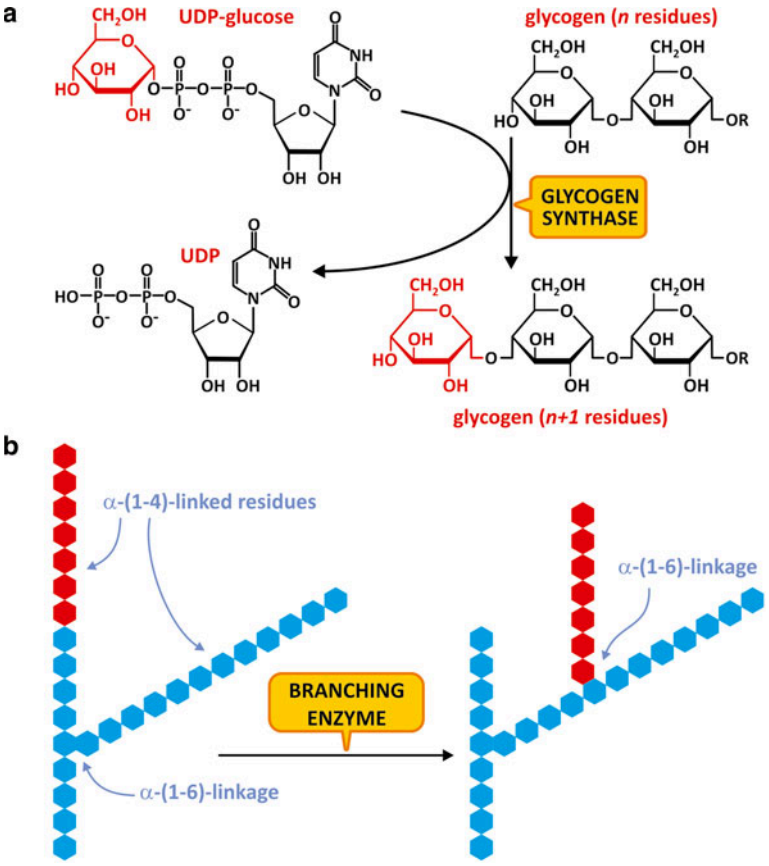


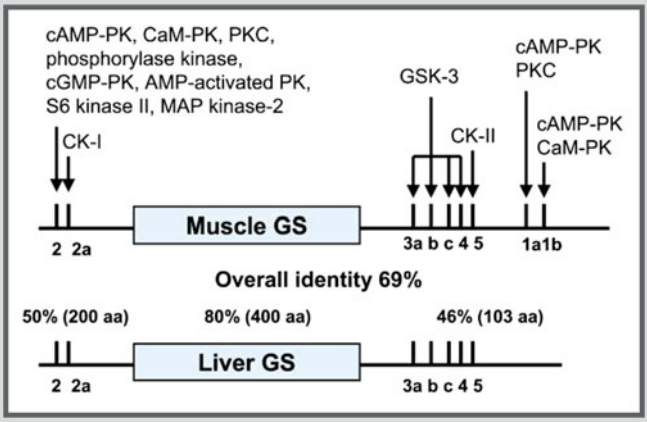
Fig. 8.8 (**a**) Reaction for glycogen chain elongation, catalyzed by GS. (**b**) Reaction for glycogen ramification, catalyzed by the branching enzyme

Box 8.3: The Multisite Phosphorylation in GS Inactivation

In humans, there are two isoforms of GS with 70 % identity: the muscle isoform, which is expressed in most tissues, and the liver isoform, whose expression is tissue specific. The catalytic site of the enzyme is located at the central region of its primary sequence and presents the highest degree of identity between the two isoforms (80 %). The N- and C-terminal ends show lower degree of homology between the isoforms (50 and 46 %, respectively) and contain multiple phosphorylation sites: 2 and 2a in the N-terminus and 3a, 3b, 3c, 4, 5, 1a, and 1b in the C-terminus (see figure). At least 11 different

(continued)

Box 8.3 (continued)
protein kinases are involved in GS phosphorylation, which results in enzyme inactivation: cAMP-dependent protein kinase (PKA), Ca^{2+}-calmodulin protein kinase (CaMK), protein kinase C (PKC), phosphorylase kinase (PK), cGMP-dependent protein kinase (PKG), AMP-activated protein kinase (AMPK), ribosomal protein S6 protein kinase II (S6KII), mitogen-associated protein kinase 2 (MAPK2), casein kinase I (CKI), glycogen synthase kinase 3 (GSK3), and casein kinase II (CKII). It is interesting to note that this mechanism of GS regulation involving multiple phosphorylations is very different from that of GP, the enzyme that catalyzes glycogen breakdown, which is activated by phosphorylation on a single serine residue by phosphorylase kinase (see Sect. 9.2.2).



Schematic representation of the primary sequence of the muscle and liver GS isoforms showing the phosphorylation sites and the possible protein kinases involved in phosphorylation. (Reproduced from Ferrer et al. FEBS Lett. 546:127–132, 2003, with permission from Elsevier)

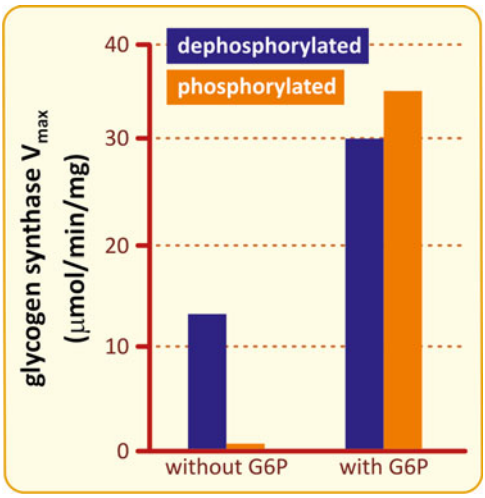


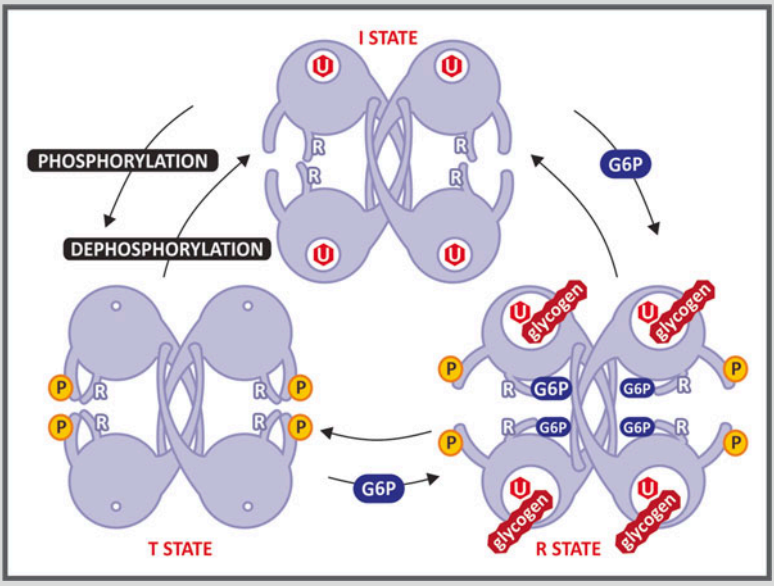
Fig. 8.9 Effects of phosphorylation and glucose-6-phosphate on GS activity. GS is activated by glucose-6-phosphate (G6P) either when it is phosphorylated (*orange*) or dephosphorylated (*blue*), but without G6P, the basal activity is inhibited by phosphorylation (Based on data from Pederson et al. J. Biol. Chem. 275:27753–27761, 2000)

The molecular mechanisms that underlie the switch between the inhibitory effect of phosphorylation and the enzyme activation by glucose-6-phosphate depend on a cluster of six conserved Arg residues located in the C-terminal region of the enzyme (Box 8.4).

The major protein kinase involved in GS inactivation is the glycogen synthase kinase 3 (GSK3), which catalyzes the sequential incorporation of phosphate groups in adjacent phosphorylation sites of the enzyme. However, evidence accumulated during the 1980s indicated that GSK3 alone cannot phosphorylate and inactivate GS, being necessary a concerted action of more than one protein kinase (Box 8.5).

Box 8.4: The Three-State Model for GS Regulation

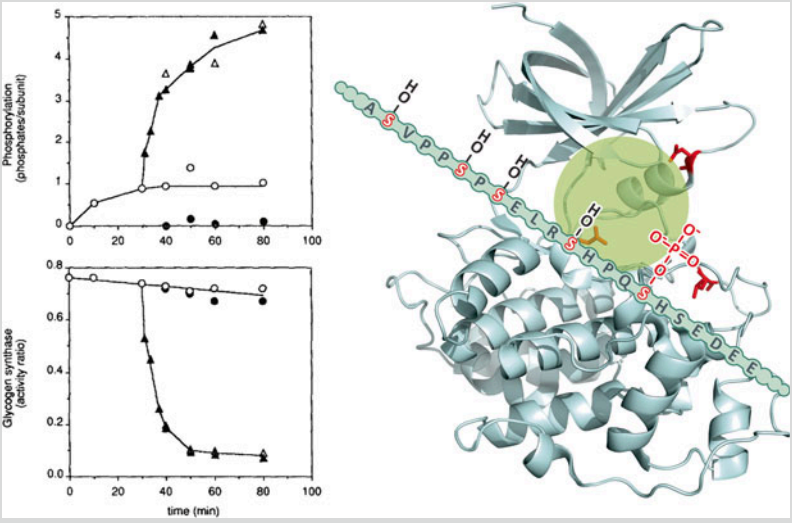
The crystal structure of yeast GS revealed that the eukaryotic enzymes exist as tetramers. The location of the helix containing the conserved Arg residues indicates that these residues interact with the phosphate groups of either glucose-6-phosphate or the phosphorylated sites, suggesting a three-state model for the regulation of GS activity (see figure). The I state, which corresponds to the dephosphorylated form, shows an intermediate basal activity due to its low affinity for glycogen. The T state is the phosphorylated form of the enzyme, in which the interaction between the phosphorylated residues and the conserved Arg locks the active site cleft, leading to enzyme inactivation. The R state is achieved by the binding of glucose-6-phosphate, whose phosphate group interacts with two conserved Arg residues, resulting in the opening of the active site cleft, allowing UDP-glucose and glycogen to bind and react.



Schematic representation of GS conformational states proposed in Baskaran et al. Proc Natl Acad Sci USA 107:17563–17568, 2010. The regulatory Arg residues (labeled with an R) interact with the phosphate groups of Tyr 668 in the phosphorylated form, locking the enzyme in the T state. Glucose-6-phosphate (G6P) binding frees this constraint, leading the enzyme to the R state, which is fully active. The hexagon labeled with U represents the UDP-glucose molecule

Box 8.5: Inactivation of GS by GSK3

The results of in vitro phosphorylation experiments, performed with the recombinant muscle GS, clearly demonstrated that GSK3 alone cannot phosphorylate and inactivate GS, requiring previous phosphorylation by the casein kinase II (see figure). Casein kinase II is able to incorporate one phosphate group (at the phosphorylation site 5; see Box 8.3) per subunit of GS, but this phosphorylation alone has little effect on the enzyme activity. On the other hand, once phosphorylated by casein kinase II, GS becomes an effective substrate for GSK3, which introduces additional four phosphate groups in the enzyme (at the phosphorylation sites 4, 3c, 3b, and 3a), resulting in a great decrease of its activity. This occurs because GSK3 needs to bind to a priming phosphate in order to catalyze the phosphorylation of the subsequent phosphorylation site (see the schematic representation in the figure of GSK3 structure). This latter site, when phosphorylated, becomes another site for GSK3 to bind and to act on the next available site. This sequential phosphorylation continues until five phosphate groups are incorporated in the GS C-terminal phosphorylation sites.



Left: quantification of phosphate groups incorporated (*upper panel*) and the effect on enzyme activity (*lower panel*) after incubation with casein kinase II alone (*open circles*), GSK3 alone (*filled circles*), or GSK3 added after 30 min incubation with casein kinase II (*triangles*) (Reproduced from Zhang et al. Arch. Biochem. Biophys. 304:219–225, 1993, with permission from Elsevier). *Right*: GSK3 structure (PDB 1H8F). The positively charged residues Arg96, Arg180, and Lys 205 (lateral chains shown in *red*) interact with the phosphate group incorporated in GS by casein kinase II, allowing the occupation of GSK3 active site (highlighted in *green*) with the GS subsequent phosphorylation. Thus, GS slides allowing the incorporated phosphate to interact with the positively charged GSK3 residues, allowing the next phosphorylation site to occupy the active site

(continued)

Box 8.5 (continued)

It is interesting to point out that although GSK3 was firstly discovered as acting in insulin-mediated activation of GS, it is now recognized as a constitutively active protein kinase that phosphorylates a wide range of substrates. GSK3 is involved in many cellular processes, such as cell proliferation, neuronal function, oncogenesis, and embryonic development, besides glycogen metabolism and insulin signaling. Interestingly, many of GSK3 substrates, besides GS, need this "priming phosphorylation" in a Ser/Thr residue located +4 residues of the site of GSK3 phosphorylation.

Since GSK3 is a constitutive enzyme, GS is maintained constantly phosphorylated and inactive until GSK3 is inhibited. GSK3 is inhibited by phosphorylation mediated by insulin action (see the detailed mechanism in Sect. 8.4). Thus, when glycemia increases and insulin is secreted, GSK3 becomes inhibited, allowing GS to be dephosphorylated and activated. Besides mediating the inhibition of GSK3 activity, insulin action also stimulates glycogen synthesis by inducing the dephosphorylation of GS. GS dephosphorylation is catalyzed by the protein phosphatase 1 (PP1), the major protein Ser/Thr phosphatase in eukaryotic cells, which associates to glycogen granules in response to insulin signaling pathway and also acts on GP and on phosphorylase kinase, leading to their inactivation. Thus, PP1 promotes the simultaneous activation of GS and inactivation of GP, favoring glycogen accumulation inside the cells.

It is interesting to note that PP1 does not act on all of its substrates in the cell at the same time. Its activity depends on regulatory proteins, which work as substrate-specifying subunits, recruiting PP1 to specific targets inside the cell. PP1 is associated to glycogen granules through a family of these target subunits, known as G target subunits, which mediate PP1 activity on GS, GP, and phosphorylase kinase (Fig. 8.10).

In muscle, PP1 binds to glycogen essentially through the target subunit G_M, which increases its activity against GS, GP, and phosphorylase kinase (Fig. 8.10). In the liver G_L and G_C (or PTG) are expressed at the same levels. G_L contains a binding site to GP*a*, which inhibits PP1 activity. Glucose binding to GP, besides inhibiting its activity, also promotes its release from G_L, activating PP1, and, consequently, the dephosphorylation and activation of GS. Thus, during hyperglycemic conditions, the increase in glucose concentration inside the hepatocytes directly and indirectly inhibits glycogen degradation and activates glycogen synthesis (Fig. 8.10).

Muscle and liver GS also differ in their intracellular localization. In muscle cells, when intracellular glucose concentration is low, GS accumulates in the nucleus. On the other hand, when insulin is released in the bloodstream, and glucose concentration increases inside the cell due to the insulin-dependent glucose uptake by GLUT4, the enzyme is translocated to the cytosol, where it becomes active, both by the direct

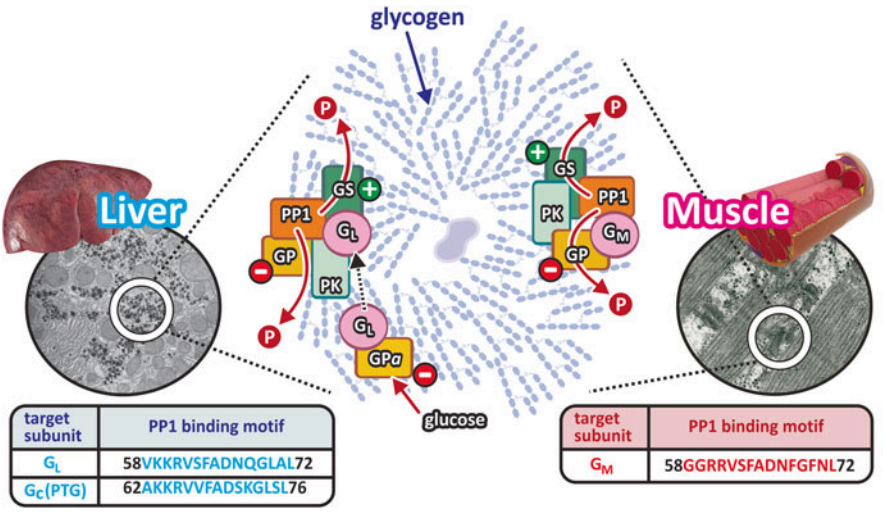


Fig. 8.10 The enzymes of glycogen metabolism (GS, GP, and phosphorylase kinase, PK) are associated to glycogen granules in the liver (*left*) or muscle (*right*) cells. In liver cells, the increase in intracellular glucose concentration inactivates GP and induces the dissociation of G_L, which binds to glycogen granule and recruits PP1, which, in turn, dephosphorylates GS, activating it, and GP and PK, inactivating them. In muscle, G_M recruits PP1, increasing its activity against GS, GP, and PK. PP1-binding motifs in each of the target subunits (as identified in the work by Fong et al. J. Biol. Chem 275:35034–35039, 2000) are represented in the *boxes*

binding of glucose-6-phosphate and due to its dephosphorylation mediated by insulin. In liver cells, GS moves from a diffuse distribution in the cytosol to the cell periphery in response to elevation of the intracellular glucose concentration, which in this case, reflects directly the increase in glycemia due to GLUT2 and GK properties. As glycogen synthesis proceeds, glycogen deposits grow from the periphery toward the center of the cell, and GS maintains its co-localization with glycogen molecules.

8.3 Biosynthesis of Lipids

The major energy reserve in humans is constituted of lipids, which are stored as triacylglycerols in lipid droplets inside the adipocytes, the main cellular type in the adipose tissue (Fig. 8.11). Adipose tissue corresponds to 12–20 % of the body weight of a nonobese man and may yield more than 100,000 kcal upon degradation.

Additionally, other lipids play equally important roles in human organisms, such as the phospholipids and cholesterol that are constituents of cellular membranes, the steroids that act as hormones, the eicosanoids that are extra- and intracellular messengers, and vitamin K that acts as cofactor of enzymatic reactions, among others.

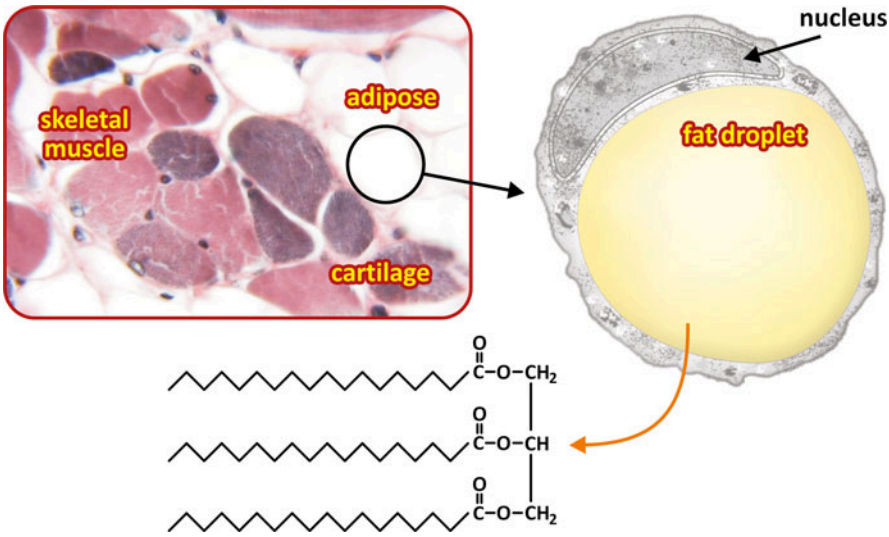


Fig. 8.11 Histological section showing the adipose tissue (Reprinted with the permission of Instituto de Histologia e Biologia do Desenvolvimento, Faculdade de Medicina, Universidade de Lisboa, FMUL). In detail, an electron microscopy of an adipocyte, showing the lipid droplet inside, which stores triacylglycerol molecules

Lipids have been regarded for a long time as relatively inert components of the body, at least in part due to the fact that the earlier methodologies to study metabolism were developed for aqueous systems. This picture started to change significantly after the pioneering studies of Rudolf Schoenheimer, who revolutionized the concept of metabolic activity by merging atomic physics and physiology through the incorporation of isotopic atoms into specific positions of organic molecules. This approach allows the pathway and the fate of different molecules to be traced, overcoming the limitations of the attachment of detectable chemical groups, as in the experiments previously performed by Franz Knoop (see Sect. 7.4), since the binding of these chemical groups would introduce structural changes in the target molecules and would alter them greatly in comparison to the natural analogs.

Schoenheimer fed mice with deuterated fatty acids and found that ingested lipids interchanged with those of the tissues, and even in mice that lost weight, a large proportion of the labeled fatty acids was deposited, indicating that fats were not catabolized directly following absorption, but they were mobilized from the adipose stores. From these results, Schoenheimer concluded: "The fat tissues are generally regarded as storage for a time of need and have frequently been compared with a dead storage or food cellar for emergencies. On the basis of the new findings, the fat tissues might better be compared with the refrigerator in which excess is stored for the short interval between meals."

Rudolf Schoenheimer (1898-1941)

Schoenheimer's findings provided a new view of metabolism, with the concept of a "dynamic state of body constituents," which, although already noticed by Claude Bernard in the nineteenth century (see Sect. 9.2), was only clearly proved with Schoenheimer's experiments.

Since the isotopic labeling has revealed the rapid metabolic turnover of body fat, it becomes clear that the conversion of carbohydrate to fat would be a major route of glucose metabolism. Indeed, several experiments in which animals were fed with a fat-free diet containing labeled glucose demonstrated the incorporation of glucose-derived atoms into fat.

In this section, we will present the metabolic pathway for the synthesis of fatty acids and show how these molecules are incorporated into triacylglycerols. Additionally, the integration of carbohydrate metabolism and fatty acid synthesis will be discussed, including how glucose is converted in fatty acids and how a shunt of the glycolytic pathway, the pentose-phosphate pathway, is essential for generating the reducing power necessary for the lipid biosynthesis.

8.3.1 Synthesis of Fatty Acids

Until the decade of the 1950s, fatty acid synthesis was thought to be the reversal of β-oxidation. This idea was sustained by the fact that, at that time, all the enzymes of β-oxidation had been purified and each reaction had been shown to be reversible. However, now it is known that, although the synthesis of fatty acid involves the sequential addition of acetyl units into a nascent fatty acid chain, this pathway occurs in the cytosol in contrast to the mitochondrial localization of β-oxidation, and it is catalyzed by different enzymes, two unusually large multifunctional enzymes, acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS). Regarding tissue localization, fatty acid synthesis occurs mainly in liver and adipose tissue, as well as in mammary gland during lactation.

8.3.1.1 Reactions for Fatty Acid Synthesis

Incorporation of acetyl units into the nascent fatty acid chain requires the previous formation of a three-carbon molecule, malonyl-CoA. This molecule, besides being the substrate for fatty acid synthesis, is also the negative regulator of fatty acid oxidation by inhibiting the carnitine acyltransferase 1 and, thus, blocking the transport of the fatty acyl-CoA into the mitochondria (see Sect. 7.4.3).

Malonyl-CoA is generated by the carboxylation of acetyl-CoA in a reaction catalyzed by the enzyme ACC (Fig. 8.12). ACC is one of the biotin-dependent carboxylases, a group of enzymes that use the same biochemical strategy to overcome the high energy barrier of the carboxylation reaction. These enzymes have two enzymatic activities catalyzed by their biotin carboxylase (BC) and carboxyltransferase (CT) components and perform a two step-reaction in which the enzyme-linked biotin is carboxylated in an ATP-dependent reaction (Box 8.6). Human ACC is a ~250 kD protein in which BC and CT activities are separated in two domains of the same polypeptide chain. A third domain of the enzyme, the biotin-carboxyl carrier protein (BCCP) component, contains the biotin prosthetic group covalently linked to a Lys residue. The structural organization of the enzyme allows that biotin move from one catalytic site to the other during catalysis.

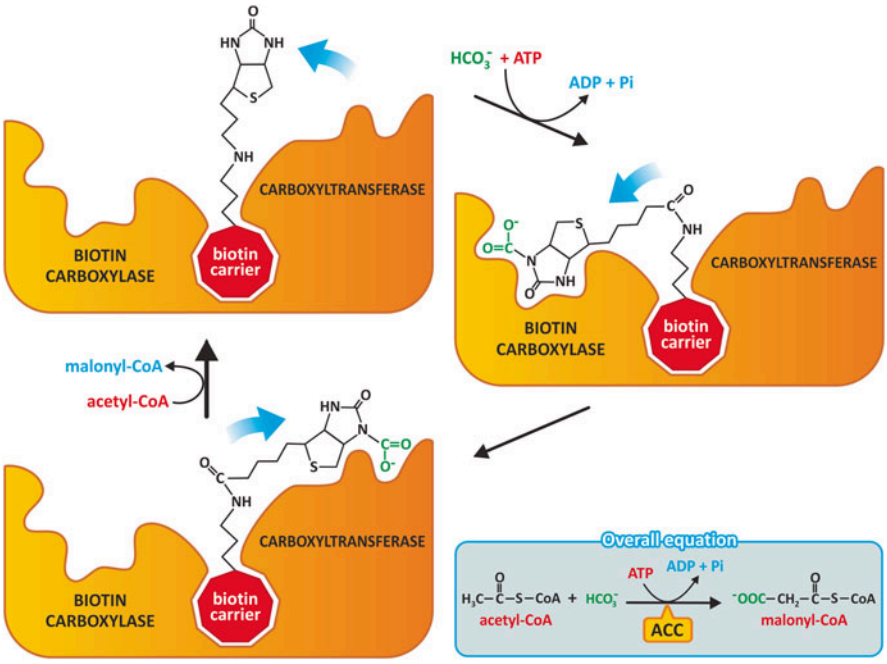
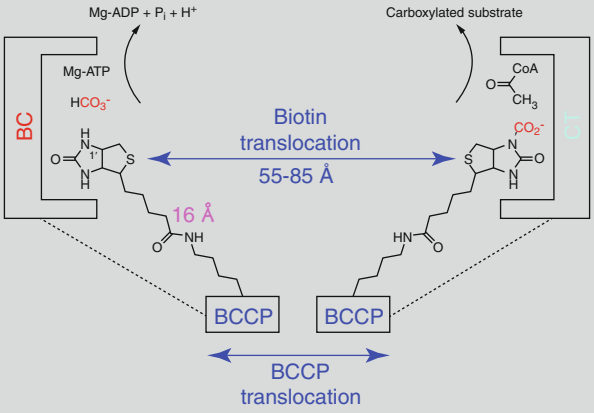


Fig. 8.12 Reaction catalyzed by ACC. First the BC component of the enzyme catalyzes the ATP-dependent carboxylation of the biotin prosthetic group linked to the BCCP component of the enzyme. Then, the biotin arm moves the carboxybiotin to the CT component of the enzyme, which catalyzes the transfer of the carboxyl group from the biotin to acetyl-CoA, forming malonyl-CoA

Box 8.6: The Biotin-Dependent Carboxylases

Biotin-dependent carboxylases include acetyl-CoA carboxylase (ACC), propionyl-CoA carboxylase (PCC), 3-methylcrotonyl-CoA carboxylase (MCC), geranyl-CoA carboxylase, pyruvate carboxylase (PC; see Sect. 9.3.1), and urea carboxylase (UC). They contain three distinct components in their structure: the biotin carboxylase (BC), carboxyltransferase (CT), and biotin-carboxyl carrier protein (BCCP) components, which occur, depending on the organism, as separate subunits (usually in bacteria) or part of a multi-domain protein (in eukaryotes), with some intermediate structural organizations also found in nature. These enzymes contain a biotin prosthetic group covalently linked to a Lys residue of the BCCP component and display two distinct enzymatic activities. First, the BC component catalyzes carboxylation of the biotin cofactor, using bicarbonate as the CO_2 donor, and then the CT component catalyzes the CO_2 transfer from carboxybiotin to the substrate (see figure).

Biotin linkage to the Lys side chain in BCCP forms a highly flexible 16 Å-length arm composed of eight methylene groups with ten rotatable single bonds, leading to the suggestion of a "swinging-arm model" for the mechanism for the translocation of biotin from BC to CT active sites, in which arm flexibility allows biotin to move from BC to CT sites. However, structure determination of several biotin-dependent carboxylases revealed that the distance between BC and CT active sites ranges from 55 to 85 Å, suggesting that besides the movement of the flexible biotin arm itself, a translocation of the BCCP domain is also necessary for biotin to visit the BC and CT active sites, in what was called a "swinging-domain model" for the catalytic activity of the biotin-dependent carboxylases.



Schematic representation of the biotin-dependent carboxylase-catalyzed reactions. First BC component catalyzes the carboxylation of the biotin prosthetic group linked to BCCP component of the enzyme, in a reaction dependent on Mg^{2+}-ATP hydrolysis. Then, biotin arm flexibility together with BCCP translocation allows carboxylated biotin to move in the direction of the TC component, which catalyzes the transfer of carboxyl group attached to the biotin to the substrate (in this example acetyl-CoA), generating the carboxylated molecule of carboxlated molecule. Figure reproduced with permission from Tong, Cell. Mol. Life Sci. 70:863–891, 2013

Two human ACC isoforms, presenting 73 % identity of amino acid sequences, were identified. They are highly segregated within the cell and play different roles in metabolism (Fig. 8.13). ACC1 is expressed in all tissues but in high levels in the liver, adipose tissue, and lactating mammary gland and produces malonyl-CoA for fatty acid synthesis. ACC2 is highly expressed in the skeletal muscle and heart and is associated to the mitochondrial membrane, where the synthesized malonyl-CoA acts as an inhibitor of carnitine/palmitoyl shuttle system, impairing fatty acid oxidation. The implications of the fate of malonyl-CoA produced by each ACC isoform in the regulation of lipid metabolism will be discussed in the next section.

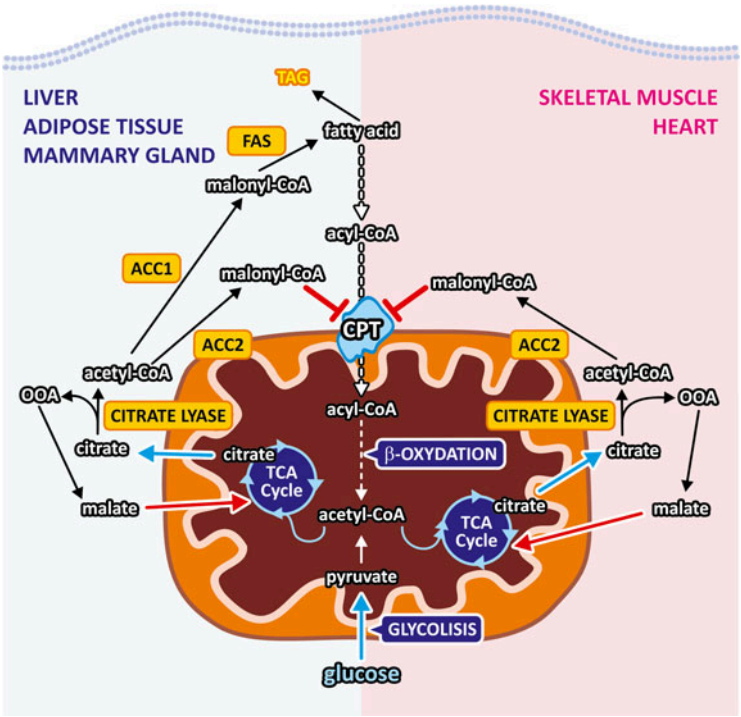


Fig. 8.13 ACC isoforms and their intracellular localization. ACC1 is highly expressed in the cytosol of liver and adipose tissue and lactating mammary gland cells, where it converts acetyl-CoA to malonyl-CoA, which is used as substrate for fatty acid synthase (FAS) in the fatty acid synthesis. In contrast, since ACC2 is associated to the mitochondrial membrane, the malonyl-CoA produced acts as an inhibitor of the carnitine/palmitoyl transferase (CPT), impairing the transport of the acyl-CoA to mitochondrial matrix and, thus, its oxidation. The substrate for both ACC isoforms, acetyl-CoA, is produced in the cytoplasm from citrate, which leaves the mitochondrial matrix after its accumulation due to the high rate of glucose metabolization. The *dashed lines* indicate inhibited reactions

Malonyl-CoA produced by ACC1 is the immediate substrate for fatty acid synthesis, which has as the main end product palmitate, a 16-carbon saturated fatty acid (see Sect. 3.1). Fatty acids with other chain length and/or containing insaturations are produced by modification of this previously synthesized palmitate.

The overall process of palmitate synthesis is catalyzed by multifunctional enzyme FAS (see Box 8.7) and occurs through sequential condensation of seven two-carbon units derived from malonyl-CoA to a primer acetyl group derived from acetyl-CoA.

Box 8.7: The FAS Isoforms

Although fatty acid biosynthesis occurs through a conserved set of chemical reactions in all organisms, the structural organization of FAS varies. In animals and fungi, FAS is a huge cytosolic multifunctional enzyme referred as type I FAS, whereas in plant and bacteria the different enzymatic activities for fatty acid synthesis are accomplished by separate polypeptide chains, known as type II FAS system. It is interesting to note that this is similar to what is observed for bacterial ACC, which shows its three components as separate subunits, in contrast to the multi-domain protein from eukaryotes (see Box 8.6). It is believed that the ancestor of type I FAS resembled the dissociated type II FAS, and gene duplication, loss of function, and gene fusion gave rise to the multienzyme of mammals.

The human FAS is a cytosolic homodimer of 270 kDa monomers, highly expressed in the liver, adipose tissue, and mammary glands during lactation, although it is also detected in some level in almost all tissues of the human body. It performs seven enzymatic activities comprised in six domains of the same polypeptide chain. Additionally, a seventh domain, the acyl carrier protein, contains the prosthetic group phosphopantetheine, to which the intermediates are linked during the synthesis reactions. The seven domains are named accordingly to their respective enzymatic activities and occur in the following order from the enzyme N-terminus: β-ketoacyl synthase (KS); malonyl/acetyl transferase (MAT), which is a bifunctional domain catalyzing acetyl and malonyl transferase reactions; β-hydroxyacyl dehydratase (DH); enoyl reductase (ER); β-ketoacyl reductase (KR); acyl carrier protein (ACP); and thioesterase (TE) (Fig. 8.14a). Although each monomer contains all the activities, only the dimeric form is functional. This is explained by the fact that in the dimer, the monomers form an intertwined, X-shaped, head-to-head homodimer, in a way that a complete cycle of reactions depends on activities located at distinct monomers (Fig. 8.14b, c).

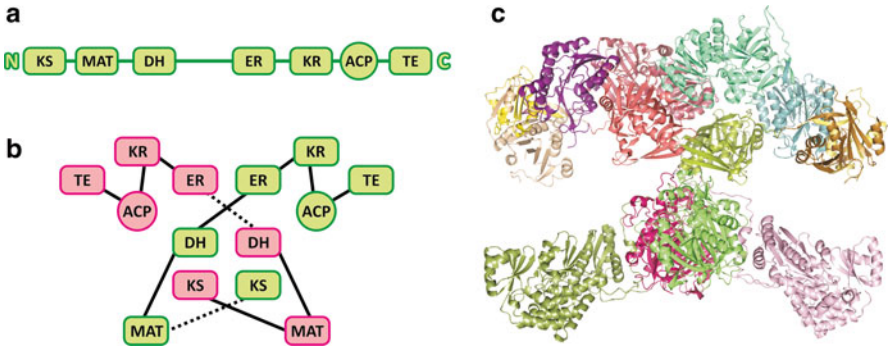


Fig. 8.14 (**a**) Primary structure of FAS showing the position of the seven domains from the N- to the C-terminus of the sequence: β-ketoacyl synthase (KS), malonyl/acetyl transferase (MAT), β-hydroxyacyl dehydratase (DH), enoyl reductase (ER), β-ketoacyl reductase (KR), acyl carrier protein (ACP), and thioesterase (TE). (**b**) Schematic representation of the X-shaped dimeric form of FAS. Each monomer is shown in a *different color*. (**c**) Crystallographic structure of FAS (natively purified from pigs). The solved structure covers five catalytic domains (the ACP and TE domains remained unresolved). One subunit is colored by different shades of *purple pink* and the other of *blue green*. Two additional nonenzymatic domains were identified in the crystal structure: a pseudo-ketoreductase (ΨKR, colored in *yellow*) and a pseudo-methyltransferase (ΨME, colored in *beige*) that is probably a remnant of an ancestral methyltransferase domain maintained in some related enzymes (Adapted from Liu et al. Int J Biochem Mol Biol 1:69–89, 2010, using PDB file 2VZ8)

Palmitate synthesis starts with an acetyl and a malonyl groups attached to the enzyme through thioester linkages. The acetyl group comes from acetyl-CoA and is transferred to a thiol group of a Cys residue of the KS portion of the enzyme by the acetyl transferase activity of FAS MAT domain. The malonyl group is derived from malonyl-CoA and is transferred to the thiol group at the end of the phosphopantetheine cofactor in the ACP domain of the enzyme by the malonyl transferase activity of FAS MAT domain (Fig. 8.15a).

A decarboxylative condensation reaction between acetyl and malonyl groups, with the elimination of CO_2, forms an acetoacetyl group bound to ACP thiol group. This step is catalyzed by FAS KS activity and is energetically favored by the decarboxylation of the malonyl group (Fig. 8.15a).

The acetoacetyl-ACP is reduced to β-hydroxybutyryl-ACP by the FAS KR activity, with concomitant oxidation of NADPH to $NADP^+$. β-hydroxybutyryl-ACP is dehydrated, generating a double bond that forms the transbutenoyl-ACP, in a reaction catalyzed by the FAS DH activity. Then, the double bond is reduced by the FAS ER activity, in a reaction coupled to oxidation of another NADPH molecule to $NADP^+$, which generates the saturated butyryl-ACP (Fig. 8.15a). Note that these three last steps correspond to the reverse of β-oxidation first reactions (see Sect. 7.4.4), except that the electrons come from NADPH instead of being transferred to NAD^+ or FAD.

After the first cycle of synthesis, the butyryl group bound to ACP is transferred to the thiol group of the Cys residue of KS (Fig. 8.15a), without dissociating from

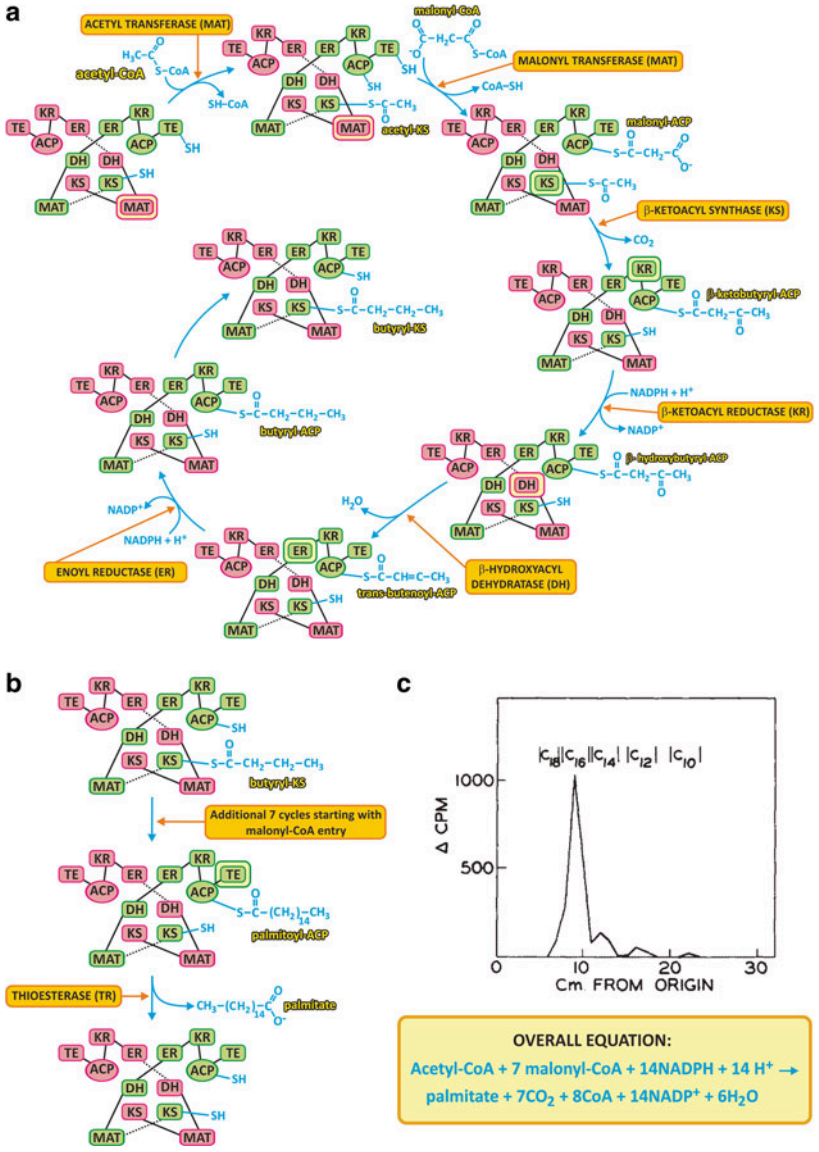


Fig. 8.15 (**a**) Schematic representation of fatty acid synthesis, starting with the transfer of acetyl and malonyl groups from acetyl-CoA and malonyl-CoA molecules to the SH groups of KS and ACP domains, respectively. KS activity condenses the acetyl and malonyl groups, generating β-ketobutyryl bound to ACP, which is sequentially reduced, dehydrated, and reduced, by the action of the KR, DH, and ER activities, respectively. In the reduction reactions the electron donor molecule is NADPH. These series of reactions generate a butyryl group bound to ACP, which is transferred to the SH group of KS, allowing the onset of the second round of fatty acid synthesis by making the ACP free to receive another malonyl group from malonyl-CoA. (**b**) After seven cycles starting with malonyl transfer to ACP, palmitate is released by the TE activity. (**c**) Distribution of ^{14}C-long-chain fatty acid synthesized in pigeon liver preparation incubated with ^{14}C-acetate after separation by a paper chromatography system (Reproduced from Porter & Tietz, BBA 25:41–50, 1957 with permission from Elsevier), the experiment that showed that only when the fatty acid reaches 16 carbons atoms it is released form the enzyme. The FAS enzymatic activities operating in each step are shown in *yellow boxes*, with the respective position highlighted in the dimer structure

the enzyme. Then, another malonyl-CoA is transferred to the unoccupied thiol group of the phosphopantetheine in the ACP, starting the second round of the fatty acid synthesis, which gives rise to a six-carbon intermediate by its condensation with the butyryl group attached to the thiol group of KS, to which the subsequent reactions are repeated.

After seven cycles of condensation and reduction, the palmitoyl group is released from ACP by the FAS TE activity, generating free palmitate in the cytosol (Fig. 8.15b), which may be incorporated into triacylglycerol or phospholipid molecules.

The long and flexible structure of the phosphopantetheine group bound to ACP allows the delivering of the intermediates to the active sites of all catalytic domains. Throughout the synthesis, the intermediates remain covalently bound as thioesters to a thiol group in the enzyme, either in KS or in ACP. The first evidence for this was obtained in the decade of the 1950s, in experiments in which after the incubation of an enzyme preparation from pigeon liver with isotopically labeled acetate, the radioactivity was found associated only to fatty acids with 16 carbons and not uniformly distributed among intermediates of different chain lengths (Fig. 8.15c).

It is interesting to note that the three-dimensional structure of FAS dimer shows that the whole structure can be divided into two portions: the condensing portion, containing KS and MAT domains, and the modifying portion, containing DH, ER, and KR domains (see Fig. 8.14c).

It is important to note that biosynthesis of fatty acid, as well as other biosynthetic processes, takes place entirely in the cytosol, in opposition to most of the oxidation reactions that occur in the mitochondrial matrix. The use of NADPH instead of NADH as the electron donor in the fatty acid synthesis is very important in this context, since glycolysis, which occurs simultaneously to fatty acid synthesis in the cytosol, requires a low NADH/NAD^+ ratio to allow the oxidation glyceraldehyde-3-phosphate to 1,3-biphosphoglycerate, a NAD^+-dependent reaction catalyzed by glyceraldehyde-3-phosphate dehydrogenase (see Sect. 6.1). Thus, cytosolic high NADPH/$NADP^+$ and low NADH/NAD^+ ratios enable the oxidative reaction of glycolysis to occur at the same time and cellular compartment that the reductive fatty acid synthesis. It is interesting to note that the phosphate group in NADPH/$NAPD^+$ does not interfere with the redox reaction itself. It only grants specificity for enzyme recognition (see Sect. 5.5).

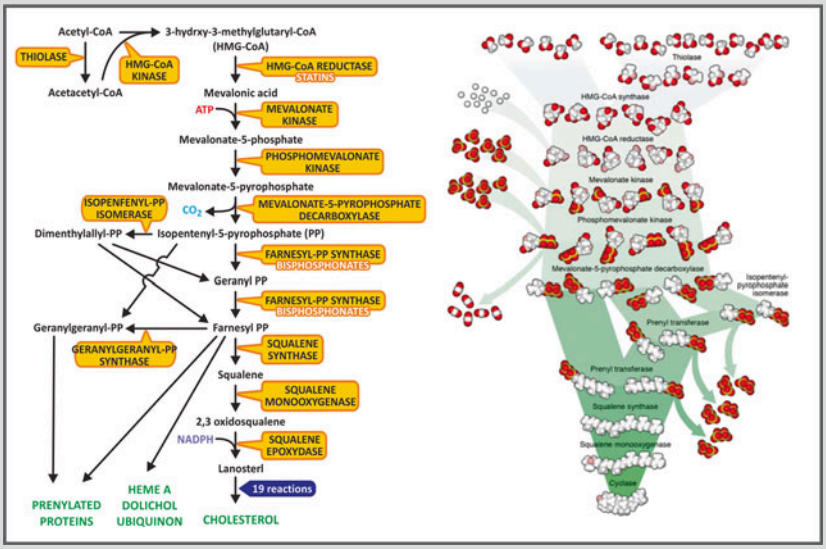
Acetyl-CoA, besides being the immediate substrate for fatty acid synthesis (both as substrate for ACC, generating malonyl-CoA, and as the donor of the first acetyl unit transferred to FAS KS), is also the precursor for the cholesterol synthesis (Box 8.8). The precursor of acetyl-CoA molecules used for these biosynthetic processes is mainly the ingested carbohydrate, as it will be discussed in the next topic.

8.3.1.2 Origin of the Acetyl-CoA for the Fatty Acid Synthesis

Fatty acid synthesis occurs mainly in liver and adipose tissue due to the high expression of both ACC1 and FAS in hepatic and adipose cells. In both tissues, the use of glucose is greatly increased as glycemia rises, either by kinetic properties of GLUT2/GK in the liver (see Sect. 8.1) or by the insulin-dependent glucose uptake in adipose cells (see Sect. 8.4.3).

Box 8.8: Cholesterol Biosynthesis

Cholesterol is a 27-carbon steroid with key roles in the metabolism. It is an essential structural component of animal cell membranes, modulating their fluidity and permeability (see Sect. 3.1), and is also the precursor of the steroid hormones, bile acids, and vitamin D. Cholesterol synthesis is very complex (see figure) and may be didactically divided in three parts. In the first phase, three acetyl units are used to synthesize the isopentenyl pyrophosphate, which act as the building block of cholesterol synthesis. This phase includes the key step in the regulation of the process, the formation of 3-hydroxyl-3-metylglutaryl-CoA from acetyl-CoA and acetoacetyl-CoA, catalyzed by the enzyme 3-hydroxyl-3-metylglutaryl-CoA reductase. In the second phase, six molecules of isopentenyl pyrophosphate are condensed to form squalene, a 30-carbon isoprenoid. In the third phase, squalene cyclizes to form a tetracyclic structure, which rearranges to form lanosterol. Lanosterol is then converted to cholesterol through 19 reactions, catalyzed by enzymes associated with endoplasmic reticulum membrane, including some members of the cytochrome P450 superfamily (see Box 8.9).



Cholesterol biosynthesis with the molecular transformations shown in the right and the reactions depicted in left. The names of the enzymes are shown in yellow boxes, with some inhibitors indicated. Figure reproduced from Goodsell DS. The machinery of life. Springer, New York, 2009

As glycolysis proceeds and pyruvate is completely oxidized in TCA cycle, the resulting NADH and $FADH_2$ feed the electron transport chain, whose activity culminates with ATP synthesis through oxidative phosphorylation (see Chap. 6) (Fig. 8.16). When the ATP/ADP ratio is high, ATP synthesis decreases due to the lack ADP, resulting in the accumulation of reduced electron carriers (NADH and $FADH_2$) in mitochondrial matrix. This leads to the inhibition of the TCA cycle dehydrogenases, which depend on the oxidized electron carriers (NAD^+ and FAD)

in order to proceed. The final consequence of this situation is the accumulation of citrate, which leaves mitochondria through the citrate transporter (Fig. 8.16).

In the cytosol, citrate is broken to acetyl-CoA and oxaloacetate, through an ATP-dependent reaction catalyzed by citrate lyase. This acetyl-CoA molecule is then used as a substrate for the fatty acid synthesis.

Oxaloacetate does not directly return to mitochondria due to the absence of a transporter in the mitochondrial membrane. Thus, it is firstly converted by the cytosolic malate dehydrogenase to malate, which enters the mitochondria, where it regenerates oxaloacetate. Malate dehydrogenase reaction is also important to reoxidize the NADH produced in glycolysis (in the reaction catalyzed by glyceraldehyde dehydrogenase; see Sect. 6.1.3), allowing the maintenance of a high NAD^+/NADH ratio in the cytosol, necessary for glycolytic pathway to proceed (Fig. 8.16).

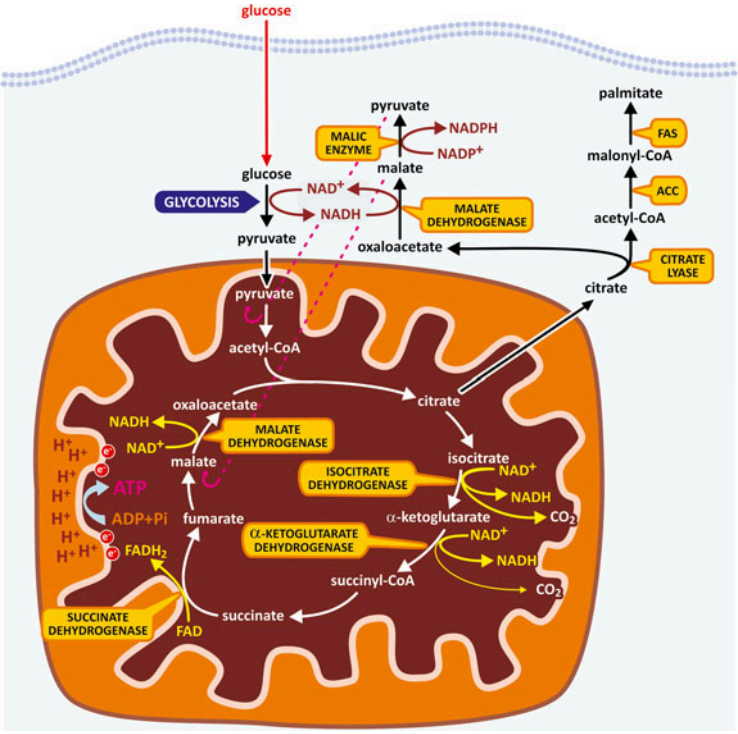


Fig. 8.16 Schematic representation of the transformation of glucose in fatty acids in liver and adipose tissue. Complete oxidation of glucose results in NADH and $FADH_2$ that feed the electron transport chain, whose activity generates ATP. When the ATP/ADP ratio is high, ATP synthesis decreases due to the lack ADP, resulting in the accumulation of NADH and $FADH_2$ in mitochondrial matrix, inhibiting TCA cycle dehydrogenases, which depend on NAD^+ and FAD to proceed, and thus leading to citrate accumulation. Citrate leaves mitochondria, being broken to acetyl-CoA and oxaloacetate by citrate lyase in the cytosol. Acetyl-CoA is used in the fatty acid synthesis. Oxaloacetate is converted to malate, with concomitant oxidation of cytosolic NADH to NAD^+, favoring glycolysis, which requires NAD^+ for the reaction of glyceraldehyde-3-phosphate dehydrogenase. Malate may enter the mitochondria, where it regenerates oxaloacetate, or may be converted to pyruvate, by the malic enzyme, in a reaction that generates part of the NADPH necessary to the fatty acid synthesis. The names of the enzymes are highlighted in *yellow boxes*

Alternatively, malate may be converted to pyruvate, by the malic enzyme, in a reaction that uses $NADP^+$ as the electron acceptor, generating part of the NADPH necessary for fatty acid synthesis (Fig. 8.16).

8.3.1.3 Origin of NADPH for the Fatty Acid Synthesis

Most of the NADPH used in fatty acid synthesis is provided by the pentose-phosphate pathway, an alternative pathway that oxidizes glucose-6-phosphate (see also Box 8.9).

The first evidence of an oxidative pathway that converted glucose-6-phosphate in a pentose phosphate was demonstrated in yeast, in the decade of the 1930s, by pioneering studies of Otto Warburg, who also had shown that this pathway was dependent on a coenzyme different from that required in glycolysis (named by Warburg at that time as TPN, from triphosphopyridine nucleotide, which is now known as NADPH). Further work by Frank Dickens proved that this pathway occurred in many animal tissues and proceeded independently of the glycolytic route, since high concentrations of glycolytic inhibitors, such as iodoacetamide, had no effects on its activity.

The pentose-phosphate pathway may be divided in two phases: (a) an oxidative phase, in which glucose-6-phosphate is converted to a pentose-phosphate, with two $NADP^+$-dependent oxidation steps, the last one being coupled to a decarboxylation reaction, and (b) a non-oxidative phase, in which the carbon skeletons of the pentose phosphates are rearranged to generate intermediates of glycolytic pathway, fructose-6-phosphate and glyceraldehyde-3-phosphate. A schematic representation of these two phases is shown in Fig. 8.17.

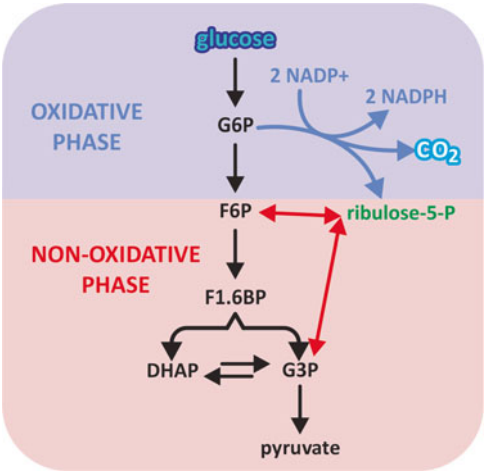


Fig. 8.17 Schematic representation of the two phases of pentose-phosphate pathway and their relationships with glycolysis

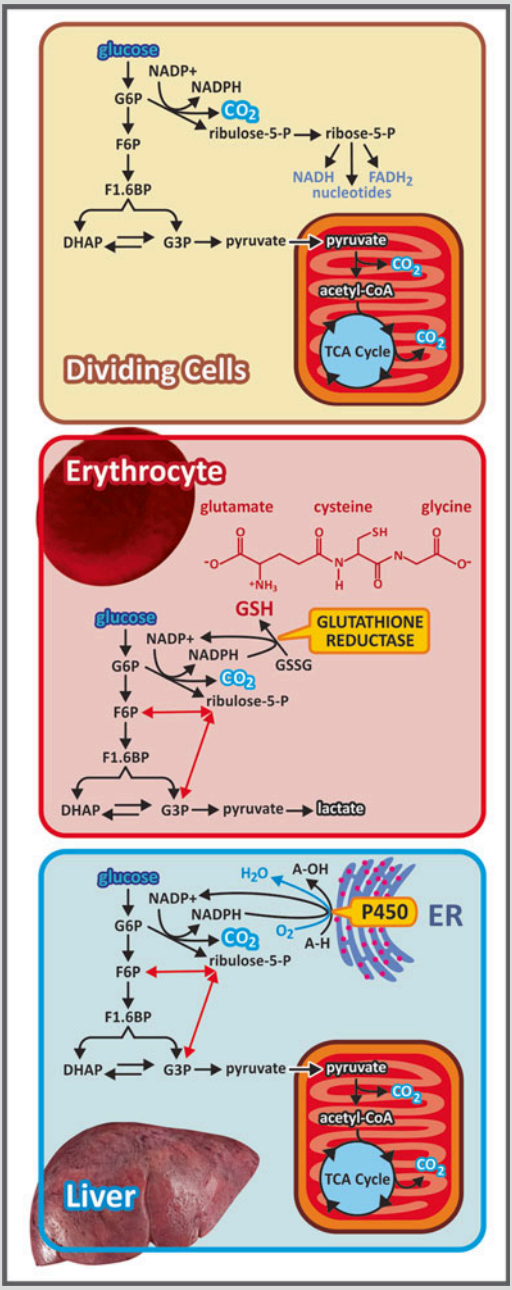
The oxidative phase starts with the oxidation of glucose-6-phosphate to 6-phosphogluconolactone, in a reaction catalyzed by glucose-6-phosphate dehydrogenase, in which $NADP^+$ is the electron acceptor, generating NADPH. Then, a hydrolysis reaction forms 6-phosphogluconate that undergoes an $NADP^+$-dependent oxidation and decarboxylation, catalyzed 6-phosphogluconate dehydrogenase, which forms ribulose-5-phosphate and the second molecule of NADPH (Fig. 8.18).

Box 8.9: Other Roles of the Pentose-Phosphate Pathway

Besides yielding the reducing power in the form of NADPH for the biosynthetic processes, the pentose-phosphate pathway plays other important roles in human metabolism. In cells with a rapid rate of division, such as the bone marrow, skin, and intestinal mucosa cells, glucose-6-phosphate follows the oxidative phase of the pentose-phosphate pathway, generating ribose-5-phosphate, which is used in the synthesis of nucleotides and nucleic acids as well as of NADH, $FADH_2$, and coenzyme A (see figure in the top). Another important role of the NADPH produced in the pentose-phosphate pathway is to prevent oxidative damage. This is the case of the erythrocytes, which are directly exposed to oxygen and are highly dependent on glutathione reduction as a protective mechanism against oxidative stress (see figure in the middle). Glutathione is a tripeptide composed of Glu, Cys, and Gly that occurs in a high intracellular concentration (~5 mM). The sulfhydryl group of the Cys residue acts as a reducing agent, which is oxidized with the formation of a disulfide bridge that links two glutathione molecules (represented as GSSG), preventing oxidative damage to cell molecules. Glutathione is converted back to its reduced form (GSH) by the enzyme glutathione reductase, in a reaction dependent on NADPH. Thus, to maintain glutathione in the reduced form, erythrocytes are highly dependent on the pentose-phosphate pathway to generate NADPH. Additionally, the metabolization of a number of drugs and other toxic chemicals, as well as endogenous compounds such as lipids and steroidal hormones, is accomplished by a family of enzymes that uses NADPH produced in the pentose-phosphate pathway as the electron donor. These enzymes are heme-containing monooxygenases known as cytochromes P450 (abbreviated as CYP). CYPs catalyze the hydroxylation of organic substrates using an oxygen atom from O_2 and electrons from two NADPH molecules (see figure in the bottom), in a reaction that involves the iron atom of the enzyme heme group (see complete cycle in Fig. 2.6). In the human genome, 57 genes codifying cytochrome P450 enzymes are found. Most of them are expressed in the liver, where CYPs play crucial roles in drug metabolization and detoxification, but these enzymes are also found in extrahepatic tissues where their main function is processing endogenous substrates such as steroids and vitamins. Most CYPs are located on the endoplasmic reticulum membranes, but mitochondrial isoforms are also found, especially those involved in the steroidal hormones processing in the adrenals.

(continued)

Box 8.9 (continued)



Roles of pentose-phosphate pathway in providing ribose-5-phosphate for nucleotide synthesis in dividing cells (*top*), NADPH for the glutathione reductase reaction that protects erythrocytes against oxidative damage (*middle*), and the cytochrome P450 reaction cycle responsible for drug and endogenous substrates metabolization in the liver (*bottom*)

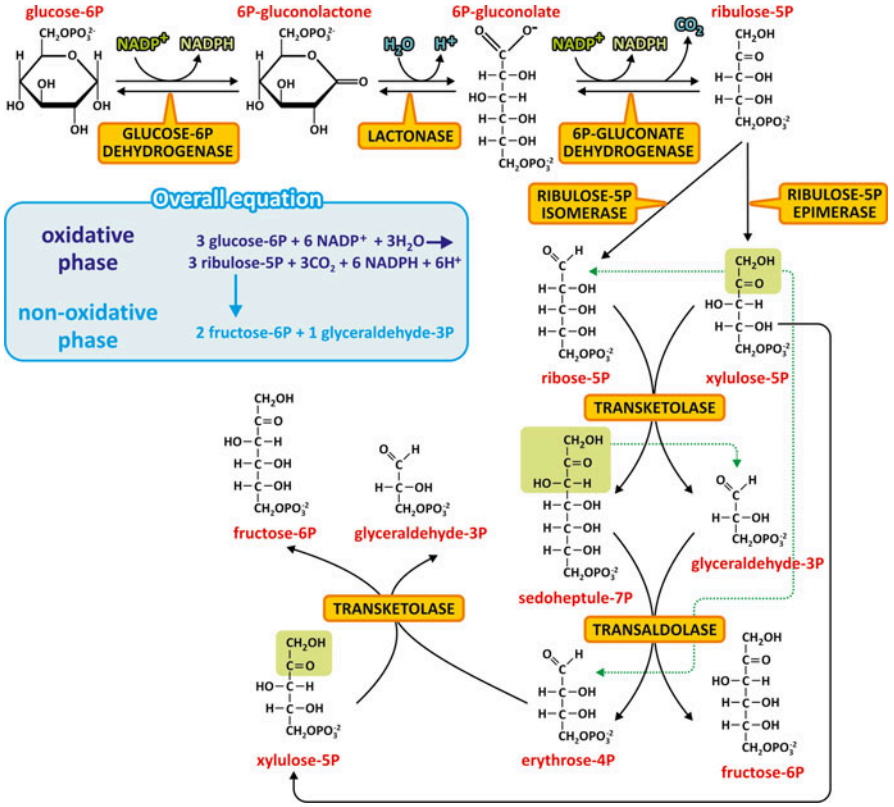


Fig. 8.18 Reactions of the pentose-phosphate pathway. In the oxidative phase, $NADP^+$-dependent oxidation of glucose-6-phosphate generates NADPH and 6-phosphogluconolactone, which is hydrolyzed into 6-phosphogluconate, which in turn undergoes an oxidative decarboxylation generating CO_2, NADPH, and ribulose-5-phosphate. Ribulose-5-phosphate may be isomerized or epimerized to ribose-5-phosphate or xilulose-5-phosphate, respectively, which follow the non-oxidative phase in which two- or three-carbon fragments are interchanged to finally form fructose-6-phosphate and glyceraldehyde-3-phosphate. The reaction stoichiometry is shown in the *box*. The names of the enzymes are shown in *yellow boxes*

Ribulose-5-phosphate may be converted to its isomer ribose-5-phosphate by the enzyme phosphopentose isomerase or may be epimerized to xilulose-5-phosphate, in a reaction catalyzed by pentose-5-phosphate epimerase. Both ribose-5-phosphate and xilulose-5-phosphate follow the non-oxidative phase of the pentose-phosphate pathway (Fig. 8.18). It is important to note that if ribose-5-phosphate is required, for example, for nucleotide synthesis, the pentose-phosphate pathway ends at this point.

The non-oxidative phase comprises two types of reactions: (a) the transfer of a two-carbon fragment from a ketose donor to an aldose acceptor, catalyzed by the enzyme transketolase, and (b) the transfer of a three-carbon fragment also from a ketose donor to an aldose acceptor, catalyzed by the enzyme transaldolase.

Transketolase first transfers a two-carbon fragment of xylulose-5-phosphate to ribose-5-phosphate, forming the seven-carbon product sedoheptulose-7-phosphate

and glyceraldehyde-3-phosphate. Then, transaldolase transfers a three-carbon fragment from sedoheptulose-7-phosphate to glyceraldehyde-3-phosphate, forming fructose-6-phosphate and erythrose-4-phosphate. Finally, transketolase transfers the two-carbon fragment from another xylulose-5-phosphate this time to erythrose-4-phosphate, forming fructose-6-phosphate and glyceraldehyde-3-phosphate, which may return to the glycolytic pathway (Fig. 8.18).

8.3.1.4 Regulation of Fatty Acid Synthesis

ACC reaction is the main point of control of fatty acid synthesis. Both ACC isoforms are allosterically regulated in a similar way, being activated by citrate and inhibited by long-chain saturated fatty acyl-CoA.

In the presence of citrate, ACC polymerizes into long filaments, which consist in the active form of the enzyme.

Citrate is, indeed, an important signal for nutrient storage. The increase in its concentration in the cytosol is a result of the inhibition of the dehydrogenases of the TCA cycle, which occurs due to a high ATP/ADP ratio that, in turn, is a consequence of the high availability of glucose (see previous section). Besides activating ACC and, thus, favoring the storage of carbons from ingested carbohydrate as lipids, citrate also acts as an allosteric inhibitor of the glycolytic enzyme phosphofructokinase 1 (PFK1), decreasing glycolytic flow and allowing part of glucose-6-phosphate to be incorporated into glycogen or metabolized through the pentose-phosphate pathway, which yields NADPH for the fatty acid synthesis (see previous section) (Fig. 8.19).

It important to note that the products of the non-oxidative phase of pentose-phosphate pathway, fructose-6-phosphate and glyceraldehyde-3-phosphate, may return to glycolytic pathway, maintaining the carbon flow in the direction of fatty acid synthesis, even with a reduction in PFK1 activity (Fig. 8.19).

ACC is also regulated by phosphorylation/dephosphorylation. Phosphorylation leads to ACC dissociation into monomers and loss of activity both triggered by hormones (mediated by PKA, see Chaps. 9 and 10 for details on this signaling pathway) or by the changes in cellular energy charge (mediated by AMP-activated protein kinase, AMPK, see Chap. 10 for detail on this kinase). Insulin mediates ACC dephosphorylation, allowing citrate-induced polymerization.

PKA phosphorylates ACC1 isoform at Ser1200. Thus, when the effects of glucagon (in hypoglycemia; see Chap. 9) or adrenaline (during intense physical activity or stress; see Chap. 10) predominate, ACC1 is phosphorylated and, consequently, inhibited. This results in the blockage of fatty acid synthesis, the main fate of malonyl-CoA produced by this ACC isoform.

AMPK phosphorylates ACC1 isoform at Ser80 and Ser81 and ACC2 isoform at Ser219 and Ser220. Thus, the decrease in cellular energy charge, which indicates that nutrients are not in excess, leads to the inhibition of malonyl-CoA formation by ACC1, whose fate is fatty acid storage, as well as the malonyl-CoA formation by ACC2, whose decrease in cytosolic concentration stops the inhibition of carnitine/

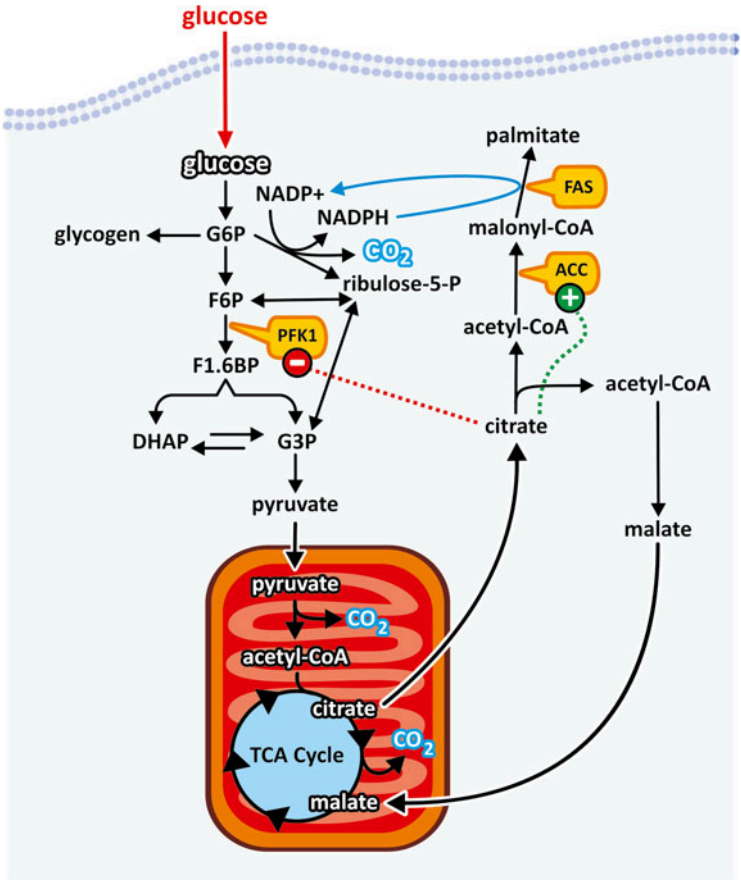


Fig. 8.19 Role of cytosolic citrate in the regulation of metabolic pathways. As a consequence of the high ATP/ADP ratio due to nutrient availability, NADH and $FADH_2$ accumulate in mitochondrial matrix, inhibiting dehydrogenases of the TCA cycle and leading to citrate accumulation. Citrate leaves mitochondria where it acts as the allosteric activator of ACC, inducing its polymerization to the active polymeric form. Additionally, citrate acts as an allosteric inhibitor of PFK1, decreasing glycolytic flow and allowing glucose-6-phosphate to be used in glycogen synthesis or to be oxidized in the pentose-phosphate pathway, generating NADPH for fatty acid synthesis

palmitoyl shuttle system (see Sect. 7.4.3), allowing the transport of fatty acyl-CoA molecules into mitochondria where they are oxidized.

Fatty acid synthesis is also regulated at the gene expression level, both by dietary components and by hormones. A diet rich in carbohydrates induces the transcription of ACC1, ACC2, and FAS. Additionally, insulin upregulates the ACC1 promoter, while glucagon downregulates it. Thus, a high ingestion of carbohydrates, directly or indirectly (through insulin action) induces the expression of the enzymes of the fatty acid synthesis pathway, leading to lipid accumulation.

8.3.2 *Synthesis of Triacylglycerols*

Most of fatty acids synthesized in the organism are incorporated into triacylglycerols, which are fatty acyl esters of glycerol (see Sect. 3.1). To form the triacylglycerol molecule, fatty acids are firstly esterified to coenzyme A in a reaction catalyzed by fatty acyl-CoA synthetase (Fig. 8.20), the same enzyme that activates fatty acids to undergo β-oxidation (see Sect. 7.4.4).

It is important to note that fatty acyl-CoA synthetase does not discriminate fatty acid in the cytosol coming either from synthesis or from mobilization of the adipose tissue, reinforcing the importance of the regulatory mechanisms triggered by the physiological situation, such as the inhibitory effect of malonyl-CoA on CPT1 (see Sect. 8.3.1), which avoids the oxidation of the newly synthesized fatty acid by impairing its transport into the mitochondrial matrix. Thus, after palmitate or other fatty acids are synthesized, the first step of triacylglycerol synthesis is their conversion to fatty acyl-CoA (Fig. 8.20).

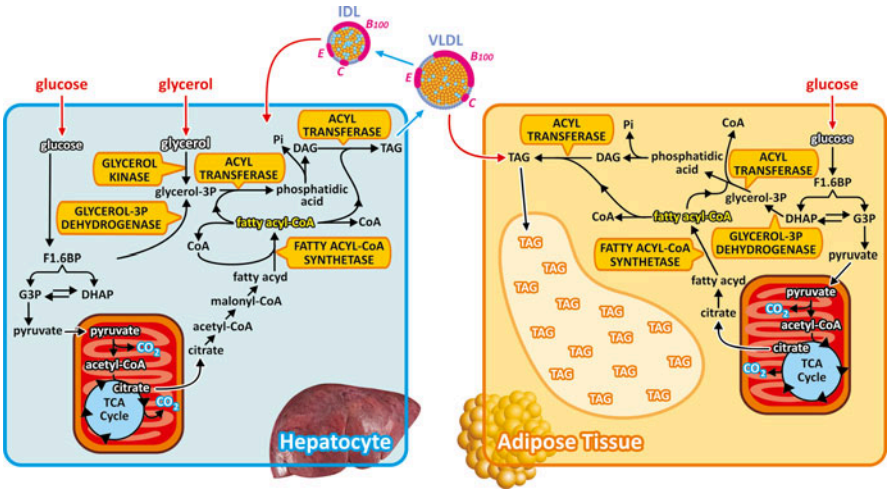


Fig. 8.20 Schematic representation of triacylglycerol synthesis in the liver and adipose tissue. Fatty acids are synthesized in both tissues from the excess of glucose. To be incorporated into the triacylglycerol molecule, the main human energy store, fatty acids are firstly esterified to coenzyme A by fatty acyl-CoA synthetase. Part of the dihydroxyacetone-phosphate (DHAP) molecules formed in glycolysis may be used to generate glycerol-3-phosphate (glycerol-3P), in a reaction catalyzed by the glycerol-3P dehydrogenase. In the liver, glycerol-3P may also be formed by the phosphorylation of glycerol by glycerol kinase. The acyl groups of two molecules of fatty acyl-CoA are transferred to each of the free hydroxyl groups of a glycerol-3P molecule, generating the phosphatidic acid. Then, the phosphate group of the phosphatidic acid molecule is hydrolyzed and a fatty acyl group of another fatty acyl-CoA is transferred to the resulting hydroxyl group of the diacylglycerol (DAG) molecule formed, generating a triacylglycerol (TAG) molecule. In adipose tissue, TAGs are directly stored in lipid droplets (LD). TAG molecules synthesized in the liver are incorporated in VLDL to be transported to the adipose tissue. After delivering TAGs in adipose tissue, VLDL becomes an IDL, which returns to the liver

The glycerol moiety of the triacylglycerol molecule comes from glycerol-3-phosphate, which is formed from dihydroxyacetone phosphate, an intermediate of glycolysis, in an NADH-dependent reaction catalyzed by glycerol-3-phosphate dehydrogenase. Liver cells also have the enzyme glycerol kinase, which catalyzes the formation of glycerol-3-phosphate from glycerol (Fig. 8.20).

The two free hydroxyl groups of glycerol-3-phosphate are firstly acylated by two molecules of fatty acyl-CoA, in a reaction catalyzed by the acyl transferase, yielding diacylglycerol-3-phosphate, also called phosphatidic acid. The phosphate group of phosphatidic acid is then hydrolyzed by phosphatidic acid phosphatase to form a 1,2-diacylglycerol, which is esterified with a third fatty acyl-CoA to form the triacylglycerol molecule.

When synthesized in adipocytes, triacylglycerols are directly stored in the lipid droplets inside these cells (Fig. 8.20).

Triacylglycerols synthesized in the liver must be transported to adipose tissue to be stored, which is not a trivial task due to their extremely low solubility in the aqueous blood environment. To overcome this barrier, they are incorporated into the very low-density lipoprotein (VLDL), which travels through the bloodstream from the liver to the adipose tissue, where it delivers part of their lipid content, becoming a lipoprotein of intermediate density (IDL) (Fig. 8.20). IDL returns to the liver or is converted to LDL (see details about lipoproteins' structure in Sect. 3.1.2).

8.4 Hormonal Responses to Hyperglycemia: Role of Insulin

The main hormonal response to hyperglycemia is insulin secretion, whose immediate global effect is to reduce blood glucose concentration. The loss of insulin-mediated effects results in an incapacity of controlling glycemia, which characterizes the diseases known as diabetes mellitus.

The role of insulin in reducing glycemia can be illustrated by comparing the result of the oral glucose tolerance test between nondiabetic and diabetic subjects (Fig. 8.21). While in nondiabetic subjects the glycemia promptly recovers the basal levels due to insulin action, diabetic subjects experience high glycemia during a long period after glucose ingestion.

In this section, we will discuss how insulin, through a complex signaling cascade, controls the metabolism in different tissues, promoting a rapid use of glucose that results in the decrease of its concentration in the bloodstream. The main metabolic responses to insulin will be described in detail, including (a) glucose uptake in muscle and adipose tissue, (b) glucose utilization as energy source by all the cells in the organism, (c) glucose storage as glycogen in the muscle and liver, (d) glucose conversion into storage lipids in liver and adipose tissue, and (e) inhibition of glucose production through gluconeogenesis and glycogenolysis in the liver. It is important to bear in mind that, besides these metabolic effects, insulin controls several other biological processes, including growth and differentiation, which will not be the focus of this chapter.

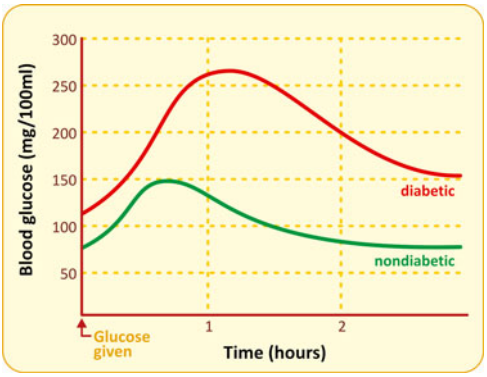


Fig. 8.21 Comparison of glucose tolerance test results between a diabetic and a nondiabetic subjects. In this test, a solution with a high concentration of glucose is ingested and blood samples are taken afterward to determine how quickly it is cleared from the blood

8.4.1 Discovery of Insulin

Until the beginning of twentieth century, insulin deficiency, which is known as type I diabetes, was a devastating disease. The impact of insulin discovery can be realized by reading the introduction of the Michael Bliss´s book, in which he states that the discovery of insulin was "one of the most dramatic events in the history of the treatment of disease … and those who watched the first starved, sometimes comatose, diabetic receive insulin and return to life saw one of the genuine miracles of modern medicine, … the closest approach of the resurrection of the body… the elixir of life for millions of people around the world" (Bliss M. *The discovery of insulin*. University of Chicago Press, 1982).

The history of insulin discovery is indeed remarkable. In the 1880s, Josef von Mering and Oskar Minkowski showed that the total pancreatectomy in experimental animals, usually dogs, resulted in the development of acute hyperglycemia and glycosuria, leading the dogs to die from severe diabetes. This raised the possibility that glucose metabolism was promoted by an "internal secretion" produced by the pancreas (in opposition to the digestive enzymes secreted by the pancreas into the gut—the "external secretion"). During more than 30 years, several researchers have tried to isolate this substance, which has already been named insulin due to the speculation that it was produced by the Langerhans islets, but all the efforts were unsuccessful probably due to its proteolytic digestion by the pancreatic enzymes. The turning point in the history occurred when Frederick G. Banting, a young surgeon, intuited, while studying the pancreatic function to prepare a lecture, that the ligation of pancreatic ducts would allow the isolation of pancreatic internal secretion free of proteolysis.

In 1921, Banting, together with the medical student Charles H. Best, with the support of John J. K. Macleod, a prominent Canadian scientist in the field of carbohydrate metabolism, performed decisive experiments that definitely demonstrated that a substance secreted by the pancreatic islets of Langerhans was able to drop plasma glucose

levels of diabetic dogs. In just one year, this substance was (a) isolated and tested in dogs; (b) further purified by James Bertram Collip, who joined the group to make the preparation suitable to be injected in humans; and (c) successfully used in the first patient, Leonard Thompson, a 14-year-old boy, who recovered from a very severe diabetic state. In the same year, the preparation was used to treat other diabetic volunteers. In 1923, only 2 years after Banting idealized the first experiments, Banting and Macleod were awarded the Nobel Prize in Physiology or Medicine, in a controversial ceremony in which Banting announced that he would share his half of the award with Best, which led Macleod to announce that he would share his half with Collip.

James Bertram Collip (1892-1965)
Charles Herbert Best (1899-1978)
Frederick Grant Banting (1891-1941)

8.4.2 Mechanisms of Insulin Action

Insulin is a small protein whose mature form is composed of two polypeptide chains (A and B) linked by disulfide bonds (Fig. 8.22; see also Sect. 3.3.1). It is synthesized as a single polypeptide called preproinsulin. Its N-terminal sequence, which consists in a signal peptide, is cleaved as the polypeptide is translocated into ER. This proteolytic cleavage induces the formation of three disulfide bonds, yielding the proinsulin, which is transported to the trans-Golgi network. The action of prohormone convertases, endopeptidases that hydrolyzes a proinsulin internal segment named C peptide as well as the carboxypeptidase E, generates the mature insulin. Insulin is packaged inside intracellular granules that are secreted through exocytosis together with the C peptide upon stimulation (see below).

Insulin secretion is dependent on β-cells' energetic metabolism. As discussed in Sect. 8.1, glucose utilization by β-cells occurs in parallel with the increase in glycemia due to GLUT2 and glucokinase high K_M values. Thus, the enhancement in the blood glucose concentration is followed by an increase in the ATP/ADP ratio inside

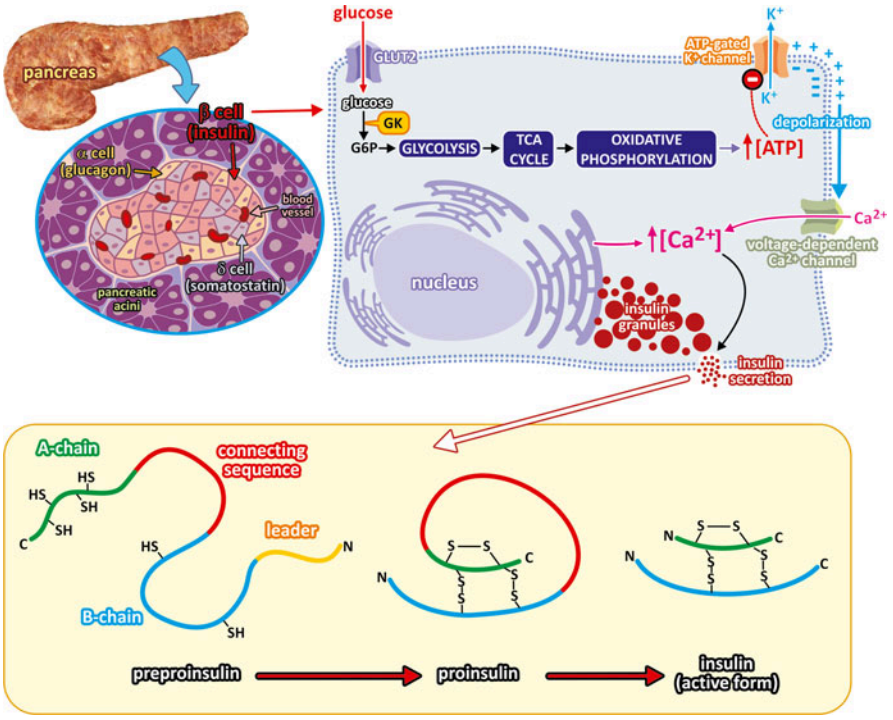


Fig. 8.22 Schematic representation of a Langerhans islet, showing β-cell localization (*left*) and the mechanism of insulin secretion in detail (*right*). The increase in ATP levels in β-cells due to the increase in glucose uptake and metabolization inhibits the ATP-sensitive K^+ channels, leading to membrane depolarization and Ca^{2+} release from ER and uptake from the extracellular medium. The high cytosolic Ca^{2+} concentration stimulates the secretory pathway resulting in insulin release from β-cell. Mature insulin is formed by proteolytic processing of the preproinsulin (*bottom*). First, preproinsulin is converted in proinsulin by the cleavage of its N-terminal signal peptide, which leads the formation of three disulfide bonds. Then, mature insulin is formed by removal of the C peptide segment through the action of the prohormone convertases

β-cells (Fig. 8.22). The high intracellular concentration of ATP inhibits the ATP-sensitive K^+ channels in β-cell plasma membrane, resulting in K^+ retention inside the cell. The elevation of intracellular K^+ concentration leads to membrane depolarization, which favors the opening of the voltage-dependent Ca^{2+} channels, allowing the increase in the cytosolic concentration of Ca^{2+}, which activates the secretory pathway, leading to insulin release from β-cells (Fig. 8.22). It is important to have in mind that the preexistence of intracellular vesicles loaded with insulin allows a very rapid insulin secretion upon stimulation.

The mechanism of insulin action on its target cells is complex and initiates with insulin binding to its receptor on cell surface. Insulin receptor is expressed in virtually all the cells, but differences in the components of the intracellular signaling pathway as well as the presence or not of the target enzymes or their specific isoforms result in distinct effects of insulin in each tissue.

The insulin receptor is a tetrameric protein composed of two extracellular α-subunits, which contain the insulin-binding site, and two transmembrane β-subunits, which display an intrinsic tyrosine kinase activity (Fig. 8.23). Insulin binding to α-subunit promotes a conformational change that is transmitted to the β-subunit, causing its autophosphorylation and further activation. The phosphorylated β-subunit recruits and phosphorylates a family of proteins known as insulin receptor substrates (IRS), which connect insulin receptor activation with the downstream signaling pathways (see Box 8.10), regulating a broad array of physiological functions.

Although very complex, insulin-mediated effects on cellular function may be summarized in a simplified double pathway: (a) the pathway mediated by phosphatidylinositide 3-kinase (PI3K), which controls metabolism, and (b) the pathway mediated by mitogen-activated protein kinases (MAPK), which regulates gene expression and cell proliferation (Fig. 8.23). The pathway-mediated MAPK will not be discussed in this book.

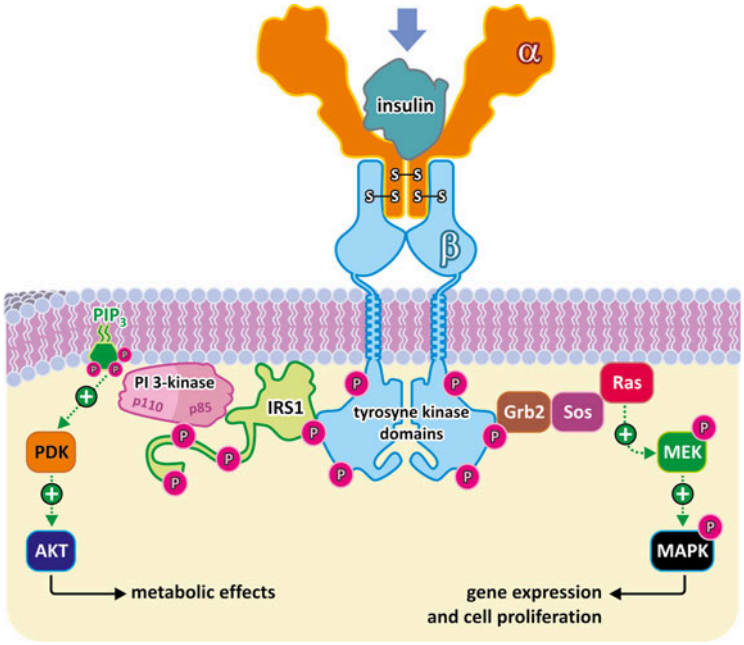
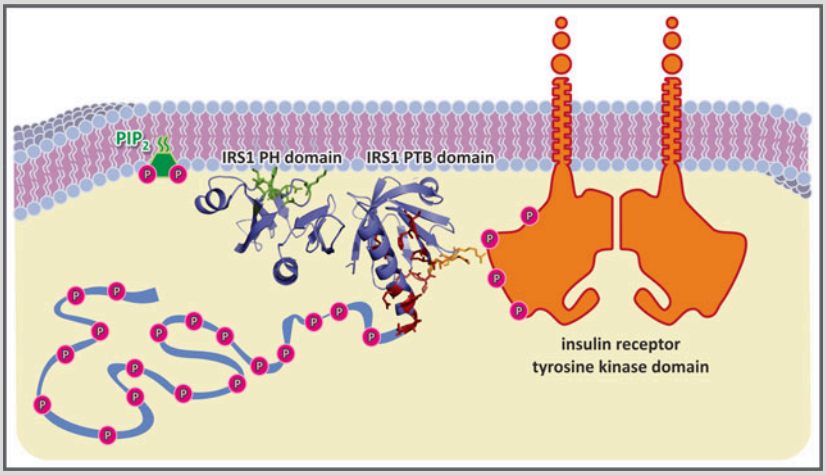


Fig. 8.23 Overview of the insulin signaling pathway. Insulin receptor is composed of α-subunits, which contain the insulin-binding site, and β-subunits, which display tyrosine kinase activity responsible for receptor autophosphorylation. Phosphorylated β-subunit recruits and phosphorylates insulin receptor substrates (IRS), which mediate two main pathways in the target cells, one that results in regulation of gene expression and other that modifies the activity of metabolic enzymes. The metabolic effects are mediated by recruitment of p85, the regulatory subunit of PI3K, which binds and activates the PI3K catalytic p110 subunit. Active PI3K phosphorylates the plasma membrane phosphatidylinositol 4,5-bisphosphate (PIP_2) at the position 3 of the inositol ring, generating phosphatidylinositol 3,4,5-trisphosphate (PIP_3), which mediates many metabolic effects of insulin action. The alterations in gene expression are mainly mediated by the activation of the mitogen-activated protein kinases (MAPK). The Grb2 protein associates to the phosphorylated receptor and recruits the protein Sos, which in turn activates Ras that activates Raf. Raf is a protein kinase that phosphorylates and activates another protein kinase, MEK, which, in turn, phosphorylates and activates MAPK

Box 8.10: IRS Proteins, Adapter Proteins in Insulin Signaling

IRS proteins do not have enzymatic activity but play a key role in connecting insulin receptor activation to the downstream enzymes that regulate a number of cellular functions. IRS proteins are characterized by three major structural elements that allow them to transmit insulin signal. The N-terminal end folds in two conserved domains: a "pleckstrin homology" domain (PH), which contains conserved basic residues that allow interaction with anionic phospholipids recruiting the protein to the cellular membrane, and a phosphotyrosine-binding domain (PTB), through which the protein interacts with the phosphorylated insulin receptor (see figure).

The C-terminus contains several Tyr phosphorylation motifs that when phosphorylated are binding sites to enzymes or proteins containing another type of protein–protein interaction domain, the "Src homology 2" (SH2) domains. SH2 proteins, in turn, frequently possess SH3 domains that recognize phosphorylated motifs in other intracellular proteins, leading to further downstream signal transduction.



The ability of PH domain to interact with phospholipids not only facilitates IRS proteins to bind insulin receptor but also allows the recruitment of phosphatidylinositide 3-kinase (PI3K), an SH2 enzyme, to the plasma membrane, where its substrate, phosphatidylinositol 4,5-bisphosphate (PIP_2), is located (see Sect. 8.4.3). Additionally, the C-terminal end also contains Ser/Thr-rich regions that may be recognized by kinases that act as negative regulators of insulin action (see Sect. 11.3.1).

8.4.3 Effects of Insulin on Energy Metabolism

Insulin effects on metabolism are mainly mediated by PI3K, an enzyme composed of two subunits, a regulatory subunit p85 and a catalytic subunit p110. When p85 binds to the Tyr-phosphorylated IRS, it recruits and activates the p110 catalytic subunit, which phosphorylate phosphatidylinositol 4,5-bisphosphate (PIP_2) at the position 3 of the inositol ring, generating phosphatidylinositol 3,4,5-trisphosphate (PIP_3) (Fig. 8.23). PIP_3 recruits several Ser/Thr kinases to the plasma membrane, including 3-phosphoinisitide-dependent protein kinase (PDK). At the plasma membrane, PDK phosphorylates and activates another Ser/Thr kinase, AKT (also known as protein kinase B, PKB), which, in turn, phosphorylates several target proteins, such as GSK3, leading to the activation of glycogen synthesis (see Sect. 8.2.4), and AS160, leading to the increase in glucose uptake through GLUT4 in muscle and adipose tissue (see next section).

8.4.3.1 Effects of Insulin on Glucose Uptake by Muscle and Adipose Tissue

In muscle and adipose tissue, glucose is transported across the plasma membrane by GLUT4. This GLUT isoform is not constitutively present on the cell surface but remains sequestered in intracellular vesicles until the insulin signaling pathway is activated (Fig. 8.24). Thus, while blood glucose concentration is maintained around the basal levels, these tissues are not able to uptake glucose from the bloodstream and their main metabolic substrates are the fatty acids. However, after a carbohydrate-rich meal, when blood glucose rises and insulin is secreted, this picture changes completely. Insulin binding to its receptor in muscle and adipose tissue cells triggers the sequence of events described above, leading to AKT (or PKB) activation. One of the AKT substrates is the protein AS160 (AKT substrate 160), which negatively regulates the migration and fusion of intracellular vesicles to plasma membrane. AS160 is inactivated by insulin-mediated phosphorylation, thus allowing the translocation of the GLUT4-containing vesicles to the cell surface (Fig. 8.24).

8.4.3.2 Effects of Insulin on Metabolic Pathways

Insulin signaling transmitted through the activation of PI3K alters the activity of several enzymes in different metabolic pathways.

Glycogen is stored due to the simultaneous activation of its synthesis and inhibition of its degradation. This occurs through the combined effects of the phosphorylation of GSK3 by AKT, which results in its inhibition, and the activation of PP1. PP1 is bound to glycogen granules, where it catalyzes the dephosphorylation of GS, promoting its activation, and also acts on GP and on phosphorylase kinase, leading to their inactivation (see details in Sect. 8.2.4). Thus, GS is maintained active through its dephosphorylation by PP1 together with the inhibition of its

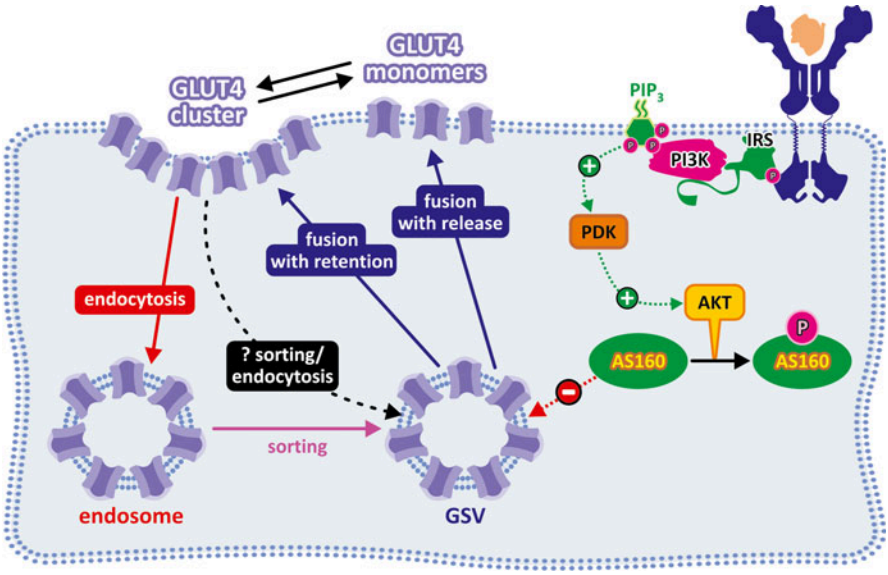


Fig. 8.24 Schematic diagram of insulin-mediated exposure of GLUT4 on the surface of muscle and adipose tissue cells. Insulin signaling cascade (described in Fig. 8.23) results in the activation of PDK, which phosphorylates AKT, leading to its activation. AKT catalyzes the phosphorylation and inactivation of AS160, which negatively regulates GLUT4 translocation to cell surface

phosphorylation by GSK3, leading to glycogen synthesis both in the liver and muscle (Fig. 8.25).

Glycolysis is stimulated in the liver due to the dephosphorylation of bifunctional enzyme phosphofructokinase-2/fructose-2,6-bisphosphatase (PFK-2/F2,6-BPase; see Sect. 9.4.1 for details), which results in the activation of the PFK2 and in the inhibition of the F2,6BPase activities, leading to a great increase in the concentration of fructose-2,6-bisphostate that in turn activates PFK1 and inhibits fructose-1,6-bisphosphatase, favoring glycolysis and stopping gluconeogenesis (Fig. 8.25).

Fatty acid and triacylglycerol synthesis is stimulated by insulin, whose signaling pathway leads to ACC dephosphorylation, allowing citrate-induced polymerization and activation both in the liver and adipose tissue (Fig. 8.25). Gene expression regulation by insulin also favors lipid biosynthesis since it induces the expression of ACC gene.

8.5 Metabolic Interplay in Response to Hyperglycemia

Responses to hyperglycemia start with liver and β-cells sensing the higher levels of blood glucose through GLUT2/GK sensor (see Sect. 8.1). β-cells respond to the increase in glycemia by secreting insulin, which acts on tissues and organs stimulating further glucose utilization and storage.

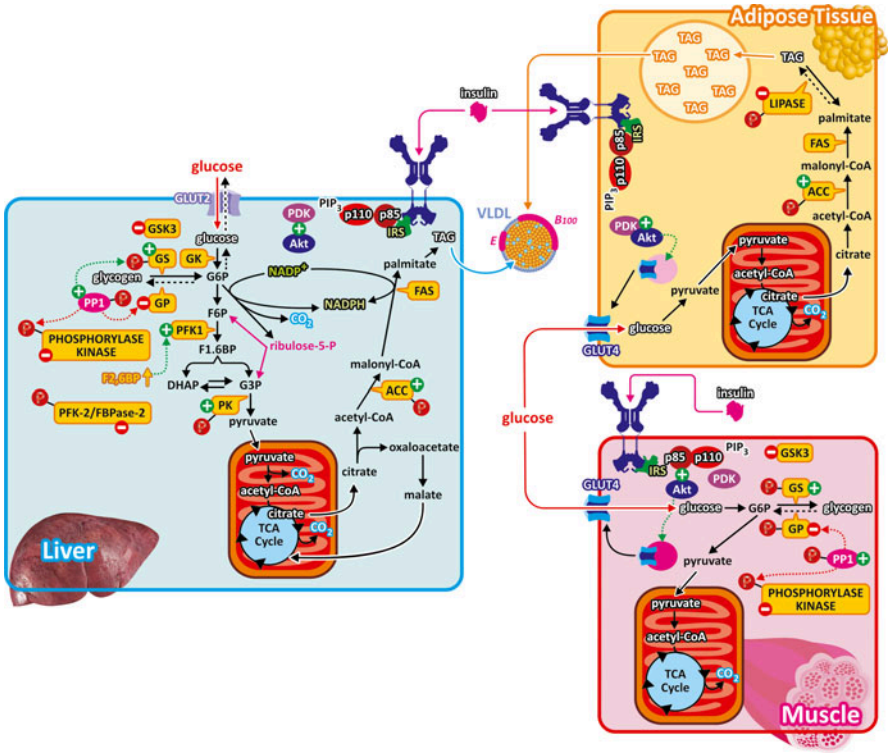


Fig. 8.25 Metabolic interplay in hyperglycemia, showing the enzymes and the metabolic pathways regulated in each cell

In liver cells, blood glucose is internalized by GLUT2 and converted to glucose-6-phosphate, which has at least four direct fates: (a) conversion to glycogen (see Sect. 8.2), (b) degradation through glycolysis (see Sect. 7.4), (c) metabolization through the pentose-phosphate pathway (see Sect. 8.3), or (d) reconversion to glucose by glucose-6-phosphatase. Insulin action on hepatocytes results in an increase in hepatic accumulation of glycogen and in a stimulus to glycolysis with the subsequent transformation of glucose in fatty acids, which are then incorporated into triacylglycerol molecules. Triacylglycerols travel in the bloodstream after their incorporation to VLDL to reach the adipose tissue, where they are stored (Fig. 8.25).

GLUT4 is the major glucose transporter in muscle and adipose tissue. Thus, the insulin-dependent translocation of GLUT4 to the cell surface results in a great enhancement in glucose uptake by these cells. Insulin action also results in a rapid switch to glucose usage as the major metabolic substrate in these cells. Since muscle and adipose tissues together represent more than 60 % of the body weight and contribute to almost one third of the basal metabolic rate of the organism (Table 8.2), their metabolic activity in this situation causes a rapid removal of glucose from the bloodstream with its storage as glycogen in muscle and triacylglycerol in adipose tissue (Fig. 8.25).

Table 8.2 Weight distribution and estimated contribution of different organs and tissues to the basal metabolic rate

Tissue	Weight (kg)	% body weight	% basal metabolic rate
Muscle	28	40	22
Adipose tissue	15	21.4	4
Liver	1.8	2.6	21
Brain	1.4	2.0	20
Heart	0.33	0.5	9
Kidney	0.31	0.4	8
Others (skin, intestine, bones, glandules, etc.)	23.16	33.1	16
Total	70	100	100

Adapted from Elia, Nutr. Res. Rev. 4:3–31, 1991, with permission

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Chapter 9
Regulation and Integration of Metabolism During Hypoglycemia

In humans, blood glucose is the main reporter of fed and fasting states. Its concentration directly regulates the secretion of hormones, including glucagon and insulin by the pancreas and glucocorticoids by the adrenal cortex. These hormones, in a coordinated action, regulate the energy metabolism in different organs, allowing the blood concentration of glucose to be maintained within a narrow range by a precise system balancing glucose production by the liver and its utilization by peripheral tissues.

The basis for this major role of glucose in controlling human metabolism may be discussed in the context of human evolution. About 4 million years ago, carbohydrates were important components of the diet of primates and prehuman ancestors, which possibly favored brain and reproductive tissues to develop a specific requirement for glucose as their primary fuel. The subsequent periods of human evolution were dominated by severe Ice Ages that selected the hominids who developed hunting and fishing abilities and consumed high-protein and low-carbohydrate diets. This led to metabolic adaptations to protect the brain and embryonic tissues from the low-glucose availability, resulting in the increased efficiency of hepatic glucose production and the decrease in peripheral glucose utilization. Additionally, the alternate periods of food scarcity and abundance selected metabolic mechanisms to increase deposition of energy reserves during periods of plenty for subsequent use when food was not available, favoring lipid accumulation and the development of the adipose tissue. However, the advent of agriculture after the last Ice Age greatly modified the quantity of carbohydrates consumed by humans, and the Industrial Revolution in the nineteenth century dramatically changed the quality of the carbohydrate ingested (see Box 9.1). These events together would result in an increase of postprandial glycemia and insulinemia, probably contributing to the predisposition of the modern diseases known as metabolic syndrome (see Chap. 11).

Nowadays, in Western cultures, humans have three main meals per day, and the amount of carbohydrates in a regular meal is about 50–60 %. After the digestion and absorption of the carbohydrates, glucose reaches the bloodstream and glycemia rapidly rises from the basal value of 4 mM to around 10 mM (see Fig. 8.2). In Chap. 8,

A.T. Da Poian, M.A.R.B. Castanho, *Integrative Human Biochemistry*,
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we discussed the biochemical mechanisms that explain why plasma glucose concentration sharply falls down between the first and the second hour after a meal.

In this chapter, we will turn our attention to the subsequent hours, which are characterized by a slow decrease in glycemia. We will focus on the metabolic pathways and the mechanisms involved in the maintenance of blood glucose concentration when carbohydrates are not ingested, which include the periods in between meals as well as during low-carbohydrate diets or prolonged starvation. Additionally, the metabolic interrelationships that take place in the different organs and the hormonal regulation in this situation will also be discussed.

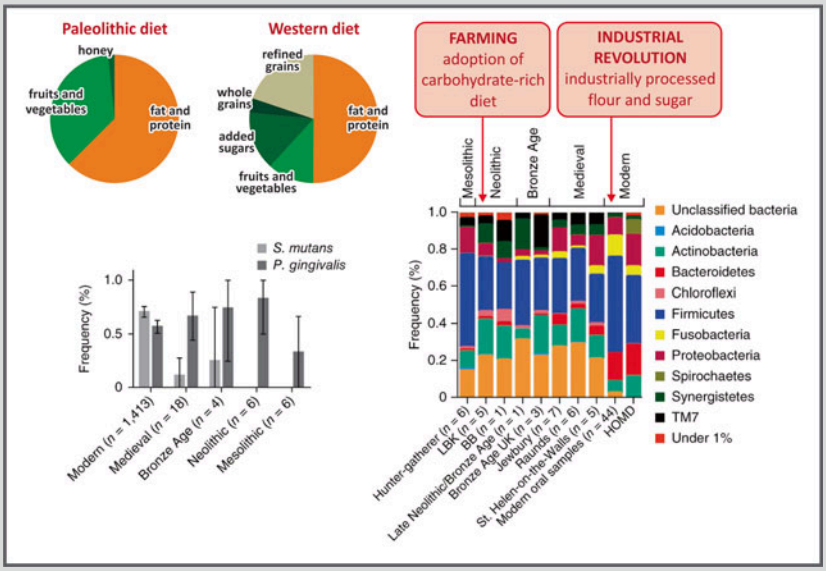
Box 9.1: The Evolution of Human Diet

Paleolithic and current Western diets show profound differences in composition, especially regarding carbohydrate content and quality (see figure). Several studies suggest that two major dietary shifts occurred during human evolution: (a) in the Neolithic (~10,000 years ago), when agriculture emerged, leading to an increase in the consumption of domesticated cereals and the adoption of carbohydrate-rich diet, and (b) after the Industrial Revolution (~1850), with the introduction of industrially processed flour and sugar. This led to the hypothesis, proposed in a classical article published by Eaton and Konner in 1985, that predisposition of the modern diseases known as metabolic syndrome, resulted from an evolutionary discordance between the adaptations established in the Paleolithic era (2.6 million to 12,000 years ago) and the way of life in the industrialized world. Based on this, the authors proposed the "Paleolithic diet" as a reference for human nutrition. However, this view is being questioned nowadays, especially because (a) it implies that human genetic background has not changed since Paleolithic, although humans continued evolving in the Neolithic period, with genetic changes directly related to diet variations, such as on the genes that code for amylase (enzyme that degrades starch) production, and (b) it does not take into account the non-genomic form of inheritance, such as the epigenetic regulation of gene expression, which alters fitness in short-term environmental shifts, such as during in utero development.

The dietary impacts during human evolution can be exemplified with one interesting study that analyzed the genetic diversity of oral microbiota of calcified dental plaque obtained from prehistoric European human skeletons, including the remains of the last hunter-gatherers in Poland and the earliest farming culture in Europe (the Linear Pottery Culture, LBK), as well as late Neolithic (Bell-Beaker culture), early and later Bronze Age, and medieval rural and urban populations (see in the figure an example of phylum frequencies obtained comparing a specific genome region). This study revealed that oral microbiota became markedly less diverse in the modern times, with the dominance of the potentially cariogenic bacteria, like *S. mutans* (see figure).

(continued)

Box 9.1 (continued)



Carbohydrate consumption by man's ancestors corresponds to about 35 % of the total energy intake, which comes mainly from fruits and vegetables, while 50 % daily energy in a Western diet is obtained from carbohydrate, of which about 15 % comes from sugar added to food during processing or consumption, and about 20 % comes from refined cereals (Based on data from Eaton SB. Proc. Nutr. Soc. 65:1–6, 2006). The changes in carbohydrate content and quality in human diet impact in the diversity of oral microbiota (Reproduced by permission from Macmillan Publishers Ltd: Adler et al., Nat. Genet. 45:450–456, 2013)

The maintenance of glycemia may be explained by two distinct phenomena: the decrease in glucose utilization by different tissues and the increase in its production and release into the bloodstream by the liver and kidney (Fig. 9.1). When plasma glucose concentration is above the basal levels, insulin secretion is stimulated and the action of this hormone allows for rapid glucose utilization by all the tissues (see Chap. 8). However, as glycemia decreases, glucagon secretion will predominate, leading to a complete change in metabolism, which is characterized by a decrease in glucose utilization, especially by the muscle and adipose tissues, and a continuous release of glucose in the bloodstream by the liver and, in smaller quantities, by the kidney cortex, as we will discuss in the next sections.

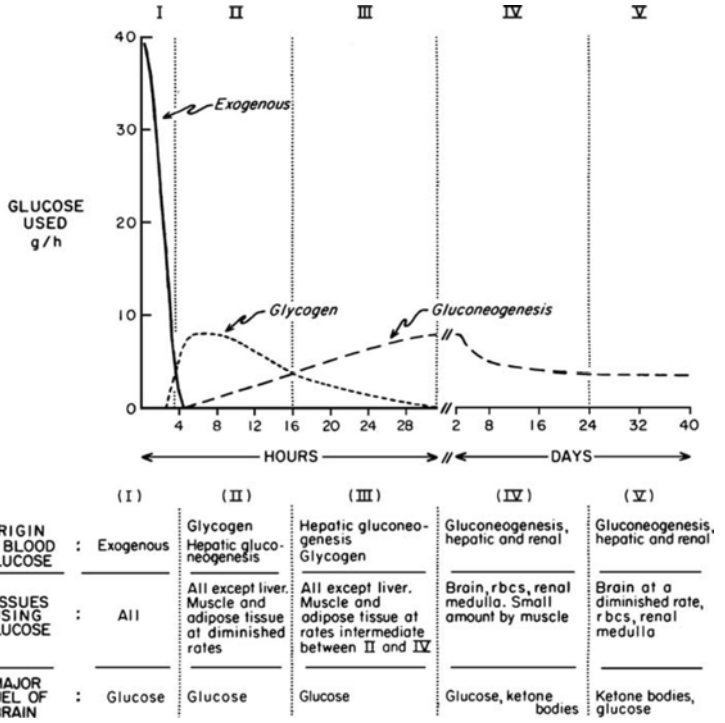


Fig. 9.1 Classical figure by Dr. George Cahill in which he describes five metabolic stages between the postabsorptive state and the near-steady state of prolonged starvation. The figure was constructed based on studies performed by Dr. Cahill and his group with patients submitted to therapeutic starvation in the 1960s (see Box 9.2). *rbcs* red blood cells (Reproduced with permission from Cahill. Ann. Rev. Nutr. 26:1–22, 2006)

9.1 Overview of Metabolism During Fasting: Exemplifying with Studies on Therapeutic Starvation

To introduce the issue of glycemia control, we will take advantage of some studies carried out between the 1950s and 1960s, when therapeutic starvation was used as a strategy to treat obesity (Box 9.2). It is important to note that this extreme situation was chosen as an example for clarity, but the adaptations and the metabolic pathways that will be discussed along this chapter also occur in more common situations, such as low-carbohydrate diets, overnight fasting, or even during the periods in between meals.

Box 9.2: Therapeutic Starvation

The studies used here to exemplify the metabolic adaptations to hypoglycemia have been performed by the group of Dr. George F. Cahill (1927–2012) during the 1960s. As pointed out by Richard W. Hanson in a retrospective article on Dr. Cahill's contributions to the understanding of human metabolism, the fact that he was not a biochemist but a physician–scientist made his approach to research integrative and not reductionist in nature. This integrative view allows him to make a crucial discovery regarding brain metabolism during fasting that explained how humans can survive for more than 60 days without food: ketone bodies, molecules derived from fatty acid metabolism, supply most of the energy requirements of the brain during fasting (see details in Sect. 9.3.4). This finding "resulted in a total reappraisal of the hierarchy of fuels used by different tissues of humans," as stated by Dr. Oliver E. Owen, who worked with Dr. Cahill on these classical experiments. In the 1950s and 1960s, therapeutic starvation of obese subjects was in vogue. The studies described here were performed in the Peter Bent Brigham Hospital, which has a National Institutes of Health-supported clinical research center where the patients were housed and continuously observed during experimental protocols. The patients spent 5–6 weeks fasting with total withdrawal of calories, when the daily intake consisted of one multivitamin capsule, water, and salt replacement. Dr. Owen tells in one of his articles that when someone asked him why he chose a 6-week period for starvation, he answered citing St. Matthew 4:2: "Jesus fasted forty days and forty nights and afterward he hungered." During treatment, the patients volunteered for blood and urine collections for measurements of the plasma concentrations of different metabolites. Furthermore, some of them underwent catheterization to determine the consumption or the production of metabolites by different organs by measuring the arterial-venous differences of these substances. Some of the data originated from these studies will be used in this chapter to discuss several aspects of human adaptation to fasting.

The variation of blood concentration of different metabolites during fasting will be used as the starting point for our discussion on the metabolic adaptations to hypoglycemia (Fig. 9.2).

As seen in Fig. 9.2, the concentration of fatty acids in the blood, after a slight increase, remains constant during all the period of fasting due to the equilibrium between its use as energy source by many tissues and its mobilization from triacylglycerol stored in the adipose tissue. Indeed, the content of triacylglycerol stored in the human body may provide energy for approximately 2 months of fasting (Table 9.1).

As ketone bodies are the main product of fatty acid oxidation in the liver (see Sect. 7.4.6), it is expected that the concentration of these metabolites increases

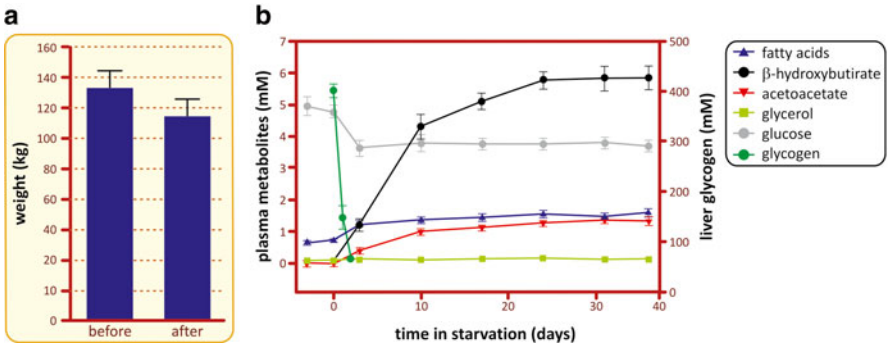


Fig. 9.2 (**a**) Average weight of a group of 11 obese patients before and after they were subjected to the treatment at the Clinical Center of the Peter Bent Brigham Hospital. (**b**) Concentrations of different metabolites in the plasma and glycogen in the liver during fasting (Based on data from Owen et al. J. Clin. Invest. 48, 574–583, 1969)

Table 9.1 Human nutrient stores (typical of a 70 kg man)

Molecule	Weight (g)	Energetic value (kcal)	Period as single energy source (days)
Triglyceride (adipose tissue)	9,000–15,000	~108,000	60
Glycogen (liver)	90	360	0.2
Glycogen (muscle)	250	1000	0.55
Glucose (blood and body fluids)	20	80	0.044
Proteins (mainly muscle)[a]	8000	32,000	17.8

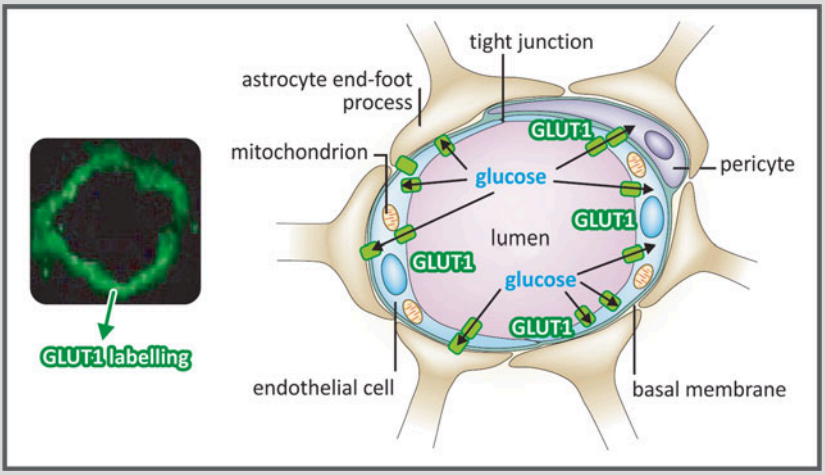
[a]It should be stressed that most of the muscular proteins are not readily available for mobilization

along fasting, as observed in Fig. 9.2. It is possible to note two different phases in the profile of the blood concentration of ketone bodies. In the first 10 days of fasting, there is a constant increase in their concentration, which then gradually reaches a plateau after 25 days. This second phase may be explained by the increase in ketone bodies' consumption and/or excretion by the organism. In fact, brain adaptation to the use of ketone bodies as its main source of energy contributes to their removal from the bloodstream as well as decreasing whole body glucose requirements (for more details, see Fig. 9.16).

A remarkable observation regarding the profile of plasma metabolites during fasting is that glucose concentration, after a small decrease in the first 3 days, is maintained constant during all the period of starvation. From this observation, immediate questions arise: Why is it necessary to maintain glycemia? Why not use only fatty acids as the energy source during fasting since they are, quantitatively, the major fuel reserve in the body (see Table 9.1)? The answer lies in the fact that some cells depend on glucose as their exclusive or preferential source of energy. This is the case of cells that lack mitochondria, as erythrocytes and the cells from crystalline lens, which depend on the anaerobic metabolism (glucose fermentation, see Sect. 6.1) to survive. It is also the case of the cells from the nervous system and

the embryonic tissues, which are isolated from the systemic circulation by blood barriers that do not allow the uptake of fatty acids since these molecules are bound to albumin, their major form of transport in the bloodstream (Box 9.3). Therefore, it is essential that glucose is constantly produced and released in the bloodstream during the periods in which it is not ingested. The metabolic pathways involved in the maintenance of glycemia will be discussed in the next sections.

Box 9.3: Glucose Transport Through the Blood–Brain Barrier (BBB)
BBB is a highly selective permeability barrier that restricts the passage of most substances from the circulating blood to the central nervous system (CNS) fluids (see also Sect. 3.3.4.1). It is formed by three cellular components (see figure): (a) the brain capillary endothelial cells; (b) the astrocytic end feet, which cover the vessel wall maintaining the endothelial barrier; and (c) the pericytes. The brain capillary endothelial cells are connected by tight junctions that make the paracellular transport of substances through BBB negligible under physiological conditions. Additionally, these cells express a number of drug efflux transporters, such as the glycoprotein P (Pgp) and several members of the multidrug resistance (MDR) protein family, which prevent the entry or remove drugs and other substances from the CNS. Glucose transport through BBB is mediated by GLUT1 (see Sect. 8.1). It is important to remind that GLUTs are facilitated-diffusion transporters, which means that glucose cannot be transported against a gradient from bloodstream to CNS. Additionally, GLUT1 K_M for glucose transport is about 1–2 mM. Thus, the human brain cannot be supplied with glucose when its blood concentration is low (normoglycemia is about 5 mM), so that the only strategy to protect brain cells from starvation is to prevent hypoglycemia.



Micrograph of a brain microvessel section showing GLUT1 labeled with a green fluorescent probe (*left*) and the schematic representation of the BBB with its three cellular components (*right*), highlighting the glucose transport through GLUT1 (represented as *green rectangles*). (Reproduced by permission from Macmillan Publishers Ltd: Löscher & Potschka. Nature Rev Neurosci 6:591–602, 2005)

9.2 Glycogen Degradation in the Liver

The crucial role of liver metabolism in producing glucose to maintain glycemia started to be elucidated in the middle of the nineteenth century, with the pioneering studies developed by Claude Bernard (Box 9.4).

In 1853, Claude Bernard showed that the liver was able to release glucose in the bloodstream even when carbohydrate was not present in the diet. This finding completely changed the current idea about animal nutrition. It contradicted the accepted concept that animals always decompose complex substances obtained from food and for the first time suggested that organism functions would be maintained by a metabolic interplay among different tissues. Some years later, Claude Bernard isolated from the liver a substance that he named "la matière glycogène" (the substance that generates glucose), the glycogen.

At that time, it was not yet clear that glucose was released from the liver by a combination of two processes: the degradation of glycogen and the synthesis of glucose from noncarbohydrate precursors. Glycogen may be seen as a transient storage of the ingested sugar that is mobilized when necessary, while different non-glycidic

Box 9.4: Claude Bernard: The Founder of Experimental Medicine
Maybe the most important accomplishment of Claude Bernard was to introduce the scientific methodology into medicine and physiology, establishing the basic rules of experimentation in the life sciences. This can be noticed in one of this letters to his friend Mme. Raffalovich: "The scientist must have imagination, but he must master this imagination and coldly probe the unknown. However, if he lets himself be carried away by his imagination, he will be overcome by vertigo and, like Faust and others, fall into the chasm of magic and succumb to phantoms of the mind." Early in his career, he aimed to follow the fate of the sugar absorbed from the food in the animal body. The hypothesis at that time was that the ingested sugar was burned in the lungs, passing the liver through the hepatic portal system (see Box 8.1) without any processing to reach the bloodstream. To confirm this hypothesis (and also to discard that the sugar was not destroyed in the liver, as it seems that he suspected), Claude Bernard measured the amount of sugar in the portal and hepatic veins of a dog fed with sweet milk. He would be convinced when he found a large amount of glucose in the blood that had passed through the liver, but he was not, as he pointed out on his book *Introduction à l´Étude de la Medicine Expérimentale*: "More than one researcher would have stopped here and would have thought that any control experiment was useless. But I performed a control experiment because I am convinced that in physiology

(continued)

Box 9.4 (continued)
you should always doubt even if the doubt doesn't seem to be permitted." The control was a similar experiment in which the dog was fed only with meat. For his surprise, he found a large amount of sugar in the hepatic vein, which led him to state: "I don't understand anything anymore."


Claude Bernard (1813-1878)

This unexpected finding led Claude Bernard to isolate glycogen some years later, but his contribution to the understanding of animal metabolism did not stop there. The observation that liver releases glucose into the blood led him to establish the concept of "internal secretion." Additionally, Claude Bernard was the first to express the idea that the animals have an inner milieu, different from the external environment, whose constancy would be a requirement for life maintenance (the basis from which the principle of homeostasis emerged), as he stated: "la fixité du milieu intérieur est la condition d'une vie libre et indépendent" (the stability of the internal environment is the condition for a free and independent life).

molecules may be converted to glucose by a pathway named gluconeogenesis. This will be discussed in the next sections of this chapter.

In many tissues, especially in the liver and muscles, glycogen is the storage form of glucose, being observed as dense granules at the microscope (see Fig. 8.5). Liver glycogen content may correspond to about 10 % of wet weight of this organ in well-fed humans. In the muscles, glycogen content can account for 1–2 % of their wet

weight, but since the muscles occupy a much larger area of the body than the liver, total muscle glycogen content is twice as high as that of liver.

The process of glycogen degradation, known as glycogenolysis, was almost entirely elucidated by Carl and Gerty Cori. They discovered (a) the nature of glycogen degradation reaction, a phosphorolysis reaction; (b) the enzyme that catalyzes it, glycogen phosphorylase; and (c) its product, glucose-1-phosphate. Due to this great contribution to carbohydrate metabolism, Carl and Gerty Cori were awarded the Nobel Prize in Physiology or Medicine in 1947.

Carl Ferdinand Cori (1896-1984)
Gerty Theresa Cori (1896-1957)

9.2.1 Reactions of Glycogen Degradation

Glycogen is a highly branched polymer of glucose containing α1,6-glycoside linkages in the branch points, with 10–12 glucose residues linked by α1,4-glycoside linkages between each branch (see Sect. 3.2.1). Its degradation depends on two enzymes, the glycogen phosphorylase (GP) and the debranching enzyme.

GP catalyzes the phosphorolysis of the α1,4 glycoside linkage at a terminal glucose unit from the nonreducing ends of the molecule, yielding glucose-1-phosphate (Fig. 9.3). In their first studies on glycogen degradation, the Coris showed that the reaction catalyzed by GP was reversible in vitro. However, now it is clear that phosphorolysis is greatly favored in vivo due to the very high intracellular ratio [Pi]/[glucose-1-phosphate], and glycogen synthesis has to occur through another pathway (see Sect. 8.2).

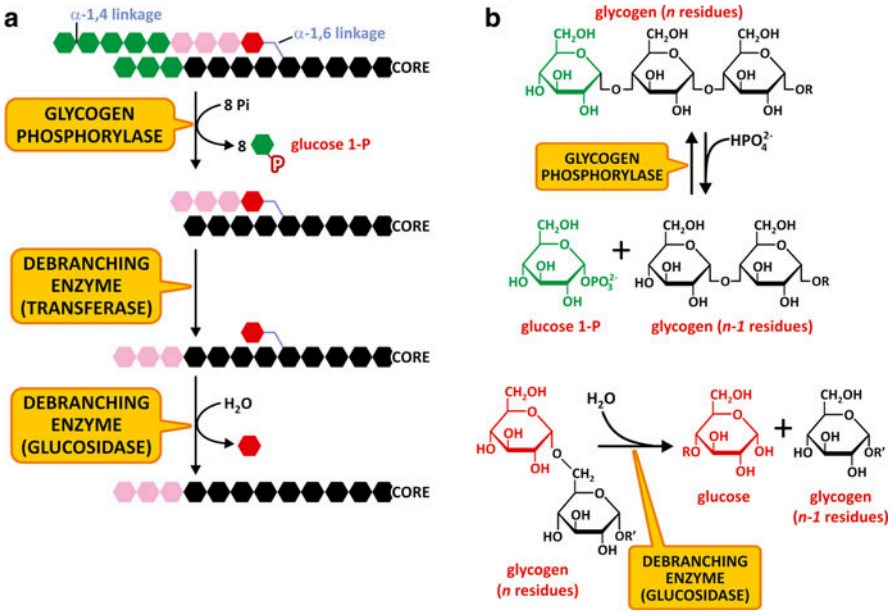


Fig. 9.3 (**a**) Schematic representation of glycogen degradation. (**b**) Reactions catalyzed by glycogen phosphorylase (*top*) and the glucosidase activity of the debranching enzyme (*bottom*)

The branched structure of glycogen enables its rapid degradation, since about 50 % of glucose units are in the outer branches. Glucose units are removed sequentially until four units before the branch point, where the enzyme loses its activity leaving what is known as a limit dextrin (a molecule with short branches of four glucose units in length).

Further removal of the glucose units from a glycogen molecule depends on the activity of the debranching enzyme. This enzyme has two different activities in the same polypeptide chain. The first is a glucosyltransferase activity, in which a trisaccharide unit at the end of a branch is transferred through an α1,4-linkage to a nonreducing end of another chain, resulting in an extended chain susceptible to GP action (Fig. 9.3). The second activity is an amylo-1,6-glucosidase, in which the α1,6-glycoside linkage in the branch points is hydrolyzed to form glucose. It is interesting to note that due to this activity, about 7 % of the glucose residues in glycogen are released as glucose and not as glucose-1-phosphate.

Glucose-1-phosphate produced during glycogenolysis may be converted to glucose-6-phosphate by the action of the enzyme phosphoglucomutase (Fig. 9.3).

In the liver, glucose-6-phosphate may be dephosphorylated through the action of glucose-6-phosphatase (G6Pase), allowing glucose units to be removed from glyco-

gen and released in the bloodstream. G6Pase is an integral protein of the endoplasmic reticulum membrane with the active site on the luminal side of this compartment (Fig. 9.4). It is expressed only in the liver and kidney, and because muscle cells lack this enzyme, the glucose units removed from muscular glycogen are metabolized in muscle tissue itself.

In their first physiological studies on carbohydrate metabolism, the Coris proposed that the lactate produced in muscle from glycogen degradation (and subsequent glycolysis) reached the liver through the bloodstream being then reconverted to glycogen, in a cycle that became known as the "Cori cycle." However, although the idea of cycling metabolites between tissues is a very important concept, the "Cori cycle," exactly as it was proposed, does not occur physiologically. Glucose produced in the liver from muscle lactate, instead of being converted to glycogen, is released into the bloodstream to be used by the brain, as well as by erythrocytes and other fermentation-dependent cells. Additionally, it is very unlikely that glucose produced in the liver goes to the muscles, since its internalization in muscle cells depends on the insulin action, which is not operating in this situation (see Sect. 8.4). In fact, we can construct a more complex picture of glycogen/glucose-lactate cycling in different organs, as presented in Fig. 9.5.

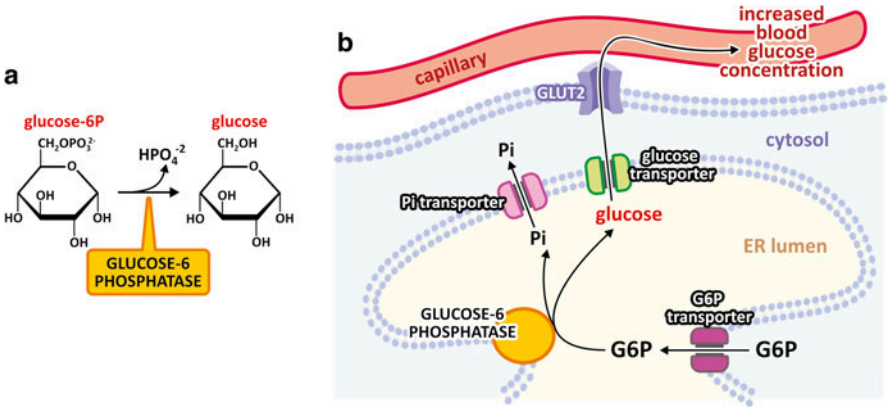


Fig. 9.4 (**a**) Glucose-6-phosphatase reaction. (**b**) Intracellular localization of glucose-6-phosphatase. The enzyme is an integral protein in the endoplasmic reticulum (ER) membrane that catalyzes the dephosphorylation of glucose-6-phosphate in the lumen of this organelle. The enzyme substrate (glucose-6-phosphate) and products (glucose and inorganic phosphate) are transported across ER membrane through specific transporters

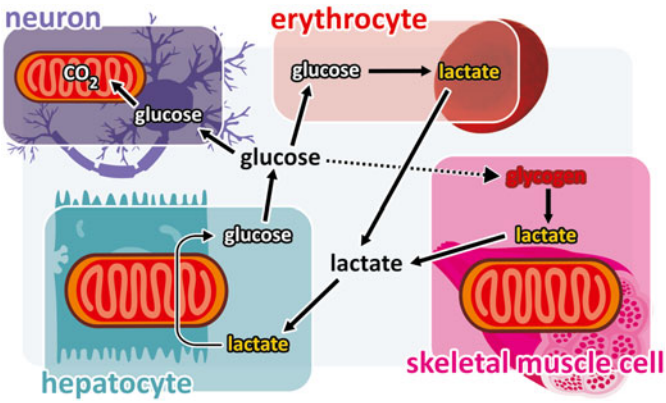


Fig. 9.5 Glycogen/glucose-lactate cycling: glycogen degradation in the muscles forms lactate, which is released in the bloodstream and enters the liver, where it is converted to glucose by gluconeogenesis. Glucose released from the liver into the bloodstream is taken up by the glucose-dependent cells, such as those from the brain and the erythrocytes. Uptake of glucose by the skeletal muscle (forming the so-called Cori cycle) is hypothetical because the physiological conditions that favor lactate production by muscle cells do not favor glucose uptake by these cells (see text)

9.2.2 Regulation of Glycogen Degradation in the Liver

Liver glycogen content largely varies in response to food intake (Fig. 9.6). Glycogen accumulates rapidly after carbohydrate ingestion and then it is gradually mobilized to generate free glucose in between the meals.

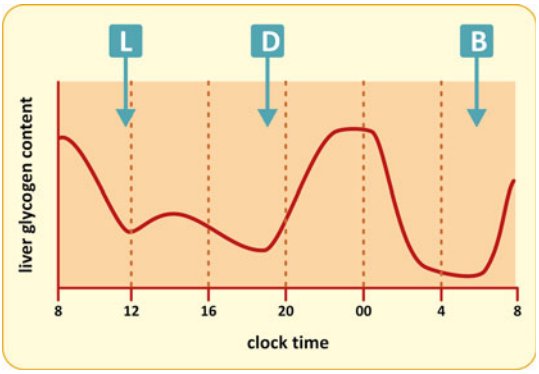


Fig. 9.6 Variation in the liver glycogen content after each meal

Glycogen degradation is mainly controlled by regulating GP activity. This enzyme is a dimer that exists in two different conformations, one more active, named phosphorylase *a*, and one much less active, named phosphorylase *b* (Fig. 9.7). These two forms are interconvertible by phosphorylation/dephosphorylation of a serine residue (Ser14), induced by hormone action (see Sect. 9.3.3).

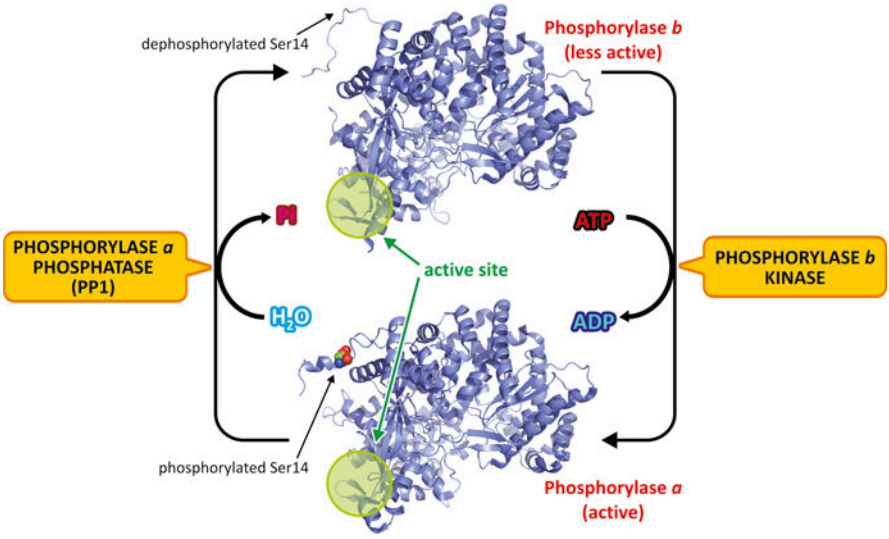


Fig. 9.7 Regulation of GP activity by phosphorylation. In the liver, glucagon action triggers the phosphorylation of the less active form of GP, named phosphorylase *b* (PDB 1FC0), in its Ser14, which promotes a structural change to the active form, phosphorylase *a* (PDB 1FA9). The N-terminal segment, which contains the phosphorylation site (Ser14), converts from a completely disordered conformation to a well-ordered structure, and several structural transitions occur in the active site (highlighted in *green*). To facilitate the visualization of enzyme conformational changes, the monomer structures are shown in the figure, although the enzyme exists as homodimers

The major allosteric modulator of the liver isoform of GP is glucose, which shifts the equilibrium between the conformational states to phosphorylase *b* (Fig. 9.7). Thus, when the intracellular concentration of glucose is high, glycogen degradation is inhibited. The allosteric regulation of the muscle isoform of GP is more complex and will be discussed in Chap. 7.

Glycogen degradation is also controlled by hormones. In a situation of hypoglycemia, insulin secretion by the pancreatic β-cells is inhibited, leading to an increase in glucagon secretion by the α-cells. The action of glucagon on liver tissue (detailed in Sect. 9.4) results in the activation of the enzyme phosphorylase kinase, which catalyzes the phosphorylation of GP. Phosphorylation maintains GP in the active form (Fig. 9.7), favoring glycogen degradation. The hormone adrenaline also controls glycogen degradation in the liver (discussed in the exercise situation in Chap. 10).

It is important to note that in humans glycogen stored in the liver lasts between 12 and 24 h during fasting (see Fig. 9.2). Therefore, the contribution of liver glycogenolysis to the control of glycemia is limited and another glucose-producing pathway is required to maintain blood glucose concentration.

9.3 Gluconeogenesis

Gluconeogenesis is the synthesis of glucose (and other carbohydrates) from non-glycidic compounds. This pathway occurs mainly in the liver, but also in the kidney cortex.

For many years it was thought that gluconeogenesis occurred as a reversal of the glycolytic pathway. However, some of the glycolysis reactions are highly exergonic (Fig. 9.8), which makes it very unlike that they could be reversed within the cells.

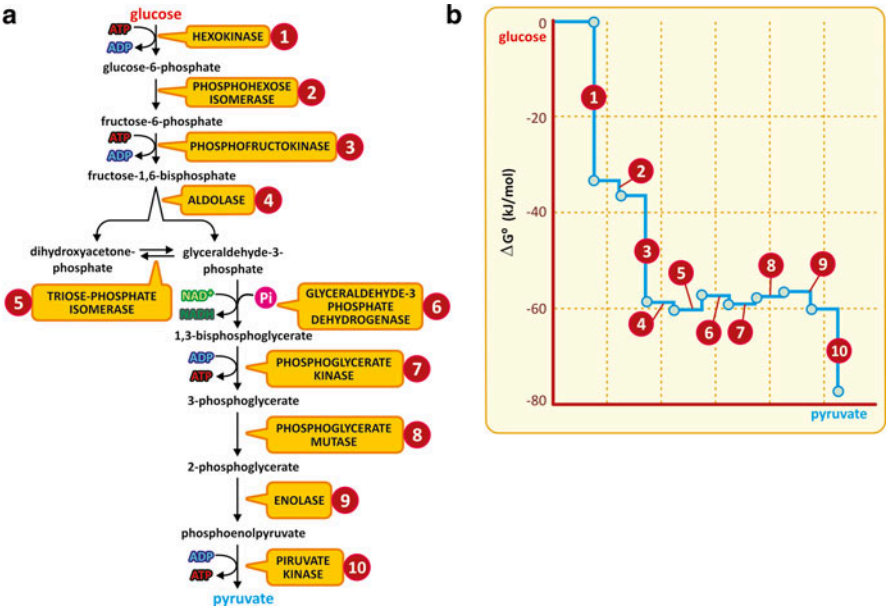


Fig. 9.8 (**a**) Glycolysis reactions (for more details, see Chap. 6). (**b**) Free energy variation between each step of glycolysis. The numbers correspond to the reactions indicated in (**a**)

This energy barrier impairs reversibility at three points of the glycolytic pathway: the conversion of (a) pyruvate in phosphoenolpyruvate (PEP), (b) fructose-1,6-bisphosphate in fructose-6-phosphate, and (c) glucose-6-phosphate in glucose. For these reactions to occur, glycolytic enzymes should be bypassed.

9.3.1 Gluconeogenesis Reactions

The first bypass is the conversion of pyruvate in PEP, which requires two reactions involving two enzymes: pyruvate carboxylase (PC) and PEP carboxykinase (PEPCK).

PC is a mitochondrial enzyme that catalyzes the biotin-dependent carboxylation of pyruvate to produce oxaloacetate. Human PC is active in the tetrameric form and contains three functional domains in the same polypeptide chain: biotin-carboxyl carrier protein (BCCP), biotin carboxylase (BC), and carboxyltransferase (CT) domains (Fig. 9.9; see also Box 8.6 for more information on biotin-dependent carboxylases).

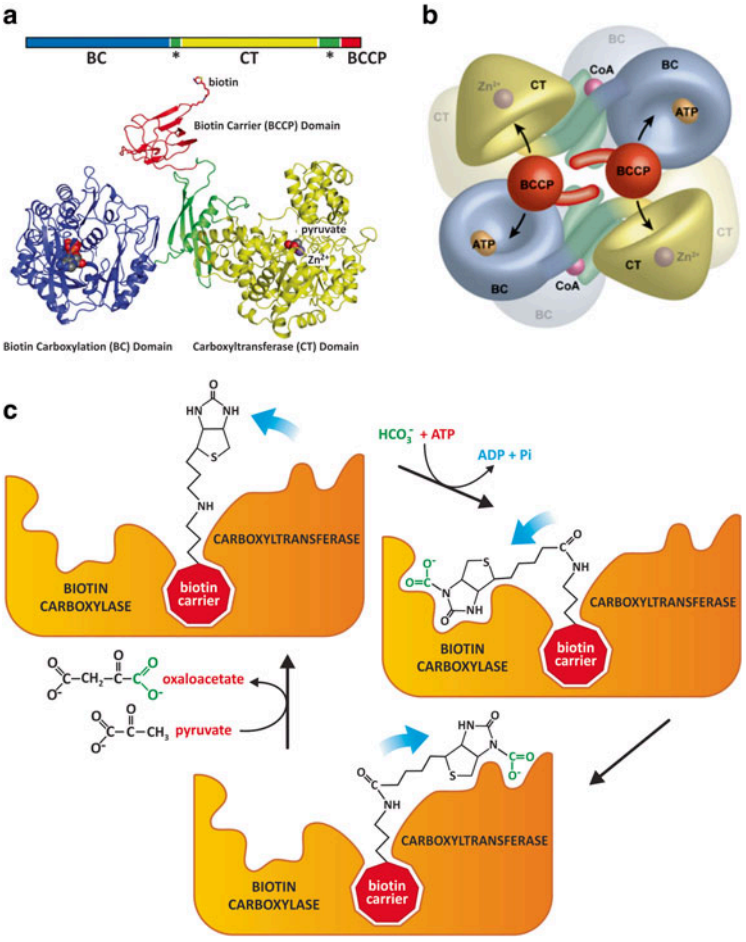


Fig. 9.9 (**a**) PC primary structure and the structure of the *Staphylococcus aureus* PC monomer highlighting the three functional domains: BC (*blue*), CT (*yellow*), and BCCT (*red*), and the allosteric domain (*green*). (**b**) Schematic representation of PC tetramer showing the movement of BCCP domain between neighboring BC and CT active sites in opposite polypeptide chains (Reproduced from Jitrapakdee et al. Biochem. J. 413:369–387, 2008. Portland Press Ltd, London). (**c**) Reactions catalyzed by PC: first bicarbonate is used to carboxylate the enzyme-linked biotin in an ATP hydrolysis-dependent reaction; then the carboxyl group is transferred to pyruvate generating oxaloacetate

Additionally, there is a fourth central structural domain that contains the binding site of the allosteric activator acetyl-CoA. Biotin is covalently linked to a Lys residue in the BCCP domain. The reaction occurs in two steps. First, the BC domain catalyzes the carboxylation of biotin in a reaction that uses bicarbonate as a substrate and requires the hydrolysis of one ATP molecule, generating ADP and Pi (Fig. 9.9). Then, the carboxyl group from carboxybiotin is transferred to pyruvate to form oxaloacetate in a reaction catalyzed by the CT domain of the enzyme. In PC tetrameric form, the domains are arranged in such a way that carboxybiotin is transferred from BC domain to the neighboring CT domain on opposing polypeptide chains, explaining why the enzyme is only active as a tetramer (Fig. 9.9).

Biotin-dependent carboxylation occurs in other metabolic pathways, such as the synthesis of fatty acids (see Sect. 8.3), in which acetyl-CoA carboxylase BC domain shares sequence homology with the BC domain of PC.

It is important to point out that besides its crucial role in gluconeogenesis, the PC reaction plays an important anaplerotic function, replenishing oxaloacetate that is withdrawn from TCA cycle in many metabolic situations (see Sect. 7.3).

The formation of PEP from oxaloacetate is catalyzed by PEP carboxykinase (PEPCK), in a reaction that requires the transfer of a phosphoryl group from GTP (Fig. 9.10).

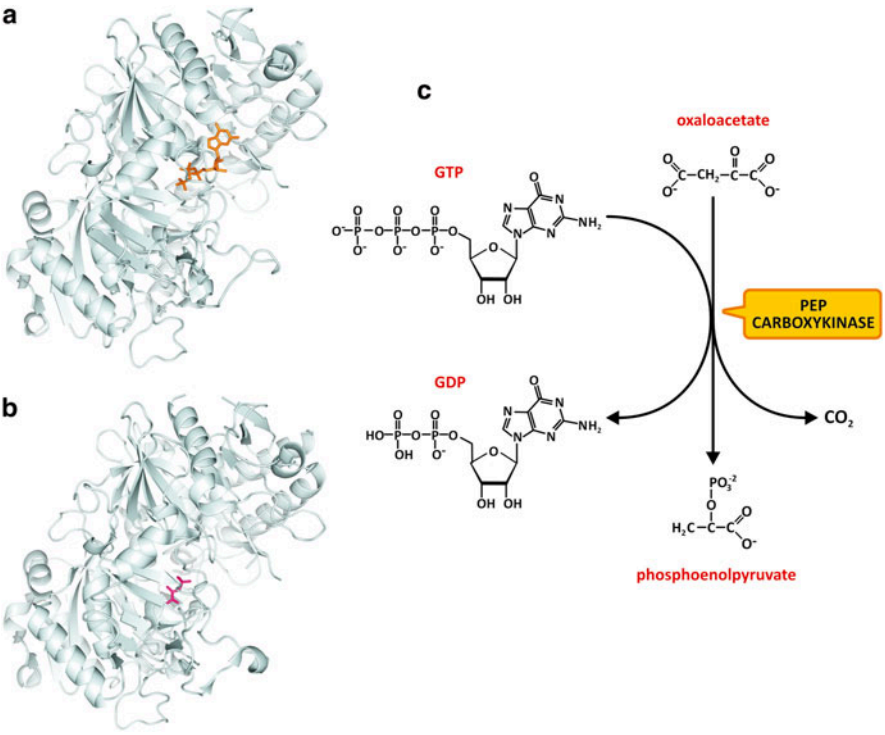


Fig. 9.10 Structure of human cytosolic PEPCK: (**a**) complexed with a non-hydrolysable GTP analogue (PDB 1KHE) and (**b**) complexed with PEP (PDB 1KHF). (**c**) Conversion of oxaloacetate in PEP, using GTP as the phosphate donor, the reaction catalyzed by PEPCK from animals. Differently, bacterial, fungal, and plant PEPCKs use ATP as the phosphate donor

In human cells, PEPCK is distributed between mitochondria and the cytosol, and the isoform used will depend indirectly on the [NADH]/[NAD⁺] ratio in the cytosol. The reason for this is that the reaction catalyzed by glyceraldehyde-3-phosphate dehydrogenase requires NADH to occur in the reverse direction of glycolysis, from 3-phosphoglycerate to glyceraldehyde-3-phosphate (Fig. 9.11).

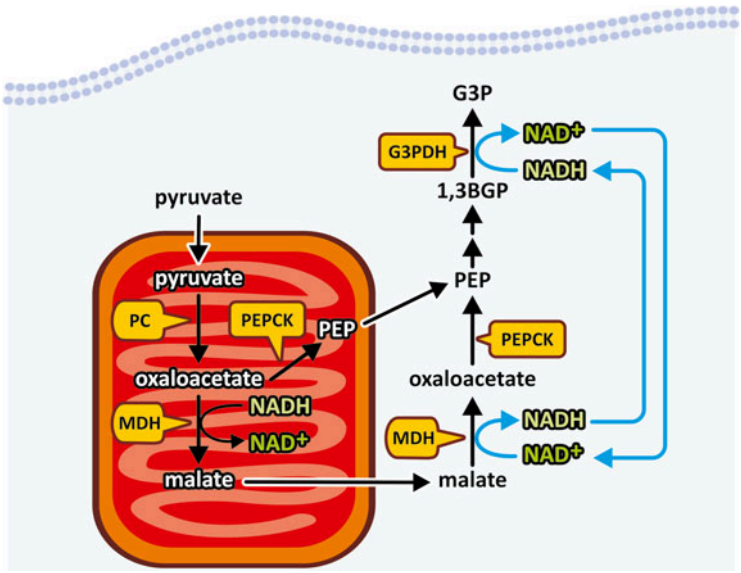


Fig. 9.11 Intracellular location of the first gluconeogenesis bypass. PC is a mitochondrial enzyme, and thus pyruvate is converted to oxaloacetate inside the mitochondria. PEPCK is distributed equally in cytosol and mitochondria, so oxaloacetate may be converted to PEP in both cellular compartments. When cytosolic [NADH]/[NAD⁺] ratio allows the reaction catalyzed by glyceraldehyde-3-phosphate dehydrogenase (G3PDH) to occur from 1,3-bisphosphoglycerate (1,3-BPG) to glyceraldehyde-3-phosphate (G3P), the mitochondrial isoform may be used. When the formation of additional NADH is required, oxaloacetate is converted to malate in the mitochondrial matrix by malate dehydrogenase (MDH) with NADH oxidation. Malate is transported to the cytoplasm where it is converted to oxaloacetate by the cytosolic MDH, generating NADH for the G3PDH reaction

When [NADH]/[NAD⁺] ratio is very low, NADH equivalents are transported from the mitochondrial matrix to the cytosol by the malate–oxaloacetate shuttle. Oxaloacetate is converted to malate by the mitochondrial malate dehydrogenase, with NADH oxidation to NAD⁺ into the mitochondria. Malate crosses the mitochondrial membranes and reaches the cytosol where it is reconverted into oxaloacetate through the action of the cytosolic malate dehydrogenase, with NADH formation in the cytosol.

Therefore, in this situation, cytosolic PEPCK is used to generate PEP, while mitochondrial PEPCK is preferred when cytosolic [NADH]/[NAD^+] ratio allows glyceraldehyde dehydrogenase reaction to occur in the direction of glyceraldehyde-3-phosphate formation (Fig. 9.11).

The second bypass is the conversion of fructose-1,6-bisphosphate to fructose-6-phosphate. In this reaction, the phosphoryl group associated to carbon 1 of fructose-1,6-bisphosphate is removed by hydrolysis through the action of the enzyme fructose-1,6-bisphosphatase.

Finally, the third bypass is the conversion of glucose-6-phosphate to glucose. As discussed in the previous section, in the liver as well as in the kidneys, the enzyme glucose-6-phosphatase catalyzes the dephosphorylation of glucose-6-phosphate generating glucose, which can be released into the bloodstream (see Fig. 9.4).

9.3.2 Precursors for the Synthesis of Glucose

At this point it is already possible to visualize how different precursors can enter the gluconeogenesis pathway. As detailed below, the main precursors are lactate, amino acids, and glycerol. Additionally, propionyl-CoA formed in the oxidation of odd-chain fatty acids can also contribute to glucose synthesis, although in a much lower scale due to the low availability of odd-chain fatty acids in human metabolism.

Several studies in the beginning of the twentieth century showed that lactate could be converted to glucose (and glycogen) in liver tissue. At that time, the interest was focused on the synthesis of glycogen in the body and less attention was given to the reactions involved in the conversion of lactate to glucose. Now we know that lactate conversion to glucose (gluconeogenesis) and glucose conversion to glycogen (glycogenogenesis) are processes that do not occur simultaneously (see the last section of this chapter). Probably as a result of this, there were many controversial observations at that time.

Lactate enters the gluconeogenesis pathway through its conversion to pyruvate in a reaction catalyzed by the enzyme lactate dehydrogenase (Fig. 9.12). In this reaction, cytosolic NAD^+ is reduced to NADH, increasing [NADH]/[NAD^+] ratio in the cytosol and favoring PEP formation inside the mitochondria (see previous section).

Evidence that amino acids can act as substrates for glucose synthesis came from the early studies on glycogen formation by Claude Bernard in the nineteenth century, in which he fed dogs with meals consisting solely of meat and found an increase of glucose released by the liver (see Box 9.4).

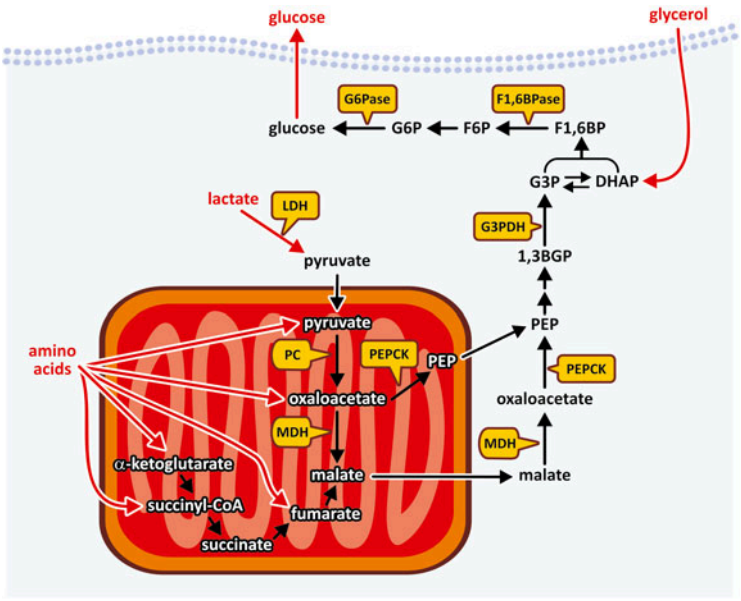


Fig. 9.12 Entry of precursors in the gluconeogenesis pathway. Lactate is converted to pyruvate by lactate dehydrogenase (LDH). Amino acids have their amino groups removed by transamination and/or deamination generating TCA cycle intermediates that converge to malate, which is converted to oxaloacetate and then to PEP in the cytosol. Glycerol is converted to glycerol phosphate and then to dihydroxyacetone phosphate (DHAP), entering gluconeogenesis. F1,6BP, fructose-1,6-bisphosphate; F1,6BPase, fructose-1,6-bisphosphatase; F6P, fructose-6-phosphate; G6P, glucose-6-phosphate; G6Pase, glucose-6-phosphatase; other abbreviations are the same as in Fig. 9.11. The names of the enzymes are highlighted in *yellow boxes*

To enter gluconeogenesis, the amino group of the amino acids should be removed by transamination and/or deamination. After this metabolization, eighteen from the twenty more common amino acids generate α-ketoglutarate, succinyl-CoA, fumarate, oxaloacetate, or pyruvate (see Sect. 7.5). The TCA cycle intermediates converge to malate through the action of the enzymes of the cycle. Malate leaves mitochondria and is converted to oxaloacetate, which then forms PEP in the cytosol (Fig. 9.12).

The product of the metabolization of leucine or lysine is acetyl-CoA, which cannot be used to synthesize glucose since its two carbon atoms are completely oxidized to CO_2 in TCA cycle, thus impairing the net accumulation of carbons to be incorporated in the newly synthesized glucose molecule. This is also the reason why fatty acids cannot be transformed in glucose in animals.

Finally, glycerol, which is converted to glycerol phosphate after being transported to the cells, is converted to dihydroxyacetone phosphate by glycerol-phosphate dehydrogenase, entering gluconeogenesis at this point (Fig. 9.12).

9.3.3 *Regulation of Gluconeogenesis*

Glycolysis and gluconeogenesis share many reactions and both pathways are exergonic in the intracellular conditions. Thus, these pathways must be reciprocally regulated to allow one of them to predominate over the other in each specific situation. As a matter of fact, in the liver, the rates of glycolysis and gluconeogenesis are adjusted to maintain the blood glucose concentration stable.

The main point of the reciprocal regulation of glycolysis and gluconeogenesis is the interconversion between fructose-6-phosphate and fructose-1,6-bisphosphate by the enzymes phosphofructokinase-1 (PFK-1) in glycolysis and fructose-1,6-bisphosphatase (F1,6BPase) in gluconeogenesis. This point is controlled by hormonal action with a key role of the molecule fructose-2,6-bisphosphate (Fig. 9.13).

Fructose-2,6-bisphosphate in submicromolar concentrations simultaneously activates PFK-1 and inhibits F1,6BPase. This molecule is synthesized through the phosphorylation of fructose-6-phosphate in a reaction similar to that catalyzed by PFK-1, but with the transfer of the phosphoryl group of ATP to the carbon 2 instead of the carbon 1 of fructose. The enzyme that catalyzes this reaction was named phosphofructokinase-2 (PFK-2) to avoid confusion with the classic PFK-1. Fructose-2,6-bisphosphate is hydrolyzed to fructose-6-phosphate by the enzyme fructose-2,6-bisphosphatase (F2,6BPase).

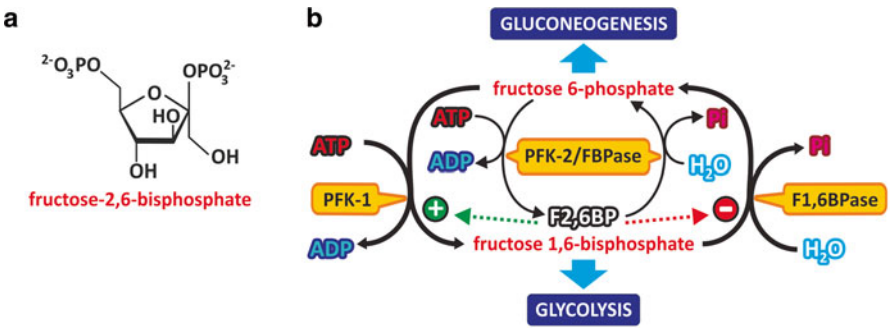
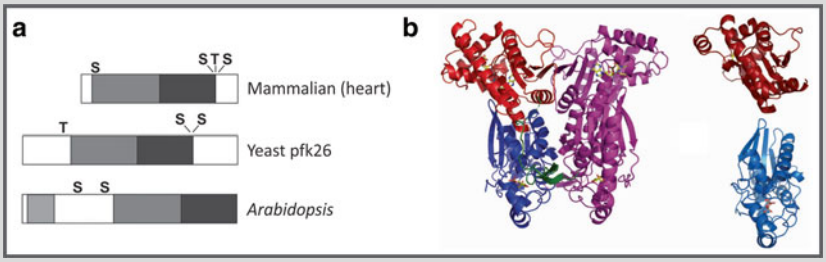


Fig. 9.13 (**a**) Fructose-2,6-bisphosphate structure. (**b**) Synthesis and degradation of fructose-2,6-bisphosphate and its effect on the activities of PFK-1 and F1,6BPase in the liver

It is very interesting to note that these two antagonic activities, PFK-2 and F2,6BPase, are present in the same polypeptide chain, an example of a bifunctional enzyme (Box 9.5).

Box 9.5: Evolution of the Bifunctional Enzyme PFK-2/F2,6BPase

It is believed that the bifunctional enzyme PFK-2/F2,6BPase resulted from the fusion of genes encoding different enzymes. This seems to have occurred very early during evolution in a common ancestor of all eukaryotes. In all taxonomic groups, the enzyme has a central catalytic core with the PFK-2 and F2,6BPase domains in tandem and extensions in N- and C-terminals [see examples in (**a**)]. In some cases, one of the activities is damaged by deletions or insertions. The kinase and the phosphatase domains are structurally similar to one-domain kinases or phosphatases (see figure).



(**a**) Schematic representation of domain localization of PFK-2 and F2,6BPase primary sequence in different organisms. (**b**) Comparison of the structure dimeric human bifunctional enzyme with gluconate kinase, in *red*, and PhoE phosphatase, in *blue*. Reproduced with permission from Michels & Rigden. IUBMB Life 58:133–141, 2006

The bifunctional enzyme presents a regulatory domain in its N-terminal end, which contains a serine residue (Ser32) that can be phosphorylated or dephosphorylated in response to the action of glucagon (see Sect. 9.4.1) or insulin (see Sect. 8.4), respectively. When Ser32 is phosphorylated, the enzyme undergoes a conformational change that favors the F2,6BPase activity. In contrast, when it is dephosphorylated, the enzyme activity is turned to PFK-2.

Gluconeogenesis is also regulated at the first bypass level. Carboxylation of pyruvate to oxaloacetate is completely dependent on the presence of acetyl-CoA, which acts as a specific activator of PC by its reversible binding to the allosteric domain of the enzyme (see PC structure in Fig. 9.9).

This is an interesting example of how the information of a physiological situation can be transmitted locally to a specific cellular compartment by means of an allosteric modulator. In hypoglycemia, triacylglycerides stored in the adipose tissue are mobilized, generating glycerol and fatty acids (see Sect. 7.4.1). The increased availability of fatty acids in the bloodstream allows them to be used as energy source for many tissues, including the liver. β-oxidation of fatty acids, which occurs within the mitochondria (see Sect. 7.4.4), increases the concentration of acetyl-CoA

in the mitochondrial matrix where PC is located. Thus, the mobilization of triglycerides as a response to hypoglycemia results in the generation of an essential activator of an important enzyme for the synthesis of glucose, a crucial pathway in this situation.

The activity of PEPCK is regulated only at the transcriptional level. The gene that encodes the cytosolic form of PEPCK in the liver contains several hormone response elements, including glucocorticoid, cyclic AMP, and insulin response units, besides thyroid hormones and retinoic acid response units. Through different mechanisms of actions, glucocorticoids and glucagon enhance the transcription of PEPCK gene (see Sect. 9.4), while insulin represses its basal and hormone-induced expression.

9.3.4 Dynamic Utilization of Gluconeogenesis Precursors

Daily glucose requirement for the adult human organism is around 120 g. In a fasting situation, in which no glucose is ingested, the contribution of each of the gluconeogenesis precursors is shown in Table 9.2.

Table 9.2 Contribution of each gluconeogenesis precursors to glucose production

	Amount of glucose produced daily (g)	
Precursor	1-day starvation	5 weeks' starvation
Lactate	39	39
Glycerol	19	19
Amino acids	60	16

Taking into account that each precursor is originated in a specific tissue, the blood concentration of glucose can be maintained due to the interplay among different tissues of the organism (Fig. 9.14). Glycerol is generated by the hydrolysis of triacylglycerols in the adipose tissue. Proteolysis of the contractile proteins in muscle cells generates amino acids that undergo transamination, mainly with pyruvate or α-ketoglutarate within muscle cells, forming alanine and glutamine. Lactate is continuously produced by the fermentation-dependent cells such as the erythrocytes.

It is important to note that this situation changes if glucose is not ingested for longer periods. If protein mobilization was maintained at the same rate as in the beginning of fasting, after about one month without food ingestion, half of the body proteins would be consumed. This can be calculated from the data shown in Tables 9.1 and 9.2 (for these calculations, the stoichiometry of glucose synthesis from amino acids must be taken into account: to produce one gram of glucose, about two grams of amino acids are necessary).

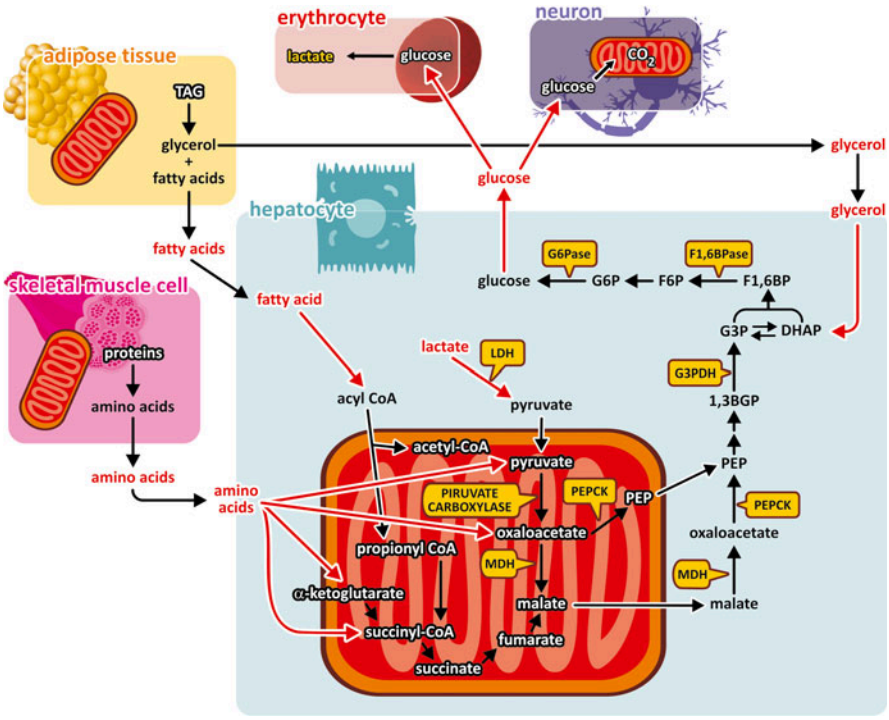
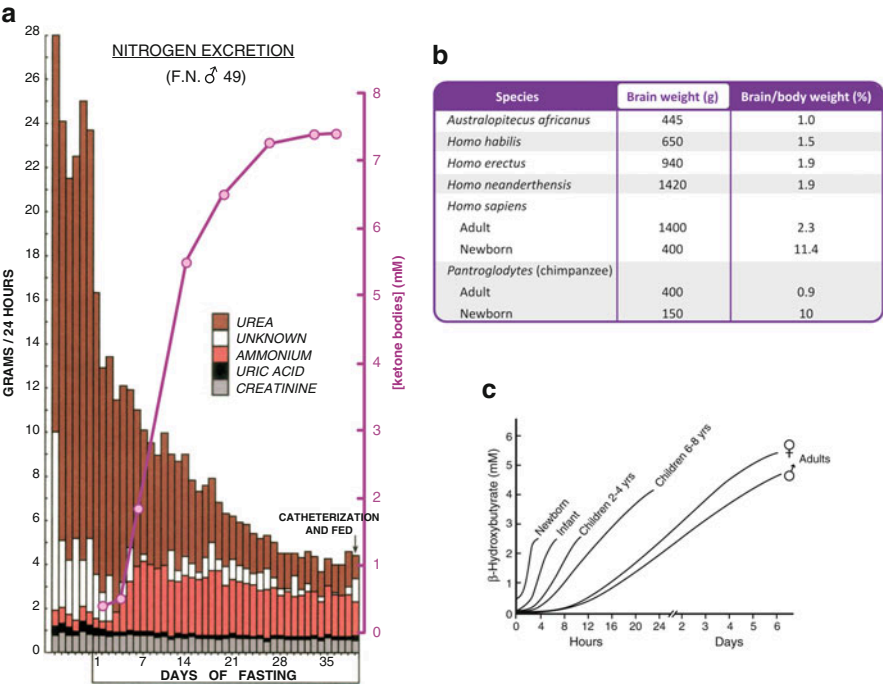


Fig. 9.14 Metabolite interplay among the organs. Hydrolysis of triacylglycerols in the adipose tissue generates glycerol and fatty acids that are released in the bloodstream. Even-chain fatty acids form acetyl-CoA, while each molecule of odd-chain fatty acid forms one molecule of propionyl-CoA besides acetyl-CoA. Propionyl-CoA may enter gluconeogenesis after its conversion to succinyl-CoA and then malate through TCA cycle reactions. Glycerol enters the liver cells where it is converted to DHAP and then to glucose. Proteolysis of the contractile proteins in muscle cells generates amino acids that are released in the bloodstream entering the liver, where they undergo transamination/deamination generating pyruvate or TCA cycle intermediates that ultimately form malate to enter the gluconeogenesis pathway. Lactate is continuously produced by and released from the fermentative cells such as the erythrocytes, reaching the liver where it is converted to pyruvate, which enters the gluconeogenesis pathway. Glucose produced is mainly used by the cells from central nervous systems and by the fermentation-dependent cells. Abbreviations are the same as used in Fig. 9.12

However, the human organism is adapted to survive much longer periods of starvation. Even in the therapeutic starvation for obesity treatment as presented in the beginning of this chapter, the patients spent more than one month without any food ingestion. The studies performed with these patients made possible to understand this adaptation. Catheterization of brain vessels demonstrated that two thirds of brain fuel consumption in long starvation corresponded to the metabolization of β-hydroxybutyrate and acetoacetate, markedly diminishing the need of glucose production and, consequently, muscle proteolysis to provide gluconeogenic precursors. In fact, the decrease in protein degradation during fasting can be inferred

by observing the profile of nitrogen excretion of a patient submitted to therapeutic starvation. It is interesting to note that the decrease in nitrogen excretion shows a clear correlation with the increase in ketone bodies' concentration in the blood (Fig. 9.15a).



Species	Brain weight (g)	Brain/body weight (%)
Australopitecus africanus	445	1.0
Homo habilis	650	1.5
Homo erectus	940	1.9
Homo neanderthensis	1420	1.9
Homo sapiens		
Adult	1400	2.3
Newborn	400	11.4
Pantroglodytes (chimpanzee)		
Adult	400	0.9
Newborn	150	10

Fig. 9.15 (**a**) Excretion of different nitrogenated compounds by a human subject during starvation (Reproduced from Owen, Biochem. Mol. Biol. Educ. 33:246–251, 2005) and its correlation to the concentration of ketone bodies (β-hydroxybutyrate and acetoacetate) in the blood. (**b**) Encephalization during human evolution (Reproduced from Cunnane & Crawford, Comp. Biochem. Physiol. 136:17–26, 2003, with permission from Elsevier). (**c**) Age dependence of β-hydroxybutyrate concentration in blood during fasting (Reproduced with permission from: Cahill. Ann. Rev. Nutr. 26:1–22, 2006)

To better understand this adaptation, it is important to have some information regarding the brain uptake of ketone bodies. Ketone bodies are transported across the blood–brain barrier through the monocarboxylate transporters (MCTs), especially MCT1, which is highly expressed in endothelial cells that form blood–brain barrier vessels. MCT1 transports a wide range of short-chain monocarboxylates, including lactate, pyruvate, acetoacetate, and β-hydroxybutyrate. The K_M values for these substrates are in the range of 5–10 mM. Thus, the increase in ketone bodies concentration in the blood during fasting greatly favors the transport of these molecules across the blood–brain barrier. As seen in Fig. 9.15a, blood concentration of

β-hydroxybutyrate reaches the range of the K_M for MCT1 after the first days of fasting, in such a way that this molecule becomes increasingly available to brain cells as fasting proceeds. Additionally, prolonged ketonemia, as it occurs in starvation or low-carbohydrate diets, induces MCT1 gene expression, also contributing to the increase of MCT1-mediated ketone bodies' transport to the cells of the central nervous system. Thus, along the first week of fasting, the use of ketone bodies by central nervous system cells greatly decreases the requirement of glucose as the energy supply for these cells. Since brain metabolism accounts for most of the use of glucose in the body (approximately 100 g of the 120 g necessary daily), it is easy to imagine that gluconeogenesis rate can be considerably reduced as the blood concentration of ketone bodies increases.

The use of ketone bodies by the brain seems also to have been very important during human evolution, which was characterized by a remarkable increase of brain weight (Fig. 9.15b). However, the "cost" of encephalization is the increase in energy demands. The brain of a modern human adult corresponds to 2.3 % of its body weight but accounts for 23 % of energy consumption of the organism. In children, the energy demand for the brain is even greater (approximately 75 % of the total organism energy demand). The fact that the brain/body ratio is similar between humans and chimpanzees suggests that primates have in general the potential to have large brains. During development, however, the brain/body ratio in chimpanzees becomes less than a half of that of humans. An interesting observation that may explain this fact is that humans are the only among the primates that are born fat. This probably enables them to produce ketone bodies for brain use during the newborn development. As a matter of fact, human newborn metabolism is essentially ketotic, since the larger the brain/body ratio is, the more rapidly ketosis develops (Fig. 9.15c).

9.4 Hormonal Responses to Hypoglycemia

One of the major metabolic adaptations to hypoglycemia is the production of glucose by the liver. As discussed throughout the chapter, this is possible by the combination of hepatic glycogen degradation (in the first hours) and glucose production from non-glycidic precursors. Additionally, mobilization of triacylglycerol in the adipose tissue occurs simultaneously, ensuring the energy supplies for most of the tissues.

The simultaneous activation of these pathways is mainly regulated by the action of glucagon and glucocorticoids. The secretion of these hormones is enhanced by the decrease in blood glucose concentration and, through their action, the information about the hypoglycemia situation is transmitted to the different cells and organs.

In this section, the signaling pathways as well as the effects of glucagon and glucocorticoids on their main target cells will be detailed.

9.4.1 *Glucagon: Mechanism of Action and Effects on Energy Metabolism*

Glucagon is a peptidic hormone of 29 amino acid residues, secreted by the pancreatic α-cells, which compose the Langerhans islets together with β-cells that secrete insulin (see Sect. 8.4) and γ-cells that secrete somatostatin (Fig. 9.16).

Glucagon was discovered in the decade of the 1920s as a hyperglycemic factor produced by the pancreas. This finding was correlated to glycogen degradation in the liver, a subject extensively studied at that time (discussed in the beginning of this chapter), giving glucagon its original name of "hyperglycemic-glycogenolytic factor." During the twentieth century, the structure of the gene that encodes glucagon and the complex processing of its product as well as the glucagon signaling pathway and its effects on the target cells have been elucidated.

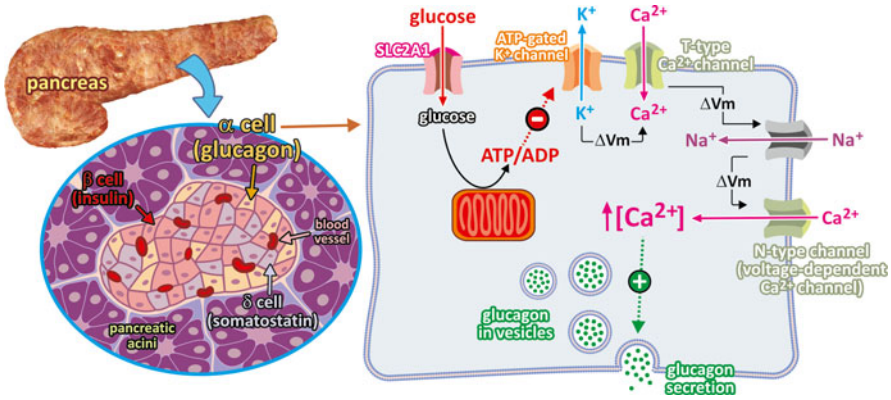


Fig. 9.16 Representation of a Langerhans islet, showing the α-cell and the mechanism of glucagon secretion in detail. Secretion of glucagon is controlled by a set of α-cell ion channels that generate action potentials of Na^+ and Ca^{2+}. At low levels of glucose, the activity of the ATP-sensitive K^+ channels renders a membrane potential that stimulates T-type Ca^{2+} channels to open, leading to membrane depolarization that, in turn, activates Na^+ and N-type Ca^{2+} channels. Intracellular Ca^{2+} waves caused by Ca^{2+} entry through N-type Ca^{2+} channels induce the exocytosis of glucagon granules. K^+ flow through A-type channels mediate membrane repolarization, so that this oscillatory electrical activity results in a pulsatile pattern of glucagon secretion. An increase in glucose consumption by α-cells rises the intracellular ATP/ADP ratio, blocking the ATP-sensitive K^+ channels. This causes membrane depolarization and decrease in Ca^{2+} influx, inhibiting glucagon secretion

At low levels of glucose, a set of ion channels on α-cell membrane generates action potentials that activate voltage-dependent L-type Ca^{2+} channels, leading to intracellular Ca^{2+} waves that induce the exocytosis of glucagon granules as well as

the expression of glucagon gene (Fig. 9.16). Membrane repolarization by K^+ flowing through A-type channels triggers oscillatory Ca^{2+} signals, so that glucagon secretion follows a pulsatile pattern. Although it seems clear that glucagon is released constitutively from α-cells, some factors, such as catecholamines and amino acids (mainly arginine), can act as positive regulators of its secretion by increasing the amplitude of the electric pulses in α-cells. However, the main control of blood concentration of glucagon occurs through the inhibition of its secretion. Glucose and insulin are the major negative regulators of glucagon secretion, so that the profile of blood glucagon concentration is the mirror image of that of glycemia. Therefore, glucagon transmits to the different organs in the body the information of hypoglycemia, leading to appropriate responses of specific tissues that allow the organism to deal with this situation.

Glucagon, like other hydrophilic hormones, acts on its target cells by binding to a receptor on the cell surface (see Chap. 5). Glucagon receptor is not uniformly distributed among the different tissues: it is expressed in high levels in the liver, kidneys, and pancreas and has also been detected in the adipose tissue and the heart, but it is absent in the skeletal muscle cells.

Glucagon receptor belongs to the superfamily of G protein-coupled receptors (see also Sect. 10.5.1.1). The main effects of glucagon on its target tissues are mediated by the increase of the intracellular levels of cyclic AMP (cAMP), through the classic mechanism of action of G protein-coupled receptors (Fig. 9.17). G proteins are composed of three subunits, α, β, and γ. α-Subunit binds GTP and catalyzes its hydrolysis, whose product, GDP, remains bound to the protein maintaining it in an inactive state, associated to β- and γ-subunits. When glucagon binds to its receptor, it undergoes a conformational change that is transmitted to G protein, leading to the replacement of GDP by GTP. When bound to GTP, the α-subunit dissociates from βγ-subunits and moves freely on the inner side of cellular membrane until it reaches the enzyme adenylate cyclase. This enzyme is activated by G protein α-subunit and catalyzes the conversion of ATP in cAMP, leading to an increase in the intracellular concentration of this molecule. The affinity of the α-subunit to adenylate cyclase decreases after GTP hydrolysis, leading the α-subunit itself to dissociate from adenylate cyclase and to reassociate to βγ-subunits.

The increase in the intracellular concentrations of cAMP as a response to glucagon binding to its receptor promotes the activation of an important enzyme, the cAMP-dependent protein kinase (PKA) (Fig. 9.17). This kinase is composed of two regulatory and two catalytic subunits. cAMP binds to the regulatory subunits, which dissociate from the catalytic ones that become free to catalyze the phosphorylation of several important enzymes, thus modulating their activities (as summarized in Fig. 9.17).

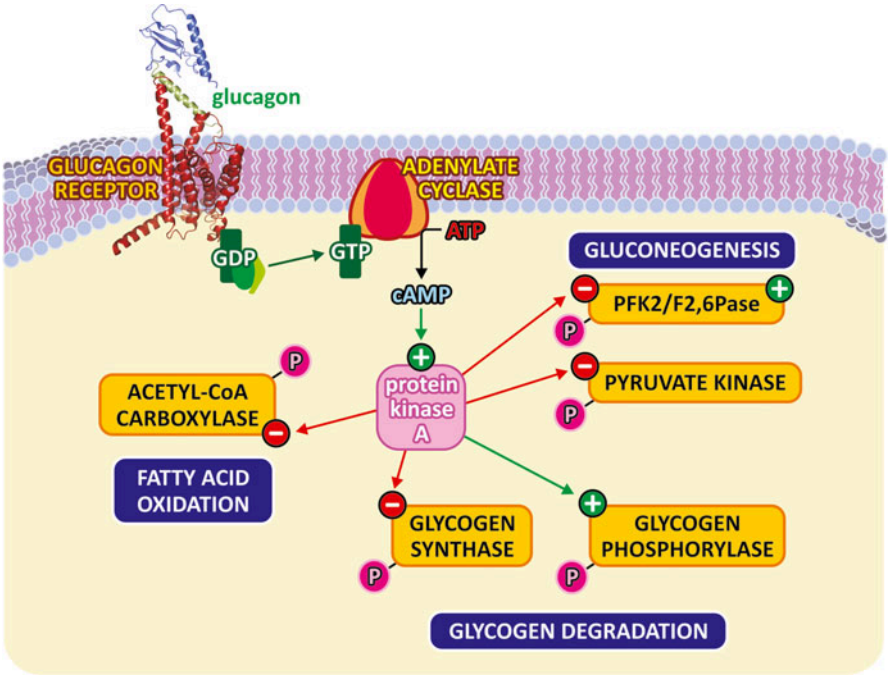


Fig. 9.17 Mechanism of glucagon action, showing its effects on enzyme activities in the liver. Glucagon (structure shown in green, PDB 1GCN) binds to its G protein-coupled receptor (PDB 4ERS and 4L6R), promoting the replacement of GDP by GTP in the G protein α-subunit, which dissociates from βγ-subunits and associates to adenylate cyclase, leading to an increase in the intracellular concentration of cAMP. This second messenger binds to the regulatory subunits (not represented in this simplified figure) of the protein kinase A (PKA), leading to the release of the active catalytic subunits. Active PKA catalyzes the phosphorylation of several enzymes (represented by the "P" in the *pink circle*), changing their activities as indicated by *green* "+," for activation, and the *red* "–," for inhibition. The pathways that are activated due to these changes in the enzyme activities are shown in the *blue boxes*

9.4.1.1 Effects of Glucagon on Liver Metabolism

Liver metabolism is drastically affected by glucagon. Hepatic glycogenolysis is activated, while glycogen synthesis is inhibited. Simultaneously, gluconeogenesis is activated and glycolysis inhibited. As a result, glucose is produced and released in the bloodstream. Additionally, fatty acid synthesis is inhibited (see Sect. 8.3), allowing the incoming fatty acids to undergo β-oxidation, ensuring ATP generation. The coordinated control of these different metabolic pathways is possible because key enzymes of each of these pathways have their activities modulated by phosphorylation promoted directly or indirectly by PKA (Fig. 9.18). Additionally, the increase in the intracellular concentration of cAMP can also regulate gene expression, since several genes present in their promoter region a cAMP-responsive element (CRE).

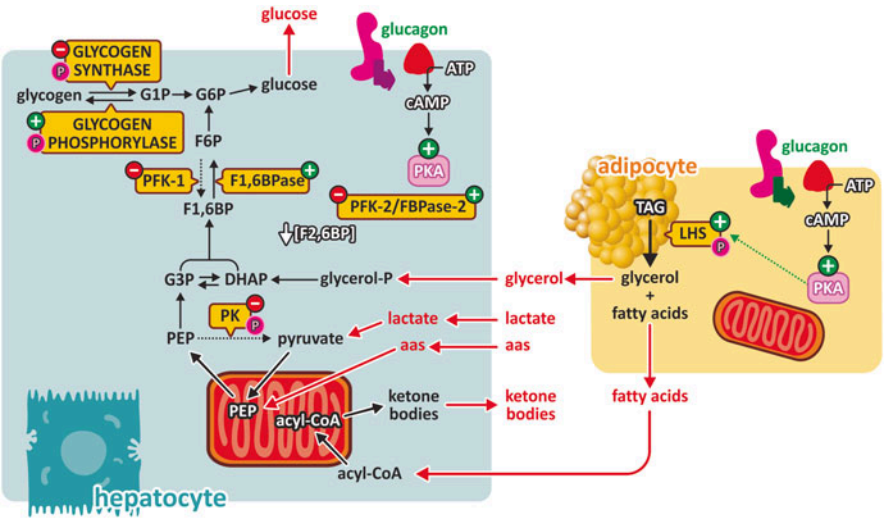


Fig. 9.18 Integration of the effects of glucagon on liver and adipose tissue, showing the enzymes and the metabolic pathways regulated in each cell. The P in the *pink circle* represents the phosphate group introduced in the enzyme by PKA; + and − indicate the activated or inhibited states, respectively. The *dashed lines* represent inhibited reactions. PFK-1, phosphofructokinase-1; F1,6BPase, fructose-1,6-bisphosphatase; PFK-2/F2,6BPase, phosphofructokinase-2/fructose-2,6-bisphosphatase F2,6BP, fructose-2,6-bisphosphate; LHS, hormone-sensitive lipase. Other abbreviations are the same as used in Figs. 9.11, 9.12, and 9.14

The conversion of the key enzyme of glycogen degradation, GP, to its active form occurs through the phosphorylation of Ser14, catalyzed by PK (see Sect. 9.2.2). This enzyme, in turn, is also activated by phosphorylation, in this case catalyzed by PKA. Thus, PKA activation in response to glucagon binding to liver cells results in the phosphorylation of GP, which becomes active and promotes glycogen degradation (Fig. 9.18). The signaling pathway triggered by glucagon not only stimulates glycogen degradation but also impairs its synthesis. This is possible because the activity of the key enzyme in glycogen synthesis, glycogen synthase (GS), is also regulated by PKA-mediated phosphorylation (for details on glycogen synthesis pathway, see Sect. 8.2). However, the phosphorylation in this case causes the inhibition of the enzyme activity (Fig. 9.18). PKA can directly phosphorylate GS or can phosphorylate another protein kinase, the glycogen synthase kinase 3 (GSK3), which also phosphorylates GS. Thus, the hyperphosphorylation of GS impairs glycogen synthesis.

As discussed in Sect. 9.3.3, fructose-2,6-bisphosphate is a key molecule in the reciprocal regulation of glycolysis/gluconeogenesis. This compound is formed/degraded by the action of the bifunctional enzyme PFK-2/F2,6BPase, which is also a substrate for PKA. PKA-mediated phosphorylation of Ser32 of the bifunctional enzyme stimulates the F2,6BPase and inhibits the PFK-2 activities, leading to

fructose-2,6-bisphosphate degradation and, therefore, to activation of gluconeogenesis and inhibition of glycolysis (Fig. 9.18).

The uptake and the ability of the liver to use amino acids are also increased in response to glucagon. This seems to occur mainly at the transcriptional level, due to the induction of the expression of the genes encoding the hepatic alanine transporter and different transaminases, such as alanine aminotransferase, aspartate aminotransferase, and tyrosine aminotransferase. This control enables the more efficient use of the amino acids as precursors of glucose synthesis through the gluconeogenesis pathway.

Lipid metabolism in the liver is also regulated by PKA-dependent phosphorylation. The enzyme that has its activity regulated is acetyl-CoA carboxylase (ACC), a key enzyme in the fatty acid synthesis pathway (see Sect. 8.3). The phosphorylated ACC is inactive, impairing fatty acid synthesis. Additionally, malonyl-CoA, the product of the reaction catalyzed by ACC and an important intermediate of this pathway, is a potent inhibitor of the transport of acyl-CoA molecules across the mitochondria membranes. Since malonyl-CoA is not formed due to glucagon-mediated inhibition of ACC, liver β-oxidation can proceed during the hypoglycemia situation.

9.4.1.2 Effects of Glucagon on the Adipose Tissue

An important effect of glucagon on the adipose tissue is the activation of lipolysis, which ensures the increase of fatty acid concentration in the blood, making these molecules available to different tissues as the main energetic supply. The phosphorylation of the adipocyte enzyme hormone-sensitive lipase by PKA activates it, increasing the hydrolysis of triacylglycerols to glycerol and fatty acids, which are then released in the bloodstream (Fig. 9.18; see also Sect. 7.4.1). Glycerol may be used as substrate for gluconeogenesis, whereas fatty acids undergo β-oxidation in different tissues.

9.4.2 Glucocorticoids: Mechanism of Action and Effects on Energy Metabolism

Glucocorticoids are steroid hormones synthesized from cholesterol in the cortex of adrenal glands (Fig. 9.19). In humans, the main glucocorticoids produced are cortisol (80–90 %) and corticosterone.

The synthesis of the glucocorticoids is stimulated by the adrenocorticotropic hormone (ACTH), a peptidic hormone produced in the anterior pituitary gland. The release of ACTH by the pituitary is, in turn, stimulated by another hormone, the corticotropin-releasing hormone (CRH). This hormone is produced by the hypothalamus and reaches the pituitary through a portal system (Fig. 9.19). Thus, the synthesis and secretion of glucocorticoids by the adrenal glands are under control of the hypothalamic–pituitary–adrenal axis, in a classical example of the integration of nervous and endocrine systems. Furthermore, there is an efficient feedback control

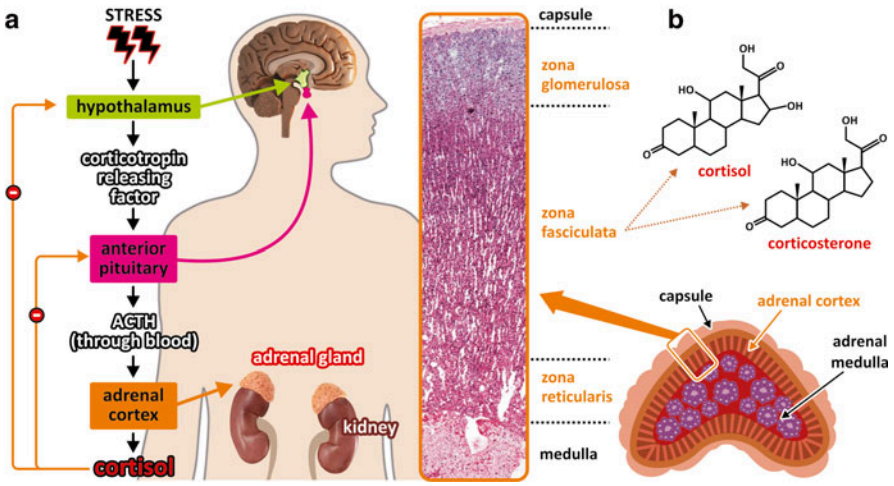


Fig. 9.19 (**a**) Hypothalamic–pituitary–adrenal axis and glucocorticoid secretion. (**b**) Representation of the adrenal gland, showing the two distinct parts: the adrenal cortex and the adrenal medulla and the three zones of the cortex, the zona glomerulosa, the zona fasciculata, and the zona reticularis. Cortisol and corticosterone, the natural human glucocorticoids, synthesized in the zona fasciculata and in the zona reticularis (The histological image was reprinted with the permission of Instituto de Histologia e Biologia do Desenvolvimento, Faculdade de Medicina, Universidade de Lisboa, FMUL)

of glucocorticoid production, in which the release of both ACTH and CRH is suppressed when circulating glucocorticoid levels reach a specific threshold.

Glucocorticoid secretion may be regulated in two levels. The first level is constitutive and follows the circadian rhythm, leading to a peak in cortisol concentration in the blood in the beginning of the morning. This pattern is adjusted by individual habits through the light/dark cycles. The second level corresponds to a response to virtually all types of physical or mental stress (Fig. 9.19), in which hypoglycemia can be included.

Due to their hydrophobic nature, the glucocorticoids enter the cells and bind to intracellular receptors (see Chap. 5). The hormone-receptor complex migrates to the nucleus, where it interacts with specific regions of the genome, inducing or repressing the expression of several genes.

Glucocorticoid receptor belongs to the superfamily of the nuclear hormones receptors, which also includes the receptors for mineralocorticoids, androgen, progesterone, thyroid hormones, retinoic acid, and retinol. The structure of all these receptors shows three distinct domains: an N-terminal domain that interacts with the DNA and/or with other transcriptional factors, a central domain with two zinc-finger motifs responsible for the recognition and binding to specific DNA sequences, and a C-terminal domain that binds to the hormone.

When not bound to the hormone, the glucocorticoid receptor associates to a multiprotein complex, which includes some of the heat-shock proteins (Hsp). The interaction with the glucocorticoid results in receptor dissociation from this

complex and in its hyperphosphorylation, which exposes nuclear localization sequences that allow the hormone-receptor complex, if in the cytosol, to migrate to the nucleus. In the nucleus, the hormone-receptor complex forms dimers and the DNA-binding segments become exposed, enabling the regulation of gene expression (Fig. 9.20). The sequences to which the glucocorticoid-receptor complex binds are small palindromic sequences of 15 nucleotides known as the glucocorticoid-responsive elements.

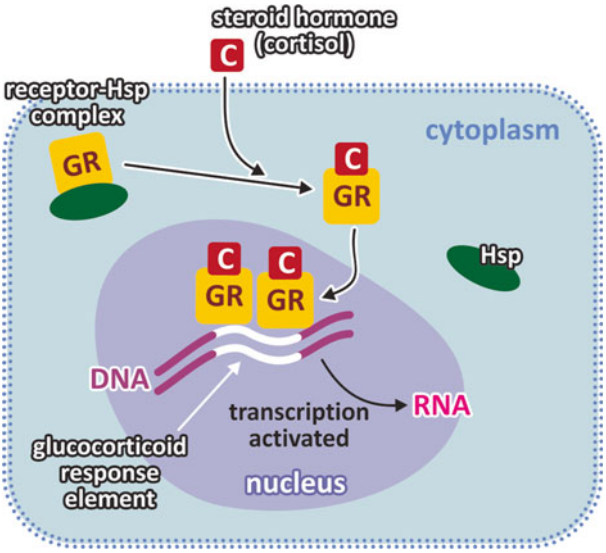


Fig. 9.20 Mechanism of action of the glucocorticoids. Intracellular binding of glucocorticoid to its receptor promotes receptor dissociation from its inhibitory multiprotein complex (Hsp - heat shock proteins) and ita migration to the nucleus where the hormone-receptor complex binds to segments in DNA containing the glucocorticoid response elements, leading to gene expression

9.4.2.1 Effects of Glucocorticoids on Muscle Metabolism

The regulation of protein and amino acid metabolisms by glucocorticoids are among the first actions of these hormones to be characterized. Glucocorticoids seem to have a crucial role in the mobilization of muscular proteins in hypoglycemia, yielding amino acids for liver and kidney gluconeogenesis. Indeed, several studies have shown that the glucocorticoids stimulate protein degradation. The mechanisms of this regulation are still not completely understood, but glucocorticoid-induced proteolysis seems to be restricted to the muscles.

Measurements of the arteriovenous differences in the muscles during fasting revealed that a net release of amino acids from the muscles occurred and, more interestingly, that 60 % of them were alanine and glutamine (Table 9.3), although

Table 9.3 Amino acid released from the skeletal muscle during fasting in man (based on data from Felig. Annu. Rev. Biochem. 44:933–955, 1975)

Amino acid	Arteriovenous difference (μmol/L)	Percentage of total
Alanine	−70	30
Glutamine	−70	30
Glycine	−24	10
Lysine	−20	9
Proline	−16	7
Threonine	−10	4
Histidine	−10	4
Leucine	−10	4
Valine	−8	3
Arginine	−5	2
Phenylalanine	−5	2
Tyrosine	−4	2
Methionine	−4	2
Isoleucine	−4	2
Cysteine	+10	–
Serine	+10	–

these two amino acids correspond to approximately 10 % of muscle proteins' content.

Therefore, it seems clear that the different amino acids produced by muscle proteolysis should be preferentially converted into Ala and Gln before being released from muscle cells. At this point, the role of glucocorticoids is very important. The genes encoding the different transaminases have glucocorticoid-responsive elements and, therefore, have their expression induced by these hormones. The increase in transaminase synthesis and consequently in their activities allows the interconversion of different amino acids into Ala and Glu (see Sect. 7.5), with the subsequent amination of Glu to generate Gln (Fig. 9.21).

Ala and Gln released from the muscles into the bloodstream during fasting or low-carbohydrate diets are used as precursors for gluconeogenesis. Quantitatively, Ala is the most important amino acid that is used as a gluconeogenic precursor in the liver, whereas Gln is preferentially used by the kidney (Fig. 9.21).

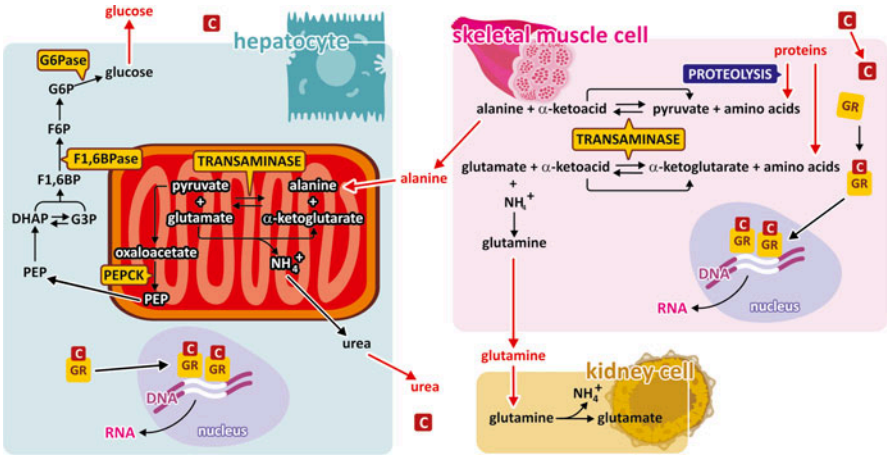


Fig. 9.21 Integration of the effects of glucocorticoids in different organs, showing the regulation of metabolic pathways in each cell type. The *red square* with a C represents cortisol; the *yellow squares* represent the glucocorticoid receptor; the enzymes in yellow boxes are the ones whose syntheses are induced by cortisol

9.4.2.2 Effects of Glucocorticoids on Liver Metabolism

The induction of the expression of the genes encoding the transaminases also occurs in the liver as a result of glucocorticoid action. This facilitates transamination of the available amino acids allowing them to enter gluconeogenesis (Fig. 9.21). Additionally, the genes of different gluconeogenic enzymes contain glucocorticoid-responsive elements and are under the control of these hormones, so that glucocorticoids induce the synthesis of PEPCK, F1,6BPase, and glucose-6-phosphatase, whose concentrations greatly increase in the liver cells. Thus, glucocorticoid action on liver cells enhances the hepatic capacity to perform gluconeogenesis.

However, it is important to take into account that glucocorticoid action alone is not sufficient to induce gluconeogenesis, since it is not only the presence of a given enzyme in high concentrations that will ensure its activation. Depending on the enzyme, changes in its phosphorylation state or the presence of an allosteric modulator is also necessary to allow the enzyme to operate. But since glucagon and glucocorticoids are both secreted as a response to hypoglycemia, in the case of gluconeogenesis, additive effects of these hormones occur, ensuring the activation of this metabolic pathway.

In addition, some effects of glucocorticoids represent another type of metabolic control. This is the case, for example, of their action on the genes encoding ACC

(a key enzyme in the synthesis of fatty acid) and GS (a key enzyme in the synthesis of glycogen). Glucocorticoids induce the expression of both enzymes, but the effect of glucagon, which is operating simultaneously, leads to the phosphorylation and inactivation of these enzymes. However, when glycemia increases (e.g., after a meal), glucagon action stops and these enzymes, in high concentration due to glucocorticoids effect, become active. Thus, the longer the period in hypoglycemia, the higher the concentrations of these enzymes. This will ensure the storage of the nutrient after a meal. Therefore, glucocorticoids prepare the organism to become more efficient in using the nutrients after a period of scarcity. Certainly, this role of glucocorticoids was very important for human beings in the past. However, today, when food is easily available, this adaptation to starvation may be one of the causes of the alarming increase in obesity in certain areas of the globe.

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Chapter 10
Regulation and Integration of Metabolism During Physical Activity

The survival of our ancestors was highly dependent on hunting, gathering, and fighting, behaviors that demanded intense physical activity. A sedentary lifestyle in that environment would certainly result in the elimination of the individuals. This situation imposed a selective pressure directed to adaptations of human physiology to a high capacity of physical activity, resulting in the development of a very efficient locomotor system, in which the skeletal muscles correspond to about 40 % of the body mass and account for a great proportion of the average energy consumption of the organism. The present-day sedentarism is dissonant with the human genetic background selected to favor a physically active lifestyle and probably consists in one of the main causes of the increasing incidence of modern chronic diseases, such as hypertension, obesity, and insulin resistance.

Muscles are tissues specialized in producing force and movement due to an amazing ability to convert the chemical energy of ATP phosphate bonds to mechanical work. This energy interconversion is performed by an array of proteins that forms a very organized structure inside the cells. The muscle mechanical activity may change very fast so that the energy sources and the metabolic pathways used to maintain cellular functions need to be finely regulated.

In this chapter, we will discuss the metabolic adaptations to physical exercise, with special attention to the metabolism of the skeletal muscle cells. We will start with a brief review of the structure of the contractile apparatus and the mechanism of muscle contraction, followed by the description of the energy sources and the metabolic pathways involved in muscle activity during physical exercise. We finish the chapter with the mechanism of action and the main metabolic effects of adrenaline, the major hormone secreted during exercise.

A.T. Da Poian, M.A.R.B. Castanho, *Integrative Human Biochemistry*,
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10.1 Muscle Contraction

Muscles are used either for locomotion or for the movements associated to the functions of the internal organs and are classified in three groups according to the type of movement they generate (Fig. 10.1). The skeletal muscles can be contracted voluntarily allowing the body to move and to maintain the posture, while rhythmic involuntary muscle contractions, such as heart contraction or peristalsis and other autonomous motilities, are performed by cardiac and smooth muscles, respectively.

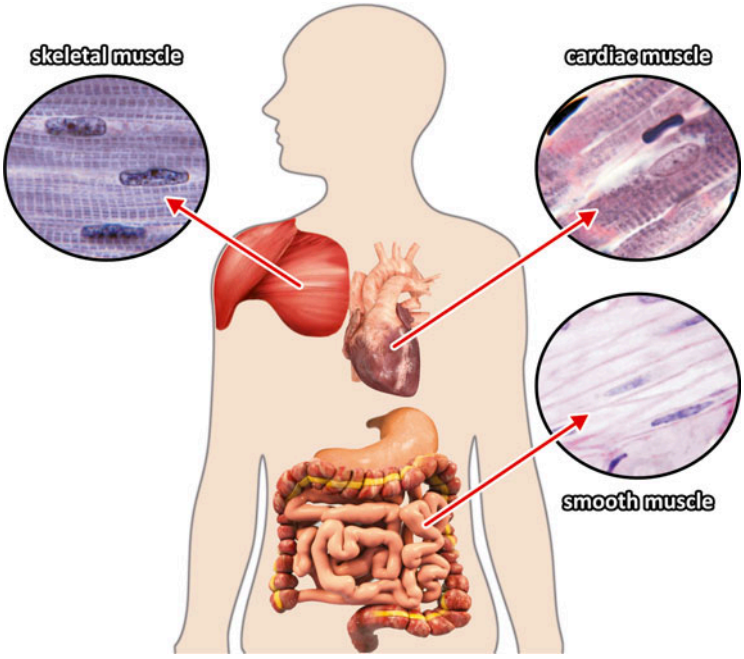


Fig. 10.1 The three types of muscle tissues. The details show histological images of skeletal, cardiac, and smooth muscles (Reprinted with the permission of Instituto de Histologia e Biologia do Desenvolvimento, Faculdade de Medicina, Universidade de Lisboa, FMUL)

10.1.1 Structural Organization of the Contractile Apparatus

In this chapter, we will focus our attention in skeletal muscles. This tissue is composed of parallel bundles of large multinucleated cells called muscle fibers. The fibers have 10–100 μm diameter and sometimes can extend over the full length of the muscle, reaching several centimeters. Most of the intracellular volume of the muscle fiber is occupied by 2 μm-thick myofibrils formed by the contractile array of proteins (Fig. 10.2a). Observed at the light microscope, the fiber presents a typical pattern that alternates light and dark bands caused by a regular arrangement of molecules of different densities (Fig. 10.2b).

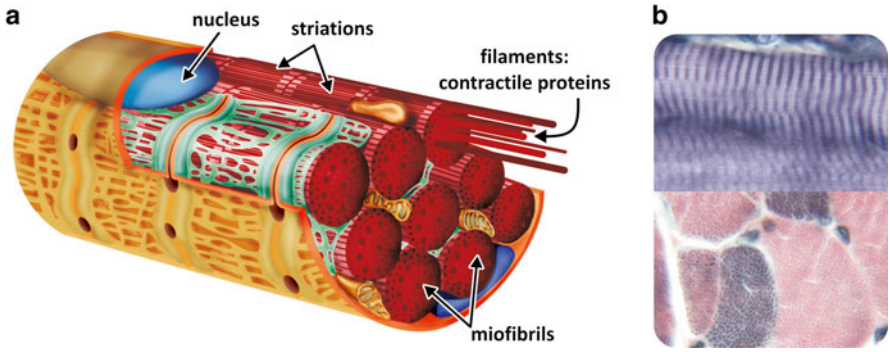


Fig. 10.2 (**a**) Schematic representation of a muscle fiber with the nucleus (*blue*) in the periphery of the cell and myofibrils formed by the contractile proteins. (**b**) Histological image of longitudinal (*top*) and transversal (*bottom*) sections of skeletal muscle tissue showing the striated pattern (Reprinted with the permission of Instituto de Histologia e Biologia do Desenvolvimento, Faculdade de Medicina, Universidade de Lisboa, FMUL)

The organization of the contractile proteins in the myofibril explains the striated pattern observed at the light microscope: they are arranged in an ordered structure forming thin and thick filaments, clearly seen by electron microscopy, as firstly observed by Hugh Huxley in the 1950s (Fig. 10.3). The different regions were named according to their characteristics. The dense regions are called A (from anisotropic) bands, while the less dense regions are named I (from isotropic) bands. A dark line in the medium point of each I band is also observed and is designated the as Z line (from the German word *zwischenscheibe*, which means "the disk in between"). A denser line is observed in the middle of the A band, called M line (from the German word *mittelscheibe*, which means "the disk in the middle"). In the resting muscle, the central region of the A band shows a lighter area, which is called H zone (from the German word *heller,* which means "brighter"). The I band is a region containing only thin filaments, while the A band contains both type of filaments. In the resting muscle, the thin filaments do not reach the center of the A band, explaining the H zone, which contains only thick filaments (Fig. 10.3).

The striated pattern occurs in skeletal and cardiac muscles, which are called striated muscles, while the smooth muscles do not present striations.

The thin filaments are inserted in the Z line and are composed of three proteins: actin, tropomyosin, and troponin. The thick filaments are formed by a protein named myosin (see Box 10.1). The region comprised between the Z lines is called sarcomere and corresponds to the contractile unit of the myofibril. It is important to mention that the components of muscle cells are usually designated by a specific nomenclature having the prefix *sarco* (from a Greek root meaning "flesh"): the plasma membrane is known as sarcolemma, the cytoplasm is known as sarcoplasm, and the endoplasmic reticulum is called sarcoplasmic reticulum.

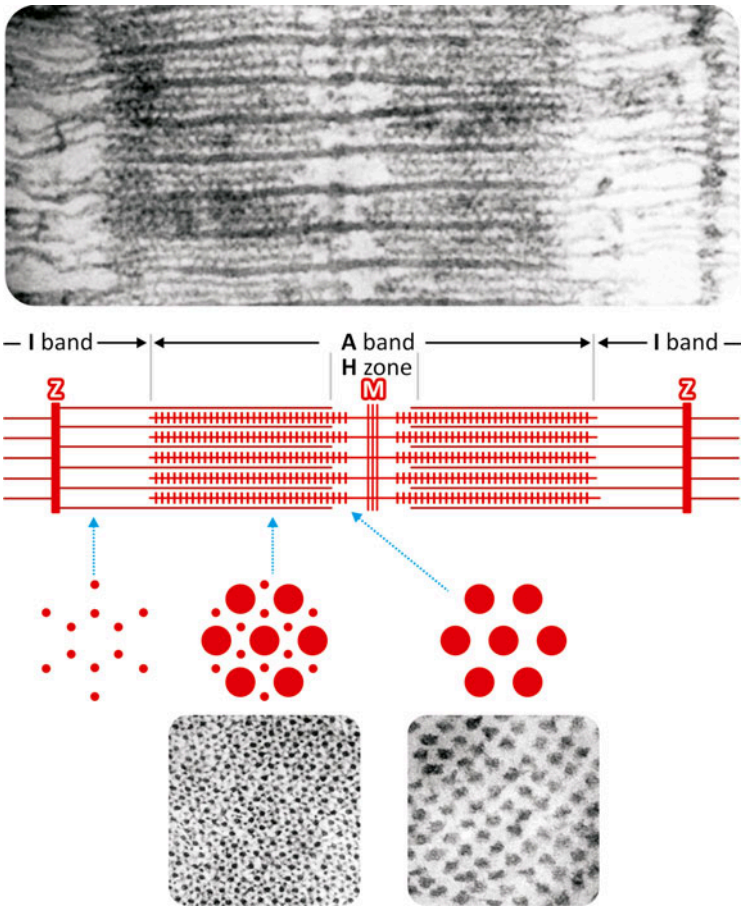


Fig. 10.3 Electron micrographs of a skeletal muscle (*top*) longitudinal section showing the thin and the thick filaments and (*bottom*) transverse sections of A band and H zone (Reproduced with permission from Huxley, J. Biophys. Biochem. Cytol. 3:631–648, 1957). The schematic representation shows filaments' organization in the region in between two Z lines and transverse sections of the I band (*left*), the A band (*medium*), and the H zone (*right*). The region of 2–3 μm in between two Z lines is called sarcomere, which is the contractile unit of the myofibril

Box 10.1: Isolation of Myosin

Since the middle of the nineteenth century, it was known that the disruption of muscle cells resulted in the precipitation of an insoluble material in a much higher amount than that observed for homogenates of other tissues. Still in the nineteenth century, this insoluble material was called "myosin" by Wilhelm Kuehne, but only in the 1940s, with the studies performed mainly by Albert Szent-Gyorgyi, this term was specifically used to name the proteic

(continued)

Box 10.1 (continued)
component that could be solubilized from the insoluble material by treatment with a high ionic strength solution (0.6 M KCl, an ionic strength much higher than the physiological 0.15 M). Another proteic component could be solubilized from the remaining insoluble material of muscle homogenate by lowering the ionic strength below the physiological range, and this protein was named "actin" (due to its ability to activate the ATPase activity of myosin, as it will be explained in the next section). It is important to note that both myosin and actin are insoluble at the physiological ionic strength, forming the organized filaments seen by electron microscopy.

10.1.1.1 The Main Proteic Components of the Contractile Apparatus

Myosin represents up to 65 % of the total protein that constitute the myofibrils. A landmark finding regarding the role of myosin in contraction came from the work of W. A. Engelhardt and M. N. Lyubimova, who demonstrated that it displays enzymatic activity hydrolyzing ATP. It was also shown that the volume of myosin in its insoluble state (at ionic strength lower than 0.6 M; see Box 10.1) contracted after addition of ATP, leading to the supposition that the hydrolysis of ATP by myosin would be the driving force for muscle contraction. This is indeed the basis for the chemical–mechanical energy conversion during muscle contraction, as we will discuss in the next section.

The first electron microscopy images of isolated myosin filaments showed a very peculiar morphology. They present typical projections regularly spaced from each other along the extension of the filament, except for the central part, which is known as the "bare zone" (Fig. 10.4a). The projections are the contact points with the thin filaments.

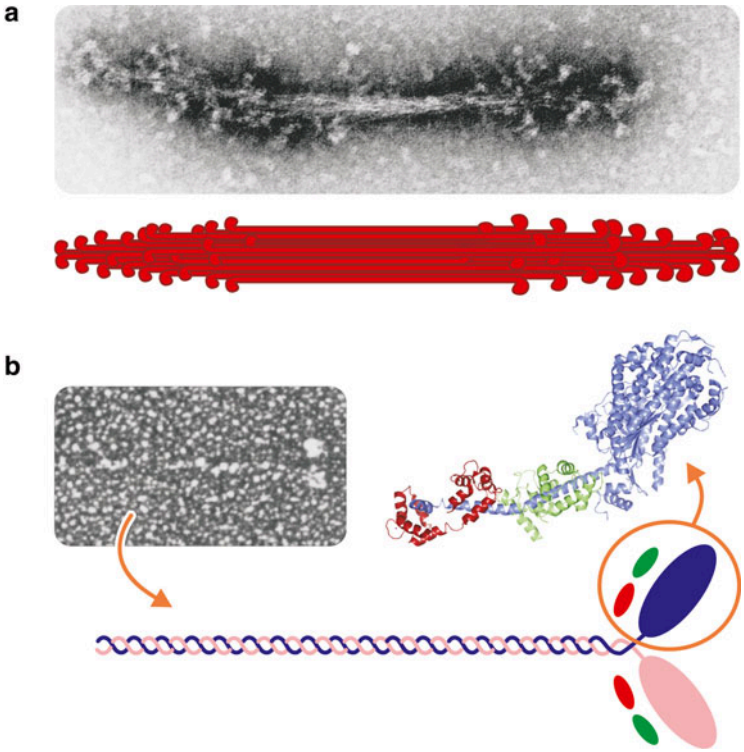


Fig. 10.4 Structure of the myosin filament. (**a**) Electron micrograph of a reconstituted myosin filament (Reproduced with permission from Huxley, Science 164:1356–1366, 1969) and its schematic representation. (**b**) Electron micrograph of an isolated myosin molecule (Reproduced from Slayter & Lowey, Proc. Natl. Acad. Sci. USA 58:1611–1618, 1967) and a schematic representation of its structure, which is composed of two heavy chains (*purple* and *pink*) associated to four light chains (*green* and *red*). The three-dimensional structure of the head, or S1 subfragment, is also shown, with the light chains highlighted in *green* and *red* (PDB 1DFL)

The morphology of the thick filament can be explained by the structure of myosin units. Each myosin unit in the thick filament shows a golf club shape, with two well-defined regions: a globular "head" from which a "tail" of about 150 nm long extends (Fig. 10.4b). It is a hexameric protein containing two identical heavy chains (with ~220 kDa each) and four light chains (each with ~20 kDa). The head can be separated from the whole myosin by brief digestion with proteases. The generated fragment, containing the N-terminal end of the heavy chains associated with the light chains, is called subfragment 1 (S1) and comprises the ATPase activity and the actin-binding site. The tail is composed of the C-terminal region of the two heavy chains intertwined to form a coiled coil.

Actin is the major component of the thin filament and corresponds to about 20–25 % of the muscle proteins. Actin is a protein of 42 kDa that is called G-actin

(in a reference of its globular structure) in its monomeric form. To form the thin filaments, actin monomers polymerize in a helical structure, in which it is named F-actin (Fig. 10.5).

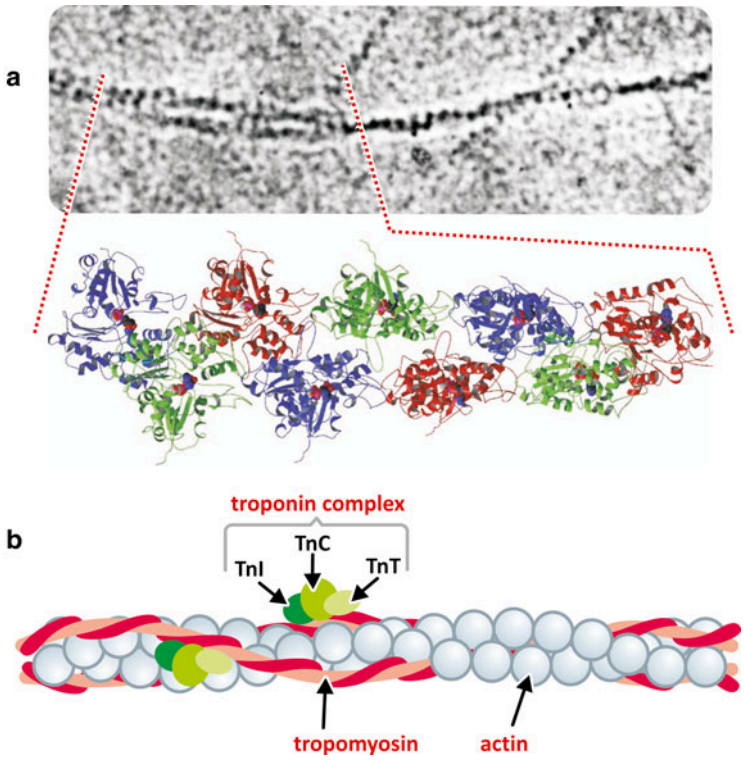


Fig. 10.5 Structure of the thin filament and its components. (**a**) Section of an electron micrograph from the article that firstly described the organization of an actin filament (Reproduced from Hanson & Lowy, J. Mol. Biol. 6:46–60, 1963, with permission from Elsevier), with the correspondent molecular structure determined by X-ray crystallography showing 13 actin monomers arranged on six left-handed turns repeated every 36 nm (Reproduced with permission from Geeves & Holmes, Ann. Rev. Biochem. 68:687–728, 1999). (**b**) Schematic representation of an actin filament with tropomyosin and troponin bound

Other proteins are associated to the thin filament. Among them, the most important are the tropomyosin and troponin, both involved in the regulation of contraction. Tropomyosin is a dimeric protein of 65 kDa that associates to actin as twisted α-helices that interacts in a tail-to-head manner forming long rods along the thin filament (Fig. 10.5b). Troponin is a globular protein of 78 kDa composed of three subunits, Tn-I, Tn-C, and Tn-T. Tn-I (I from inhibiting) binds actin fixing the tropomyosin-troponin complex on the actin surface and blocking myosin binding to thin filament. Tn-C (C from calcium binding) binds calcium ions (Ca^{2+}), the main

regulator of contraction (see Sect. 10.1.3). Tn-T (T from tropomyosin binding) promotes the association of the two other subunits of troponin and the binding of them to tropomyosin (Fig. 10.5b).

10.1.2 Mechanism of Muscle Contraction

After the discovery that the contractile apparatus was constituted mainly by two proteins, actin and myosin, many hypotheses have emerged to explain the contraction, most of them suggesting that muscle shortening during contraction was due to changes in protein structure leading to a more packed folding or coiling of the filament. However, the visualization of two separate sets of longitudinal filaments that overlap in certain regions in a series of studies performed during the 1950s led to a completely new model to explain the process.

10.1.2.1 The Sliding Filaments Model

The theory accepted today to explain myofibril shortening during muscle contraction was proposed in 1954, independently by H. E. Huxley and J. Hanson, and A. F. Huxley and R. M. Niedergierke, both studies published simultaneously. These scientists observed that either in contracted muscles or after their complete stretching, the length of the A band remained constant, while the length of the I band or the H zone varied according to the extent of contraction (Fig. 10.6). Based on these observations, they proposed that contraction occurred through the sliding of the filaments along each other.

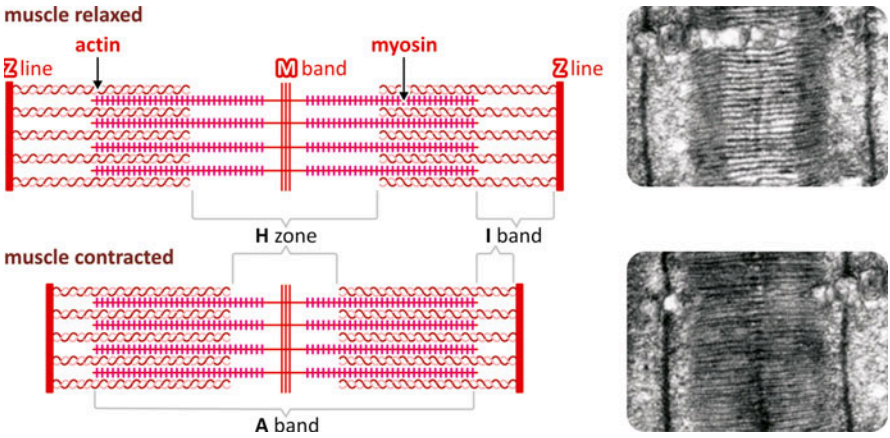


Fig. 10.6 Schematic representation of the slide of the filaments showing the relaxed and the contracted pattern with the corresponding electron micrographs (Reproduced with permission from Huxley. J. Biophys. Biochem. Cytol. 3:631–648, 1957)

One important finding that contributed to the elucidation of the mechanism that allows the filaments to slide was the observation that when the thin filaments were incubated with myosin S1, these subfragments form crossbridges with the thin filament in two orientations: perpendicular to the filament or with an inclination of approximately 45°. When inclined, all myosin heads show the same orientation on one side of the Z line but the opposite orientation on the other side of the Z line, which means that on each side of the Z line, the myosin heads point to opposite directions (Fig. 10.7a). This information could be correlated to the fact that during contraction the thin filaments on each side of the Z line are pulled to the center of the sarcomere.

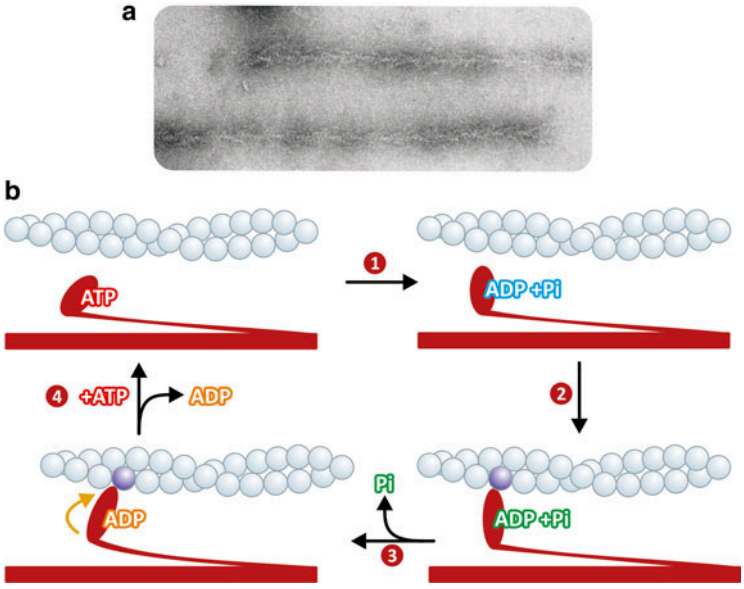


Fig. 10.7 (**a**) Actin filaments "decorated" with myosin heads, which show an arrowhead appearance (Reproduced with permission from Huxley, Science 164:1356–1366, 1969). (**b**) Schematic representation of actomyosin cycle: (1) myosin head hydrolyzes ATP to ADP and Pi; (2) myosin heads bind to the actin molecule in a perpendicular orientation, in a pre-force-generating state; (3) Pi is released, leading to a conformational change that makes myosin head to be at a 45° orientation in relation to the filaments, allowing myosin to perform work; (4) ADP is replaced by ATP leading to myosin dissociation from actin

The current knowledge on the molecular mechanism that leads to the sliding of the filaments is based on the model proposed by R. W. Lymn and E. Taylor, known as the Lymn–Taylor actomyosin ATPase cycle, according to which contraction occurs as myosin heads bind and detach repeatedly to the thin filaments with concomitant ATP hydrolysis, each cycle with the binding occurring in a position closer to the Z line. The sequence of events that are repeated in each cycle can be summarized as follows, starting with ATP binding to the myosin head for convenience (Fig. 10.7b):

1. Myosin hydrolyzes ATP to ADP and Pi, which remain tightly bound to the protein. The hydrolysis induces a structural change in the myosin head, which points to the thin filament in a perpendicular orientation.

2. Myosin heads bind to the actin molecule in a pre-force-generating state.
3. Binding of the myosin head to actin induces Pi to be released, leading to a conformational change that makes the myosin head to be at a 45° orientation in relation to the filaments. This conformational change allows myosin to perform work.
4. ADP dissociates from myosin being replaced by ATP, whose binding makes myosin heads detach from the actin.

Thus, as the cycle is repeated, the thick filaments move along the thin filaments in the direction of the Z line, so that the H zone becomes shorter and the Z lines get nearer to each other.

It is important to note that if ATP is not available, myosin keeps strongly attached to the thin filaments, in a condition known as *rigor*, as occurs after death, when all the body muscles become rigid, which is called *rigor mortis*. In living muscle cells, on the other hand, there is always an excess of ATP due to a constant cycle of hydrolysis and resynthesis and thus myosin heads remain bound to actin only for short periods of time.

10.1.3 Regulation of Muscle Contraction

Knowing that ATP is constantly hydrolyzed and resynthesized within the cells, so that its levels remain almost unchanged, one would ask how muscle contraction can be triggered exactly when it is required and how it is stopped when a specific task has already been performed.

The answer resides in the fact that the process is controlled by the central nervous system. This means that although the contraction is sustained by ATP hydrolysis, the ATP levels inside the muscle cells do not regulate the process extensively. Conversely, ATP concentrations are maintained high by different metabolic pathways that will be discussed in Sect. 10.2. Thus, it becomes clear that an additional player has to be called in to translate the brain signal to a biochemical response. This role is played by Ca^{2+}, whose concentration transiently increases in the sarcoplasm inducing the start of the contraction.

To understand how Ca^{2+} concentration is modulated in the sarcoplasm to control contraction, we need firstly to look at the morphological organization of the muscle fibers. One particularity of the skeletal muscle cell is that its sarcolemma invaginates perpendicularly to the length of the cell forming what is called the transverse (T) tubules (Fig. 10.8). Inside the cell, the myofibrils are surrounded by a system of membranous vesicles called sarcoplasmic reticulum (SR), which contains enlarged areas, known as the SR terminal cisternae, where Ca^{2+} is stored in a high concentration (~10^{-3} M). The SR terminal cisternae are connected to the T-tubules by a complex of two proteins, the dihydropyridine receptor (DHPR, inserted in the T-tubule membrane) and the ryanodine receptor (RyR, inserted in the terminal cisternae membrane) (Fig. 10.8).

A nerve impulse induces the opening of ion channels in the sarcolemma leading to an inflow of Na^+ into the cell. This causes membrane depolarization due to the dissipation of the membrane potential maintained by the Na^+/K^+-ATPase (see Box 10.2).

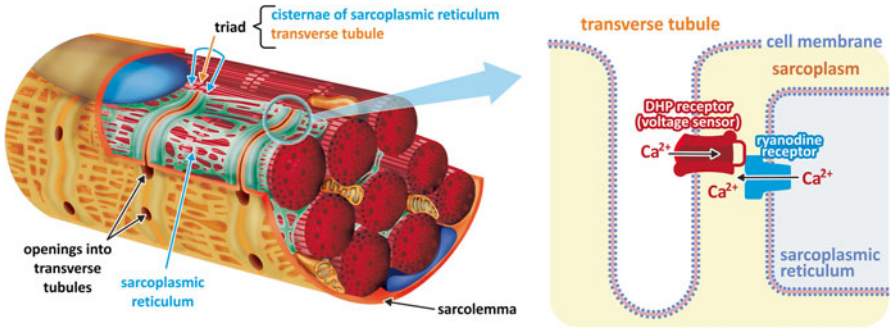
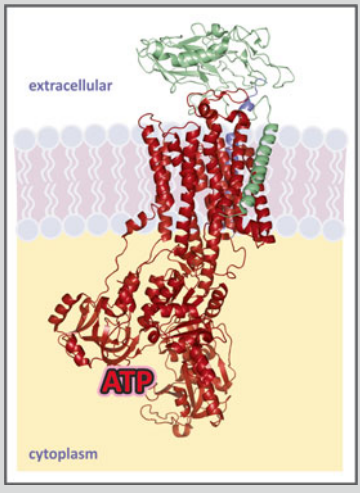


Fig. 10.8 Schematic diagram of a skeletal muscle fiber section showing the sarcolemma invaginations into the T-tubules and the sarcoplasmic reticulum with its terminal cisternae. Dihydropyridine receptor (DHPR), a voltage-dependent Ca^{2+} channel inserted in the T-tubule membrane, interacts with ryanodine receptor (RyR), also a Ca^{2+} channel, inserted in SR terminal cisternae membrane. Propagation of the action potential over the T-tubules activates DHPR, which in turn induces RyR opening, leading to Ca^{2+} release from SR lumen to the cytosol

Box 10.2: Na^+/K^+-ATPase and the Maintenance of Cellular Membrane Potential

In living cells, the distribution of ions inside and outside the plasma membrane is asymmetric, resulting in an electrical voltage between the two sides of the membrane, which is called membrane potential. Membrane potential is regulated by the combined action of ion channels, which depolarize the membrane, and ion pumps, which actively exchange ions across the membrane, restoring polarization. Membrane potential mainly arises from the exchange of Na^+ and K^+ through the activity of the Na^+/K^+-ATPase. This enzyme is an integral protein in the plasma membrane (see figure) that pumps 3 Na^+ out in exchange of 2 K^+ in, at the expense of ATP hydrolysis. Its activity accounts for a great part of cellular energy expenditure, being estimated that it is responsible for from 1/3 to 2/3 of ATP hydrolysis in the cells.



Crystal structure of Na^+/K^+-ATPase (PDB 3A3Y), with its three subunits, the α (catalytic, in *red*), β (in *green*), and regulatory (in *blue*). ATP is drawn in its binding site

Membrane depolarization propagates along the sarcolemma from the fiber surface to the T-tubules. DHPR is a voltage-dependent Ca^{2+} channel that is activated by the action potential propagation over the T-tubules. DHPR interaction with RyR in the closely apposed SR membrane causes RyR opening, leading to Ca^{2+} release from SR lumen to the cytosol. The increase of cytosolic Ca^{2+} concentration itself also activates RyR, causing further Ca^{2+} release, in a process known as Ca^{2+}-induced Ca^{2+} release. At higher concentrations in the sarcoplasm, Ca^{2+} binds to troponin, resulting in a conformational change that induces the displacement of tropomyosin, allowing myosin heads to bind actin and starting the contraction cycle (Fig. 10.9).

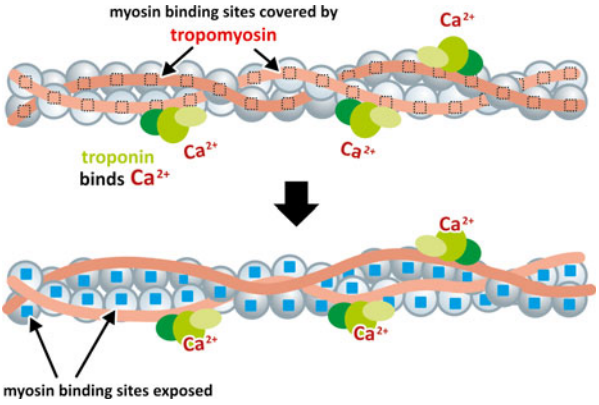


Fig. 10.9 Model for the Ca^{2+} regulation of contraction. At low concentration of Ca^{2+} in the sarcoplasm, the complex troponin–tropomyosin blocks the myosin-binding sites in actin. When Ca^{2+} is released from the SR and its concentration increases in the sarcoplasm, it binds troponin inducing a conformational change that ultimately leads tropomyosin to move away from the myosin-binding sites

When the nervous stimulus ceases, Ca^{2+} concentration in the sarcoplasm decreases due to the activity of Ca^{2+}-ATPase, an SR membrane enzyme that pumps Ca^{2+} from the cytosol to the SR lumen. Thus, in resting muscle, the concentration of Ca^{2+} in the sarcoplasm is maintained very low, in the range of 10^{-7} to 10^{-8} M, since almost all the intracellular Ca^{2+} is stored inside the SR. In this situation, the complex troponin–tropomyosin is bound to actin in a way that prevents myosin binding (Fig. 10.9). Thus, even in high ATP concentrations, its hydrolysis by myosin occurs in a very slow rate and the muscle remains relaxed.

10.2 Different Metabolic Profiles of the Skeletal Muscle Fibers

Skeletal muscles are used to perform very different kinds of activities. Some of these activities require that muscle cells work in their maximal capacity, such as when an elite athlete runs a 100 m sprint, but also in more usual situations, as when you have

to quickly run to take a bus that just started to leave the bus stop. Other activities demand muscle work for a long time, such as in running a marathon but also in prolonged walks, riding a bicycle, or cleaning the house. The different metabolic demands required in these diverse activities can be achieved due to the existence of distinct types of muscle fibers, characterized by specific metabolic adaptations that include the type of nutrient metabolized and the metabolic pathways used to synthesize ATP (Fig. 10.10).

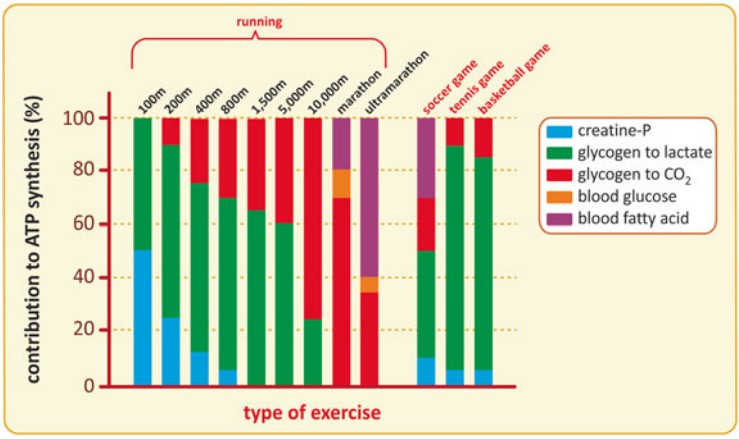


Fig. 10.10 Contribution of distinct metabolites to ATP synthesis during different types of exercise. The transfer of the phosphate group of phosphocreatine to ADP is the fastest way to regenerate ATP in muscle cells (see next section for details), but the content of phosphocreatine is limited and sustains only short duration exercises. Muscle glycogen is the main energy source used for ATP synthesis during short- or medium-duration exercises. Depending on the type of exercise, muscle glycogen may be used anaerobically (for instance, in short-distance runs), generating lactate as the end product, or aerobically (as in long-distance runs), being oxidized to CO_2. Aerobic metabolism becomes gradually more important in long-duration exercises, with also an increasing requirement of fatty acid oxidation as glycogen is depleted. The use of blood glucose by muscle during exercise is almost irrelevant, especially because its transport into the muscle cells is dependent on insulin (see Sect. 8.4). This guarantees glucose availability for the cells that use this nutrient preferentially or exclusively, such as brain cells or erythrocytes, respectively. Ball games, such as soccer or tennis, may be long in duration but they consist in short and intense runs alternating with resting periods, which makes them having metabolite use profiles closer to short-distance running (Based on data from Newsholme & Leech, Functional biochemistry in health and disease, chap. 13. P. 291, 2010)

From the physiological point of view, there are two major types of muscle fibers: the red or slow-twitch fibers and the white or fast-twitch fibers. They are classified according to the expression profile of myosin heavy chain isoforms, which correlate with their physiological role and their biochemical adaptations. Slow-twitch fibers are also known as type I fibers due to the predominant expression of type I myosin heavy chain, while fast-twitch fibers are called type II fibers due to the predominance of myosin heavy chains type IIa and IIb. There is an association between fiber

type and mitochondrial content, with type IIb fibers tending to have the lowest and type I fibers the highest abundance of mitochondria.

Slow-twitch fibers are adapted to prolonged work and are very resistant to fatigue, although they provide relatively low force upon contraction. Their metabolism is mainly dependent on oxidative phosphorylation and thus requires an adequate supply of O_2. These cells are very rich in mitochondria and myoglobin and are irrigated by a large amount of blood vessels. The cytochromes in the mitochondria and the high content of myoglobin inside the cells, as well as the hemoglobin in the surrounding blood, give to red muscle its characteristic red color. Myoglobin has a much higher affinity for O_2 than hemoglobin (see Sect. 3.3.3) and thus receives O_2 from blood to supply the aerobic activity of these muscle fibers. The fatty acids are the main nutrient used by red fibers, but they can also use ketone bodies and degrade glycogen and glucose aerobically.

Fast-twitch fibers are adapted to work at low levels of O_2. These cells contain a low number of mitochondria and are less supplied by blood vessels than type I fibers. Their metabolism is mainly anaerobic, using muscle stocks of glycogen as the major metabolic substrate. Although this type of fiber contracts quickly and powerfully, it undergoes fatigue very rapidly as the low ATP yield provided by anaerobic metabolism cannot sustain the ATP demand for long periods of contraction.

There is a genetic component that determines the amount of red and white muscles in the body, but exercise training influences the expression profile of contractile proteins resulting in changes in the proportion of fiber types in the muscles.

10.3 Overview of ATP Synthesis in the Muscle Cells

ATP consumption in muscle cells may increase 100-fold from resting to vigorous activity. During intense contractile activity, ATP is hydrolyzed to ADP and Pi mainly as a result of three ATPase activities: (a) the ATPase activity of myosin head, which is directly involved in the sliding of the filaments and thus in the contraction; (b) the Na^+/K^+-ATPase activity that maintains the Na^+/K^+ gradient across the sarcolemma and T-tubules, allowing the membrane potential to be restored; and (c) the SR Ca^{2+}-ATPase activity, responsible for pumping Ca^{2+} against the concentration gradient from the sarcoplasm into the lumen of SR. The high rate of ATP hydrolysis during contraction demands that ATP levels are continuously restored within the muscle cells.

ATP is supplied to muscle cells through different pathways depending on the type of the fiber (Fig. 10.11). In type I fibers, oxidative phosphorylation is the major mechanism of ATP synthesis, with the fatty acids being the main metabolic substrate used. In contrast, in type II fibers most of the ATP synthesized comes from the substrate-level phosphorylation in glycolysis, being the muscle glycogen the main source of glucose-6-phosphate for glycolysis, and lactates the major end product of this pathway.

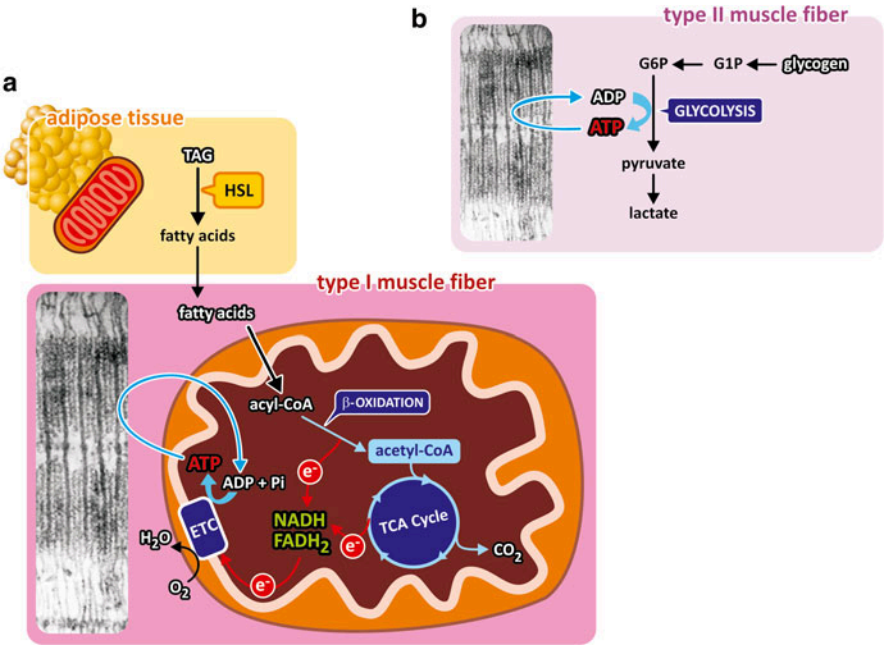


Fig. 10.11 Main metabolic pathways for ATP synthesis that sustain contraction in muscle fibers. (**a**) Type I fibers use fatty acids as the main metabolic substrate. The fatty acids are mobilized by the hydrolysis triacylglycerols (TAGs) coming mainly from the adipose tissue adjacent to muscles, in a reaction catalyzed by the enzyme hormone-sensitive lipase (HSL). Fatty acids are oxidized through the β-oxidation pathway followed by complete oxidation of the resultant acetyl-CoA in TCA cycle. The electrons transferred to the coenzymes NADH and $FADH_2$ are then transported in the electron transport chain, ultimately reducing O_2 to H_2O. Electron transport promotes the formation of an electrochemical gradient that is the driving force for ATP synthesis. (**b**) Type II fibers use muscle glycogen as the main metabolic substrate. Glycogen degradation forms glucose-1-phosphate (G1P), which is converted in glucose-6-phosphate (G6P), which in turn enters glycolysis. ATP is formed by substrate-level phosphorylation in glycolysis. In low availability of O_2, the product of glycolysis, pyruvate, is reduced to lactate

Additionally, skeletal muscle contains another mechanism to transiently and rapidly generate ATP. This is possible due to a large amount of phosphocreatine (10–30 mM) in muscle cells (Fig. 10.12). The enzyme creatine kinase catalyzes the transfer of phosphate group of phosphocreatine to ADP, regenerating the ATP hydrolyzed in contraction. The resulted creatine can be phosphorylated again during recovery, when the ATP levels increase in the cells. The same enzyme, creatine kinase, catalyzes the reverse reaction using ATP to phosphorylate creatine, restoring the levels of phosphocreatine in muscle cells.

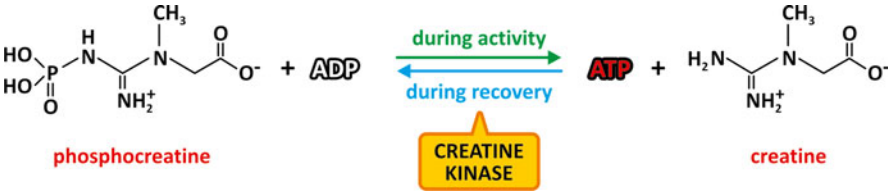


Fig. 10.12 Creatine kinase catalyzes the reversible reaction of phosphate transfer from phosphocreatine to ADP, generating ATP and creatine during muscle contraction, or from ATP to creatine, forming phosphocreatine and ADP, during recovery

10.4 Muscle Cell Metabolism During Physical Activity

Due to the extremely high demand for ATP imposed by the contractile activity during intense exercise, a rapid metabolic adaptation to regenerate ATP is required in muscle cells, in which the available substrates are driven to the catabolic pathways. In this section we will discuss the main metabolic steps that are regulated in muscle cell to maintain ATP concentration within the adequate ranges required for cellular functions.

10.4.1 Role of the Cellular Energy Charge in the Muscle Cell Metabolism

Before focusing specifically on muscle cells, we will start by discussing a basic level of metabolic regulation that occurs in all the cells of the body. The idea of such metabolic control emerged in the decade of the 1960s, when Daniel E. Atkinson proposed that the regeneration and the expenditure of ATP would be regulated by the cellular energy balance itself. This concept has proven to be true for all the cells, at least as a first level of metabolic control. To formalize this idea, Atkinson developed a parameter to describe the cellular energy status based on the relative concentrations of the adenine nucleotides within the total cellular pool in a given moment or situation. He termed this parameter as the "energy charge of the adenylate system," whose value represents half of the average number of anhydride-bound phosphates per adenosine moiety (Box 10.3). A value of energy charge of 0 would mean that only AMP is present in the cell, whereas if all the adenine nucleotide were in the form of ATP the cellular energy charge would be 1. In most cells, the energy charge value ranges from 0.8 to 0.95.

Box 10.3: Atkinson's Concept of Cellular Energy Charge
Daniel E. Atkinson proposed that the energy stored into the cell in the form of adenine nucleotides, referred by him as the adenylate system (AMP+ADP+ATP), resembles an electrochemical storage cell in its ability to accept, store, and supply energy. Based on this view, the adenylate system is fully discharged when all adenylate is in the form of AMP and fully charged when only ATP is present, with the number of anhydride-bound phosphates per adenosine moiety varying from 0 to 2. To have a parameter varying from 0 to 1, he divided the number of anhydride bonds per adenosine nucleotide by 2. Since ATP contains 2 of anhydride-bound phosphate groups and ADP contains 1, Atkinson defined the cellular "energy charge" as the actual concentrations of ATP+½ADP in the total adenylate system:

$$\frac{[\mathrm{ATP}]+½[\mathrm{ADP}]}{[\mathrm{ATP}]+[\mathrm{ADP}]+[\mathrm{AMP}]}$$

It is important to note that during intense contraction, AMP accumulates as a result of the combination of ATP hydrolysis (which generates ADP and Pi) and the reaction catalyzed by the enzyme adenylate kinase (which converts two molecules of ADP in one ATP and one AMP). Therefore, each ATP is ultimately converted in one AMP and two Pi molecules (Fig. 10.13a). Considering the reaction catalyzed by adenylate kinase at equilibrium, Atkinson represented the variation of the concentrations of AMP, ADP, and ATP as a function of the energy charge (Fig. 10.13b). Observing this graph, it becomes clear that AMP is a very sensitive indicator of metabolic status, since its concentration varies in a much greater amplitude when compared to ADP concentration variation (during exercise, AMP concentration may rise more than 100-fold, while no more than a 10-fold increase is observed for ADP concentration).

AMP is indeed an important activator of the pathways for ATP synthesis in muscle cells. It regulates muscle metabolism acting as an allosteric modulator of many enzymes, such as activating the muscle isoform of the glycogen phosphorylase (GP) and the glycolytic enzyme phosphofructokinase (PFK) and inhibiting fructose-1,6-bisphosphatase (see Sect. 10.4.2 for details).

Besides acting through its direct binding to several enzymes in different metabolic pathways, another regulatory role has been attributed to AMP: it is the activator of an important regulatory enzyme, the AMP-activated protein kinase (AMPK; note that this is not the *cyclic AMP-dependent protein kinase*, PKA, but an enzyme activated by the 5′-AMP).

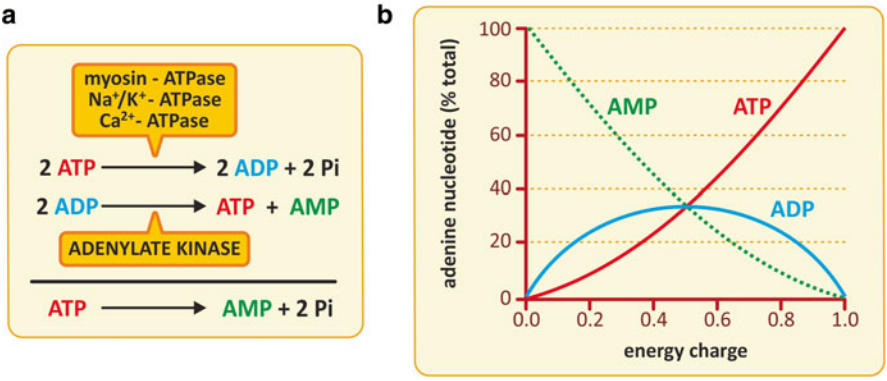


Fig. 10.13 (**a**) AMP production in muscle cells. ATP is hydrolyzed to ADP and Pi by the ATPase activities of myosin, Na^+/K^+-ATPase and Ca^{2+}-ATPase. The resulting ADP may be converted to ATP and AMP by the action of the enzyme adenylate kinase. (**b**) Considering the reaction catalyzed by adenylate kinase at equilibrium (using the calculated equilibrium constant of 0.8), the concentrations of the adenine nucleotides in the cell can be represented as a function of the cellular energy charge (Reproduced from Oakhill et al. Trends Endocrinol. Metab. 23:125–132, 2012, with permission from Elsevier)

10.4.1.1 The AMP-Activated Protein Kinase: A Cellular Energy Sensor

AMPK is a heterotrimeric protein composed of one catalytic subunit (α) and two regulatory subunits (β and γ) (Fig. 10.14a). In the γ-subunit, there are four adenine nucleotide-binding sites (sites 1–4). Site 2 seems to be unoccupied in mammalian enzymes and site 4 has a non-exchangeable AMP molecule bound. This indicates that only sites 1 and 3 are involved in AMPK regulation, exchanging ATP for ADP or AMP as the cellular energy charge varies. The N-terminal end of the β-subunit is myristoylated, being this modification important for nucleotide binding to γ-subunit.

AMPK activity is regulated mainly through phosphorylation/dephosphorylation of the Thr172 in the catalytic subunit (Fig. 10.14b). The main protein kinases involved in AMPK phosphorylation are the LKB1 complex and the Ca^{2+}/calmodulin-dependent kinase kinase-β (CaMKKβ). Phosphorylation of Thr172 results in an increase of more than 100-fold in AMPK activity. On the other hand, its dephosphorylation catalyzed by phosphatases, such as the protein phosphatase 2C (PP2C), leads to AMPK inactivation.

Binding of ADP or AMP to site 3 of the γ-subunit facilitates AMPK phosphorylation and inhibits its dephosphorylation, maintaining the enzyme in its active state, while binding of ATP to this site causes the opposite effect. This is an interesting example of a posttranslational modification that is modulated allosterically, since

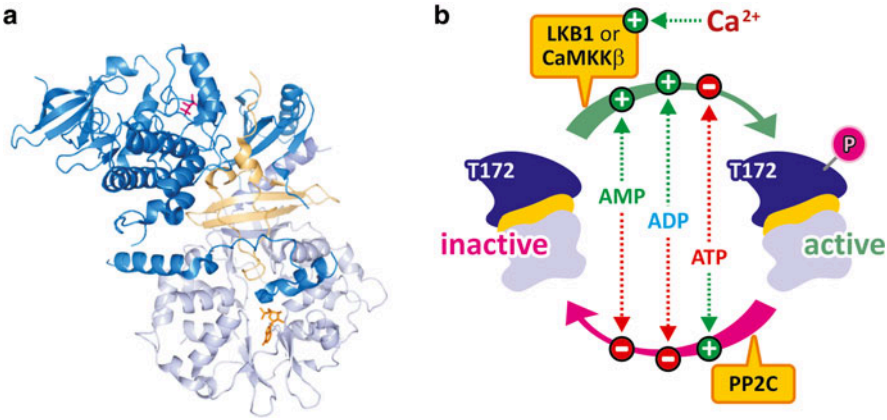


Fig. 10.14 (**a**) Structure of mammalian AMPK. The structure represents a composition of the available structures of the rat α-subunit (*blue*), with the phosphorylated Thr shown in *red*; the human β-subunit (*light yellow*); and the rat γ-subunit (*lilac*), with an AMP molecule bound shown in *orange* (PDB 4CFH). (**b**) Schematic representation of AMPK regulation. The increase in ADP/AMP concentration triggers Thr172 phosphorylation by the upstream kinases LKB1 and CaMKKβ and simultaneously inhibits Thr172 dephosphorylation by phosphoprotein phosphatase 2C (PP2C). ATP antagonizes this ADP/AMP effect. AMP acts directly as an allosteric activator of AMPK

binding of the adenine nucleotide to γ-subunit makes the enzyme a better or a worse substrate to the kinases or phosphatases that will introduce or remove the phosphate group in Thr172.

Additionally, when AMPK is phosphorylated, binding of AMP to the site 1 of the γ-subunit further activates the enzyme, although this direct allosteric activation results in only two- to fivefold stimulation of AMPK activity.

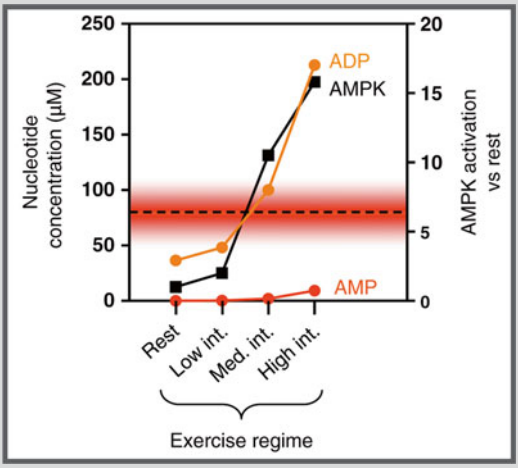
It is also interesting to note that AMPK activation/inhibition is not controlled by extracellular signals (e.g., hormones), as occurs with most of the regulatory kinases, but by the intracellular status. Therefore, AMPK can be seen as an adenylate charge-regulated protein kinase, since it detects and reacts to changes in the adenine nucleotide ratio.

It is interesting to mention that although it is unquestionable that protein phosphorylation by AMPK is an important mechanism of control of the energy metabolism in response to changes in cellular energy charge, restoring cellular ATP levels by switching off the anabolic pathways and switching on the catabolic pathways, recent evidence suggest that the actual physiological activator of this enzyme seems to be ADP rather than AMP (see Box 10.4).

In muscle cells, the main metabolic pathways regulated by AMPK are the β-oxidation of fatty acids and the glucose uptake via its transport by GLUT4 (see next section for details).

Box 10.4: ADP and the AMPK Activation

Recent studies that measured the nucleotide-binding affinity to AMPK revealed that the binding constants (K_d) of AMP and ADP to the site 3 of AMPK γ-subunit are 80 and 50 μM, respectively. Although during exercise AMP concentration rises dramatically (up to more than 100-fold over the resting levels), the highest AMP concentration reached (about 10 μM) is much lower than the K_d value. On the other hand, ADP concentration, although showing a much more modest increase (from 36 to 200 μM), overcomes the K_d value by up to fivefold. These observations led Bruce E. Kemp and colleagues to propose that ADP, rather than AMP, plays a dominant role in activating AMPK. These authors also showed that upon exercise AMPK activation clearly correlates with the increase in ADP concentration (see figure), further supporting this new view. This makes the historical name of this kinase as well as the extensive discussion in the literature regarding the role of AMP in this regulation somewhat confusing.



AMPK activation and the concentrations AMP and ADP in human muscle cells after exercises of different intensities. The *black dashed line* indicates the measured K_d for AMP binding at site 3 with the standard deviation indicated by a *red zone*. (Reproduced from Oakhill et al. Trends Endocrinol. Metab. 23:125–132, 2012, with permission from Elsevier)

10.4.2 Metabolic Pathways for ATP Synthesis in the Skeletal Muscle

10.4.2.1 Fatty Acid Oxidation in Skeletal Muscle

Fatty acids are the preferential nutrient used by muscle cells. These molecules are taken up by muscle fibers from blood or mobilized from TAG accumulated either inside the myocytes themselves or in the adipocytes dispersed between or along the fibers (Fig. 10.15).

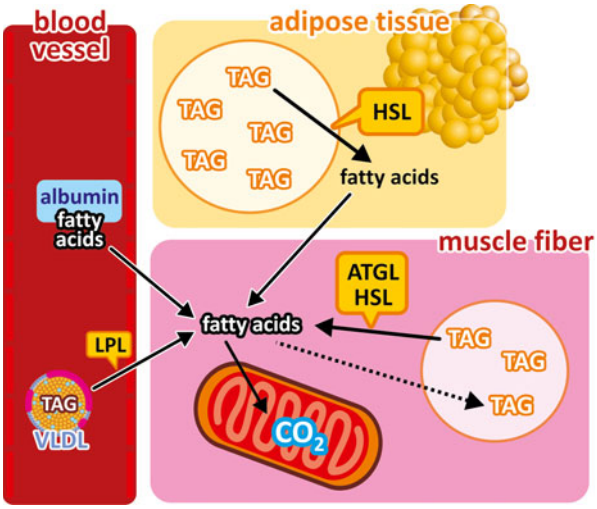


Fig. 10.15 Sources of fatty acids for muscle metabolism: Fatty acids mobilized from the adipose tissue circulate in association with albumin and are delivered to the muscle cells. VLDL transports the de novo synthesized TAG from the liver to the peripheral tissues, including muscles. VLDL-associated TAGs are hydrolyzed by the enzyme lipoprotein lipase (LPL) at the surface of the endothelium cells of the vessels that irrigate the muscles and the resulting fatty acids are transported into the fibers. The enzyme adipose triglyceride lipase (ATGL) hydrolyzes the TAG molecules stored inside the myocytes, generating DAG, which in turn is further hydrolyzed by the muscle isoform of the hormone-sensitive lipase (HSL). Finally, the TAGs stored in the adipocytes associated to the fibers are also mobilized yielding fatty acids to the muscle cell metabolism

Albumin-bound fatty acids circulate in the blood after mobilization from the adipose tissue, being the main source of fatty acids for muscle metabolism. Fatty acid can also be obtained from blood through the circulating VLDL. This lipoprotein is the main carrier of TAG in the blood in the postabsorptive state (see Sect. 8.3.2). VLDL-associated TAG is hydrolyzed by lipoprotein lipase (LPL), an enzyme present at the luminal site of endothelium cells of the capillary bed of muscles, making the resulting fatty acids available to skeletal muscle during exercise (Fig. 10.15).

Muscle cells contain a certain amount of intracellular stores of TAG, especially in type I fibers, representing a potential energy source for muscle metabolism during aerobic exercise. Intramyocellular TAGs are probably formed through the reesterification of the excess of fatty acids that are taken up from blood. It is important to note that although an increasing number of evidence support the importance of intramyocellular TAG mobilization during muscle activity, it is still difficult to unequivocally distinguish between intracellular and intercellular TAGs (those located in the adipocytes associated with the muscle cells), which should also have a role in supplying muscle cells of fatty acids during exercise. The hydrolysis of intramyocellular TAGs is attributed to the activity of two lipases, the adipose triglyceride lipase (ATGL), which catalyzes the hydrolysis of TAG to diacylglycerol (DAG), and the muscle isoform of the hormone-sensitive lipase (HSL), which exhibits a higher specificity for DAG than TAG (Fig. 10.15).

The reactions of fatty acid oxidation are described in detail in Sect. 7.4. Briefly, fatty acids are firstly activated in the cytosol by esterification with coenzyme A (CoA) in an ATP-dependent reaction catalyzed by the acyl-CoA synthetase (ACS). The resulting acyl-CoA molecules are transported into mitochondria where they undergo β-oxidation, generating acetyl-CoA, $FADH_2$, and NADH (Fig. 10.16). To be transported into mitochondria, acyl-CoA molecules are firstly converted to their acyl-carnitine derivatives that are then translocated across the mitochondrial membrane and reconverted to acyl-CoA molecules in the matrix.

The main regulation site of fatty acid oxidation is the transfer of the acyl group from CoA to carnitine, a reaction catalyzed by the carnitine/palmitoyl transferase I (CPT-I). This enzyme is located at the outer mitochondrial membrane and is strongly inhibited by malonyl-CoA. Therefore, a decrease in malonyl-CoA concentration in sarcoplasm increases the transport of the fatty acids into mitochondria, favoring their oxidation.

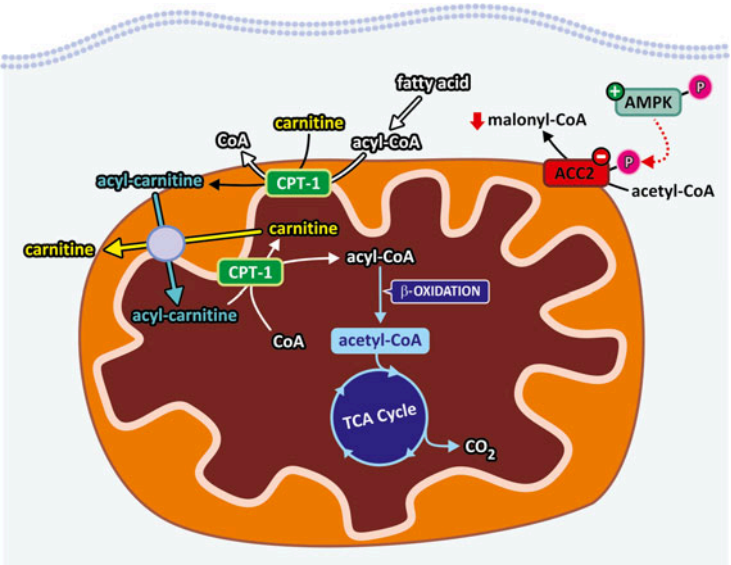


Fig. 10.16 β-oxidation of fatty acids is activated during contraction due to the decrease in the levels of malonyl-CoA, a potent inhibitor of the transport of the acyl-CoA into the mitochondrial matrix. Activated AMPK phosphorylates the isoform 2 of the acetyl-CoA carboxylase (ACC2), inhibiting the conversion of acetyl-CoA in malonyl-CoA. To be transported into the mitochondrial matrix, the acyl group of acyl-CoA is firstly transferred to carnitine, in a reaction catalyzed by the carnitine/palmitoyl transferase I (CPT-I). The acyl-carnitine is then transported across the inner mitochondrial membrane through the carnitine-acyl-carnitine transporter. In the matrix, the acyl group is transferred to coenzyme A by carnitine/palmitoyl transferase II (CPT-II), regenerating the acyl-CoA, which undergoes β-oxidation. Since malonyl-CoA is a potent inhibitor of the CPT-I, the decrease in its concentration allows the transport and the subsequent oxidation of the acyl-CoA

Malonyl-CoA is produced by the carboxylation of acetyl-CoA in the cytosol (see also Sect. 8.3.1). In muscle cells, this reaction is catalyzed by the isoform 2 of the enzyme acetyl-CoA carboxylase (ACC2), which is associated to the outer mitochondrial membrane (Fig. 10.16). ACC2 is inhibited by the phosphorylation of its Ser219 and Ser220, catalyzed by AMPK. Therefore, the activation of AMPK during contraction (see previous section) causes the decrease in the concentration of malonyl-CoA in sarcoplasm due to ACC2 inhibition, resulting in the activation of fatty acid β-oxidation (Fig. 10.16).

10.4.2.2 Insulin-Independent Glucose Uptake in the Skeletal Muscle

Glucose uptake in muscle cells occurs mainly through the isoform 4 of the glucose transporters (GLUT4). In the absence of specific stimuli, GLUT4 is sequestered in intracellular vesicles, restricting the use of blood glucose by the muscle cells. Insulin, the hormone secreted when the concentration of glucose in the blood increases, is the major signal that induces the exposure of GLUT4 on the cell surface, leading to a robust increase in glucose uptake by GLUT4-containing cells (see Sect. 8.4). In the muscle cells, contraction also regulates the migration of GLUT4-containing vesicles to the plasma membrane, allowing an increase in glucose uptake from blood during intense exercise even in the absence of insulin signaling.

The mechanisms by which contraction stimulates the glucose uptake in muscles are not completely understood, but several evidence support that AMPK is involved. The decrease in the cellular energy charge due to the intense ATP hydrolysis during contraction activates AMPK, and this can be correlated to GLUT4 exposure on the cell surface. The signaling pathway that links AMPK activity to GLUT4 translocation has not been elucidated yet, but it seems to involve the phosphorylation of AS160 (AKT substrate 160; see Sect. 8.4) (Fig. 10.17).

As mentioned in Sect. 10.1.3, upon stimulation by a nerve impulse, the membranes of the transverse tubules depolarize causing an increase in cytoplasmic Ca^{2+} concentration due to the release of this ion from the SR stores. Ca^{2+} activates the Ca^{2+}/calmodulin-dependent protein kinase kinase (CaMKK), one of the upstream kinases that phosphorylates and activates AMPK (see Sect. 10.4.1.1). Therefore, during exercise, the increase in the glucose uptake by the muscle cells occurs through a coordinated action of ADP/AMP and Ca^{2+}, which work simultaneously on the activation of AMPK: while the increase in ADP/AMP concentration makes AMPK susceptible to the action of the CaMKK, the elevation of Ca^{2+} concentration in the sarcoplasm makes this kinase active to phosphorylate and activate AMPK (Fig. 10.17).

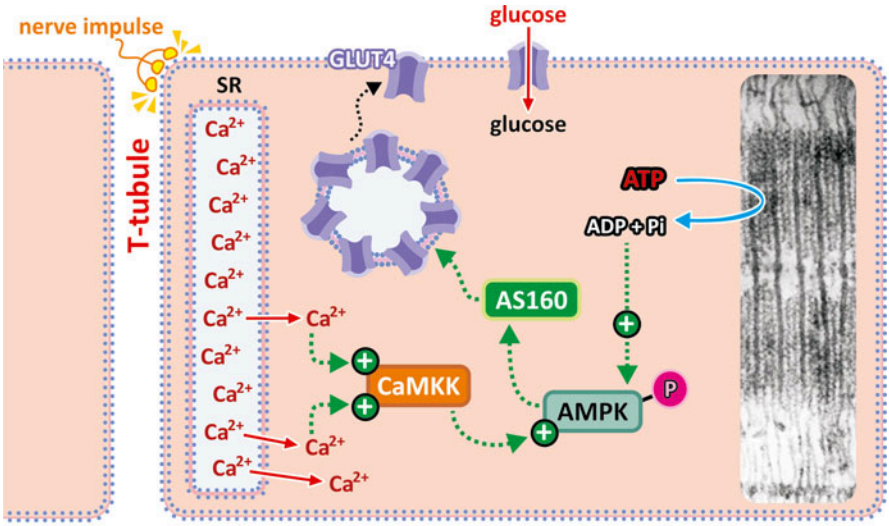


Fig. 10.17 Increase in glucose transport into the muscle cells during exercise. Intense contraction leads to a high rate of ATP hydrolysis, increasing the concentration of ADP (and AMP due to adenylate kinase activity). ADP/AMP binding to AMPK facilitates its phosphorylation and activation. Simultaneously, T-tubule membrane depolarization caused by nerve impulses induces the release of Ca^{2+} from SR, increasing its concentration in the sarcoplasm and resulting in the activation of the CaMKK, which phosphorylates AMPK. Probably through phosphorylation of AS160 by AMPK, the GLUT4 vesicles migrate to the cell surface, leading to an increase in glucose uptake

10.4.2.3 Glycogen Degradation in the Skeletal Muscle

Glycogen content in muscle cells corresponds to 1–2 % of the net weight of muscles. It consists of an important energy store especially because it can be used either aerobically or anaerobically, although in this latter case it can sustain vigorous activity only for a short period of time.

The existence of an energy source that can be use independently of the amount of O_2 available is especially important for the type II muscle fibers as they are less irrigated by blood vessels and contain a low number of mitochondria, depending largely on the anaerobic metabolism as the mechanism of ATP synthesis (see Chap. 6 for review of the mechanisms of ATP synthesis).

The detailed structure of glycogen granules and the reactions for glycogen degradation have already been presented in Sect. 9.2.1. Although in that chapter special attention was given to the liver cells, the reactions per se are the same as those that occur in the muscle cells. The differences between glycogen metabolism in the liver and muscle cells consist basically of the mechanisms of regulation of the synthesis and degradation pathways. Thus, in this section we will give only a brief description of the glycogen degradation pathway, focusing our discussion on the aspects of the regulation of the glycogen metabolism that are characteristic of the muscle cells.

Glycogen degradation depends on the activity of two enzymes, the glycogen phosphorylase (GP) and the debranching enzyme. GP sequentially removes the terminal glucose unit from the nonreducing ends of the glycogen molecule by a phosphorolysis reaction, yielding glucose-1-phosphate. When a branched point is reached, further degradation depends on the activity of the debranching enzyme.

The major site of control of glycogen degradation is the regulation of GP activity. This enzyme exists in two interconvertible conformational states, called GP*a*, the catalytically active form, and GP*b*, the less active form (see Sect. 9.2.2). In resting muscle, the predominant form is the GP*b*, which is converted to the active form by adrenaline-mediated phosphorylation, the main hormonal control that acts on muscle cells. The action of adrenaline on muscle cells (detailed in Sect. 10.4) results in the activation of the enzyme phosphorylase kinase, which catalyzes the phosphorylation of GP. Phosphorylation maintains GP in the active form, favoring glycogen degradation (Fig. 10.18).

In addition to the regulation by phosphorylation, two allosteric modulators activate GP in muscle cells: Ca^{2+} and AMP.

As described in Sect. 10.1, the nervous stimulus to contraction induces Ca^{2+} release from SR to the sarcoplasm, where the concentration of this ion increases greatly. Ca^{2+} binds to the phosphorylase kinase, activating this enzyme and leading to the phosphorylation of GP to its active form (Fig. 10.18). This coordinates the

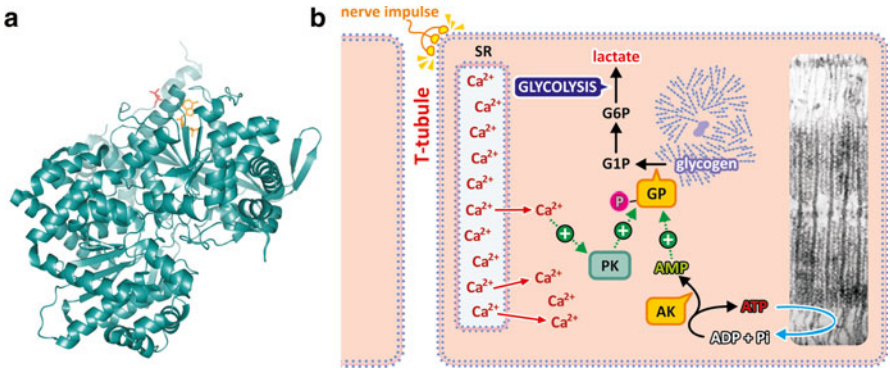


Fig. 10.18 (**a**) Structure of the human muscle GP (PDB 1Z8D) showing the phosphorylated in Ser14 (*pink*) and the AMP (*yellow*) in its binding site. (**b**) Activation of the glycogen degradation in contracting muscle. Intense contraction leads to a high rate of ATP hydrolysis, increasing the concentration of ADP, which may be converted to AMP and ATP by the adenylate kinase (AK). AMP binds to GP and facilitates glucose-1-phosphate (G1P) release from the active site, speeding the reaction of glycogen phosphorolysis. Simultaneously, T-tubule membrane depolarization caused by nerve impulses induces the release of Ca^{2+} from SR, increasing its concentration in the sarcoplasm and resulting in the activation of the phosphorylase kinase (PK), which phosphorylates and activates GP. G1P resulted from the glycogen degradation is converted to glucose-6-phosphate (G6P) by the phosphoglucomutase (PGM). G6P enters glycolysis, which in anaerobiosis generates lactate as the end product

first intracellular signal that induces contraction (Ca^{2+} release) to the mobilization of glycogen as an energy source for the process.

The other allosteric activator of glycogen degradation is AMP, which acts directly on GP, favoring glucose-1-phosphate release from the active site of the enzyme and speeding the GP reaction (Fig. 10.18). In resting muscle, ATP, which is in higher concentrations, replaces AMP in the allosteric site, inactivating the enzyme and inhibiting glycogen degradation.

The direct end product of glycogen degradation is glucose-1-phosphate (together with a small amount of glucose that is the product of the glucosidase activity of the debranching enzyme; see Sect. 9.2.1). Glucose-1-phosphate is then converted to glucose-6-phosphate by the action of the enzyme phosphoglucomutase (Fig. 10.18). It is important to remind that muscle cells lack the enzyme glucose-6-phophatase, an enzyme whose expression is restricted to the liver and kidneys. Thus, glucose-6-phosphate in muscles enters glycolysis, generating ATP to support muscle contraction.

Glycogen stores mobilized during muscle activity are replenished in muscle cells after carbohydrate ingestion. The increase in blood glucose after a carbohydrate-rich meal induces insulin secretion, whose action on muscle cells promotes glucose uptake through GLUT4 and its conversion to glycogen through the activation of the enzyme glycogen synthase (GS). The detailed reactions and regulatory aspects that take place in this situation are described in Sects. 8.2 and 8.4.

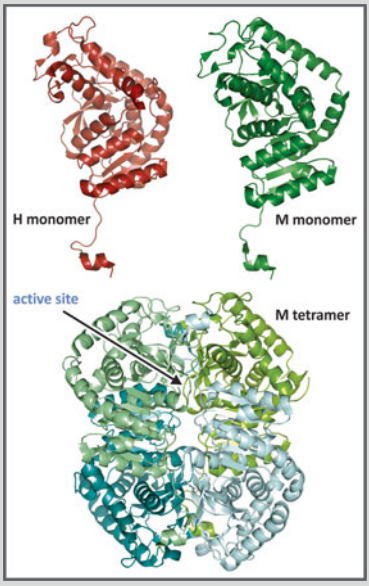
10.4.2.4 Glycolysis in the Skeletal Muscle

The glycolytic pathway in the muscle cells is also activated during physical activity. This occurs mainly by hormonal regulation, through adrenaline-induced phosphorylation of the muscle isoform of the bifunctional enzyme, which results in the activation of its phosphofructokinase-2 (PFK-2) activity, leading to an increase in the concentration of fructose-2,6-bisphosphate (see next section). Fructose-2,6-bisphosphate strongly activates the glycolytic enzyme phosphofructokinase-1 (PFK-1), increasing the metabolic flux through glycolysis.

Once glycolysis proceeds rapidly and if there is not enough O_2 available, pyruvate must be converted to lactate to allow NADH produced in glycolysis to be reoxidized (see Sect. 6.1.2 for details). The conversion of pyruvate to lactate is catalyzed by lactate dehydrogenase (LDH), an enzyme that is expressed as different isoforms depending on the tissue. The LDH isoform expressed in the skeletal muscles has a high affinity for pyruvate, making it possible a high glycolytic flow during contraction, especially in anaerobiosis (see Box 10.5).

Box 10.5: LDH Isoforms

LDH is a tetrameric enzyme that can be formed by a combination of two types of polypeptide chains: the M (from muscle) and the H (from heart) chains (see figures, PDB 1I10 and 1I0Z). Thus, there are five possible different isoforms of LDH (MMMM—see figure at the bottom—MMMH, MMHH, MHHH, HHHH). The detection of H isoform in the plasma can be used for diagnosis of heart infarction (see Sect. 3.3.4.1). The M subunits confer to the enzyme a lower K_M to pyruvate, favoring the reduction of pyruvate to lactate even when the concentration of pyruvate is low. This gives to skeletal muscle cells a high capacity of performing lactic fermentation with the pyruvate produced in glycolysis, generating a high glycolytic flow during contraction. The presence of H subunits favors the oxidation of lactate to pyruvate. Therefore, cells expressing the H chain-containing isoforms can use the lactate as a metabolic substrate, converting it to pyruvate, which in turn can be oxidized, as occurs in the heart tissue, for example. This is also important in liver cells, where lactate is converted to pyruvate to enter gluconeogenesis (see Sect. 9.3.2). The close similarity of the structures of the M and H subunits suggests that the different K_M observed for each isoforms results from variations in charge surface distribution on the active site.



10.5 Hormonal Regulation During Physical Activity: Role of Adrenaline

Adrenaline (also known as epinephrine) is the major hormone secreted when the organism is confronted with different stimuli processed in the central nervous system as indicative of an acute stressful situation, for which the capacity to perform an intense physical activity to deal with dangerous situations—the "fight-or-flight" response—was favored during evolution. Additionally, the physical activity itself promotes adrenaline secretion in a way dependent on the duration or the intensity of the exercise.

Adrenaline acts on almost all the tissues in the body, triggering many physiological and metabolic responses that prepare the organism for action. It promotes the dilatation of bronchioles, which increases the O_2 uptake, and a wide-range effect on the circulatory system, with the increase in the heart rate and blood pressure, and changes in blood flow patterns, leading to a decrease in the peripheral circulation and a reduction in the digestive system activity. These effects guarantee O_2 delivery to different organs, especially to the brain, allowing an increase in alertness. Nutrient availability is also tightly controlled by adrenaline, with the activation of glucose production by the liver and the mobilization of TAG in the adipose tissue. Finally, adrenaline prepares the skeletal muscle for contraction, with the activation of ATP-generating pathways, either through the anaerobic use of muscle glycogen or through the aerobic use of fatty acids. In this section, we will focus on the metabolic effects of adrenaline on the muscle, liver, and adipose tissues.

10.5.1 Molecular Mechanisms of Adrenaline Action

Adrenaline belongs to a group of substances known as catecholamines. It is synthesized by the adrenal gland, from which its name is derived. The adrenal glands are localized at the top of the kidneys and can be divided in two regions, the cortex, which secretes steroid hormones such as the glucocorticoids (see Sect. 9.4.2), and the medulla, where adrenaline is produced, more specifically in the chromaffin cells (Fig. 10.19A). The pathway for the adrenaline synthesis starts with the amino acid tyrosine and consists in four enzymatic steps, detailed in Fig. 10.19B.

Adrenaline secretion is triggered by a direct stimulus from the sympathetic nervous system that propagates through preganglionic nerve fibers reaching the adrenal gland (Fig. 10.19A). The concentration of adrenaline in the blood may increase more than 50-fold upon stimulation of the adrenal gland, but since the half-life of this hormone is too short, about 2 min, the effects of adrenaline on the body may be seen as acute and short-term responses to stress, which is compatible with an evolutionary adaptation to deal with "fight-or-flight" situations.

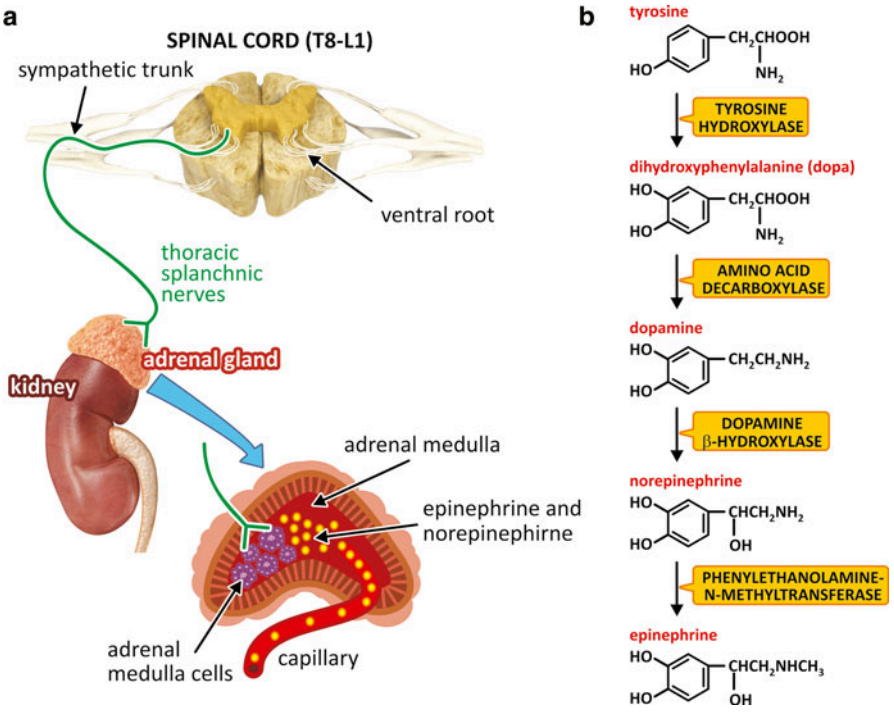


Fig. 10.19 (**a**) Representation of the adrenal gland, showing the two distinct parts: the cortex and the medulla. The medulla chromaffin cells produce and secrete adrenaline and noradrenaline. The stimulus for hormone secretion comes from the sympathetic nervous system and is transmitted to the adrenal gland through thoracic nerve fibers. (**b**) Metabolic pathway for adrenaline synthesis: the enzyme tyrosine hydroxylase converts the amino acid Tyr to L-dopa, which is decarboxylated by the enzyme aromatic amino acid decarboxylase, generating dopamine. The enzyme dopamine β-hydroxylase transforms dopamine in noradrenaline, which is converted to adrenaline by the action of the phenylethanolamine-*N*-methyl transferase (PNMT)

10.5.1.1 Cellular Receptors for Adrenaline

The action of adrenaline on its target tissues depends on the binding of the hormone to receptors present on the cell surface. There are different types of receptors for adrenaline divided in two main classes, the α- and the β-adrenergic receptors, which are in turn subdivided in α1 and α2, and β1, β2, and β3 subtypes, respectively.

The different, and sometime antagonic, effects of adrenaline on each tissue, such as the relaxation of the smooth muscles in the airways that increases the respiratory rate and the contraction of the muscles of the arterioles that causes vasoconstriction, can be explained by a tissue-specific expression of the different types of receptors. The different responses occur because each receptor subtype is coupled to distinct signaling systems, whose activation upon hormone binding triggers distinct intracellular responses (Fig. 10.20).

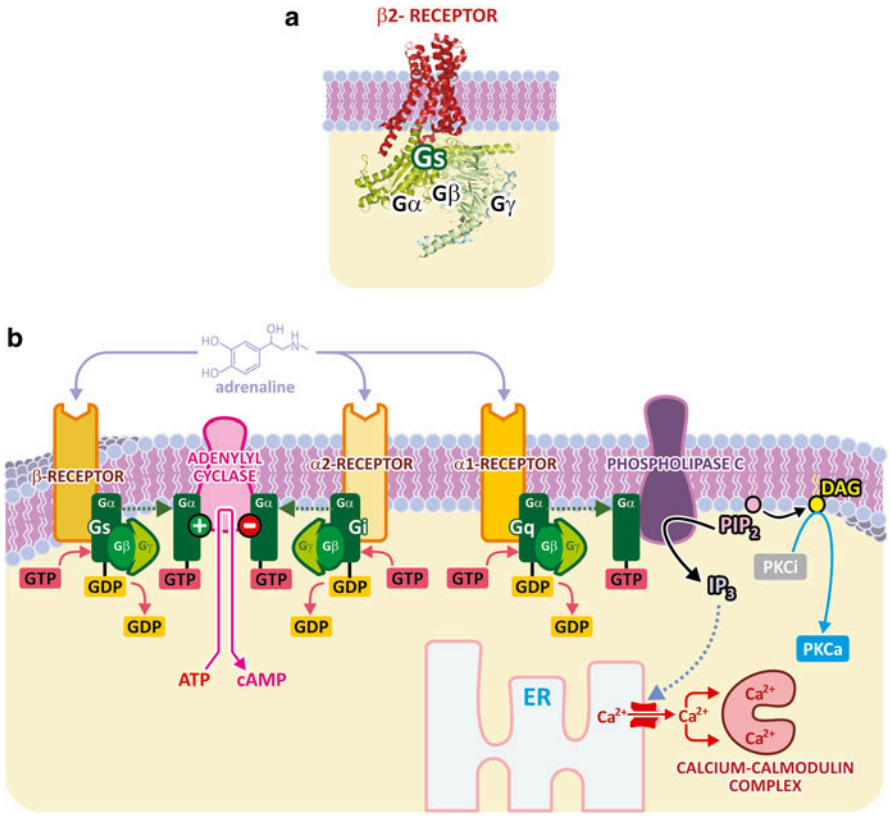


Fig. 10.20 (**a**) Crystallographic structure of the β2-receptor (*red*) bound to the trimeric G_s protein, with α (*green*)-, β (*light green*)-, and γ (*blue*)-subunits (PDB 3SN6). (**b**) Schematic representation of adrenaline signaling pathway through the different types of adrenergic receptors showing the different G protein α-subunits: G_s activates adenylate cyclase, while G_i inhibits this enzyme; G_q activates phospholipase C. β-adrenergic receptors are bound to G_s protein. When adrenaline binds to the receptor, the GDP bound to G protein is replaced by GTP and the G protein α-subunit moves on the membrane surface until it reaches adenylate cyclase, activating this enzyme, which converts ATP into cAMP. cAMP activates PKA leading to the phosphorylation of different targets in the cell. α2 receptor is bound to G_i, which inhibits the adenylate cyclase, leading to opposite effects of those mediated by G_s-coupled receptors. α1-adrenergic receptor is bound to G_q protein. When adrenaline binds to the receptor, the GDP bound to G protein is replaced by GTP and the G protein α-subunit moves on the membrane surface until it reaches the phospholipase C, which hydrolyzes the phosphatidylinositol (PIP_2) in DAG and IP_3. DAG activates PKC and IP_3 induces the release of Ca^{2+} from the SR stores to the cytoplasm

All the adrenergic receptors belong to the superfamily of G protein-coupled receptors (see also Sect. 9.4.1). They contain seven transmembrane helices and are bound to the G protein, a trimeric protein composed of α-, β-, and γ-subunits that is associated to the internal face of the plasma membrane (Fig. 10.20). The G protein α-subunit binds GDP, which maintains the protein in its inactive form, in

which it is associated to β- and γ-subunits. Upon hormone binding to the receptor, GDP is replaced by GTP, causing the α-subunit to dissociate from βγ-subunits and to move on plasma membrane until reaching a target enzyme, which may be the adenylate cyclase or the phospholipase C, depending on the type of G protein. The α-subunit has an intrinsic GTPase activity that terminates the signaling pathway through the conversion of the bound GTP in GDP, leading its reassociation to βγ-subunits.

The three subtypes of β-adrenergic receptors are linked to G_s ("s" from stimulatory G protein), which is a G protein type that activates the adenylate cyclase. The signaling pathway mediated by G_s-coupled receptors is the same as that involved in the glucagon mechanism of action, detailed in Sect. 9.4.1. Briefly, upon activation by the G_s subunit, adenylate cyclase catalyzes the conversion of ATP in cyclic AMP (cAMP), leading to an increase in the intracellular concentration of this molecule (Fig. 10.20). cAMP promotes the activation of the cAMP-dependent protein kinase (PKA), which phosphorylates several enzymes, modulating their activities. The β2-adrenergic receptors mediate the main effects of adrenaline on energy metabolism, which will be the focus of the next sections.

The α1-adrenergic receptors are mainly expressed in the smooth muscles, causing vasoconstriction and the decrease of the gastrointestinal tract motility. They are coupled to the type G_q of the G proteins, which activates the phospholipase C, an enzyme that hydrolyzes the phosphatidylinositol in the plasma membrane generating diacylglycerol (DAG) and inositol triphosphate (IP_3). DAG activates the protein kinase C (PKC), which mediates the smooth muscle contraction through the phosphorylation of proteins and ion channels. IP_3 binds to SR inducing Ca^{+2} release from the SR stores to the cytosol, stimulating muscle contraction (Fig. 10.20).

The α2 receptors are coupled to the type G_i, a G protein that inhibits the adenylate cyclase, leading to opposite effects of those mediated by G_s-coupled receptors.

10.5.2 Effects of Adrenaline on Energy Metabolism

10.5.2.1 Effects of Adrenaline on the Adipose Tissue Metabolism

The main effect of adrenaline on the adipose tissue is the activation of lipolysis, with the release of fatty acid in the bloodstream, leading to an increase of their availability to be used as an energy source. Lipolysis is activated through the PKA-mediated phosphorylation of two adipocyte proteins, the perilipin present on the surface of the lipid droplets and the hormone-sensitive lipase (HSL), which catalyzes the hydrolysis of the ester linkages of the TAG molecules. Phosphorylation activates the HSL and promotes a conformational change in perilipin that allows HSL recruitment to the surface of the lipid droplet, where it gains access to the TAG molecules (Fig. 10.21; see also Sect. 7.4.1 for details).

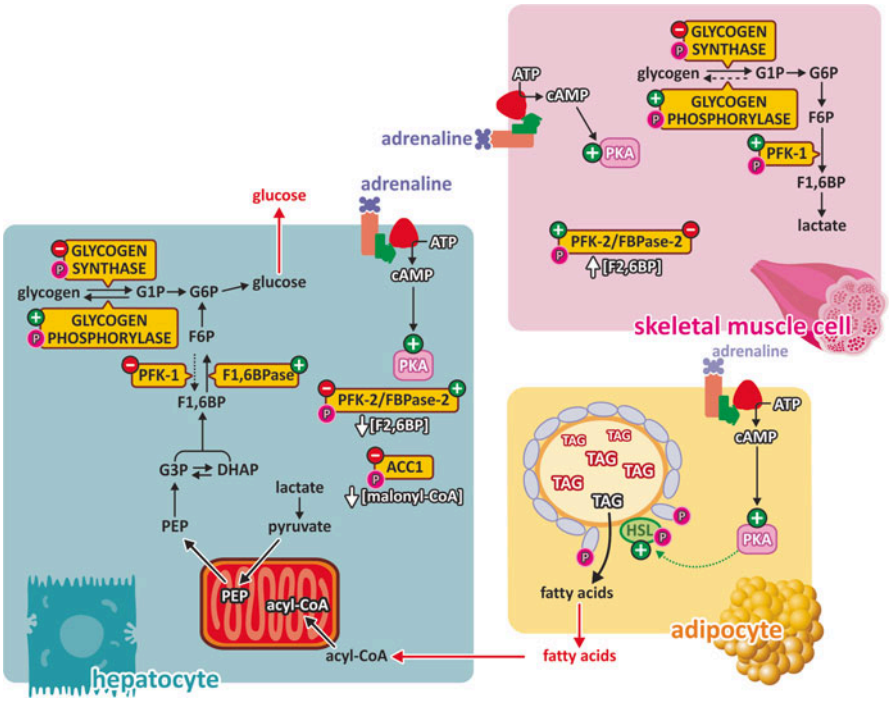


Fig. 10.21 Effects of adrenaline on energy metabolism. In liver, muscle, and adipose tissue cells, adrenalin binds to β-adrenergic receptors (*orange*) leading to G protein (*green*)-mediated adenylate cyclase (*red*) activation. The intracellular concentration of cAMP increases leading to the activation of PKA (*pink*), which phosphorylates different enzymes. In the adipocytes, PKA targets are perilipin (*light blue*) and HSL, resulting in the mobilization of TAGs. In the hepatocytes, the targets are (a) GP and GS, leading to glycogenolysis; (b) the bifunctional enzyme, leading to a decrease in the concentration of fructose-2,6-bisphosphate and activation of gluconeogenesis; and (c) ACC1, leading to the decrease in malonyl-CoA concentration and thus to the inhibition of fatty acid synthesis and the activation of the acyl-CoA transport into the mitochondrial matrix where they undergo β-oxidation. In the muscle cells, PKA targets are GP and GS, leading to glycogenolysis, and the muscle isoform of the bifunctional enzyme, in this case leading to an increase in fructose-2,6-bisphosphate concentration and activation of glycolysis

Adrenaline also interferes with the activity of the lipoprotein lipase (LPL), an enzyme involved in the transfer of lipids from the lipoproteins, especially from chylomicrons and VLDL, to the adipocyte, where they are stored after ingestion or de novo synthesis. The effects of adrenaline on LPL involve the inhibition of the translation of its mRNA, ultimately leading to a decrease in the lipid uptake by the adipose tissue. This makes the lipids available for use by the muscle cells.

10.5.2.2 Effects of Adrenaline on the Liver Metabolism

The main hepatic metabolic response upon adrenaline binding is the increase in glucose release in the bloodstream. This occurs both through the degradation of the liver glycogen and through the gluconeogenic pathway. Additionally, fatty acid synthesis is inhibited, allowing the incoming fatty acids to undergo β-oxidation. The effects of adrenaline on the liver metabolism are the result of a coordinated regulation of different metabolic pathways through the modulation of the activity of their key enzymes by phosphorylation promoted directly or indirectly by PKA.

Figure 10.21 provides a schematic overview of the adrenaline effects on liver metabolism, the results of PKA-induced phosphorylation being (a) the activation of GP and the inhibition of GS, leading to glycogenolysis; (b) the activation of the F2,6BPase activity of the bifunctional enzyme, leading to a decrease in the concentration of fructose-2,6-bisphosphate and the consequent activation of F1,6BPase and inhibition of PFK-1 with the activation of gluconeogenesis and the inhibition of glycolysis; and (c) the inhibition of ACC1, leading to the decrease in malonyl-CoA concentration and the consequent inhibition of fatty acid synthesis and the activation of the acyl-CoA transport into the mitochondrial matrix where they undergo β-oxidation. A more detailed description of the regulation of each of these key enzymes can be found in Sect. 9.4.1, since the effects of adrenaline on the liver metabolism are the same as those induced by glucagon (both β-adrenergic and glucagon receptors are G_s-coupled receptors).

10.5.2.3 Effects of Adrenaline on Muscle Metabolism

The effects of adrenaline in the muscle cells result in glycogen degradation and in a strong activation of glycolysis, adaptations that are especially important for the anaerobic metabolism of the type II fibers.

The adrenaline-mediated regulation of glycogen metabolism is similar to that occurring in the liver cells. PKA phosphorylates the phosphorylase kinase, which in turn phosphorylates and activates GP. Simultaneously, PKA-mediated phosphorylation inactivates GS. This results in intense glycogen degradation, yielding glucose-6-phosphate to the glycolytic pathway (Fig. 10.21).

Glycolysis is activated by the increase in the concentration fructose-2,6-bisphosphate, a potent activator of the glycolytic key enzyme PFK-1. Fructose-2,6-bisphosphate is synthesized through the phosphorylation of fructose-6-phosphate by the phosphofructokinase-2 (PFK-2) activity of bifunctional enzyme (see Sect. 9.3.3 for more details). The isoform of the bifunctional enzyme expressed in muscle cells differs from the liver isoform in its regulatory phosphorylation site, resulting in an opposite effect of phosphorylation on the enzyme activity in each tissue. The effect of PKA-induced phosphorylation on muscle isoform is the activation of the PFK-2 activity and in the inhibition of the fructose-2,6-bisphosphatase (F2,6BPase) activity,

leading to the synthesis of fructose-2,6-bisphosphate and the consequent PFK-1 activation, increasing the metabolic flux through glycolysis (Fig. 10.21). Furthermore, the muscle isoform of PFK-1 is itself a substrate for PKA. Phosphorylated muscle PFK-1 binds to actin filaments, resulting in further activation of the enzyme, which also becomes insensitive to the inhibitory effects of ATP, citrate or lactate. Thus, as a consequence of adrenaline action, glycolysis is strongly stimulated in muscle cells, while in the liver this hormone activates of gluconeogenesis.

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Chapter 11
Control of Body Weight and the Modern Metabolic Diseases

The increase in the body weight in the world population has become one of the most important public health problems, as it is a major risk factor for several pathologies such as cardiovascular diseases and diabetes.

A systematic analysis evaluating the worldwide changes in the body mass index (BMI, defined as the body mass divided by the square of the height) was performed by the Global Burden of Metabolic Risk of Chronic Diseases Collaborating Group. They used health examination surveys and epidemiological studies from 199 countries and territories in the world, including 9.1 million participants, and showed that the mean worldwide BMI increased by 0.4 kg/m^2 per decade for men and 0.5 kg/m^2 per decade for women. Figure 11.1 shows the results of the percentage of the obese (BMI $\geq$ 30 kg/m^2) or overweight (BMI $\geq$ 25 kg/m^2) people, between 1980 and 2008, in distinct areas of the globe. It is clear that although there are some differences among the regions, the increase in body weight seems to be a global phenomenon.

Body weight is a result of the balance between food intake and energy expenditure. Several peripheral mediators secreted by different cells in the body act on the central nervous system, influencing the feeding behavior by controlling appetite or satiety, as well as regulating body energy expenditure by changing the metabolic rate and controlling thermogenesis. In this chapter we will discuss the mechanisms by which the body weight is controlled as well as the main proposals on how the impairment of this control may cause the modern metabolic diseases.

A.T. Da Poian, M.A.R.B. Castanho, *Integrative Human Biochemistry*,
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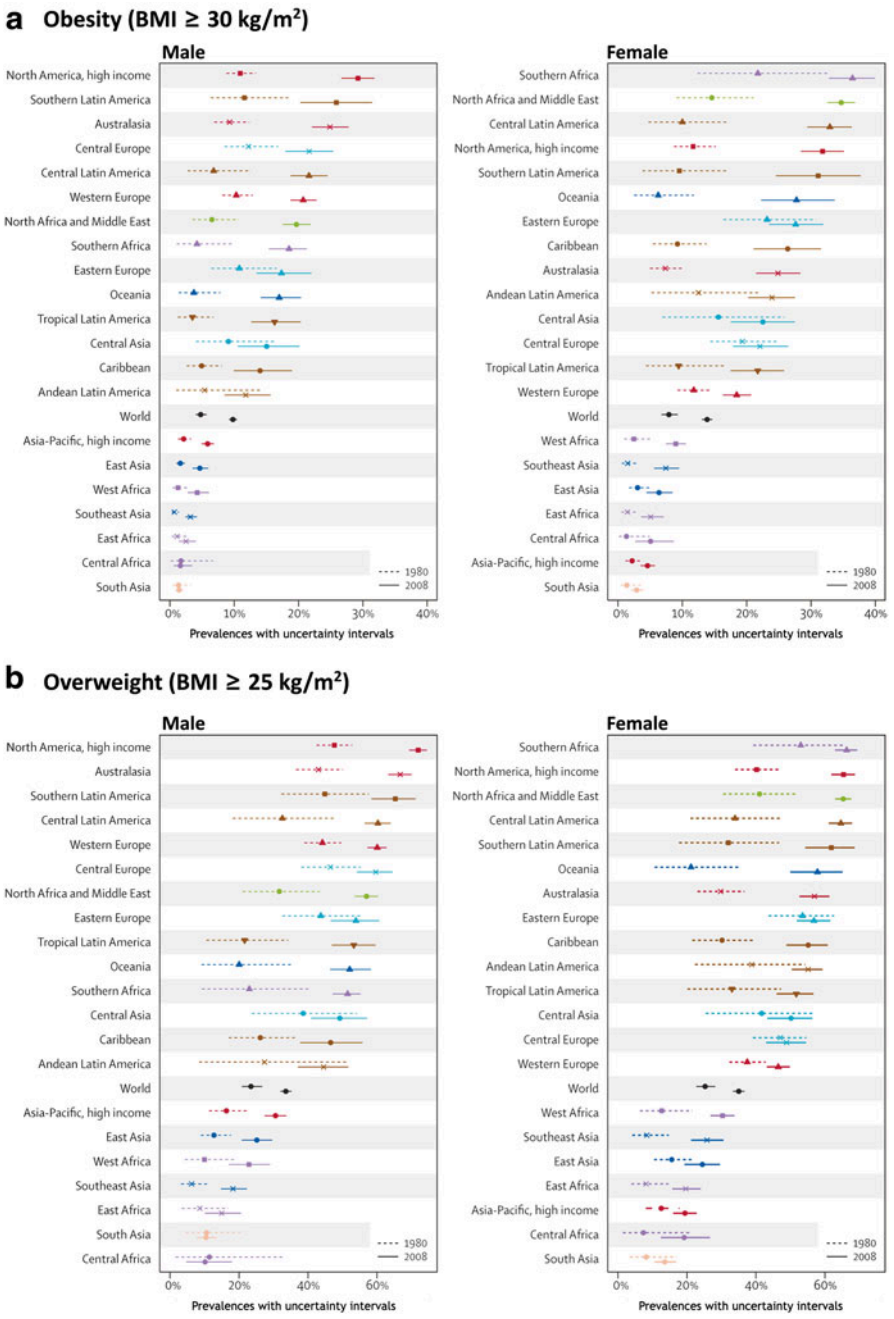


Fig. 11.1 Prevalence of obesity (**a**) or overweight (**b**) in 1980 and 2008 among males (*left*) and females (*right*) in different areas of the world (Reproduced from Finucane et al., Lancet 377:557–567, 2011, with permission from Elsevier)

11.1 Humoral Control of Food Ingestion

Although eating is a complex behavior that involves distinct areas of the brain, including the sensory areas that process the information of food taste, smell, and appearance, and the cortex, which is responsible for the psychological component of the appetite and satiety, the hypothalamus may be seen as a central player in the control of feeding.

The hypothalamus is located below the thalamus, just above the brain stem, in the ventral part of the diencephalon (Fig. 11.2). Anatomically, it is divided in several regions; the following ones are directly involved in the control of energy balance: ARC, arcuate nucleus; VMH, ventromedial hypothalamus; DMH, dorsomedial hypothalamus; PVN, paraventricular nucleus; and LH, lateral hypothalamus (Fig. 11.2).

The hypothalamus induces anorexigenic (appetite-suppressing) or orexigenic (appetite-stimulating) behaviors by sensing several substances that are secreted by different cells in the body as a response to food intake or to the increase in adiposity. For instance, leptin, insulin, cholecystokinin (CCK), and peptide YY (PYY) provide the anorexigenic signals, while ghrelin is the main orexigenic mediator, as it will be discussed in the next sections.

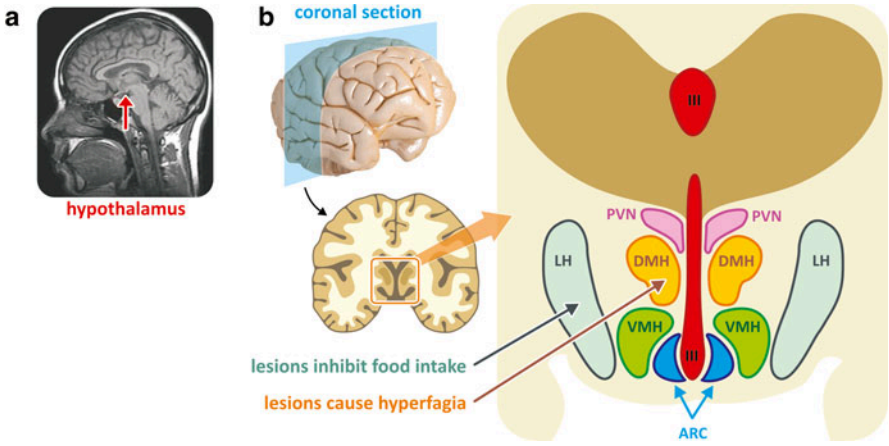


Fig. 11.2 (**a**) Magnetic resonance image of the human brain showing the localization of the hypothalamus (reproduced from the free media repository Wikimedia Commons). (**b**) Human brain section showing the anatomical localization of the hypothalamic regions. The *blue arrow* indicates the area whose lesion caused hyperphagia and the *orange arrow* indicates the area whose lesion caused the inhibition of food intake. *III* third ventricle, *ARC* arcuate nucleus, *VMH* ventromedial hypothalamus, *DMH* dorsomedial hypothalamus, *PVN* paraventricular nucleus, *LH* lateral hypothalamus

11.1.1 A Historical Perspective of the Role of Hypothalamus in Food Intake

The first evidence showing that the hypothalamus controls the body energy balance came from a number of studies conducted in the 1940s. These experiments showed that lesions in areas of the medial-basal hypothalamus caused either hyperphagia and obesity or anorexia, depending on the specific region that was lesioned (as indicated in Fig. 11.2).

More than just revealing its role in the control of food ingestion, these experiments led to the hypothesis that the hypothalamus would act as a sensor of the feeding status of the organism, stimulating or inhibiting food intake depending on the amount of the energy stored in the body. A question that was immediately raised was how this sensing mechanism would operate. An attractive hypothesis was that it occurred through the circulating metabolites detected by the hypothalamus.

At that time, a common approach to investigate the participation of a circulating mediator in a given phenomenon was through parabiosis experiments. In these experiments, the blood vessels of two animals were surgically connected to allow the exchange of circulating factors between them. In a classical experiment performed in 1959, parabiotic pairs of rats were subjected to a lesion in the hypothalamus of one of the animals. While the lesioned rats became obese, as expected, the unlesioned partners stopped eating and lost weight, indicating that a circulating factor produced by the obese animals, for which the lesion turned the animal unresponsive, inhibited food intake in the normal ones (see the result of this experiment in Fig. 11.3).

Other parabiosis experiments were performed in the 1950s taking advantage of the description of two spontaneous recessive mutations in mice that result in a phenotype of extreme obesity caused by hyperphagia. The two mutated genes were called *ob* (from the obese phenotype; Fig. 11.4) and *db* (from diabetic, and also obese, phenotype).

When a homozygotic mouse for a mutation in the *ob* gene (*ob/ob* mouse) was joined through parabiosis to a normal mouse, the obese animal stopped eating in excess and tended to normalize its weight. This suggested that the product of the *ob* gene, which is defective in the *ob/ob* mouse, would be the circulating factor that signalizes to the hypothalamus the excess of body weight, leading to the inhibition of food intake. In contrast, when a *db/db* mouse was connected to a normal mouse, only the normal animal lost weight, suggesting that the mutation caused a failure in the response to the "obesity" factor, which acts in the normal mouse inhibiting food ingestion (Fig. 11.5).

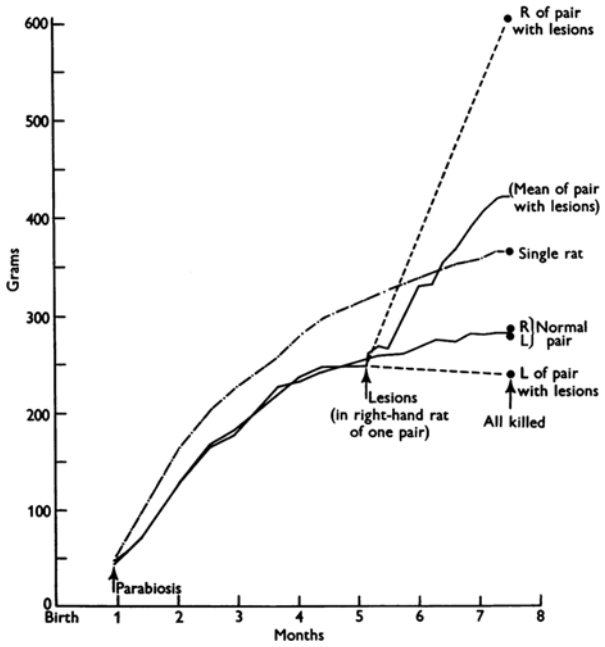


Fig. 11.3 Growth curves of parabiotic rats. When the animals were 5 month old, lesions were made in the hypothalamus of the right-hand member of the parabiotic pair. The last point in the curve corresponds to the individual body weights after death and separation of the parabiotic pairs. The *dashed lines* indicate the presumed body weight of each member of the pair based on the weight at the end of the experiment. The growth curve of a single rat is also shown for comparison. Figure reproduced with permission from Hervey, J. Physiol. 145:336–352, 1959

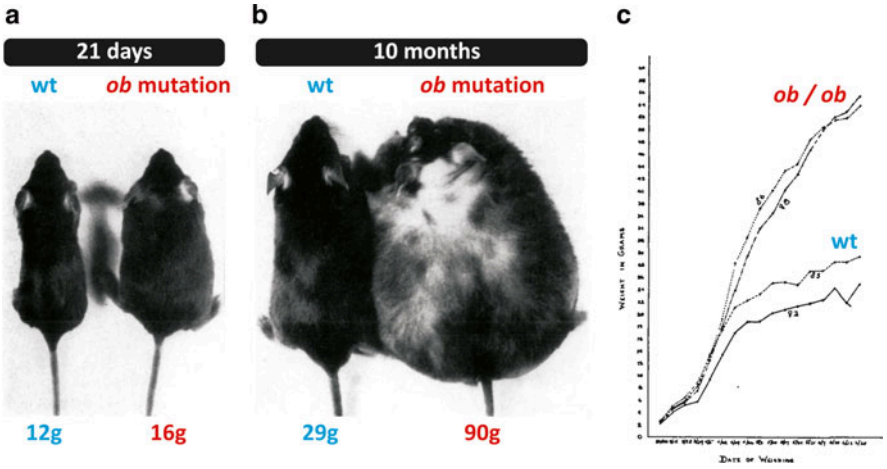


Fig. 11.4 Photographs and growth curves reproduced from the original article that described the phenotype of the animal with the spontaneous mutation in the *ob* gene. (**a**) Normal and *ob/ob* mice at 21 days of age. (**b**) Normal and *ob/ob* mice at 10 months of age. (**c**) Growth curves of two normal and two *ob/ob* mice from birth to 4 months of age. Figures reproduced with permission from Ingalls et al., J. Hered. 41:317–318, 1950

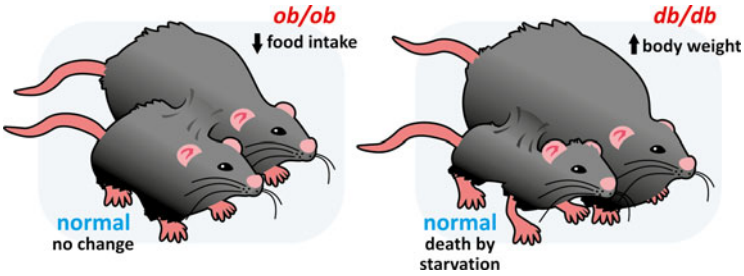


Fig. 11.5 Schematic representation of the parabiosis experiments with *ob/ob* and *db/db* mice. When an *ob/ob* mouse was connected to a normal mouse, the obese animal stopped eating in excess and lost weight, while no changes occurred in the normal mouse. When a *db/db* mouse was connected to a normal one, only the normal mouse lost weight

11.1.2 Leptin: A Hormone Indicative of Adiposity

The parabiosis experiments with the *ob/ob* and *db/db* mice described in the previous section indicated that the amount of adipose tissue accumulated in the body was regulated by the endocrine system. However, the hormone (the circulating factor) responsible for the transmission of the information of the increase in body weight to the hypothalamus remained unknown until 1994, when the product of the *ob* gene was finally discovered. More interestingly, it was found that this protein is expressed almost exclusively in the adipose tissue. This protein was named leptin (from the Greek word *leptos*, which means thin), and its discovery represented a revolution in the study of obesity both due to the expectations it raised on the pharmaceutical industry and because it revealed a complete new function for the adipose tissue, whose role changed dramatically from an inert tissue of energy storage to an endocrine organ (Box 11.1).

Immediately after the discovery of leptin, massive investments were made in the development of new treatments that envisaged the elimination of obesity by simply administrating leptin to obese people. However, as high as the expectations were the disappointments when it became clear that in humans the mutations in the leptin gene accounted for a very small number of obesity cases, and the simple administration of leptin in the remaining obesity cases did not lead to weight loss.

Despite the failure of using leptin to treat obesity, it soon became clear that the plasma levels of leptin directly correlated with the BMI and to the percentage of fat in the body, showing that the increase in the amount of adipose tissue determined the increase in plasma leptin (Fig. 11.6).

Leptin is a small protein of 167 amino acid residues forming an α-helical structure that resembles the structure of some cytokines (Fig. 11.7). Leptin effects are mediated by its binding to specific receptors expressed by neurons in the central nervous system, the inhibition of appetite being mainly due to its binding to receptors of the hypothalamic neurons (see also Sect. 11.1.5).

Box 11.1 The Adipose Tissue as an Endocrine Organ

The discovery of leptin opened the way to the identification of a series of proteins secreted by the adipose tissue, which were named adipokines (see table). These proteins act as hormones or cytokines and are mainly involved in the regulation of energy metabolism or in inflammation. This made the white adipose tissue to be recognized as a dynamic endocrine organ besides being a lipid storage tissue.

Active polypeptides secreted by the adipose tissue

Name	Molecular nature	Main features and functions
Leptin	16 kDa protein	Expressed mainly in adipocytes. Controls energy balance (see main text)
Adiponectin	30 kDa protein that forms multimeric complexes	Expressed exclusively in adipocytes. Blood levels inversely correlate to adiposity. Increases lipid catabolism and insulin sensitivity
Resistin	75 kDa protein	Expressed mainly in adipocytes. Secreted during adipogenesis. Increases insulin resistance
Visfatin	52 kDa protein	Extracellular isoform of the enzyme nicotinamide phosphoribosyltransferase of the NAD biosynthesis pathway. Stimulates insulin secretion
Apelin	55 amino acid precursor that generates several active fragments	Expressed in many cell types including adipocytes. Increases cardiac contractility and lowers blood pressure
Retinol-binding protein-4 (RBP-4)	20 kDa protein	Expressed mainly in hepatocytes and adipocytes. Transports retinol in the blood but also decreases insulin sensitivity
Tumor necrosis factor-α (TNF-α)	17 kDa protein that forms soluble trimers	Pro-inflammatory cytokine secreted mainly by macrophages but also by many other cells including adipocytes. Triggers the inflammatory response and regulates cell death, but also increases insulin resistance (see Sect. 11.3.1)
interleukin 6 (IL-6)	24 kDa protein	Produced by many cell types. Acts as a pro-inflammatory cytokine
Monocyte chemotactic protein-1 (MCP-1)	13 kDa protein	Produced by many cell types. Acts on the inflammatory process, but also impairs insulin signaling in skeletal muscle cells
Plasminogen activator inhibitor-1 (PAI-1)	47 kDa protein	Expressed in endothelium but also in other cell types such as adipocytes. Inhibits the proteases involved in the degradation of blood clots

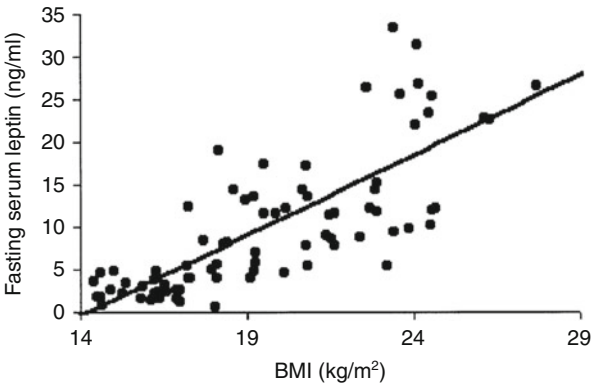


Fig. 11.6 Correlation of plasma leptin concentrations with BMI in a sample of 41 obese children (aged 6–9 years old) and the same number of nonobese children (control group), matched by age and sex (Reproduced with permission from Valle et al., Int. J. Obes. Relat. Metab. Disord. 27:13–18, 2003)

The leptin receptor is a homodimeric transmembrane protein that is constitutively associated to the enzyme Janus kinase 2 (JAK2, Box 11.2). Leptin binding promotes conformational changes in the receptor that mediate an increase in the tyrosine kinase activity of JAK2, resulting in its autophosphorylation and in the phosphorylation of different intracellular targets, leading to the activation of different signaling pathways, including the JAK/STAT3, the phosphatidylinositol-3-kinase (PI3K), and mitogen-activated protein kinase (MAPK) pathways (Fig. 11.7).

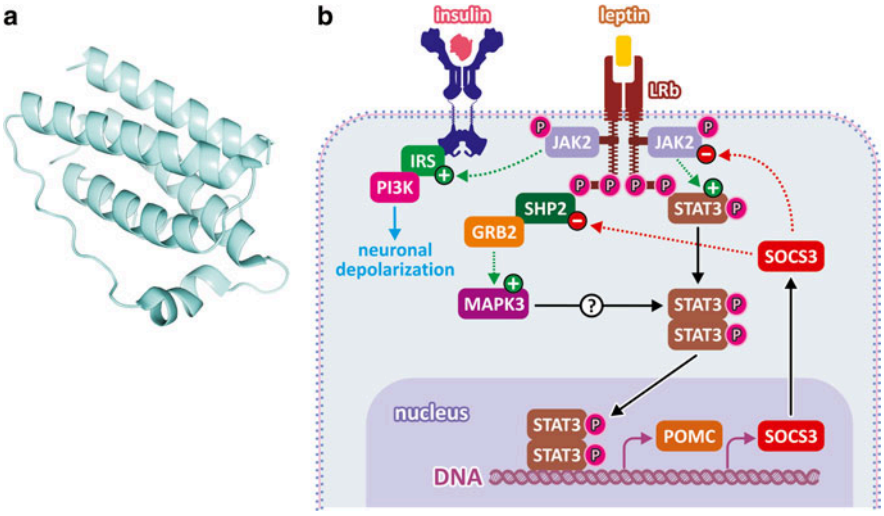


Fig. 11.7 (**a**) Leptin structure. (**b**) Leptin signaling pathway. Leptin binding to its receptors in the hypothalamus leads to the activation of JAK2 and to the recruitment of STAT3, IRS, and SHP, which become active. STAT3 migrates to the nucleus, inducing the expression of POMC and the feedback inhibitor of the pathway SOCS3. IRS mediates the activation of PI3K, which induces neuronal depolarization involved in the rapid responses independent on gene expression. Activation of MAPK pathway is mediated by SHP recruitment

JAK/STAT3 is the predominant pathway that mediates leptin effects. When STAT3 is phosphorylated by JAK2, it migrates to the nucleus, where it regulates the expression of several genes. This includes those encoding some important

Box 11.2 The Origin of the Janus Kinase Name

In the Roman mythology, Janus (in Latin, *Ianus*) is the god of the gateways, beginnings, and transitions, who keeps the door of Heaven. He is represented as having two faces looking in opposite directions (see figure with Janus representation in an old Roman coin), meaning that he can see the future and the past simultaneously. The first month of the year is named after him, as in January we look back at the last year and forward to the next. The reference to Janus in JAK's name is related to the fact that the members of this family of kinases possess two near-identical phosphate-transferring domains, one with kinase activity and the other that negatively regulates the kinase activity of the first.

Janus head represented in a Roman coin dated 225–214 BC (Reproduced from the free media repository Wikimedia Commons)

neuropeptides involved in the appetite control, such as neuropeptide Y, which has its expression inhibited, and the pro-opiomelanocortin (POMC), corticotropin-releasing hormone (CRH), and the cocaine- and amphetamine-regulated transcript (CART), which have their expression induced (see Sect. 11.1.5 for details on the role of these neuropeptides in the appetite control). STAT3 also induces the expression of feedback inhibitors of the signaling pathway, such as SOCS3 (suppressor of cytokine signaling 3) (Fig. 11.7).

The active JAK2 also recruits proteins from the family of the insulin receptor substrates (IRS), which mediate the activation of the PI3K pathway (for more details about this pathway, see the mechanism of insulin action in Sect. 8.4.2). Thus, there is a cross talk between the signaling pathways triggered by leptin and insulin at the hypothalamic level, so that the leptin action on the hypothalamus is positively modulated by insulin and vice versa. PI3K regulates the electrophysiological properties of the hypothalamic neurons, modulating the release of neurotransmitters in the synapses, which also contribute to the reduction of feeding. It is important to mention that the blood concentration of both insulin and leptin are increased in response to food ingestion. Since the hypothalamic neurons are also rich in insulin receptors, both leptin and insulin contribute to the triggering of the anorexigenic responses after feeding.

In summary, the action of leptin on the hypothalamic neurons alters the gene expression pattern in these cells and causes cell depolarization, resulting in the activation or inhibition of the secretion of different neuropeptides (see Sect. 11.1.5). It is important

to mention that leptin effects can be seen as part of the long-term effects on the control of the body weight. Leptin does not promote an abrupt interruption in food ingestion, but influences the amount of food intake and its relationship with the body energy expenditure over time. There are other hormones and mediators that directly promote the postprandial satiety or trigger appetite. These mostly short-term mediators are mainly secreted by the gastrointestinal tract, as discussed in the next sections.

11.1.3 Intestinal Peptides: Triggers of Postprandial Satiety

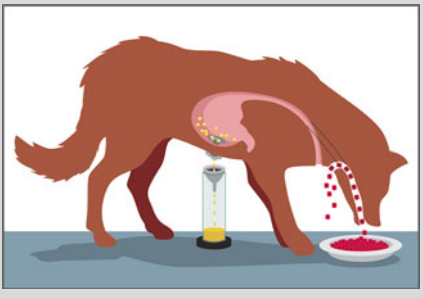
The satiety feeling just after a meal is a result of several signals, mainly arising from the gastrointestinal tract, that act on the brain, providing information regarding the quality and the quantity of food ingested. These signals are involved in the short-term regulation of food ingestion, especially the control of the meal size.

The first experiments suggesting the role of the intestine in promoting satiety were performed using a surgical procedure previously developed by Ivan P. Pavlov in his classic experiments of the decade of the1890s, which revealed the role of the central nervous system in the regulation of gastric secretion in dogs (see Box 11.3).

Box 11.3 Pavlov and the Sham Feeding Experiments

Ivan Petrovich Pavlov was a Russian scientist whose research on the physiology of digestion led him to be awarded the Nobel Prize in Physiology or Medicine in 1904. Pavlov developed a surgical method that establishes fistulas in various organs, enabling the continuous observation of their functions. This opened a new era in the development of physiology, whose procedures until then were based essentially on vivisection methods, which allowed only an instantaneous picture of the analyzed process. The classical Pavlov's experiments were performed on sham-fed dogs, to which a gastric fistula was applied (see figure). Within a few minutes after the beginning of sham feeding, gastric juice begins to flow without ceasing for hours.

Pavlov demonstrated that when the vagus nerves were lesioned, secretion of gastric juice during sham feeding was absent, showing the reflex nature of the first phase of gastric juice secretion.



Pavlov's esophagostomy associated to the application of a gastric fistula used in the sham-feeding experiments

This procedure consists of establishing an esophagic fistula in animals, so that when they eat, the food is tasted and swallowed, but does not accumulate in the stomach and does not pass into the small intestine, which is usually referred as sham feeding (Box 11.3).

A number of experiments performed in the 1970s showed that when the animals were sham fed, satiety did not occur, revealing that the satiety feeling originates from the passage of food through the gastrointestinal tract. These experiments allowed the subsequent identification of several intestinal peptides involved in triggering satiety and whose plasma levels rapidly increase after a meal (Fig. 11.8). In this section we will comment on the secretion profile and the action of three of these peptides: cholecystokinin (CCK), glucagon-like peptide 1 (GLP-1), and peptide YY (PYY).

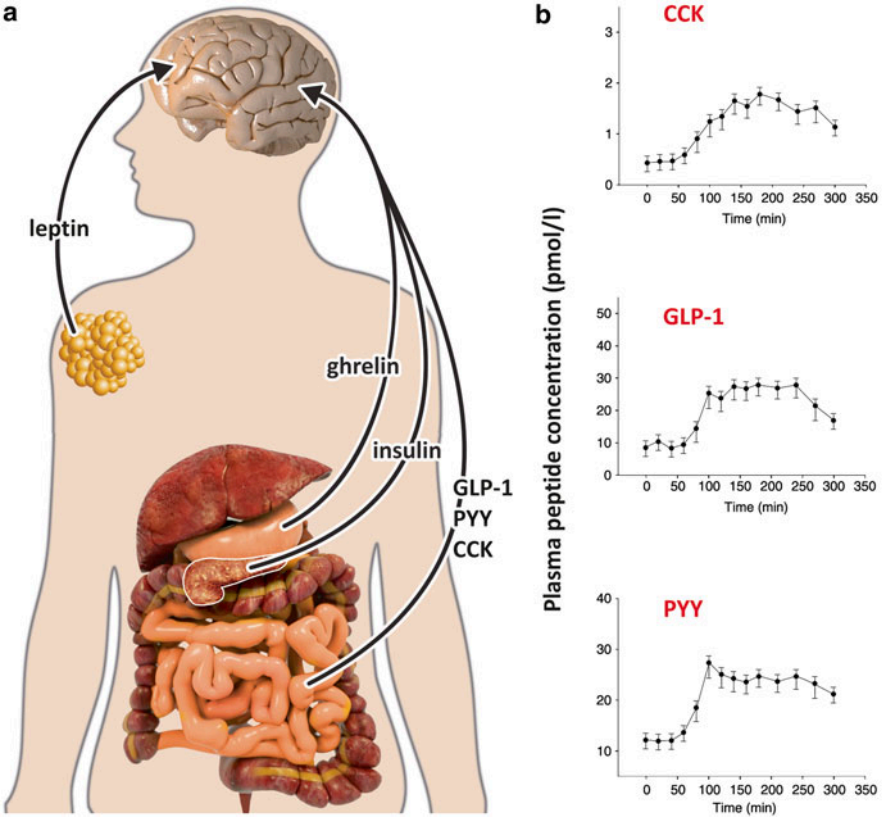


Fig. 11.8 (**a**) Sites of secretion of the peripheral factors that modify food intake and energy expenditure through direct effects on the brain: ghrelin is secreted by the stomach prior to the meals, inducing food intake (Sect. 11.1.4); CCK, GLP-1, and PYY are secreted by the intestine just after the meals, interrupting eating; leptin is secreted by the adipose tissue as a signal of adiposity (Sect. 11.1.2); and insulin is secreted by the pancreas in response to nutrient ingestion (Sect. 8.4). (**b**) Plasma concentrations of intestinal peptides in response to breakfast ingestion, which occurred 60 min after the beggining of the measurements. Data are from 12 subjects, presented as mean ± SEM. CCK, cholecystokinin; GLP-1, glucagon-like peptide-1; PYY, peptide tyrosine tyrosine (Reproduced with permission from Vidarsdottir et al., Eur. J. Endocrinol. 162:75–83, 2010)

CCK is secreted by the I cells, mainly located in the proximal duodenum, suppressing food intake and decreasing meal size. CCK was one of the first gastrointestinal peptides to be demonstrated as an important trigger of the postprandial satiety. Experiments in the 1970s using sham-fed rats, which ate continuously due to the absence of intestinal stimulation, showed that the intraperitoneal injection of CCK suppressed feeding in a dose-dependent manner (Fig. 11.9).

Long-chain fatty acids and proteins are particularly effective in inducing CCK secretion, although carbohydrate-rich meals also promote an increase in CCK plasma levels. CCK seems to act on the brain through a paracrine stimulation of the vagal afferent nerve fibers, whose terminals are proximal of the intestinal I cells, suggesting that the CCK plasma levels are not the best indicator of its potential action.

The L cells of the distal intestine secrete both GLP-1 and PYY, although the regulation of the release of each of these peptides differs. PYY is a 34 amino acid residue peptide named after its tyrosine content. PYY secretion is triggered by the direct contact of the L cells with the nutrients, as well as in response to duodenal lipids. This latter stimulus is probably mediated by CCK. PYY binds to Y receptors in the arcuate nucleus neurons inhibiting NPY release and thus causing anorexigenic effects (see Sect. 11.1.5). GLP-1 is a peptide derived from the pro-glucagon precursor and its secretion is stimulated especially after a carbohydrate-rich meal, although fats are also potent secretagogues. A body of evidence suggests that, as CCK, GLP-1 reduces food intake and promotes satiety by acting on vagal afferent nerve terminals close to the L cells. The satiety promoted by the increase in secretion of both GLP-1 and PYY seems to be the main reason for the success of bariatric surgeries, currently considered to be the most effective surgical treatment for morbid obesity (see Box 11.4).

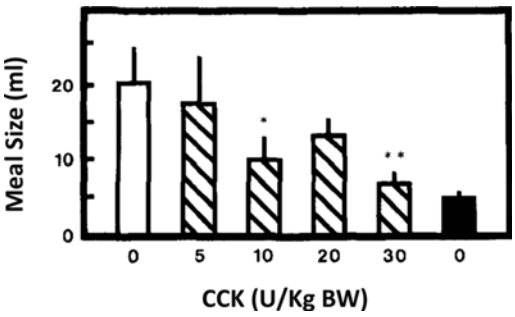
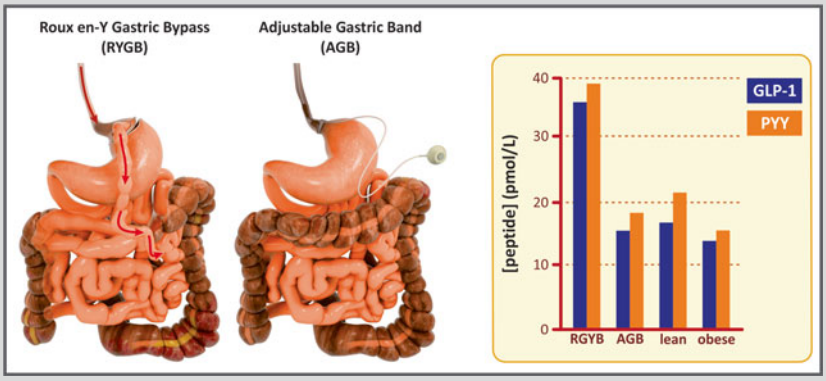


Fig. 11.9 Result of an experiment in which sham intake of a liquid diet was quantified in rats ($n=8$) upon intraperitoneal injection of CCK in the concentrations indicated in the figure (*hatched bars*), with the "0" (*white bar*) corresponding to saline injection. Animals in which the gastric fistula was maintained closed were used as control (*black bar*). The animals were submitted to food deprivation for a period of 3 h after which the ingested food volume was quantified until the animal stops eating. *Vertical lines* are the standard errors with * corresponding to $p=0.05$ and ** corresponding to $p<0.01$ for CCK vs. saline. (Copyright © 1978 by the American Psychological Association. Reproduced with permission from Kraly et al., J. Comp. Physiol. Psychol. 92:697–707, 1978)

Box 11.4 Bariatric Surgery

Bariatric surgery is a weight loss procedure based on the removal of a portion of the stomach (gastrectomy), the reduction of its size through the introduction of a medical device (gastric banding), or the creation of a small stomach pouch which is connected to the intestine skipping the duodenum (gastric bypass). It has become the treatment of choice for individuals with severe obesity, being recommended for people with BMI > 40 or BMI > 35 when serious coexisting medical conditions occur. Bariatric surgery may be classified as purely restrictive (as in the case of the adjustable gastric band) to mostly malabsorptive (as occurs in the biliopancreatic diversion), which usually results in clinical complications related to malabsorption of macronutrients. The surgical approaches predominantly used today are the Roux-en-Y gastric bypass and the adjustable gastric band (see figure). The Roux-en-Y gastric bypass produces better results in terms of weight loss, probably because it is associated to an increase of the plasma concentrations of the satiety peptides PPY and GLP-1 (see figure). More recently, a variation of this procedure was proposed, in which a gastric fundus resection is combined to the bypass. This promotes a more consistent decrease in ghrelin secretion together with an increase in PPY and GLP-1 release, which makes the response even more effective.



Schematic representation of the predominantly used bariatric surgery: the Roux-en-Y gastric bypass (*left*) and the adjustable gastric band (*right*) and changes in plasma concentrations of GLP-1 and PYY 1 h after breakfast in lean ($n = 15$) or obese ($n = 12$) subjects or patients submitted to RYGB ($n = 6$) or GB ($n = 6$) (Based on data from the study described in le-Roux et al. Ann. Surg. 243:108–114, 2006)

11.1.4 Ghrelin: The Main Orexigenic Hormone

Ghrelin was discovered in 1999 as an endogenous ligand of the *g*rowth *h*ormone secretagogue *r*eceptor, from which its name is derived. Indeed, one of the actions of ghrelin is to stimulate growth hormone release, but its role in the control of appetite seems to be independent of this previously identified function.

The active form of ghrelin is an acylated peptide of 28 amino acid residues, secreted mainly by the stomach (Fig. 11.10a). Ghrelin concentration in blood increases during fasting and reaches its lowest level just after a meal, indicating a physiological role for this hormone in meal initiation (Fig. 11.10b). Measurements of plasma ghrelin levels in gastrectomized patients confirmed that the stomach is a major source of circulating ghrelin in humans (Fig. 11.10b). Indeed, the fact that ghrelin is an important signal to start feeding may explain the decrease in feeding behavior after gastrectomy (see Box 11.4).

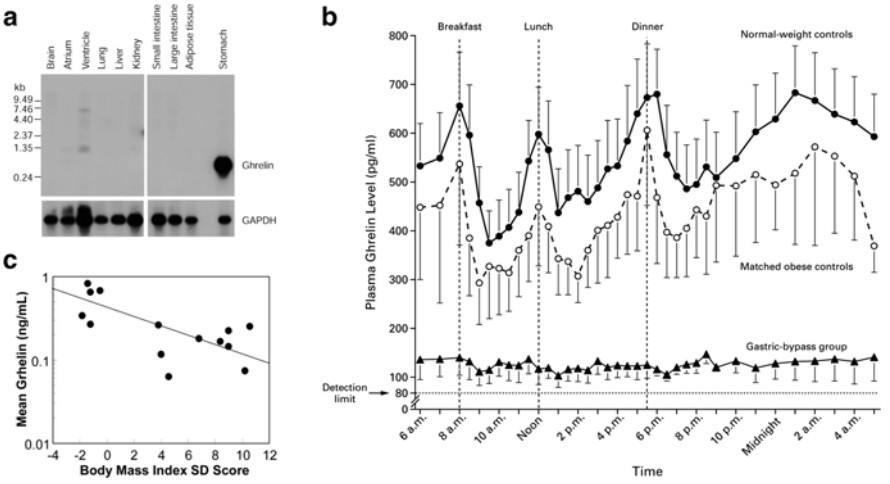


Fig. 11.10 (**a**) Analysis of the expression of ghrelin mRNA in different rat tissues. The figure shows the result of a Northern blotting, a technique in which the mRNA for a specific gene is radioactively labeled and detected by autoradiography (Reproduced with permission from Kojima et al., Nature 402:656–660, 1999). (**b**) Mean 24-h plasma ghrelin concentration during a day in 10 normal-weight human subjects and 5 obese subjects who underwent a proximal Roux-en-Y gastric bypass and 5 obese subjects who had recently lost weight by dieting and were matched to the subjects in the gastric-bypass group according to final body mass index, age, and sex. The *dashed lines* indicate breakfast, lunch, and dinner (Reproduced with permission from Cummings et al. N. Engl. J. Med. 346:1623–1630, 2002). (**c**) Relationship between the mean 24-h plasma ghrelin concentration and BMI in five lean and nine obese girls (Reproduced with permission from Foster et al. Pediatr. Res. 62:731–734, 2007)

Many studies have shown that ghrelin administration into the brain ventricle in rodents induces food intake through the increase of the expression of hypothalamic neuropeptide Y, an opposite effect of that observed for leptin. In agreement, the relationship of blood ghrelin concentrations and BMI is the opposite of that observed for leptin: plasma ghrelin concentrations inversely correlate with BMI (Fig. 11.10c).

Ghrelin receptor, the growth hormone secretagogue receptor (GHSR), belongs to the class of G protein-coupled receptors (for more details on G protein-coupled receptors and their different signaling pathways, see the description of adrenaline receptors in Sect. 10.5.1). GHSR may be coupled to different types of G protein, including type G_q, which mediates changes in ion currents in the neurons through the activation of phosphatidylinositol-specific phospholipase C. Phospholipase C hydrolyzes the phosphatidylinositol 4,5-bisphosphate (PIP_2) in inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG), which induce Ca^{2+} release from ER and the activation of protein kinase C (PKC), leading to the inhibition of K^+ channels and causing neuronal depolarization (Fig. 11.11). Additionally, GHSR, through G proteins type G_q or G_i, mediates the activation of the MAPK or PI3K pathways, leading to changes in neuronal gene expression, which includes the induction of expression of the orexigenic neuropeptide Y (NPY, see next section) (Fig. 11.11).

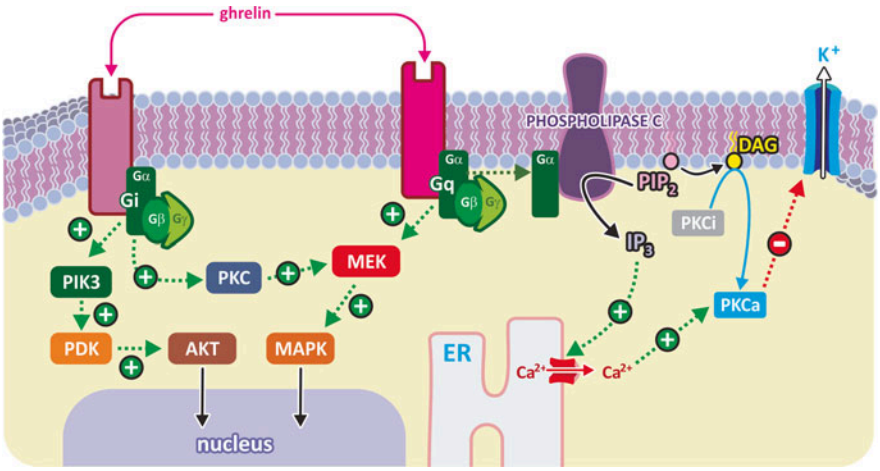


Fig. 11.11 The binding of ghrelin to GHSR results in the release of GDP and binding of GTP to the G protein α-subunit coupled to the receptor. Neuronal depolarization is triggered by ghrelin binding to GHSR coupled to G protein-type G_q, which activates PLC, generating IP_3 and DAG, resulting in an increase in the intracellular Ca^{2+} concentration and the activation of PKC, which inhibits the K^+ channels. Changes in the neuronal gene expression are achieved through ghrelin binding to GHSR coupled to G protein-type G_q or G_i, which activates MAPK or PI3K pathways

11.1.5 The Arcuate Nucleus and the Melanocortin System

The main target of the peripheral mediators in the hypothalamus is the arcuate nucleus (ARC). ARC is a region of the medial-basal hypothalamus adjacent to the third ventricle (see Fig. 11.2). The blood–brain barrier in this region seems to be more permeable, facilitating the contact of the ARC neurons with humoral and metabolic factors coming from the peripheral circulation, including the anorexigenic and the orexigenic factors discussed in the previous sections.

Two subsets of ARC neurons play a central role in the regulation of energy balance: the NPY/AgRP neurons and the POMC neurons (Fig. 11.12). It is important to mention that the name of these neuron populations is derived from the name of the neuropeptides secreted by them, which, in turn, were named as a reference to an initial function described for them, usually unrelated to their role in the energy balance control. Thus, although we will point out the origin of these peptides'

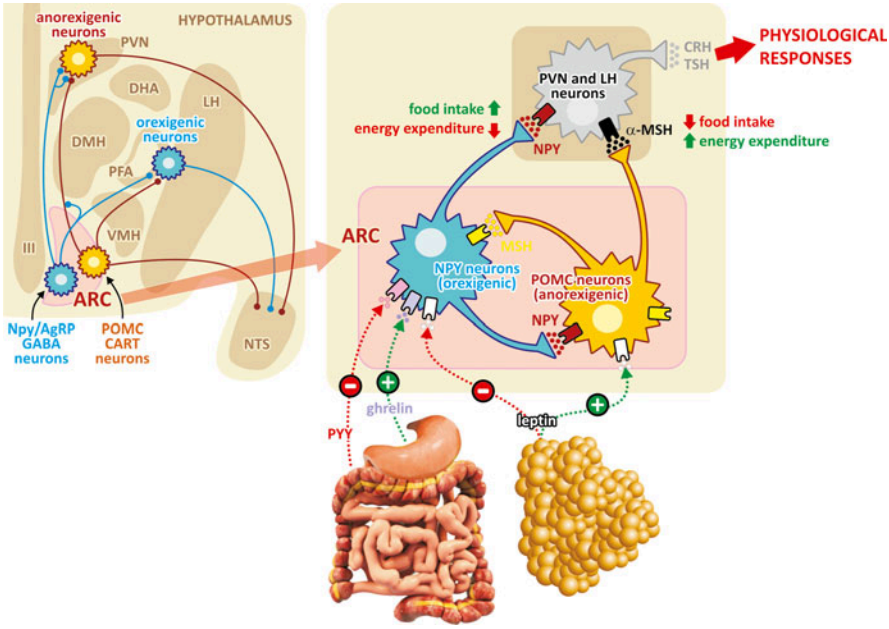


Fig. 11.12 Schematic representation of the subpopulation of the ARC neurons: the NPY/AgRP and the POMC neurons. PYY and leptin inhibit NPY/AgRP neurons, decreasing appetite by the reduction of NPY release. Leptin activates POMC neurons, leading to the release of POMC-derived peptides, such as α-MSH, which bind to melanocortin receptors (such as MC4R) in other hypothalamic areas (mainly in the PVN and LH), promoting satiety. Ghrelin stimulates NPY/AgRP neurons, inducing the expression and secretion of NPY and AgRP, which are antagonists of melanocortin receptors, triggering hunger and decreasing energy expenditure

nomenclature, the names themselves will not be useful for the understanding of their functions in the energy balance control.

The NPY/AgRP neurons express the neuropeptide Y (NPY), a neuropeptide with strong orexigenic properties, which stimulates food ingestion. These neurons also express the Agouti-related protein (AgRP), a protein related to fur pigmentation in rodents that when mutated causes obesity in mice.

The POMC neurons express pro-opiomelanocortin (POMC), whose cleavage originates several endocrine- and neuroendocrine-active peptides, including the anorexigenic peptides α- and β-melanocyte-stimulating hormones (α-MSH and β-MSH) and the adrenocorticotropic hormone (ACTH). Among these peptides, α-MSH and β-MSH are directly related to the increase in energy expenditure in the organism, as it will be detailed in Sect. 11.2.

Food ingestion behavior is established by a concerted action of the anorexigenic and orexigenic hormones on these two neuron populations. During fasting, ghrelin is secreted by the stomach cells leading to an increase in its blood level. In ARC, ghrelin binds to NPY/AgRP neurons, stimulating the synthesis and secretion of NPY, which results in an increase in appetite (Fig. 11.12). During feeding, gastrointestinal peptides, such as PYY, are released from the intestine cells. PYY also acts on NPY/AgRP neurons, but its binding to these neurons inhibits NPY release, diminishing appetite. As a response to the increase of adiposity, the adipocytes secrete leptin, which binds to both NPY/AgRP and POMC neurons. The effects of leptin binding to each of these neurons are antagonic: it inhibits the NPY/AgRP neurons and stimulates POMC neurons, stopping food ingestion by a simultaneous decrease in the NPY-induced appetite and increase in satiety promoted by anorexigenic POMC-derived peptides (Fig. 11.12).

The next question to be posed is how the ghrelin and leptin signals are deciphered by the hypothalamus producing their respective physiological responses: the changes in the feeding behavior and the energy expenditure rate in the body. The answer resides in the fact that both NPY/AgRP and POMC neurons are also connected to neurons in other hypothalamic areas (mainly in the PVN and LH; Fig. 11.12, see also Fig. 11.2), which mediate the recognition of the feeding status to a systemic response in the organism. The PVN and LH neurons express the melanocortin receptors (MCR1–MCR5), whose agonists are the POMC-derived peptides and the antagonist is NPY. Upon stimulation by POMC-derived peptides, PVN and LH neurons secrete the corticotropin-releasing hormone (CRH) and thyrotropin-releasing hormone (TRH), which mediate anorexigenic, thermogenic responses, and even physical activity (behavioral) responses.

In summary, the orexigenic and anorexigenic signals (such as ghrelin and leptin) bind to the ARC neurons leading to the secretion of POMC-derived peptides or NPY. These peptides act on the PVN and LH neurons, stimulating or inhibiting the release of CRH and TRH, which in turn regulate the anorexigenic and thermogenic responses in the organism. The integration of these events and players is known as the melanocortin system, which is considered the most efficient model to explain the neuronal control of the long-term energy balance in the organism.

11.2 Control of Energy Expenditure

Energy metabolism in animals can be resumed as the chemical processes through which the energy obtained from food—energy intake—is converted to work and heat—energy expenditure. The total energy expenditure in an organism can be subdivided for didactic purposes in three components: (a) the basal metabolic rate, which corresponds to the energy involved in all metabolic reactions required for the cellular functions; (b) the adaptive thermogenesis, which consists in the production of heat in response to cold or diet; and (c) the physical activity, both the spontaneous, such as that necessary to maintain posture, and the voluntary, including exercise in sports, leisure, and other activities (Fig. 11.13).

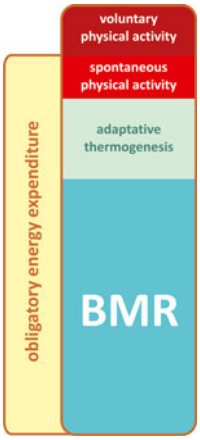


Fig. 11.13 The three components of energy expenditure: the basal metabolic rate (BMR), represented in *blue*, corresponds to energy involved in the metabolic reactions necessary to maintain cellular functions; the adaptive thermogenesis, represented in *green*, corresponds to the regulated energy release in the form of heat as a response to changes in ambient temperature or diet; and physical activity, represented in *red*, corresponds to the energy expenditure during voluntary or involuntary physical activities

11.2.1 Adaptive Thermogenesis

The adaptive thermogenesis is one of the components of energy expenditure and can be defined as the production of heat due to a regulated increase in the metabolic rate. It is controlled by the brain, both through the stimulation of the sympathetic system and through the hypothalamus–pituitary–thyroid axis, as a response to triggering signals including cold exposure and food intake (Fig. 11.14).

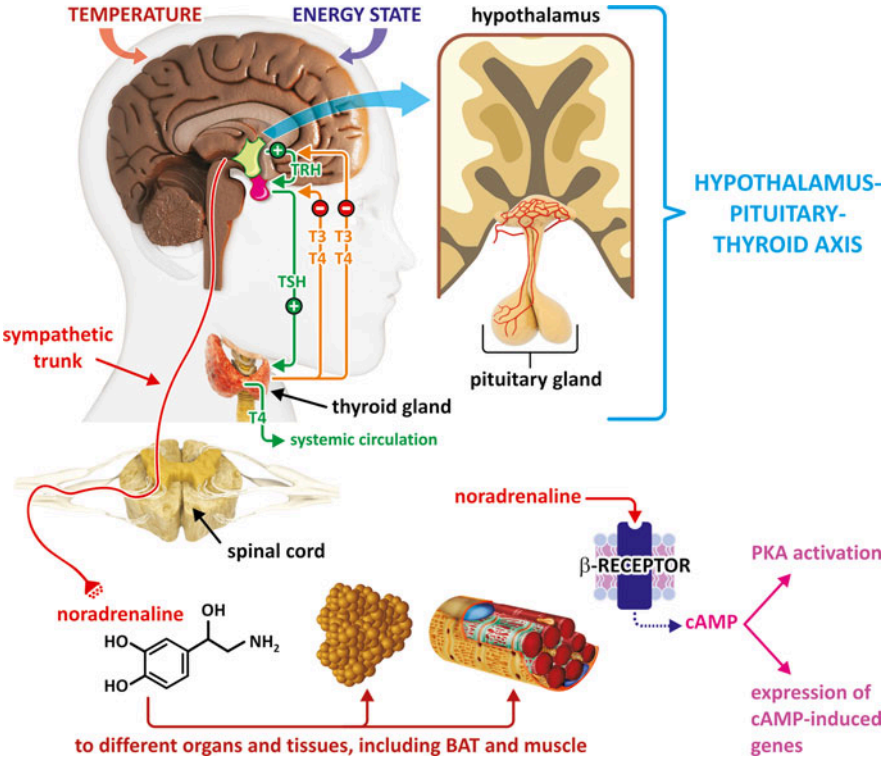


Fig. 11.14 Changes in the ambient temperature and in diet are sensed by the brain resulting in noradrenaline release by the sympathetic nerves and in the activation of hypothalamus–pituitary–thyroid axis. Noradrenaline acts on brown adipose tissue (BAT) and muscles through its binding to adrenergic receptors, which leads to an increase in intracellular AMPc concentration and the consequent modulation of the activities of some PKA-regulated enzymes as well as the induction of the expression of the AMPc-induced genes. Thyroid hormones (T3 and T4) play a major role in controlling energy expenditure (see Sect. 11.2.2). They are released by the thyroid gland as a response to the thyroid-stimulating hormone (TSH) produced by pituitary, whose secretion is stimulated by the hypothalamic thyrotropin-releasing hormone (TRH). T3 and T4 exert a negative feedback control over the hypothalamus and pituitary

11.2.1.1 Cold-Induced Thermogenesis

Shivering is one of the most primitive responses induced by exposure to cold, which is especially important to the adaptive thermogenesis in adult humans and large mammals. However, in human newborns and other small mammals, the most important cold-induced adaptive thermogenesis involves the release of noradrenaline by the sympathetic nerve terminals, particularly within the brown adipose tissue (BAT).

BAT thermogenic capacity is mainly sustained by the high activity of the uncoupling protein 1 (UCP1 or thermogenin) in this tissue (see Sect. 6.2.5). Binding of noradrenaline to BAT adrenergic receptors leads to an increase in the intracellular cAMP concentration, which in turn mediates the modulation of enzymatic activities, as well as the expression of the cAMP-induced genes (Fig. 11.14), including the gene for UCP1 and those that codify the deiodinase 2 (D2), the enzyme involved in the intracellular production of the active form of the thyroid hormone, T3 (see Sect. 11.2.2). A synergic effect between T3-dependent and cAMP-dependent actions, which includes the increase in fatty acid oxidation associated with mitochondria uncoupling, results in energy dissipation in the form of heat.

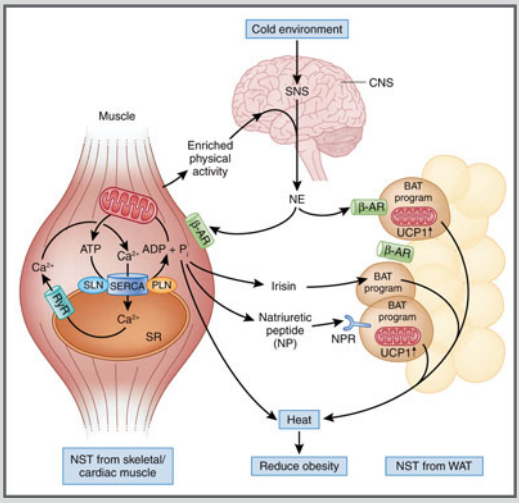
For many years, the role of BAT in the thermogenic response in humans has been considered to be relevant only in newborns due to the insignificant amounts of brown fat found in adults. However, in the last few years, a number of new findings revealed that in addition to the classical BAT, there is an inducible thermogenic adipose tissue, also referred to as beige adipocytes, which are interspersed in white fat depots in adult humans. The origin of the beige adipocytes is not completely clear, but it seems that they have a complete different origin than that of the classical brown fat cells. Conversely, strong evidence supports a common origin for classical BAT and skeletal muscle cells. Interestingly, muscle cells recently also appear as potential players in the cold- and diet-induced thermogenic responses, with a mechanism involving a controlled cycling of calcium (see Box 11.5).

Box 11.5

Exposure to cold induces norepinephrine (NE) release by the sympathetic nerve terminals within the skeletal muscle. NE binding to β-adrenergic receptors in muscle cells mediates the activation of the sarcoplasmic reticulum calcium ATPase (SERCA). Two proteins are known to associate to SERCA in muscle, phospholamban and a recently identified protein named sarcolipin. Sarcolipin uncouples SERCA-mediated ATP hydrolysis from Ca^{2+} pumping, resulting in the dissipation of energy in the form of heat. Simultaneously, NE induces the "browning" of the adipose tissue in a combined action with at least two other muscle-derived peptides: irisin and natriuretic peptide, which are secreted by skeletal and cardiac muscles, respectively. One of the main responses that lead to the "browning" of the adipocytes is the expression of UCP1 in the mitochondria, which induces heat production (see Sect. 6.2.5).

(continued)

Box 11.5 (continued)



Schematic representation of the thermogenic responses in the muscle and in the adipose tissue. *CSN* central nervous system; *SNS* sympathetic nervous system; *NE* norepinephrine; *β-AR* β-adrenergic receptor; *SERCA* sarcoplasmic reticulum Ca^{2+}-ATPase; *PLN* phospholamban; *SLN* sarcolipin; *RyR* ryanodine receptor; *SR* sarcoplasmic reticulum; *BAT* brown adipose tissue. Figure reproduced by permission from Macmillan Publishers Ltd. from Kozac LP & Young ME. Nat. Med. 18:1458–1459, 2012

11.2.1.2 Diet-Induced Thermogenesis

Adaptive thermogenesis also occurs in response to diet. The diet-induced thermogenesis can be defined as the increase in energy expenditure above the basal fasting level as a response to food intake. This phenomenon is illustrated in Fig. 11.15a, which represents the mean pattern of the diet-induced thermogenesis throughout a day. Indeed, feeding increases the metabolic rate by 25–40 %, while starvation reduces it to up to 40 %, and these compensatory changes in metabolism may be the explanation for the low efficacy of treatments for obesity. This can be exemplified by a study in which the energy expenditure was measured in obese and nonobese volunteers submitted to overfeeding or underfeeding diets that resulted in controlled gain or loss of weight, respectively. The results of this study, shown in Fig. 11.15b, revealed that a 10 % increase or 10 % decrease in body weight was associated to compensatory changes in energy expenditure in both the obese and the nonobese groups of individuals.

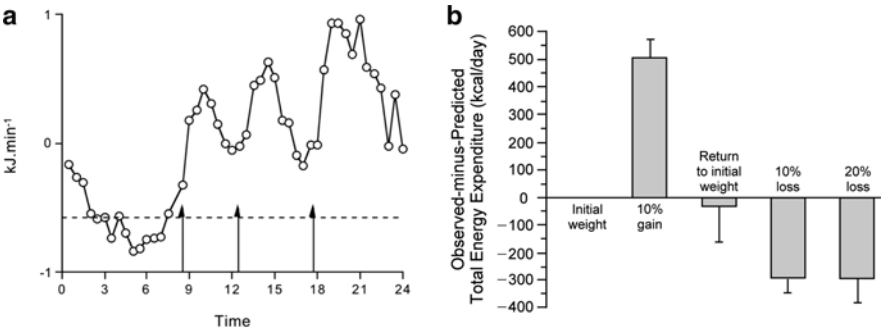


Fig. 11.15 (**a**) Mean pattern of diet-induced thermogenesis measured throughout the day in 37 subjects. The *dashed line* indicates the level of basal metabolic rate and the *arrows* indicate the time of the meals (Reproduced from Westerterp Nutr. Metab. 1:5, 2004). (**b**) Changes in the 24-h energy expenditure of 41 subjects subjected to controlled diets that resulted in 10 % weight gain or 10 % or 20 % weight loss, as well as when they returned to their original weights (Reproduced with permission from Leibel et al., N. Engl. J. Med. 332:621–628, 1995)

The mechanisms through which food intake affects the metabolic rate are not completely clear, but they seem to involve the "browning" of the adipose tissue (see previous topic) and the melanocortin system described in Sect. 11.1.5.

11.2.2 Role of Thyroid Hormones

The thyroid hormones thyroxine (T4) and triiodothyronine (T3) are iodinated hormones produced in the thyroid gland that play a major role in the control of energy expenditure by mediating the increase in the metabolic rate. This can be clearly illustrated by the fact that patients with hyperthyroidism show up to a 50 % increase in the total body energy expenditure, while in severe hypothyroidism it can fall by as much as 50 %.

The thyroid gland is a butterfly-shaped endocrine organ composed of two lobes, located on the anterior side of the neck (Fig. 11.16a). The name thyroid comes from the Greek word *thyreos*, which means shield, in a reference of its position around the larynx and trachea. Histological observation of the thyroid shows spherical follicles formed by a single layer of polarized epithelial follicular cells (Fig. 11.16b). The basolateral membrane of the follicular cells is in contact with the bloodstream from where iodide is uptaken through a sodium/iodide co-transporter (NIS, from Na^+/I^- symporter) (Fig. 11.16c). This mechanism makes the iodide concentration inside the thyroid to be 20- to 50-fold higher than in the blood. The follicular cells surround a region called follicular lumen, which is filled with a colloidal substance composed mainly by a large glycoprotein named thyroglobulin. Around the follicles, the parafollicular cells form the thyroid parenchyma, which produce the hormone calcitonin, involved in calcium homeostasis.

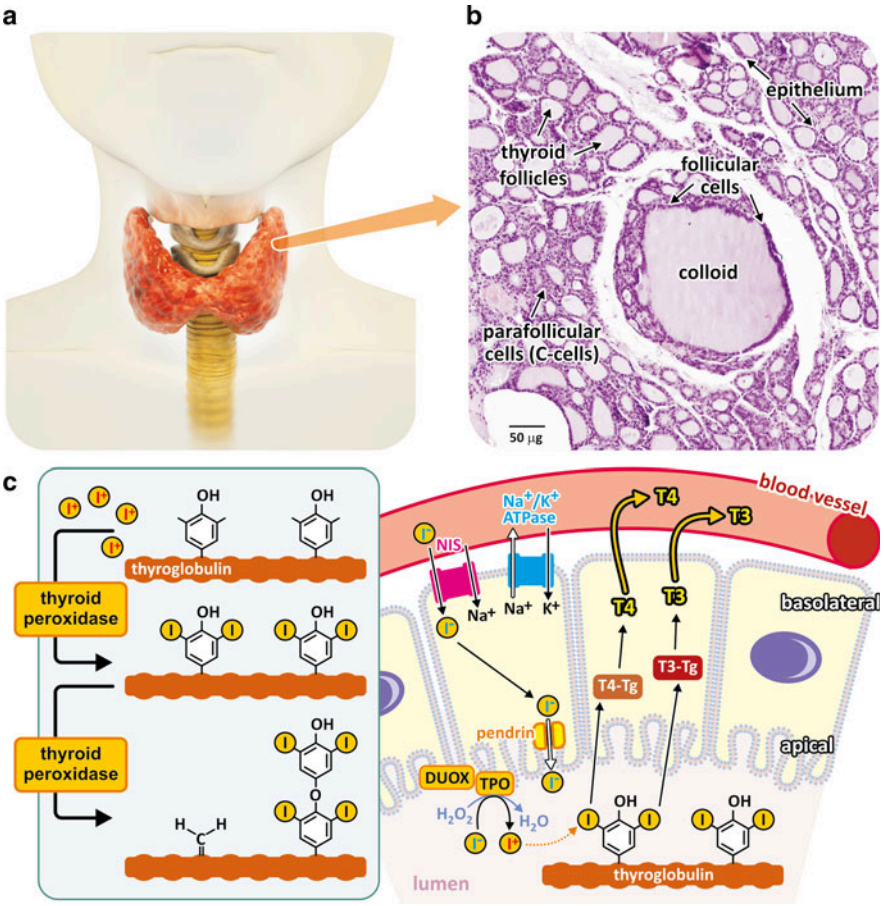


Fig. 11.16 (**a**) Anatomy of the thyroid gland. (**b**) Histological image of thyroid follicles showing the follicular cells, the colloid, and the parafollicular cells. (**c**) Iodine organification and the synthesis of thyroid hormones. The follicular cells uptake iodide from the blood through the transporter NIS (Na^+/I^- symporter) located on their basolateral membranes. The activity of the Na^+/K^+-ATPase on the follicular cell membrane maintains a low intracellular concentration of Na^+. At the apical membrane, the enzyme thyroperoxidase (TPO) oxidizes the iodide and incorporates the iodine to tyrosine residues of thyroglobulin in the lumen of the follicles. Iodinated tyrosines are conjugated forming T3 or T4 (*inset*)

The first step of thyroid hormone synthesis is called the "organification" of iodine, which consists in the iodide oxidation followed by its incorporation to tyrosine residues of the thyroglobulin (Fig. 11.16c). Thyroperoxidase (TPO), an enzyme located on the apical membrane of the follicular cells, catalyzes iodide oxidation using hydrogen peroxide as the oxidizing agent and the subsequent iodination of thyroglobulin, generating either a mono-iodinated tyrosine (MIT) or di-iodinated tyrosine (DIT). The next step in the synthesis of thyroid hormones is also catalyzed by TPO and consists of the coupling of two neighboring iodotyrosyl residues through the

formation of an ether bond between the iodophenol part of a donor iodotyrosyl and the hydroxyl group of the acceptor iodotyrosyl residue (Fig. 11.16c). The cleavage of the iodophenol group of the tyrosyl donor forms an alanine side chain that remains in the thyroglobulin polypeptide chain as dehydroalanine. The coupling of two DIT generates T4, whereas when a DIT and a MIT are coupled, the product is T3.

The secretion of the thyroid hormones depends on the endocytosis of the iodinated thyroglobulin from the colloid, which is stimulated by the thyroid-stimulating hormone (TSH) released from the pituitary gland. Thyroglobulin is then digested by lysosomal proteases, and the thyroid hormones are released in the bloodstream, where they circulate bound to specific binding proteins, mainly the thyroxine-binding globulin (TBG) and transthyretin (formerly known as thyroxine-binding prealbumin), but also by albumin.

After transport into the cells, the thyroid hormones bind to nuclear receptors (THR) that belong to the large superfamily of the steroid receptors (Fig. 11.17). The affinity of T3 for THR is about 100-fold higher than that of T4. THR acts as a hormone-activated transcription factor that regulates gene expression through binding

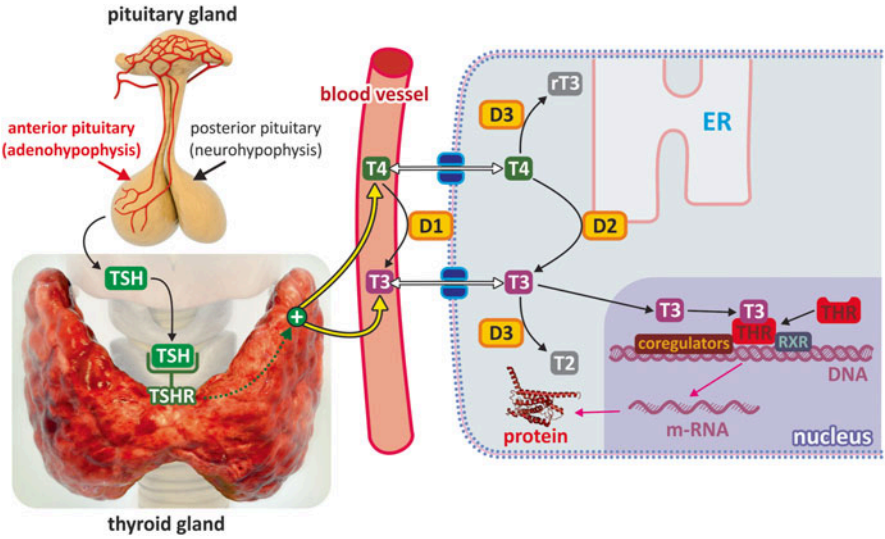


Fig. 11.17 Mechanism of action of thyroid hormones. The pituitary gland secretes the thyroid-stimulating hormone (TSH), which binds to the TSH receptor (TSHR) in the thyroid gland, stimulating the release of T4 (in higher amounts) and T3. Circulating T4 can be converted into T3 on the surface of some cells, especially liver and kidney cells, in a reaction catalyzed by the type 1 deiodinase (D1). T3 and T4 are transported into the cell. At the membrane of ER, T4 is converted to T3 through the action of the type 2 deiodinase (D2). Both T3 and T4 can also be inactivated by type 3 deiodinase (D3), which converts T4 to the inactive reverse T3 (rT3) or T3 to the inactive diiodothyronine (T2). T3 binds to the thyroid hormone receptors (THR) and the complex regulates the expression of specific genes by binding to DNA either as monomers, as homodimers, or as heterodimers with the retinoid X receptor (RXR)

to specific hormone-responsive elements in DNA (Fig. 11.17). THR can bind to DNA either as monomers, as homodimers, or as heterodimers with the retinoid X receptor (RXR), a nuclear receptor that binds 9-cis retinoic acid. The heterodimer seems to be the major functional form of the receptor. Additionally, in the absence of the hormone, the receptors function as transcription inhibitors, repressing the expression of some genes.

It is important to bear in mind that although T3 is more biologically active than T4 due to its higher affinity to THR, the major coupling reaction occurring inside the thyroid is the one that generates T4 (it is estimated that the thyroid gland secretes about 80 μg T4 and only 5 μg T3 each day). Thus, a critical step for the thyroid hormones action is the conversion of T4 into T3, which occurs mainly in the peripheral tissues. This reaction is catalyzed by the deiodinases, enzymes that remove one iodine atom from the outer ring of T4. There are three isoforms of deiodinases (Fig. 11.17). Type 1 deiodinase (D1) is expressed on the outer face of the plasma membrane of liver and kidney cells, being responsible for the production of T3 in the serum. Type 2 deiodinase (D2) is an intracellular isoform of the enzyme, expressed on the membrane of the endoplasmic reticulum, mainly in cardiac and skeletal muscles, adipose tissue, the central nervous system, and thyroid and pituitary glands. D2 is responsible for the control of cytoplasmic levels of T3. Type 3 deiodinase (D3) inactivates the thyroid hormones by catalyzing the removal of an iodine atom from the inner ring, which converts T4 to the inactive reverse T3 or T3 to inactive T2.

Thyroid hormones regulate a broad range of physiological processes including growth, development, and energy metabolism. The effects of thyroid hormones on energy metabolism can be clearly recognized in cases of hypo- or hyperthyroidism. Almost all patients with hyperthyroidism exhibit weight loss even under a high food intake diet, whereas weight gain is a very usual condition of hypothyroidism patients. This happens because the general effect of the thyroid hormones is to increase the metabolic rate by simultaneously activating, directly or indirectly, the metabolic pathways that consume and produce ATP. In this context, thyroid hormones are also critical for thermogenesis since the heat that is automatically released from the metabolic reactions is consequently increased.

The regulation of the metabolic rate by thyroid hormones occurs through different mechanisms depending on the tissue (Fig. 11.18). In muscle, their action includes the upregulation of the expression and the activity of Na^+/K^+-ATPase and sarcoplasmic reticulum Ca^{2+}-ATPase, increasing ATP consumption by these enzymes (see Sect. 10.3). Consequently, the cellular respiratory activity is increased to maintain the ATP levels. In BAT, thyroid hormones enhance noradrenaline signaling pathway as well as the expression of UCP1, sustaining the thermogenic effect mediated by the sympathetic nervous system (see Sect. 11.2.1).

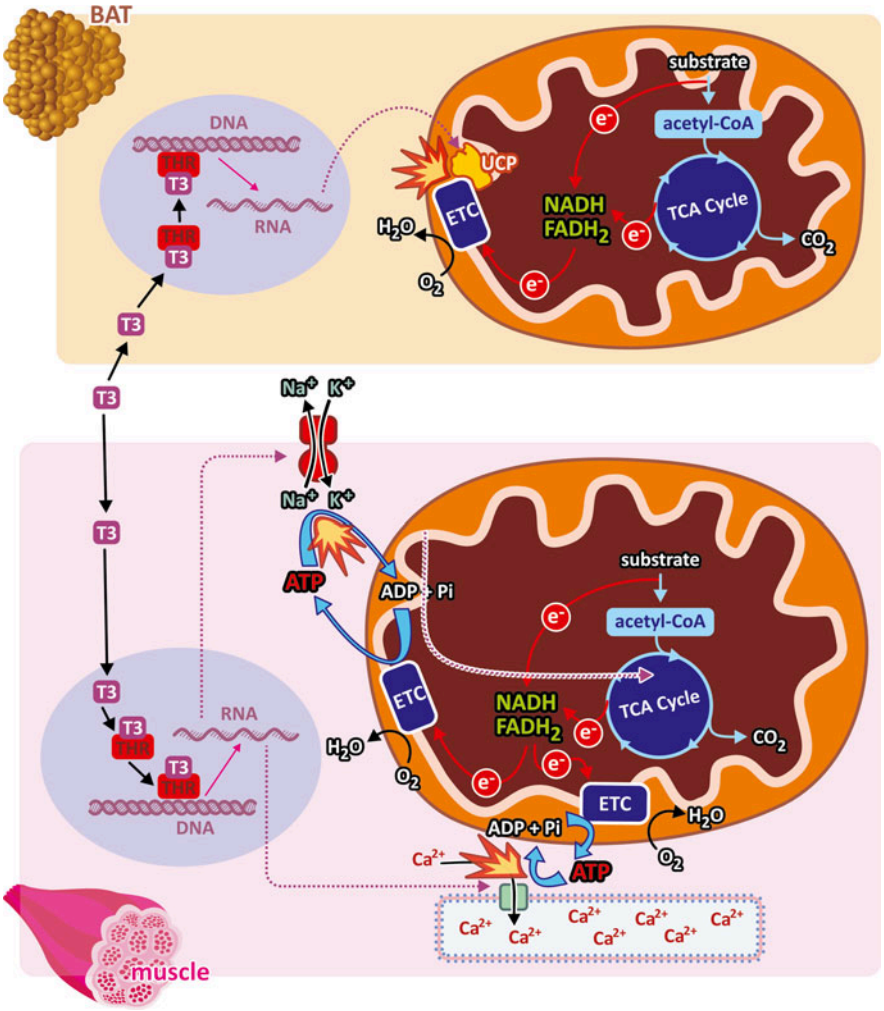


Fig. 11.18 Main metabolic effects of thyroid hormones in the peripheral tissues. Thyroid hormones induce the expression of UCP1 in BAT, increasing cellular respiratory activity, and of Na^+/K^+-ATPase and Ca^{2+}-ATPase in muscle, increasing ATP consumption by these enzymes. These effects accelerate the metabolic rate and lead to thermogenesis. The *dashed arrows* indicate the enzymes whose expression is induced by the thyroid hormones and the fire represents the thermogenic effect

Besides these actions on the peripheral tissues, it is now evident that the metabolic effects of the thyroid hormones are also mediated by their action on the central nervous system, specifically on hypothalamic areas such as ARC, VMH, and PVH (Fig. 11.19). In ARC, T3 upregulates the expression of the neuropeptides AgRP and NPY and downregulates the expression of POMC, leading to hyperphagia (see Sect. 11.1.5). Simultaneously, T3 inhibits AMPK in VMH, which activates the sympathetic nervous system leading to the enhancement of BAT thermogenic pro-

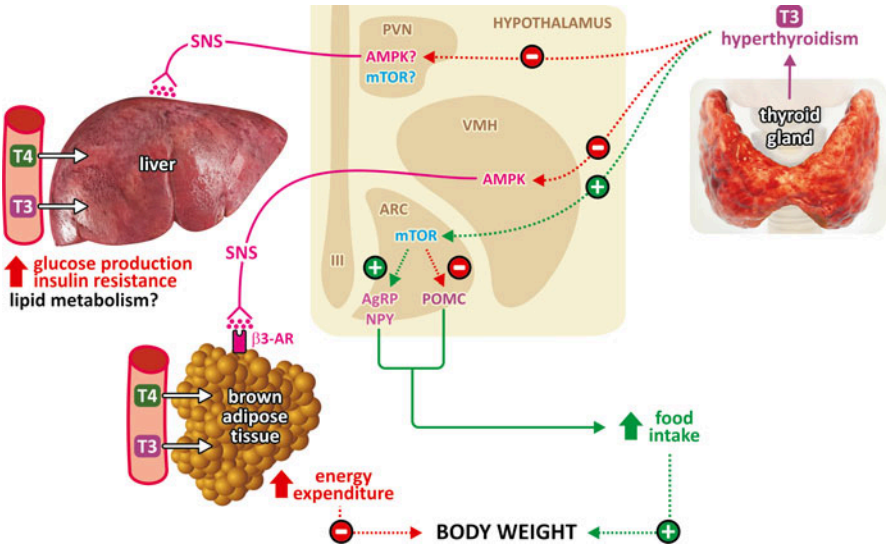


Fig. 11.19 Central actions of thyroid hormones. In ARC, T3 regulates feeding through mammalian target of rapamycin (mTOR), which upregulates the expression of AgRP and NPY and downregulates the expression of POMC, increasing food intake. In VMH, T3 inhibits AMPK, activating the sympathetic nervous system and the thermogenic program in BAT. Additionally, T3 action on PVH is probably involved in the control of hepatic glucose homeostasis

gram (see Sect. 11.2.1). Thus, these combined effects of thyroid hormones on hypothalamic regions promote an increase both in food intake and energy expenditure. Additionally, it is also evident that the central action of thyroid hormones also controls glucose metabolism in different tissues, although the mechanism through which this occurs are still unknown.

11.3 Obesity and the Metabolic Syndrome

Obesity is caused by a massive accumulation of adipose tissue, which is primarily a result of an imbalance between energy intake and energy expenditure. The increase in adipose mass is strongly associated to the development of insulin resistance and type 2 diabetes, which together with hyperlipidemia, glucose intolerance, and hypertension characterize a clinical entity sometimes referred to as metabolic syndrome.

A crucial clue for the understanding of the metabolic syndrome was the discovery that obesity itself causes an inflammatory state in the metabolic tissues, which in turn is tightly linked to the development of insulin resistance. In this section, we will discuss this metabolic-triggered inflammation, which has been recently termed metaflammation, a chronic, low-grade inflammatory response that seems to be initiated by the excess of nutrients.

11.3.1 *Chronic Inflammation and Insulin Resistance in Obesity*

Adipose tissue occupies a central position in the development of the obesity-induced inflammation. The first observation that clearly linked obesity and chronic inflammation was that the pro-inflammatory cytokine tumor necrosis factor-α (TNF) is overexpressed and secreted in higher levels by adipocytes of obese individuals (Fig. 11.20a). After this first discovery, it became clear that besides TNF, several other cytokines and inflammatory mediators were produced in high levels by adipose and also by other metabolic tissues from obese individuals, generating a chronic inflammatory state. Additionally, immune cells such as macrophages are recruited to and infiltrate these inflamed tissues, amplifying the inflammatory response.

The connection between the chronic inflammatory state in obesity and the development of insulin resistance became apparent from the discovery that the signaling pathway triggered by TNF (and by other overexpressed cytokines) blocks insulin action downstream of the activation of its receptor (Fig. 11.20b). In summary, what occurs is the following. In response to the inflammatory signals, intracellular kinases, mainly JNK (c-jun N-terminal kinase) and IKK-β (inhibitor of nuclear factor-κB (NF-κB) kinase-β), are activated. These kinases have as one of their substrates IRS (the insulin receptor substrate), which is phosphorylated by them in Ser residues instead of the Tyr residues that are phosphorylated in response to the insulin signaling pathway (see details of the insulin signaling pathway in Sect. 8.4.2).

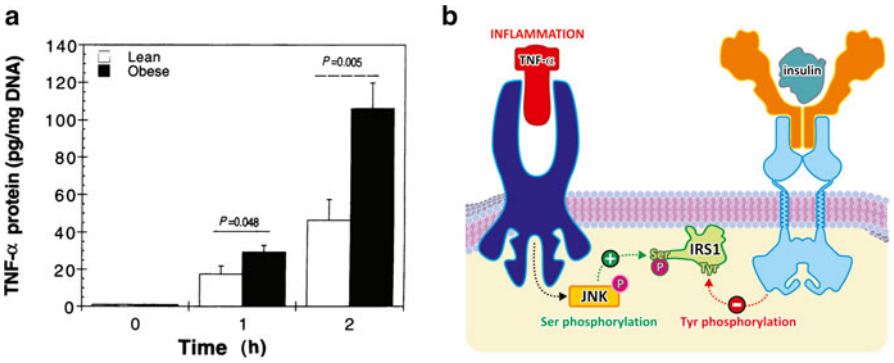


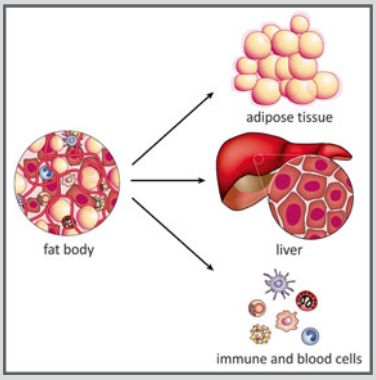
Fig. 11.20 (**a**) Comparison of TNF secretion during 2 h by the same amount of adipocytes explanted from the adipose tissue from 18 lean and 19 obese female subjects (Reproduced with permission from Hotamisligil et al., J. Clin. Invest. 95:2409–2415, 1995). (**b**) Integration of inflammation and insulin signaling pathways. Binding of TNF to its cellular receptor triggers the activation of the kinases JNK and IKK-β, which catalyze the Ser phosphorylation of IRS. IRS phosphorylation in Ser residues blocks its phosphorylation in Tyr residues by the insulin receptor, inhibiting insulin action

IRS phosphorylation in Ser residues impairs its phosphorylation in Tyr by the insulin receptor, leading to the inhibition of insulin-mediated cellular responses.

At a first glance, it is intriguing that the control of metabolism would be so tightly linked to the inflammatory process. Under an evolutionary perspective, one can understand the development of the immune and the metabolic responses as basic requirements for survival. The efficient use of the available nutrients and the ability to successfully eliminate the pathogens are essential features from the time of primitive organisms. Clues sustaining the coevolution of these processes can be found in nature, for example, in the case of the insects, in which the control of metabolic homeostasis and the immune responses are carried out by the same organ, the fat body (Box 11.6).

Box 11.6 Coevolution of Immune and Metabolic Responses

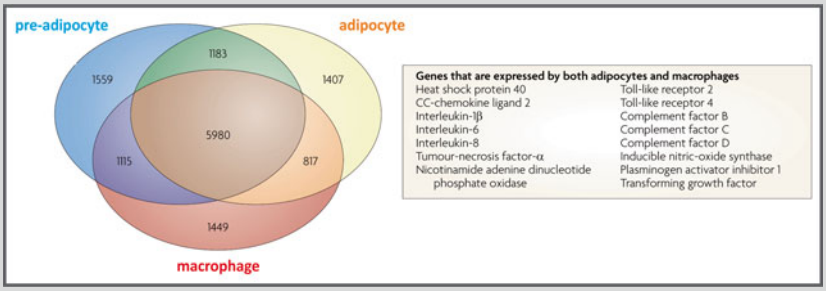
In insects, a single organ, the fat body, accumulates the functions that are carried out in mammals by the liver, the adipose tissue, and the immune cells. The fat body is the largest organ in the insect body cavity, and it is the major site of intermediate metabolism in these organisms. It metabolizes and stores lipids, carbohydrates, and proteins; it is the target for the majority of the insect hormones and also synthesizes the hemolymph proteins. Additionally, the fat body cells express the innate immune receptors, proteins that recognize molecular patterns specific of pathogens, in high levels. Through the activation of these receptors, the fat body produces and secretes a number of antimicrobial agents and regulators of cellular immune response. It is interesting to note that the metabolic organs of the more complex organisms contain resident immune cells, such as the Kupffer cells in the liver and the macrophages in the adipose tissue.



Organization of the metabolic and immune cells in insect and mammals (Reproduced with permission from Hotamisligil, Nature 444:860–867, 2006)

(continued)

Box 11.6 (continued)
Another remarkable evidence of the common evolutionary origin of the metabolic and immune responses is the fact that macrophages and adipocytes show an extensive genetic and functional overlap, with several genes expressed preferentially or approximately equally by pre-adipocytes, adipocytes, and macrophages, including many inflammatory genes (see figure below).



Transcriptional profile overlapping in pre-adipocytes, adipocytes, and macrophages (*left*) and the list of the inflammatory genes expressed in both adipocytes and macrophages (*right*) (Reproduced with permission from Hotamisligil & Erbay, Nat. Rev. Immunol. 8:923–934, 2008)

11.3.2 Origin of Inflammation in Obesity

The discovery that obesity is linked to a chronic inflammatory state raises the question of which are the triggers of inflammation in this case. This issue is now under intense investigation but the obesity-related factors that initiate the inflammatory process have not been identified so far.

The classical inflammatory response is driven by the contact of the host cells with molecular components specifically present in pathogens (known as pathogen-associated molecular patterns, PAMPs) or exposed during tissue injury (the damage-associated molecular patterns, DAMPs). These molecules are recognized by cellular receptors, known as pattern recognition receptors (PRR), which have a central role in the innate immune response. PRR can be membrane-bound receptors, such as the Toll-like receptors (TLRs), which are located on the plasma or endosomal membranes, or cytoplasmic receptors, such as the NOD-like receptors (NLRs) and the RIG-I-like receptors (RLRs). The binding of PAMPs or DAMPs to the PRR triggers a chain of signaling events that result in the activation of a number of transcription factors, which induce the expression of several pro-inflammatory cytokines, including TNF-α, and antimicrobial molecules, such as interferons (Fig. 11.21).

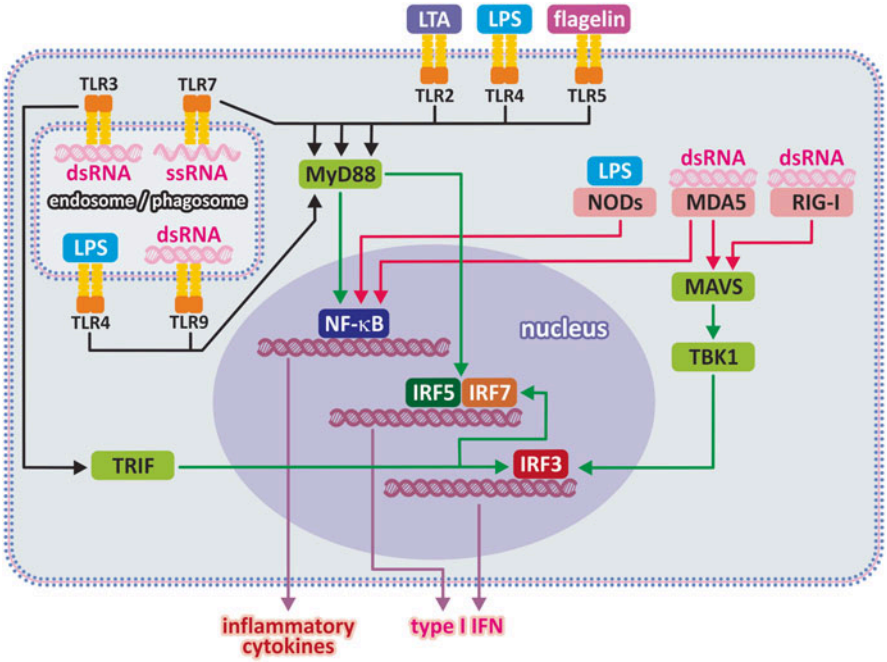


Fig. 11.21 Sensing microbial molecular patterns by host innate immune system. Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-like receptors (RLRs), and C-type lectin receptors (CLRs) are pattern recognition receptors (PRR) expressed on cellular membranes or in the cytoplasm of a variety of cells in host tissues. Ligand binding to PRR triggers different signaling pathways that culminate with the activation of transcription factors, such as the IRFs, AP-1, and NF-κB, inducing the expression of interferons α and β (IFNα, β) and several pro-inflammatory cytokines. They recognize different microbial components, such as cell walls, microbial (and modified host) nucleic acids, bacterial motor flagellin, or stress (danger)-induced molecules. The information of ligand binding to PRR is transmitted to the transcription factors through some adaptor proteins, including MyD88, TRAM, TRIF or MAVS. Additionally, some NLRs can activate caspase-1, which cleaves the pro-IL-1β and pro-IL-18 in the active IL-1β and IL-18 that are released from the cell

The starting signal for the inflammatory response caused by overfeeding is still unclear. One hypothesis is that nutrients themselves would be recognized by innate immune system, although probably this occurs only when nutrient are in excess. This would happen if nutrients were not the preferential ligands of PRR, binding to these receptors with low affinity. During normal feeding the blood levels of nutrients are maintained low due to their rapid metabolization. Thus, very few nutrient molecules would bind PRR and trigger inflammation. Conversely, as nutrient concentration is maintained high enough to allow PRR occupation, the innate immune response is triggered, resulting in the establishment of the inflammatory state. Indeed, there is evidence that saturated fatty acids are recognized by one of the PRR, the Toll-like receptor 4 (TLR4), the PRR that usually recognizes the bacterial

lipopolysaccharide (LPS). Alternatively, it is also speculated that the increase in the intestine permeability during feeding allows some pathogens or inflammatory molecules (such as LPS) to enter the organism together with the nutrients, triggering inflammation.

Another explanation for the inflammatory effect of overnutrition is related to a cellular response known as endoplasmic reticulum stress (ER stress). ER is the site of protein synthesis and the place where all the secretory and membrane proteins are assembled and folded. The accumulation of unfolded or misfolded proteins in this organelle triggers ER stress, a series of events that inhibit protein synthesis, increase protein degradation, and increase the expression of chaperone proteins, known as the unfolded protein response (UPR). Among the triggers of ER stress, we can cite the high concentrations of saturated fatty acids and glucose (characteristics of the high caloric diets) and hypoxia. UPR leads to the upregulation of the production of inflammatory mediators either directly through the activation of NF-κB or via JNK and IKK-β (Fig. 11.22). The inflammatory mediators themselves also activate JNK and IKK-β pathways, as discussed in the previous sections, amplifying inflammation and inhibiting the insulin signaling pathway.

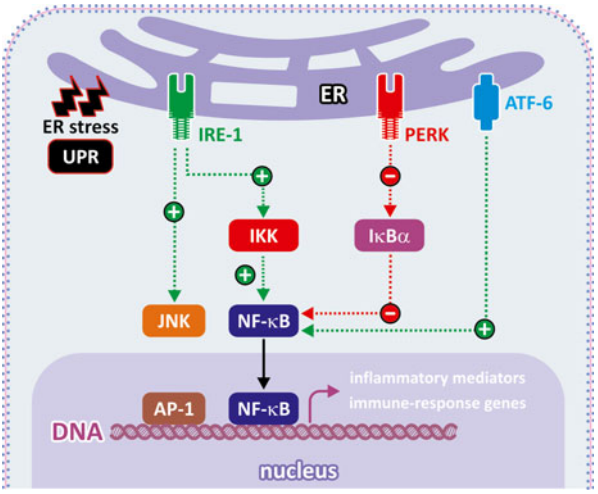


Fig. 11.22 ER stress pathways leading to inflammation. Three components of ER membrane are implicated in the inflammatory response, IRE-1, PERK, and ATF-6. IRE-1 acts either through its association with TRAF2, which activates JNK and IKK, and consequently AP-1 and NF-κB, or through the splicing of XBP1 mRNA. PERK inhibits the translation of IκBα, an inhibitor of NF-κB, thereby increasing NF-κB transcriptional activity. ATF-6 also increases NF-κB transcriptional activity

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Credits

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