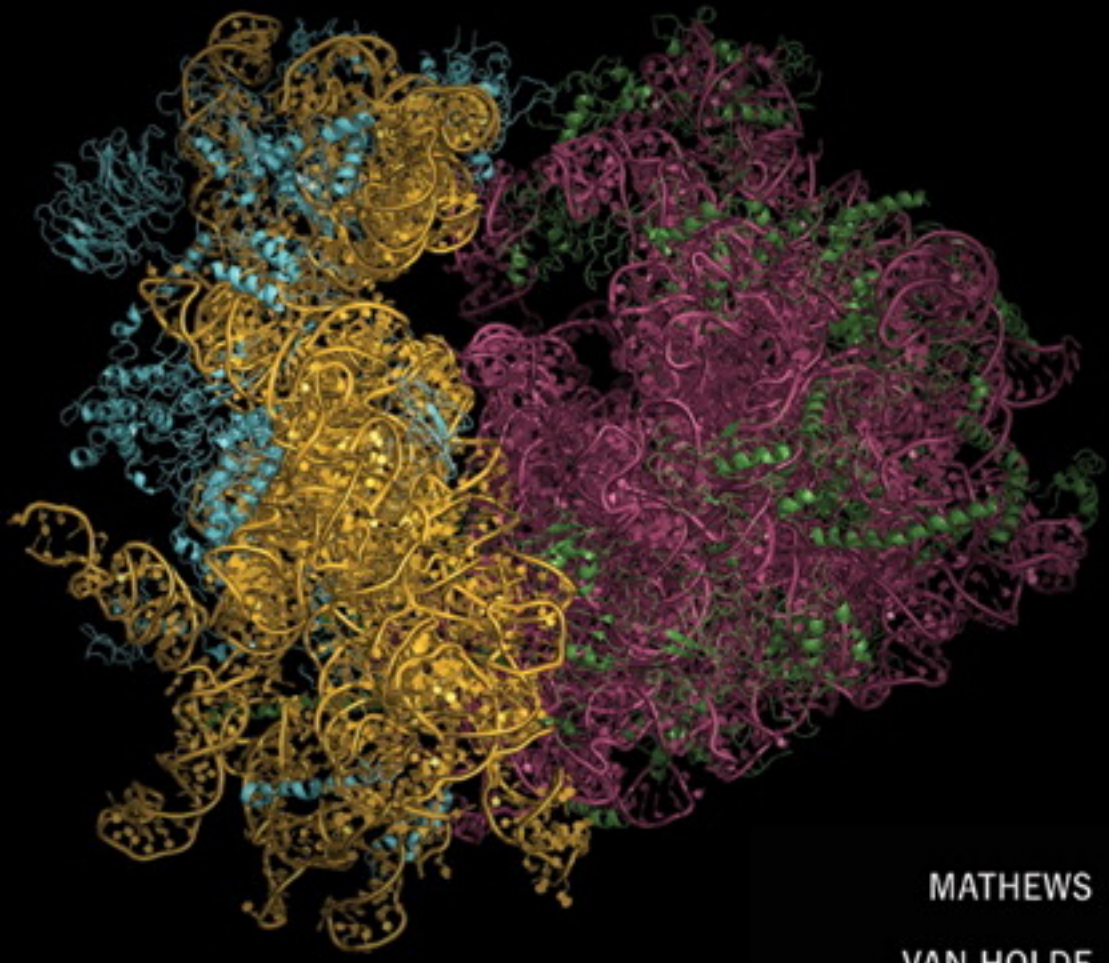
FOURTH EDITION

BIOCHEMISTRY



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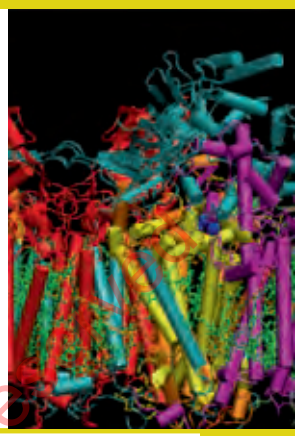


CHAPTER 12

Chemical Logic of Metabolism

A chemist carrying out an organic synthesis rarely runs more than one reaction in a single reaction vessel. This procedure is essential to prevent side reactions and to optimize the yield of the desired product. Yet a living cell carries out thousands of reactions simultaneously, with each reaction sequence controlled so that unwanted accumulations or deficiencies of intermediates and products do not occur. Reactions of great mechanistic complexity and stereochemical selectivity proceed smoothly under mild conditions—1 atm pressure, moderate temperature and osmotic pressure, and a pH near neutrality. How do cells avoid metabolic chaos? A goal of the next several chapters is to understand how cells carry out and regulate these complex reaction sequences.

A First Look at Metabolism

As noted above, a principal task of the biochemist is to understand how a cell regulates its myriad reaction sequences and, in so doing, controls its internal environment. In Chapter 11 we discussed the properties of individual enzymes and control mechanisms that affect their activity. Here we consider specific reaction sequences, or **pathways**; the relationship between each pathway and cellular architecture; the biological importance of each pathway; control mechanisms that regulate **flux**, or intracellular reaction rate; and experimental methods used to investigate metabolism. Figure 12.1, a simplified view of the processes we shall consider, illustrates two important principles. First, metabolism can be subdivided into two major categories—**catabolism**, those processes in which complex substances are degraded to simpler molecules, and **anabolism**, those processes concerned primarily with the synthesis of complex organic molecules (Figure 12.1a). Catabolism is generally accompanied by the net release of chemical energy, and anabolism requires a net input of chemical energy; we will see that these two sets of reactions are coupled together by ATP. Second, both catabolic and anabolic pathways occur in three stages of complexity—stage 1, the interconversion of

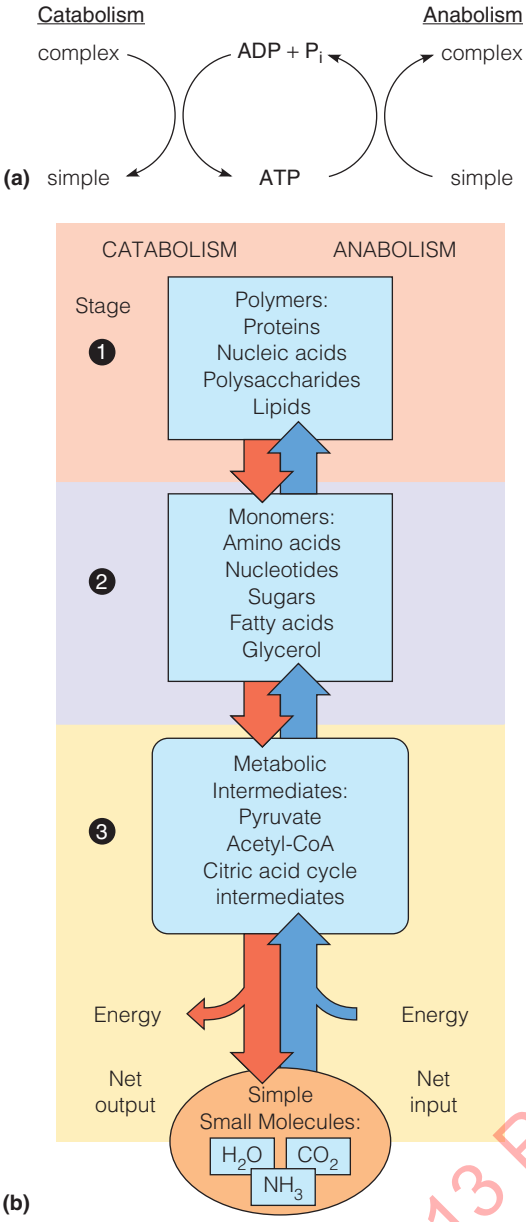


FIGURE 12.1
A brief overview of metabolism.

Intermediary metabolism refers primarily to the biosynthesis, utilization, and degradation of low-molecular-weight compounds (intermediates).

polymers and complex lipids with monomeric intermediates; stage 2, the interconversion of monomeric sugars, amino acids, and lipids with still simpler organic compounds; and stage 3, the ultimate degradation to, or synthesis from, inorganic compounds, including CO_2, H_2O, and NH_3 (Figure 12.1b). As we proceed through this chapter, we shall add detail to this figure, introducing you thereby to each major metabolic process and identifying the functions of each.

During our discussion we shall see that energy-yielding pathways also generate intermediates used in biosynthetic processes. Thus, although we will focus at first upon degradation of organic compounds to provide energy, you should be aware that metabolism is really a continuum, with many of the same reactions playing roles in both degradative and biosynthetic processes.

We shall also use the terms **intermediary metabolism, energy metabolism**, and **central pathways**. Intermediary metabolism comprises all reactions concerned with storing and generating metabolic energy and with using that energy in biosynthesis of low-molecular-weight compounds (intermediates) and energy-storage compounds. Not included are nucleic acid and protein biosynthesis from monomeric precursors. The reactions of intermediary metabolism can be thought of as those that do not involve a nucleic acid template because the information needed to specify each reaction is provided within the structure of the enzyme catalyzing that reaction. Energy metabolism is that part of intermediary metabolism consisting of pathways that store or generate metabolic energy. Chapters 13 through 22 present intermediary metabolism, and a major focus in Chapters 13 through 17 is on its energetic aspects. The central pathways of metabolism are substantially the same in many different organisms, and they account for relatively large amounts of mass transfer and energy generation within a cell; they are the quantitatively major pathways. Later we will identify and further discuss each central pathway, but for now let us focus on the overall process.

Most organisms derive both the raw materials and the energy for biosynthesis from organic fuel molecules such as glucose. The central pathways involve the oxidation of fuel molecules and the synthesis of small biomolecules from the resulting fragments; these pathways are found in all aerobic organisms. But a fundamental distinction among these organisms lies in the source of their fuel molecules.

Autotrophs (from Greek, "self-feeding") synthesize glucose and all of their other organic compounds from inorganic carbon, supplied as CO_2. In contrast, **heterotrophs** ("feeding on others") can synthesize their organic metabolites only from other organic compounds, which they must therefore consume. A primary difference between plants and animals is that plants are autotrophs and animals are heterotrophs. With the exception of rare insect-eating plants, such as the Venus flytrap, green plants obtain all of their organic carbon through photosynthetic fixation of carbon dioxide. Animals feed on plants or other animals and synthesize their metabolites by transforming the organic molecules they consume. Microorganisms exhibit a wide range of biosynthetic capabilities and sources of metabolic energy.

Microorganisms also show adaptability with respect to their ability to survive in the absence of oxygen. Virtually all multicellular organisms and many bacteria are strictly **aerobic** organisms; they depend absolutely upon **respiration**, the coupling of energy generation to the oxidation of nutrients by oxygen. By contrast, many microorganisms either can or must grow in **anaerobic** environments, deriving their metabolic energy from processes that do not involve molecular oxygen.

To the extent that biological molecules are synthesized ultimately from CO_2 that undergoes photosynthetic carbon fixation, the sun can be considered as the ultimate source of biological energy. However, this concept is not quite accurate because of the existence of a relatively large group of prokaryotic cells (both eubacteria and archaea) that derive their energy from other sources—for example, extremely **thermophilic** organisms, which live at temperatures as high as

100 °C or more, either in hydrothermal vents deep in the ocean or in geothermal vents in active volcanic regions. Although there is much to learn about metabolism in these organisms, it is clear that most of their metabolic energy does not come from sunlight.

Freeways on the Metabolic Road Map

Now let us return to the central pathways and their identification. Probably you have seen metabolic charts—those wall hangings like giant road maps that adorn biochemistry laboratories and offices. Figure 12.1 is a highly simplified metabolic chart. Figure 12.2 presents metabolism in more detail and is the basic road map for this section of the book. This figure will reappear in subsequent chapters, with the pathways presented in those chapters highlighted.

Faced with the thousands of individual reactions that constitute metabolism, how do we approach this vast topic? Our first concerns are with central pathways and with energy metabolism. Therefore, in the next several chapters we consider the degradative processes that are most important in energy generation—the catabolism of carbohydrates and lipids. We shall also consider how these substances are biosynthesized. These reactions are located in the middle of the metabolic charts and are illustrated with the biggest arrows—freeways, so to speak, on the metabolic road map.

The central pathways account for most of the mass transformations in metabolism.

The road map analogy is also useful when we consider directional flow in metabolism. Just as traffic flows from the suburbs to downtown in the morning and in the reverse direction in the evening, so also will we see that some conditions favor biosynthesis whereas others favor catabolism, and that parts of the same highways are used in both sets of processes.

Central Pathways of Energy Metabolism

Recall from our discussion of Figure 12.1 that metabolism can be subdivided into three stages of complexity of the metabolites involved. The first pathway that we present in detail (in Chapter 13) is **glycolysis**, a stage 2 pathway for degradation of carbohydrates, in either aerobic or anaerobic cells. As schematized in Figure 12.3, the major input to glycolysis is glucose, usually derived from either energy-storage polysaccharides or dietary carbohydrates. This pathway leads to pyruvate, a three-carbon α-keto acid. Anaerobic organisms reduce pyruvate to a variety of products—for example lactate, or ethanol plus carbon dioxide. These processes are called *fermentations* (see page 515). In oxidative metabolism (respiration), the major fate of pyruvate is its oxidation to a metabolically activated two-carbon fragment, **acetyl-coenzyme A**, or acetyl-CoA (see page 632). The two carbons in the acetyl group then undergo oxidation in the **citric acid cycle** (Figure 12.4). In aerobic organisms the citric acid cycle, presented in Chapter 14, is the principal stage 3 pathway. This cyclic pathway accepts simple carbon compounds, derived not only from carbohydrate but also from lipid or protein, and oxidizes them to CO_2. Using the freeway analogy again, we will see that numerous on-ramps from the highways and byways of stage 1 and stage 2 metabolism lead to the citric acid cycle. In fact, all catabolic pathways converge at this point.

In aerobic organisms all catabolic pathways converge at the citric acid cycle.

Oxidative reactions of the citric acid cycle generate reduced electron carriers whose reoxidation drives ATP biosynthesis, primarily through processes in the mitochondrial respiratory chain—**electron transport** and **oxidative phosphorylation**, shown also in Figure 12.4. As described in Chapter 15, the mitochondrial membrane uses oxidative energy to maintain a transmembrane gradient of hydrogen ion concentration (the **protonmotive force**), and discharge of this electrochemical potential energy powers the synthesis of ATP from ADP + P_i.

Stage 2 pathways other than glycolysis also deliver fuel to the citric acid cycle. Acetyl-CoA comes not only from pyruvate oxidation but also from the

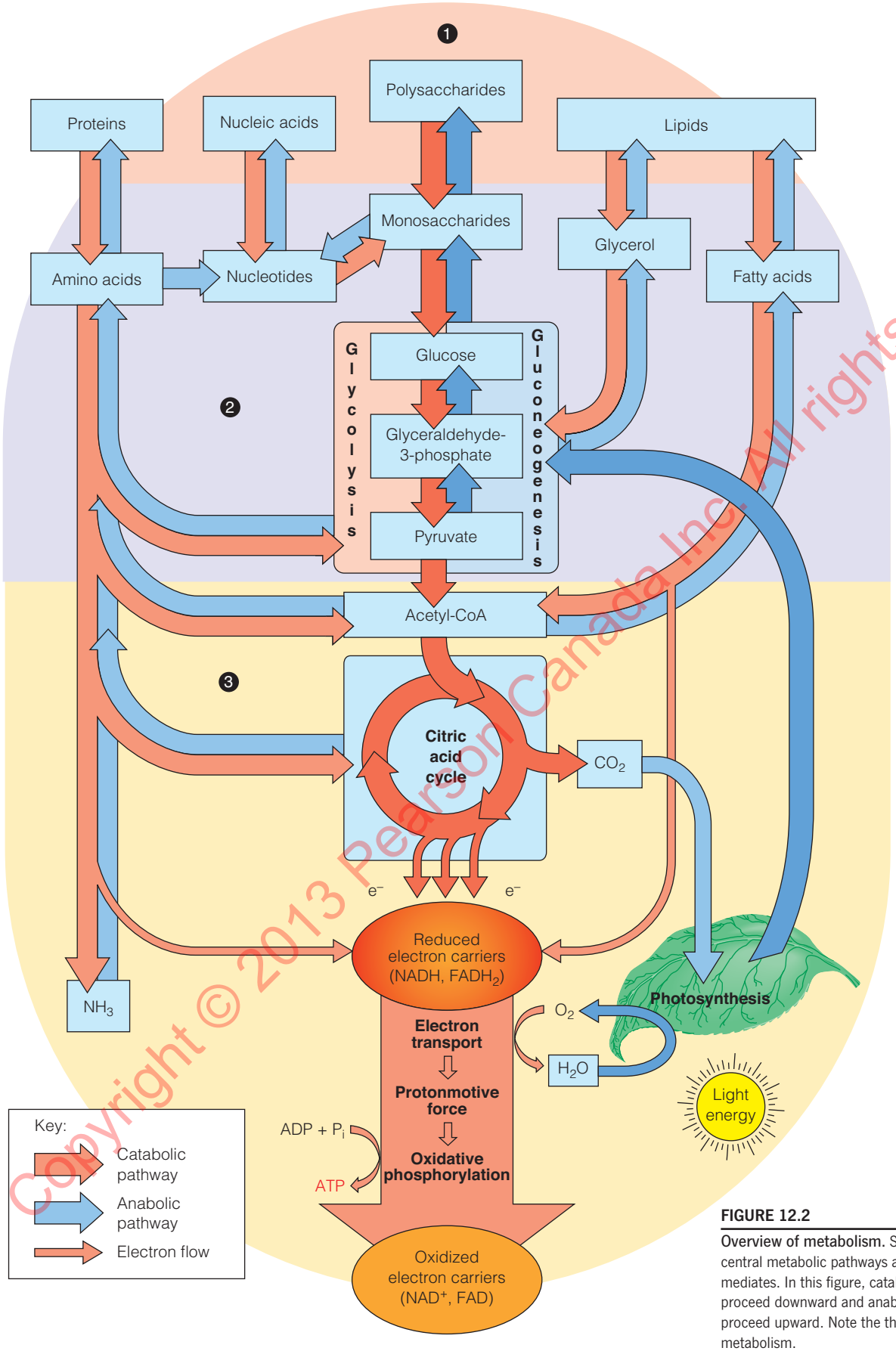


FIGURE 12.2

Overview of metabolism. Shown here are the central metabolic pathways and some key intermediates. In this figure, catabolic pathways (red) proceed downward and anabolic pathways (blue) proceed upward. Note the three stages of metabolism.

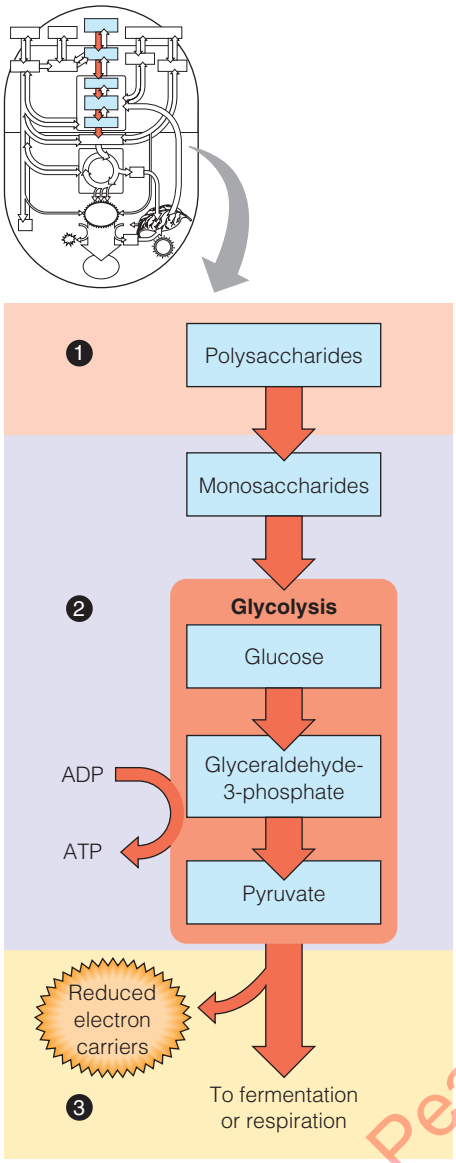


FIGURE 12.3

The initial phase of carbohydrate catabolism: glycolysis. Pyruvate either undergoes reduction in fermentation reactions or enters oxidative metabolism (respiration) via conversion to acetyl-CoA as shown in **Figure 12.4**.

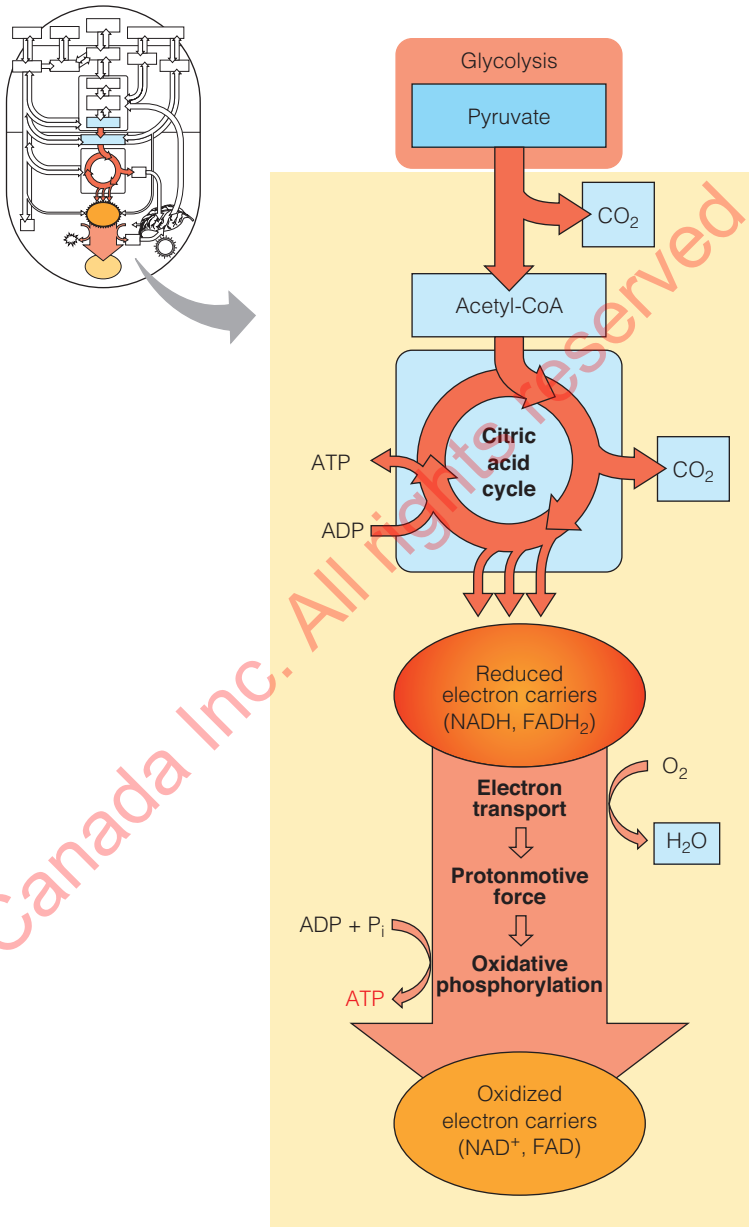


FIGURE 12.4

Oxidative metabolism. Oxidative metabolism includes pyruvate oxidation, the citric acid cycle, electron transport, and oxidative phosphorylation. Pyruvate oxidation supplies acetyl-CoA to the citric acid cycle.

breakdown of fatty acids by ***β*-oxidation** (presented in Chapter 17) and from some amino acid oxidation pathways (presented in Chapters 20 and 21). If the two carbons of acetyl-CoA are not oxidized in the citric acid cycle, they can go in the anabolic direction, providing substrates for synthesis of fatty acids and steroids (presented in Chapters 17 and 19). These and other biosynthetic processes use a reduced electron carrier, NADPH, which is structurally very similar to NADH (page 516).

Several important biosynthetic processes for carbohydrates will concern us in this book (Figure 12.5). Chapter 13 presents **gluconeogenesis**, the synthesis of glucose from noncarbohydrate precursors, and polysaccharide biosynthesis, particularly the biosynthesis of glycogen in animal cells. In Chapter 16 we

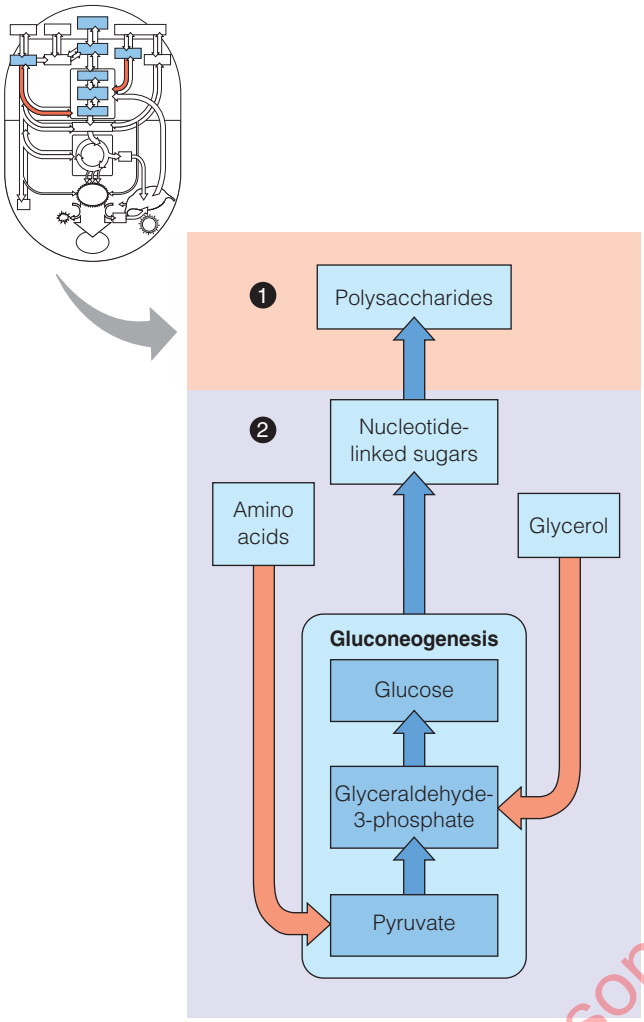


FIGURE 12.5

Carbohydrate anabolism. Biosynthesis of carbohydrates includes gluconeogenesis and polysaccharide synthesis.

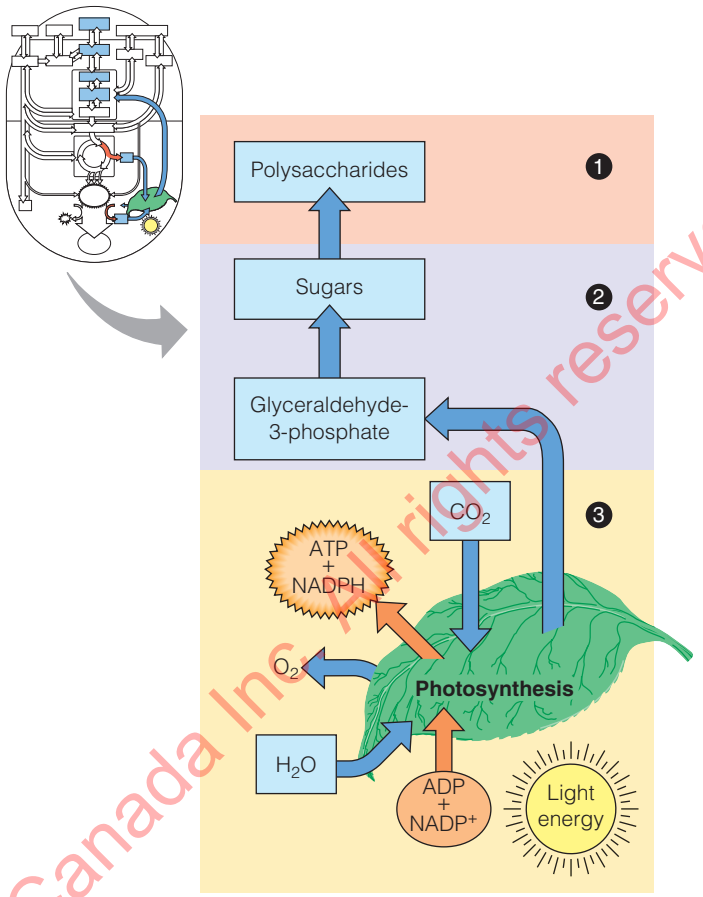


FIGURE 12.6

Photosynthesis.

present **photosynthesis** (Figure 12.6), the supremely important process by which green plants capture light energy to drive the generation of energy (ATP) and reducing power (NADPH), both of which are used for carbohydrate synthesis.

Distinct Pathways for Biosynthesis and Degradation

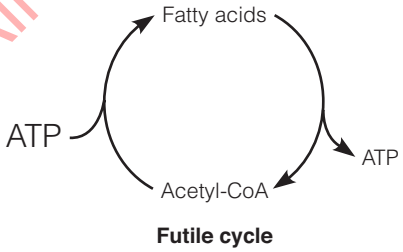
It may appear from Figure 12.2 that some pathways operate simply as the reversal of other pathways. For example, fatty acids are synthesized from acetyl-CoA, but they are also converted to acetyl-CoA by β-oxidation. Similarly, glucose-6-phosphate is synthesized from pyruvate in gluconeogenesis, which looks at first glance like a simple reversal of glycolysis. It is important to realize that in these cases the opposed pathways are quite distinct from one another. They may share some common intermediates or enzymatic reactions, but they are separate reaction sequences, regulated by distinct mechanisms and with different enzymes catalyzing their regulated reactions. They may even occur in separate cellular compartments. For example, fatty acid synthesis takes place in cytosol, whereas fatty acid oxidation takes place in mitochondria.

Biosynthetic and degradative pathways are never simple reversals of one another, even though they often begin and end with the same metabolites. The existence of separate unidirectional pathways is important for two reasons. First, to proceed in a particular direction, a pathway must be exergonic in that direction. If a pathway is strongly exergonic, then reversal of that pathway is just as strongly endergonic, and thus impossible, under the same conditions. Thus, opposed biosynthetic and degradative pathways must both be exergonic, and thus unidirectional, in their respective directions.

Degradative and biosynthetic pathways are distinct for two reasons: A pathway can be exergonic in only one direction, and pathways must be separately regulated to avoid futile cycles.

Second and equally important is the need to control the flow of metabolites in relation to the bioenergetic status of a cell. When ATP levels are high, there is less need for carbon to be oxidized in the citric acid cycle. At such times the cell can store carbon as fats and carbohydrates, so fatty acid synthesis, gluconeogenesis, and related pathways come into play. When ATP levels are low, the cell must mobilize stored carbon to generate substrates for the citric acid cycle, so carbohydrate and fat breakdown must occur. Using separate pathways for the biosynthetic and degradative processes is crucial for control, so conditions that activate one pathway tend to inhibit the opposed pathway and vice versa.

Consider what would happen, for example, if fatty acid synthesis and oxidation took place in the same cell compartment and in an uncontrolled fashion. Two-carbon fragments released by oxidation would be immediately used for resynthesis, a situation called a **futile cycle**. No useful work is done, and the net result is simply consumption of more ATP in the endergonic reactions of fatty acid synthesis than produced in the oxidation reactions. A similar futile cycle could result from the interconversion of fructose-6-phosphate with fructose-1,6-bisphosphate in carbohydrate metabolism.



Futile cycle

Fructose-6-phosphate + ATP		→	fructose-1,6-bisphosphate + ADP
Fructose-1,6-bisphosphate + H_2O		→	fructose-6-phosphate + P_i
Net:	ATP + H_2O	→	ADP + P_i

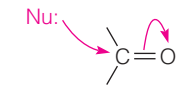
The first reaction occurs in glycolysis, and the second participates in a biosynthetic pathway, gluconeogenesis. Both processes occur in the cytosol. The net effect of carrying out both reactions simultaneously would be the wasteful hydrolysis of ATP to ADP and P_i. However, enzymes catalyzing both of the above reactions respond to allosteric effectors, such that one enzyme is inhibited by conditions that activate the other. This reciprocal control prevents the futile cycle from occurring, even though the two enzymes occupy the same cell compartment. Therefore, it is more appropriate to call this situation—two seemingly opposed cellular reactions that are independently controlled—a **substrate cycle**.

Compartmentation and allosteric control of anabolic and catabolic processes prevent futile cycles, which simply waste energy.

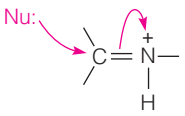
Studies on metabolic control suggest that a substrate cycle represents an efficient regulatory mechanism because a small change in the activity of either or both enzymes can have a much larger effect on the flux of metabolites in one direction or the other. You can test this idea in Problem 6 at the end of this chapter.

Biochemical Reaction Types

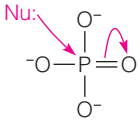
There is no metaphysics in biochemistry—the chemistry of living systems follows the same chemical and physical laws as the rest of nature. The complexity of these biochemical pathways may at first glance seem overwhelming, but only five general types of chemical transformations are commonly found in cells. Although all of these transformations are catalyzed by enzymes in cells, the reactions proceed by straightforward organic chemistry mechanisms. We will discuss these reaction types only briefly here because you previously learned about them in organic chemistry. However, you should consult your organic chemistry textbook if you need a more detailed review.



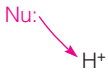
Carbonyl



Protonated imine



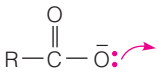
Phosphate



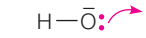
Proton

Electrophiles

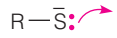
Alkoxide



Carboxylate



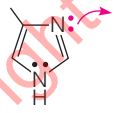
Hydroxide ion



Thiolate

Carbanion

Amine

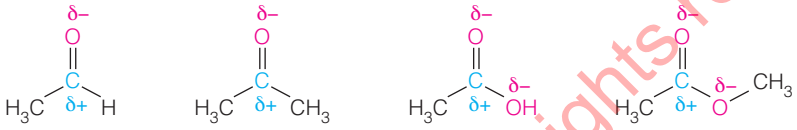


Imidazole

Nucleophiles

Nucleophilic Substitutions

Much of the chemistry of biological molecules is the chemistry of the carbonyl group (C═O) because the vast majority of biological molecules contain them. And most of the chemistry of carbonyl groups involves **nucleophiles** (abbreviated "**Nu:**") and **electrophiles**. Recall that a nucleophile is a "nucleus-loving" substance with a negatively polarized, electron-rich atom that can form a bond by donating a pair of electrons to an electron-poor atom. An electrophile is an "electron-loving" substance with a positively polarized, electron-poor atom that can form a bond by accepting a pair of electrons from an electron-rich atom. Carbonyl groups are polar, with the electron-poor C atom bearing a partial positive charge and the electron-rich O atom bearing a partial negative charge.

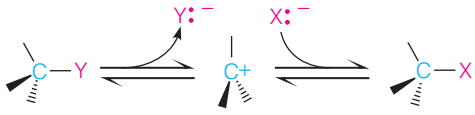


Aldehyde **Ketone** **Carboxylic acid** **Ester**

Carbonyl carbons are thus very common electrophiles in biochemical reactions. Other common electrophiles are protonated imines, phosphate groups, and protons.

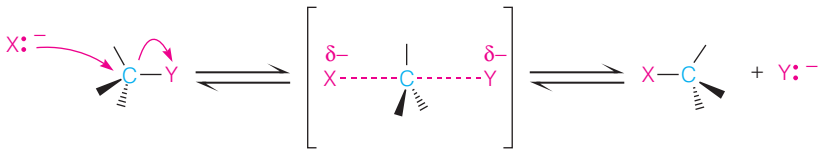
Oxyanions (e.g., hydroxide ion, alkoxides, or ionized carboxylates), thiolates (deprotonated sulfhydryls), carbanions, deprotonated amines, and the imidazole side chain of histidine are common nucleophiles in biochemical reactions.

In a nucleophilic substitution reaction, one nucleophile replaces a second nucleophile (the leaving group) on an sp^3-hybridized carbon atom. The leaving group develops a partial negative charge in the transition state, and the best leaving groups are those that are stable as anions. Thus, halides and the conjugate bases of strong acids, such as the phosphate anion, are good leaving groups. Nucleophilic substitutions proceed by either S_N1 or S_N2 mechanisms. In the S_N1 (Substitution, Nucleophilic, Unimolecular) mechanism, the leaving group ($Y:^-$) departs with the bonding electrons, generating a carbocation intermediate, *before* the attacking nucleophile ($X:^-$) arrives. S_N1 reactions result in either retention of configuration or racemization at the reacting center.



Carbocation intermediate

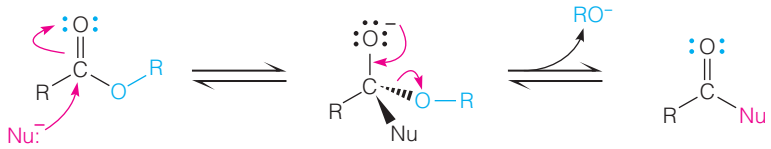
In the S_N2 (Substitution, Nucleophilic, Bimolecular) mechanism, the attacking nucleophile approaches one side of the electrophilic center while the leaving group remains partially bonded to other side, resulting in a transient pentavalent intermediate.



Pentavalent intermediate

In the S_N2 mechanism, departure of the leaving group from the opposite side results in a substituted product with inverted configuration.

A very important class of substitution reaction in biochemistry is the **nucleophilic *acyl* substitution** of carboxylic acid derivatives. Acyl substitutions occur most readily when the carbonyl carbon is bonded to an electronegative atom (such as O or N) or a highly polarizable atom (such as S) that can stabilize a negative charge and thereby act as a good leaving group. Thus, carboxylic acids and their derivatives (esters, amides, thioesters, acyl phosphates) are common substrates in acyl substitution reactions. In contrast to the S_N1 and S_N2 mechanisms of *alkyl* substitutions, *acyl* substitutions involve a tetrahedral oxyanion reaction intermediate.



Oxyanion intermediate

The planar carbonyl group is converted to a tetrahedral geometry as the carbonyl carbon rehybridizes from sp^2 to sp^3. As the electron pair moves from the oxyanion back towards the central carbon, the leaving group is expelled and the C═O bond is regenerated. Many enzymes catalyze nucleophilic acyl substitutions, such as carboxypeptidase A, in which an activated water molecule serves as the attacking nucleophile (see Figure 11.39, p. 475). This mechanism is also the basis of a variety of **group transfer reactions**, in which an acyl, glycosyl, or phosphoryl group is transferred from one nucleophile to another.

Nucleophilic Additions

Unlike carboxylic acids and their derivatives, the carbonyl carbon in aldehydes and ketones is bonded to atoms (C and H) that cannot stabilize a negative charge, and thus are not good leaving groups. These carbonyl groups typically undergo **nucleophilic addition reactions**, instead of substitution reactions. Like the nucleophilic acyl substitution mechanism, addition of a nucleophile leads to a tetrahedral oxyanion intermediate, as the electron pair from the C═O bond moves onto the oxygen. Recall from Chapter 11 that an oxyanion intermediate is formed in the initial steps of peptide bond hydrolysis by the serine proteases. The oxyanion intermediate then has several fates, depending on the nucleophile (Figure 12.7). When the attacking nucleophile is a hydride ion ($H:^-$), the oxyanion intermediate undergoes protonation to form an alcohol. Alcohols also result when the attacking nucleophile is a carbanion (R_3C^-), and this is one of the mechanisms that yield new C—C bonds. When an oxygen nucleophile adds, such as an alcohol (ROH), the oxyanion intermediate undergoes proton transfer to yield a hemiacetal. This reaction is the basis of ring formation in monosaccharides (Chapter 9). Reaction with a second equivalent of alcohol gives an acetal. When the attacking nucleophile is a primary amine ($R'NH_2$), the oxyanion intermediate picks up a proton from the amino group, giving a carbinolamine, which loses water to form an **imine** ($R_2C{=}NR'$). Imines (called **Schiff bases**) are common reaction intermediates in many biochemical reactions due to their ability to delocalize electrons.

Carbonyl Condensations

Formation of new C—C bonds is a critical element of metabolism, and the condensation of two carbonyl compounds is a common strategy used in many biosynthetic pathways. A carbonyl condensation reaction relies on the weak acidity of the carbonyl α hydrogen, producing a carbanion, which is in resonance with a nucleophilic enolate ion.

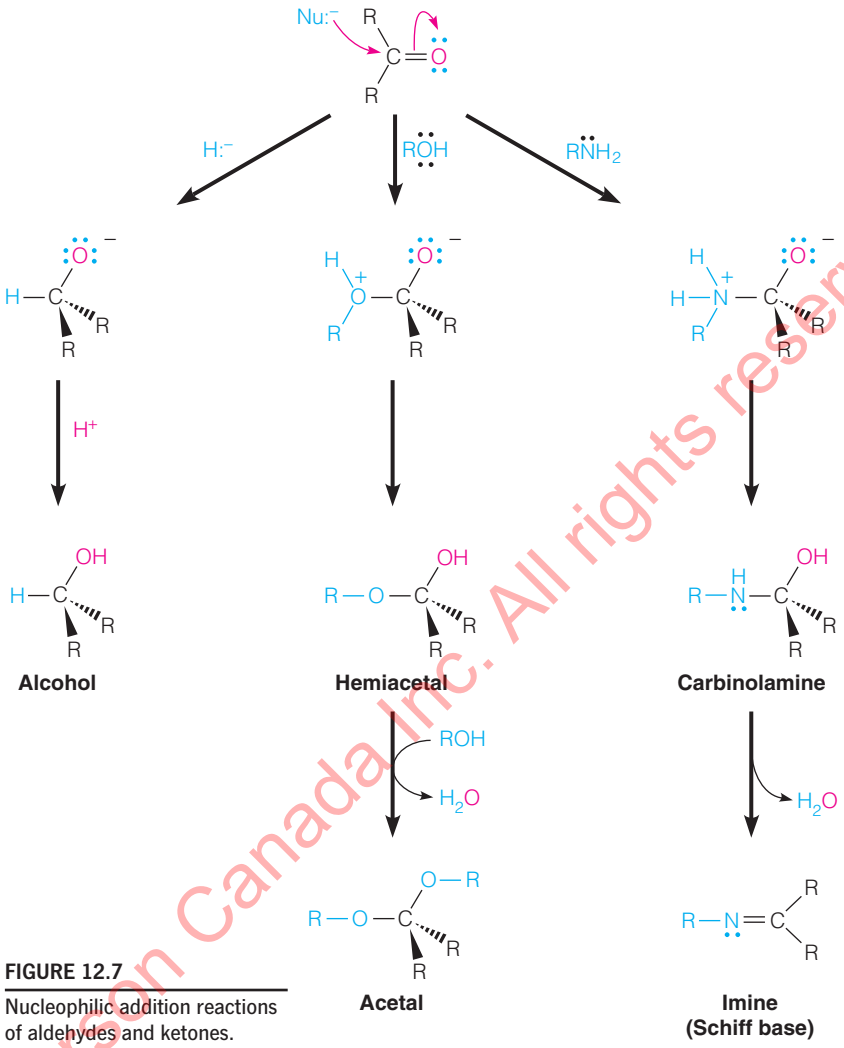
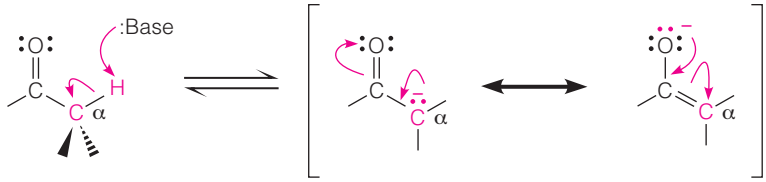


FIGURE 12.7
Nucleophilic addition reactions of aldehydes and ketones.



The enolate ion, stabilized by resonance, nucleophilically adds to the electrophilic carbon of a second carbonyl, forming a new C—C bond (Figure 12.8). If the second carbonyl is an aldehyde or ketone (an **aldol condensation**), this nucleophilic addition produces an oxyanion intermediate, which is protonated to give a β-hydroxy carbonyl product. If the second carbonyl is an ester (a **Claisen condensation**), the intermediate oxyanion expels the ester alkoxide (RO—) as the leaving group, giving a β-keto product. Carbonyl condensations thus result in a new bond formed between the carbonyl carbon of one reactant with the α carbon of the other. Aldol and Claisen condensations are both reversible, and such "retro-aldol" and "retro-Claisen" reactions are frequently used to cleave C—C bonds. Indeed, β-keto compounds readily undergo cleavage or decarboxylation by a retro-aldol mechanism, in which the electron-accepting carbonyl group two carbons away from the carboxylate stabilizes the formal negative charge of the carbanionic transition state.

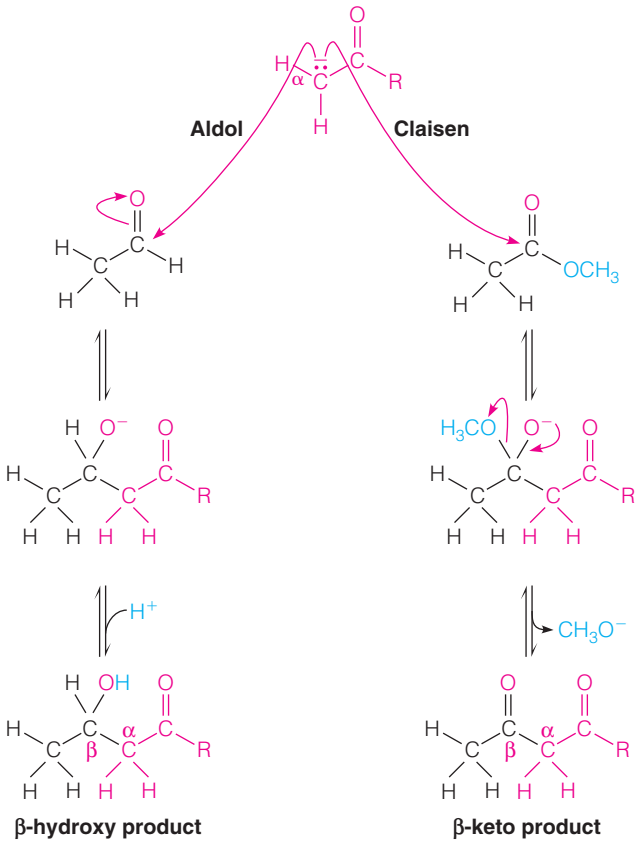
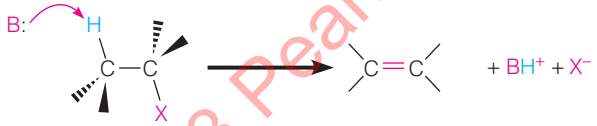


FIGURE 12.8

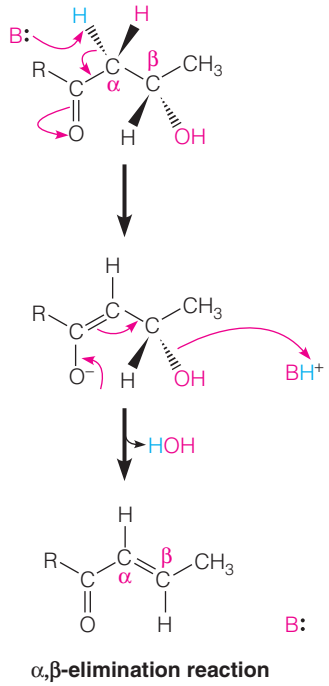
Carbonyl condensation reactions. These reactions are initiated by deprotonation of the weakly acidic α hydrogen to give a resonance-stabilized enolate ion (top). In an aldol condensation (left side), the enolate adds to an aldehyde or ketone, yielding a β-hydroxy carbonyl product. In a Claisen condensation (right side), the enolate adds to an ester, yielding a β-keto product.

Eliminations

Eliminations of the type

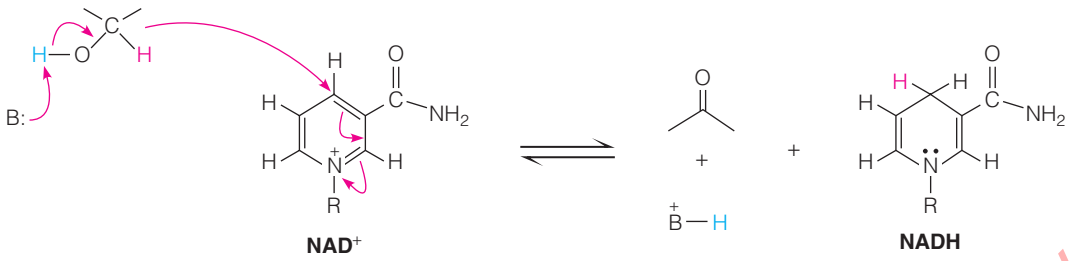


are also quite common in biochemical pathways. Elimination reactions can occur by several different mechanisms, but the most common one involves a carbanion intermediate. The reactant is often a β-hydroxy carbonyl (where X═OH) in which the H atom to be removed is made more acidic by being adjacent to a carbonyl group. A base abstracts the proton to give a carbanion intermediate (resonance-stabilized with the enolate) that loses OH^- to form the C═C double bond. β-hydroxy carbonyl compounds are readily dehydrated via these α,β-elimination reactions.



Oxidations and Reductions

Energy production in most cells involves the oxidation of fuel molecules such as glucose. Oxidation-reduction, or **redox**, chemistry thus lies at the core of metabolism. Redox reactions involve reversible electron transfer from a donor (the **reductant**) to an acceptor (the **oxidant**). Cells have evolved a number of electron carriers, such as the NAD^+ (nicotinamide adenine dinucleotide) coenzyme introduced in Chapter 11. Oxidations involving coenzymes such as NAD^+ occur by a reversible hydride (H^-) transfer mechanism, illustrated by the oxidation of an alcohol to a carbonyl compound.



A base abstracts the weakly acidic O—H proton, the electrons from that bond move to form a C=O bond, and the C—H bond is cleaved. The hydrogen *and the electron pair*, (i.e., hydride, H^-) add to NAD^+ in a nucleophilic addition reaction, reducing it to NADH. The plus sign in NAD^+ reflects the charge on the pyridine ring nitrogen in the oxidized form; this charge is lost as the electron pair moves through the ring onto this nitrogen. Because the alcohol has lost a pair of electrons and two hydrogen atoms, this type of oxidation is called a **dehydrogenation**, and enzymes that catalyze this reaction are called **dehydrogenases**. Remember, however, that redox reactions are reversible, and dehydrogenases can catalyze the reductive direction as well. While two-electron oxidations exemplified by the NAD^+-dependent dehydrogenases are the most common redox reactions in metabolism, they are certainly not the only ones. A number of redox processes involve one-electron transfers, and various electron carriers exist to handle single electrons—you'll learn more about these in Chapter 15.

There are other, less common types of reactions in biochemical pathways, such as free radical reactions, but these five represent the basic toolkit that cells use to carry out the vast majority of their chemical transformations.

Some Bioenergetic Considerations

Oxidation as a Metabolic Energy Source

Because the primary focus of the next few chapters will be on energy metabolism, we will now consider briefly how metabolic energy is generated. As we saw in Chapter 3, a thermodynamically unfavorable, or endergonic, reaction will proceed readily in the unfavored direction if it can be coupled to a thermodynamically favorable, or exergonic, reaction. In principle, any exergonic reaction can serve this purpose, provided that it releases sufficient free energy. In living systems, most of the energy needed to drive biosynthetic reactions is derived from the *oxidation* of organic substrates. Oxygen, the ultimate electron acceptor for aerobic organisms, is a strong oxidant; it has a marked tendency to attract electrons, becoming reduced in the process. Given this tendency and the abundance of oxygen in our atmosphere, it is not surprising that living systems have gained the ability to derive energy from the oxidation of organic substrates.

Biological Oxidations: Energy Release in Small Increments

In a thermodynamic sense, the biological oxidation of organic substrates is comparable to nonbiological oxidations such as the burning of wood. The free energy release is the same whether we are talking about oxidation of the glucose polymer cellulose in a wood fire, combustion of glucose in a calorimeter, or the metabolic oxidation of glucose:

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O \qquad \Delta G^{\circ\prime} = -2870\ \text{kJ/mol} \qquad (12.1)$$

Most biological energy derives from oxidation of reduced metabolites in a series of reactions, with oxygen as the final electron acceptor.

This equation reveals the conservation stoichiometry, or simple **reaction stoichiometry** of glucose combustion. Biological oxidations, however, are far more complex processes than combustion. When wood is burned, all of the energy is released as heat; useful work cannot be performed, except through the

action of a device such as a steam engine. In biological oxidations, by contrast, oxidation reactions occur without a large increase in temperature and with capture of some of the free energy as chemical energy. This energy capture occurs largely through the synthesis of ATP, and, as discussed in Chapter 3, the hydrolysis of ATP can be coupled to many processes to provide energy for biological work. In catabolism of glucose, about 40% of the released energy is used to drive the synthesis of ATP from ADP and P_i.

Unlike the oxidation of glucose by oxygen shown in the previous equation, most biological oxidations do not involve direct transfer of electrons from a reduced substrate to oxygen (Figure 12.4). Rather, a series of coupled oxidation–reduction reactions occurs, with the electrons passed to intermediate electron carriers such as NAD^+ and FAD, and finally transferred to oxygen. We have already briefly mentioned the role of NAD^+ as an electron carrier; FAD (flavin adenine dinucleotide) will be described in detail in Chapter 14. Thus, the biological oxidation of glucose might be more accurately represented by the following coupled reactions:

$$C_6H_{12}O_6 + 10NAD^+ + 2FAD + 6H_2O \rightarrow 6CO_2 + 10NADH + 10H^+ + 2FADH_2 \quad (12.2)$$

$$10NADH + 10H^+ + 2FADH_2 + 6O_2 \rightarrow 10NAD^+ + 2FAD + 12H_2O \quad (12.3)$$

$$\text{Net: } C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O \quad (12.4)$$

The net reaction of the biological oxidation process (reaction 12.4) is identical to that of direct combustion (reaction 12.1). Reactions 12.2 and 12.3 are examples of **obligate-coupling stoichiometry**. These are stoichiometric relationships fixed by the chemical nature of the process. Thus, the complete oxidation of glucose requires the transfer of 12 pairs of electrons from glucose to molecular oxygen, whether as a direct process, as in combustion, or via intermediate electron carriers, as in the biological process. In the biological process, 12 moles of electron carriers (NAD^+ and FAD) are obligately coupled to the oxidation of 1 mole of glucose to 6 moles of CO_2.

The transfer of electrons from these intermediate electron carriers to oxygen is catalyzed by the **electron transport chain**, or **respiratory chain**, and oxygen is called the **terminal electron acceptor**. Because the potential energy stored in the organic substrate is released in small increments, it is easier to control oxidation and capture some of the energy as it is released—small energy transfers waste less energy than a single large transfer does.

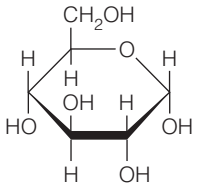
Not all metabolic energy comes from oxidation by oxygen. Substances other than oxygen can serve as terminal electron acceptors. Many microorganisms either can or must live **anaerobically** (in the absence of oxygen). For example, *Desulfovibrio* carry out anaerobic respiration using sulfate as the terminal electron acceptor:

$$SO_4^{2-} + 8e^- + 8H^+ \rightarrow S^{2-} + 4H_2O$$

Most anaerobic organisms, however, derive their energy from **fermentations**, which are energy-yielding catabolic pathways that proceed with no net change in the oxidation state of the products as compared with that of the substrates. A good example is the production of ethanol and CO_2 from glucose, presented in Chapter 13. Other anaerobic energy-yielding pathways are seen in some deep-sea hydrothermal vent bacteria, which reduce sulfur to sulfide as the terminal electron transfer reaction, and in other bacteria that reduce nitrite to ammonia. These organisms oxidize the substrates that sustain them, but they use terminal electron acceptors other than oxygen.

Energy Yields, Respiratory Quotients, and Reducing Equivalents

If metabolic energy comes primarily from oxidative reactions, it follows that the more highly reduced a substrate, the higher its potential for generating biological energy. We can use a calorimeter to measure the heat output (enthalpy) from oxidation of fat, carbohydrate, or protein. The combustion of fat provides more heat



Glucose

$CH_3(CH_2)_{14}COOH$

Palmitic acid

energy than the combustion of an equivalent mass of carbohydrate. In other words, fat has a higher **caloric content** than carbohydrate. For illustration, compare the oxidation of glucose with the oxidation of a typical saturated fatty acid, palmitic acid:

$$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O \qquad \Delta G^{\circ\prime} = -3.74\ \text{kcal/g}$$

$$C_{16}H_{32}O_2 + 23O_2 \rightarrow 16CO_2 + 16H_2O \qquad \Delta G^{\circ\prime} = -9.30\ \text{kcal/g}$$

Converting calories (the units of nutrition) to joules (the modern units of thermodynamics), we see that the oxidation of glucose yields 15.64 kJ/g, and the oxidation of palmitic acid yields 38.90 kJ/g. The carbons in fat are in general more highly reduced than those in carbohydrate; thus, they contain more protons and electrons to combine with oxygen on the path to CO_2 than do the carbons in sugar. We can see this by counting oxygen atoms. Glucose has more oxygens per carbon than does palmitic acid; each carbon in glucose is linked to at least one oxygen atom.

We can also tell that glucose is the more highly oxidized substance because its oxidation produces more moles of CO_2 per mole of O_2 consumed during oxidation, a ratio called the **respiratory quotient**, or **RQ.** The above reaction stoichiometries reveal RQ for glucose to be 1.0 ($6CO_2/6O_2$), whereas that for palmitic acid is 0.70 ($16CO_2/23O_2$). In general, the lower the RQ for a substrate, the more oxygen consumed per carbon oxidized and the greater the potential per mole of substrate for generating ATP.

Another way to express the degree of substrate oxidation is to say that more **reducing equivalents** are derived from oxidation of fat than from oxidation of carbohydrate. A reducing equivalent can be defined as 1 mole of hydrogen atoms (one proton and one electron per H atom). For example, two moles of reducing equivalents are used in the reduction of one-half mole of oxygen to water:

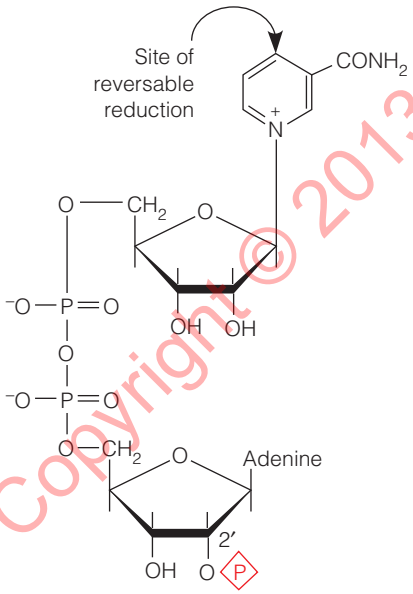
$$\tfrac{1}{2}O_2 + 2e^- + 2H^+ \rightarrow H_2O$$

Whereas the breakdown of complex organic compounds yields both energy and reducing equivalents, the biosynthesis of such compounds utilizes both. For example, we know that both carbons of acetate are used for fatty acid biosynthesis:

$$8CH_3COO^- \rightarrow\ \rightarrow\ \rightarrow CH_3(CH_2)_{14}COO^-$$

Acetate **Palmitate**

Fifteen of the 16 carbon atoms of palmitate are highly reduced—14 at the methylene level and 1 at the methyl level. Therefore, many reducing equivalents are required to complete this biosynthesis.



NADP⁺
Nicotinamide adenine dinucleotide phosphate (oxidized)

The major source of electrons for reductive biosynthesis is **NADPH**, **nicotinamide adenine dinucleotide phosphate (reduced)**. $NADP^+$ and NADPH are identical to NAD^+ and NADH, respectively, except that the former have an additional phosphate esterified at C-2 on the adenylate moiety. NAD^+ and $NADP^+$ are equivalent in their thermodynamic tendency to accept electrons; they have equal standard reduction potentials (Chapter 3). However, nicotinamide nucleotide–linked enzymes that act primarily in a catabolic direction usually use the NAD^+–NADH pair, whereas those acting primarily in anabolic pathways use $NADP^+$ and NADPH. In other words, as shown in Figure 12.9, nicotinamide nucleotide–linked enzymes that oxidize substrates (dehydrogenases) usually use NAD^+, and those enzymes that reduce substrates (reductases) usually use NADPH. Of course, both dehydrogenases and reductases catalyze reversible reactions—the directions are determined by the **redox state**, or ratio of the oxidized and reduced forms of each pair that prevail in the cell. Thus, the NAD^+/NADH pair is maintained at a more oxidized level than the $NADP^+$/NADPH pair in a healthy cell. These ratios generally drive NAD^+-linked reactions in the oxidative

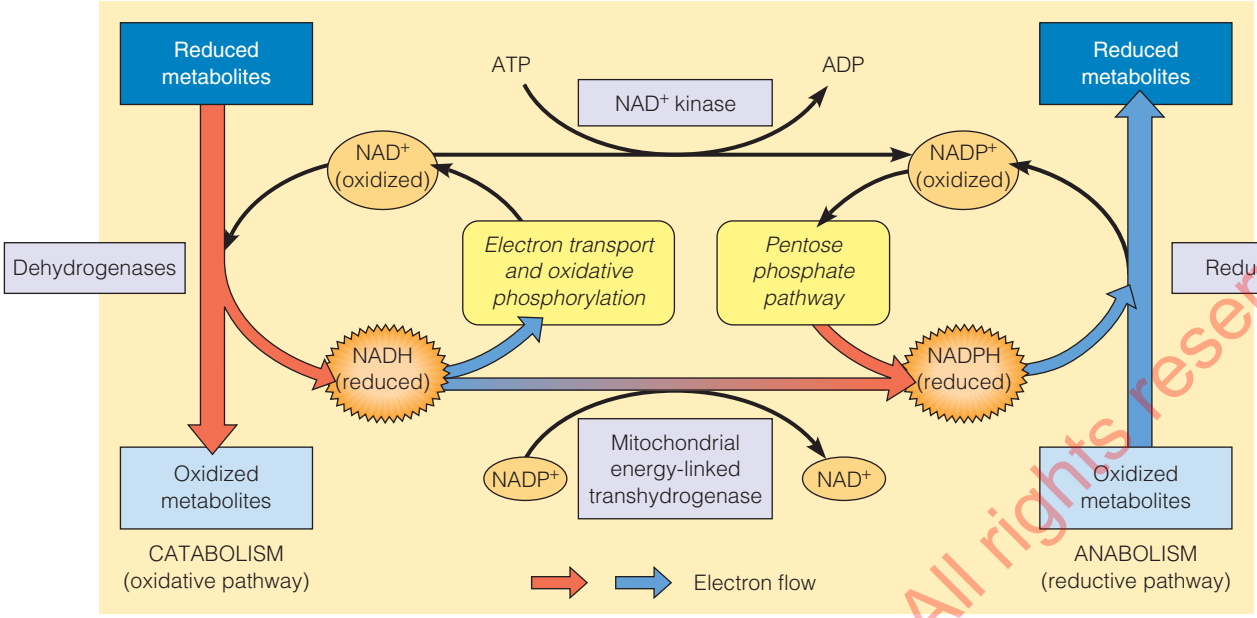


FIGURE 12.9

Nicotinamide nucleotides in catabolism and biosynthesis. NAD^+ is the cofactor for most enzymes that act in the direction of substrate oxidation (dehydrogenases), whereas NADPH usually functions as a cofactor for reductases, enzymes that catalyze substrate reduction. NADPH is regenerated either from $NADP^+$ in the pentose phosphate pathway (see Chapter 13) or from NADH through the action of mitochondrial energy-linked transhydrogenase (see Chapter 15). $NADP^+$ is synthesized from NAD^+ by an ATP-dependent kinase reaction.

direction and $NADP^+$-linked reactions in the reductive direction. An exception is two $NADP^+$-linked dehydrogenases in the **pentose phosphate pathway** (see Chapter 13), which convert $NADP^+$ to NADPH and represent a major route for regeneration of the reduced nucleotide.

NAD^+ is the cofactor for most dehydrogenases that oxidize metabolites. NADPH is the cofactor for most reductases.

ATP as a Free Energy Currency

ATP is commonly referred to as a "free energy currency." What does this mean? Currency is a medium of exchange. A $20 bill has a generally recognized value that can be readily exchanged for various goods or services—for example, dinner at a moderately priced restaurant, or about a quarter-hour's labor by a skilled auto mechanic. In the same sense, cells exchange the energy released from the breakdown of ATP to carry out essential functions, often converting the chemical energy released in ATP hydrolysis to other forms of energy—mechanical energy, for example, in muscle contraction, or electrical energy in conducting nerve impulses, or osmotic energy in transporting substances across membranes against a concentration gradient. Thus ATP serves as an immediate donor of free energy—it is continuously formed and consumed. It is estimated that a resting human turns over as much as 65 kg ATP every 24 hours, about equal to the entire body weight! (The Na^+/K^+–ATPase you learned about in Chapter 10 accounts for approximately 25% of this resting ATP consumption). During strenuous exercise, ATP turnover can be as high as 0.5 kg/min.

As summarized in Figures 12.3 and 12.4, regeneration of all this ATP is coupled to fermentative or oxidative processes. Thus, as we will see in Chapter 15, the oxidation of 1 mole of glucose during respiration is coupled to the phosphorylation of ~32 moles of ADP:

$$C_6H_{12}O_6 + 6O_2 + 32ADP + 32P_i \rightarrow 6CO_2 + 38H_2O + 32ATP \qquad (12.5)$$

The stoichiometries for ADP, P_i, and ATP in this equation are fundamentally different from the simple reaction and obligate-coupling stoichiometries we have encountered thus far. There is no chemical necessity for the production of 32 moles of ATP (in fact, there is not even a direct chemical connection between glucose oxidation and ATP synthesis). We could not predict a stoichiometry of 32 ATP from chemical considerations. This is instead an **evolved-**

coupling stoichiometry. Evolved-coupling stoichiometries are biological adaptations, phenotypic traits acquired during evolution—they are the result of compromise. So why did evolution settle on a stoichiometry of ~32 ATP? Recall from Chapter 3 that the free energy changes of coupled reactions are additive. Thus equation 12.5 can be broken into its free energy yielding and free energy requiring processes:

	ΔG (kJ/mol glucose)
$C_6H_{12}O_6 + 6O_2 \rightarrow 6CO_2 + 6H_2O$	−2900
$32ADP + 32P_i \rightarrow 32H_2O + 32ATP$	$\Delta G = +50\,kJ/mol \times 32\,mol\,ATP = +1600$
Sum: $C_6H_{12}O_6 + 6O_2 + 32ADP + 32P_i \rightarrow 6CO_2 + 38H_2O + 32ATP$	−1300

Using estimates of the Gibbs free energy changes under physiological conditions (ΔG) of −2900 kJ/mol for glucose oxidation and −50 kJ/mol for the hydrolysis of ATP, we can see that the coupled process occurs with a net ΔG of −1300 kJ/mol glucose based on an evolved-coupling stoichiometry of 32 ATP. AΔG of this magnitude represents such an immense driving force that respiration is emphatically favorable under virtually any physiological condition, and thus goes to completion. Because the ATP-coupling stoichiometry is an evolved trait, perhaps a different stoichiometry evolved in some ancient organism. Imagine a cell that mutated such that it ended up with an ATP-coupling stoichiometry for respiration of 58 instead of 32. Repeating the calculations above, the energy requiring process would have a ΔG of +2900 kJ/mol glucose (58 ATP × +50 kJ/mol), giving an overall ΔG of 0 for respiration. Thus, there would be no net driving force for the reaction. Although this hypothetical cell might have a higher yield of ATP per glucose oxidized, it would come to equilibrium before very much glucose was metabolized. This cell would probably be a very poor competitor, especially if glucose concentrations were limiting, and the mutation(s) that led to this stoichiometry would likely be selected against. The ATP-coupling stoichiometry that we actually find (~32) is thus an evolved compromise between maximizing ATP yield and ensuring that the overall process is unidirectional under any conceivable conditions the cell might encounter.

The fundamental biological role of ATP as an energy-coupling compound is to convert thermodynamically unfavorable processes into favorable processes.

Of course, as emphasized in Figure 12.1, there are many biochemical pathways that consume ATP. The role of ATP in these anabolic processes is to convert a thermodynamically unfavorable process into a favorable process. Remember that biosynthetic and degradative pathways are never simple reversals of one another. In particular, opposed pathways will always have different ATP stoichiometries. That is, the number of ATP (or ATP equivalents) produced in the catabolic direction will always be different from the number of ATP (or ATP equivalents) required in the anabolic direction. We refer to these numbers as the **ATP-coupling coefficient** of the reaction or pathway. For example, in the fructose-6-phosphate/fructose-1,6-bisphosphate substrate cycle (p. 509), the glycolytic reaction,

$$\text{Fructose-6-phosphate} + \text{ATP} \rightarrow \text{fructose-1,6-bisphosphate} + \text{ADP} \qquad (12.6)$$

has an ATP-coupling coefficient of −1. The gluconeogenic reaction,

$$\text{Fructose-1,6-bisphosphate} + H_2O \rightarrow \text{fructose-6-phosphate} + P_i \qquad (12.7)$$

has an ATP-coupling coefficient of 0. Likewise, as we will learn in Chapter 13, glycolysis has an overall ATP-coupling coefficient of +2, whereas gluconeogenesis has an ATP-coupling coefficient of −6. These differences in ATP-coupling coefficients between opposed reactions or pathways are essential for the unidirectionality of both processes.

What do we really mean when we say that ATP converts a thermodynamically unfavorable process into a favorable process? Coupling ATP hydrolysis to a pathway provides a new chemical route, using different reactions with different stoichiometries, resulting in a different overall equilibrium constant for the process.

In fact, coupling ATP hydrolysis to a process changes the equilibrium ratio of certain [reactants] to [products] by a factor of 10^8! To illustrate this, let's consider how the equilibrium ratio of [fructose-1,6-bisphosphate] to [fructose-6-phosphate] changes when we compare a direct addition of P_i to fructose-6-phosphate vs. the reaction pathway that is coupled to ATP hydrolysis. The direct addition of P_i is simply reversal of reaction 12.7:

$$\text{Fructose-6-phosphate} + P_i \rightleftharpoons \text{fructose-1,6-bisphosphate} + H_2O \qquad (12.8)$$

In this direction, the standard free energy change, $\Delta G^{\circ\prime}$, is + 16.3 kJ/mol (see Table 13.2, p. 575). Let's calculate the equilibrium constant, using Equation 3.28 from Chapter 3:

$$K = e^{\left(\frac{-\Delta G^{\circ}}{RT}\right)} = e^{\left(\frac{-16300\,\text{J/mol}}{(8.315\,\text{J/mol}\cdot\text{K})(298\text{K})}\right)} = 0.0014 = \frac{[\text{fructose-1,6-bisphosphate}]}{[\text{fructose-6-phosphate}][P_i]} \qquad (12.9)$$

In many cells, the normal intracellular concentration of P_i is ~1mM (10^{-3} M), so the equilibrium ratio of [fructose-1,6-bisphosphate]/[fructose-6-phosphate] achieved by this reaction would be $(0.0014)(10^{-3}) = 1.4 \times 10^{-6}$. Now let's compare that to a reaction that couples the phosphorylation of fructose-6-phosphate to ATP hydrolysis, as in reaction 12.6 above. $\Delta G^{\circ\prime}$ for this reaction is −14.2 kJ/mol (see Table 13.1, p. 564), and thus the equilibrium constant, *K*, for this reaction is 308.

$$K = e^{\left(\frac{14200\,\text{J/mol}}{(8.315\,\text{J/mol}\cdot\text{K})(298\,\text{K})}\right)} = 308 = \frac{[\text{fructose-1,6-bisphosphate}][\text{ADP}]}{[\text{fructose-6-phosphate}][\text{ATP}]} \qquad (12.10)$$

To compare the resulting equilibrium ratio of [fructose-1,6-bisphosphate]/[fructose-6-phosphate], we need to estimate the intracellular concentrations of ADP and ATP. In most normal, healthy cells, [ATP] is several-fold (3–10 times) higher than [ADP]. For the purposes of this calculation, let's make the simplifying assumption that the intracellular concentrations of ADP and ATP are roughly equal, that is, their concentration ratio is ~1. Thus, [ADP] and [ATP] cancel out, and the equilibrium ratio of [fructose-1,6-bisphosphate]/[fructose-6-phosphate] for this reaction is 308. This equilibrium ratio is indeed 10^8 times higher than that achieved without ATP coupling [$308/1.4 \times 10^{-6}$) = 2.2×10^8]. This effect is completely general—it depends only on the existence of a chemical mechanism that couples ATP hydrolysis to the process at hand (e.g., an enzyme like phosphofructokinase that catalyzes reaction 12.6 in glycolysis). Note that ATP is not directly hydrolyzed in this reaction, but is instead converted to ADP as its phosphate is transferred to fructose-6-phosphate. However, any process that couples the conversion of ATP to ADP gains the thermodynamic equivalent of the free energy of hydrolysis of ATP.

Metabolite concentrations and solvent capacity

The use of ATP as the fundamental coupling agent in biochemical systems has had far-reaching implications for the evolutionary design of cell metabolism. Evolution has produced a complex cell metabolism comprising thousands of enzymes and metabolic intermediates each at very low individual concentrations. There are at least a couple of reasons why a cell must maintain its components at very low concentrations. Even a simple bacterial cell contains several thousand different metabolites dissolved in the aqueous compartment. This aqueous compartment has a finite capacity for the amount of dissolved substances (metabolites and macromolecules)—this is its **solvent capacity**. Thus, individual metabolites must exist at low concentrations (10^{-3} − 10^{-6} M, or even lower) to avoid exceeding the solvent capacity of the cell. Secondly, low metabolite concentrations minimize unwanted side reactions. By way of illustration, imagine two metabolites, A and B, that can react nonenzymatically to form C. Assume that this unwanted side reaction is first-order with respect to each metabolite so that the

reaction rate is directly proportional to the product of [A] and [B] ($v = k[A][B]$). We can compare the amount of C that would be produced under two different metabolite concentrations: 1 M each vs. 10^{-5} M each. Whatever the rate constant is, the velocity of the unwanted side reaction at the two different concentrations will differ by a factor of 10^{10}. Thus, the same amount of C that would be produced in 1 sec if each metabolite is at 1 M would take ~317 years (10^{10} sec) to produce if each metabolite is at 10^{-5} M instead.

How does ATP coupling help avoid high metabolite concentrations? It does it by activating metabolic intermediates. Let's return to our previous example, comparing the phosphorylation of fructose-6-phosphate using inorganic phosphate vs. ATP. Assume that the cell needs to maintain a [fructose-1,6-bisphosphate]/[fructose-6-phosphate] ratio of 10 to thrive. What concentration of P_i is required to ensure this nonequilibrium ratio using reaction 12.8? We can solve for the required [P_i] by recognizing that ΔG for this reaction must be <0 and rearranging the relevant form of Equation 3.23:

$$\Delta G = \Delta G^{\circ\prime} + RT \ln\left(\frac{[\text{F-1,6-BP}][H_2O]}{[\text{F-6-P}][P_i]}\right) < 0$$

or

$$\ln\left(\frac{[\text{F-1,6-BP}][H_2O]}{[\text{F-6-P}][P_i]}\right) < \frac{-\Delta G^{\circ\prime}}{RT} \tag{12.11}$$

$$\ln\left(\frac{[10][1]}{[1][P_i]}\right) < \frac{\dfrac{-16.3\,\text{kJ}}{\text{mol}}}{\left(\dfrac{0.008314\,\text{kJ}}{\text{mol K}}\right)(298\,\text{K})} \tag{12.12}$$

Solving for [P_i]:

$$[P_i] > \frac{10}{0.0014} = 7143\ \text{M!}$$

Of course, there is no way to fit 7000 moles of phosphate into a liter of aqueous solution. Even if the cell could survive with a [fructose-1,6-bisphosphate]/[fructose-6-phosphate] ratio of 1, it would still need a [P_i] of > 714 M to make the reaction thermodynamically favorable (i.e., to make $\Delta G < 0$).

Now let us consider the process coupled to ATP hydrolysis, via the phosphofructokinase reaction (equation 12.6). Here, the relevant calculation is

$$\frac{[\text{F-1,6-BP}][\text{ADP}]}{[\text{F-6-P}][\text{ATP}]} < e^{\left(\frac{-\Delta G^{\circ\prime}}{RT}\right)} \tag{12.13}$$

with $\Delta G^{\circ\prime} = -14.2$ kJ/mol. If we assume an [ADP]/[ATP] ratio of ~1 under physiological conditions, we can calculate the maximum ratio of [fructose-1,6-bisphosphate]/[fructose-6-phosphate] that still makes the reaction favorable:

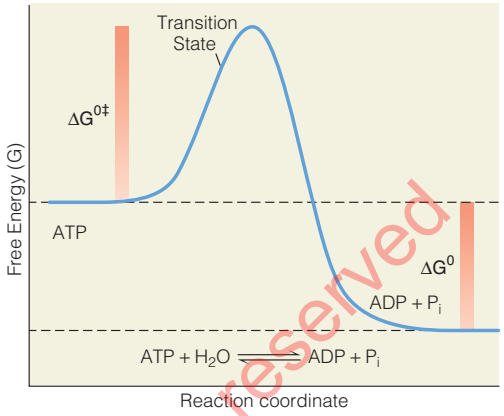
$$\frac{[\text{fructose-1,6-bisphosphate}]}{[\text{fructose-6-phosphate}]} < 308$$

Thus, by coupling the reaction to ATP hydrolysis the reaction will be thermodynamically favorable as long as the ratio of [F-1,6-BP] to [F-6-P] remains under 308—far above the desired 10. This scenario completely avoids the involvement of impossible concentrations of inorganic phosphate. In this example, ATP can be thought of as an activated form of phosphate. Cells have evolved a number of activated intermediates besides ATP, including acetyl-CoA, acyl-lipoate, and nucleoside diphosphate sugars (NDP-sugars). We will discuss each of these in later chapters, but suffice it to say here that they all function to allow reactions to occur under physiologically relevant concentrations of intermediates.

Activated intermediates, such as ATP, allow reactions to occur under physiologically relevant concentrations of metabolic intermediates.

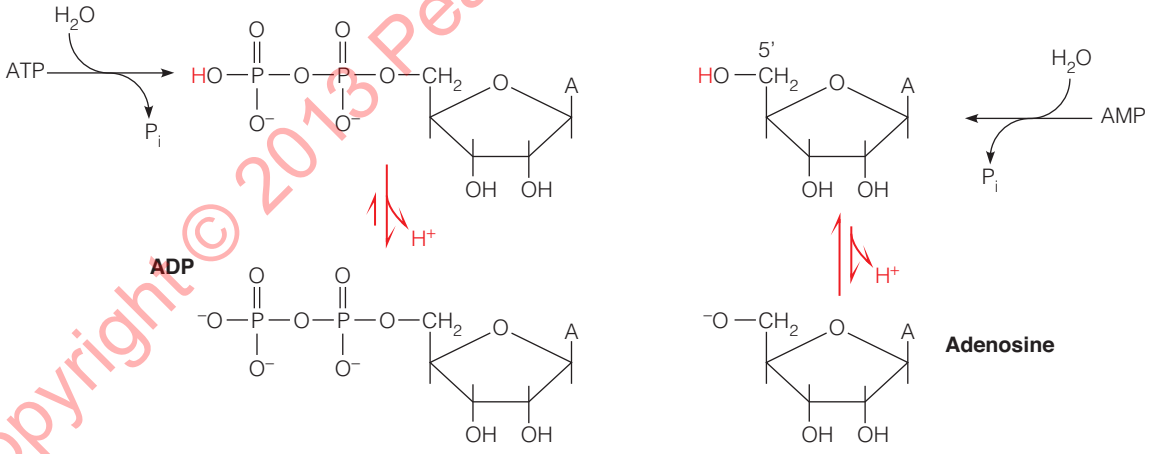
Thermodynamic Properties of ATP

What factors equip ATP for its special role as energy currency? First, there is nothing unique about the chemistry of ATP; the phosphoanhydride bonds whose breakdown can be coupled to drive endergonic reactions are shared by all other nucleoside di- and triphosphates and by several other metabolites. When we call ATP a "high-energy compound," as we did in Chapter 3, we use that term within a defined context; i.e., a high-energy compound is one containing at least one bond with a sufficiently favorable ΔG° of hydrolysis. ATP has two phosphoanhydride bonds. Cleavage of one yields adenosine diphosphate (ADP) and inorganic phosphate (P_i), and cleavage of the other gives adenosine monophosphate (AMP) and pyrophosphate (PP_i). Either reaction proceeds with a large negative $\Delta G^{\circ\prime}$ of hydrolysis (Figure 3.7, p. 81). Note, however, that calling a substance a high-energy compound does not mean that it is chemically unstable or unusually reactive. In fact, ATP is a *kinetically* stable compound; its spontaneous hydrolysis is slow, but when hydrolysis does occur, whether spontaneously or enzyme-catalyzed, substantial free energy is released. Be aware, however, that utilization of that energy to drive endergonic reactions usually does *not* involve hydrolysis. Rather, ATP breakdown is usually coupled with a thermodynamically unfavorable reaction, such as the synthesis of glucose-6-phosphate from glucose. In this case the phosphate released from ATP does not become P_i but instead is transferred directly to glucose, forming the esterified phosphate of glucose-6-phosphate. Thus, as discussed in Chapter 3, it is more accurate to say that ATP has a "high phosphoryl group transfer potential" than to call it a high-energy compound. However, so long as you understand the context in which the term is used, the concept of a high-energy compound is quite useful.



Phosphoanhydride bonds are *thermodynamically* unstable, but *kinetically* stable - large free energies of activation require enzymes to lower the activation barrier.

Recall from Chapter 3 that several factors contribute to the thermodynamic stability of a hydrolyzable bond and determine whether $\Delta G^{\circ\prime}$ is highly favorable, as in the phosphoric anhydride bonds of ATP, ADP, or pyrophosphate, or less favorable, as in the phosphoric ester bonds of glucose-6-phosphate or AMP. These factors include electrostatic repulsion among the negative charges in the molecule before hydrolysis, resonance stabilization of the products of hydrolysis, and the tendency of the hydrolysis products to deprotonate (see page 83). By contrast, the hydrolysis of a phosphate ester, such as AMP, generates an alcohol (the sugar 5′ hydroxyl), which has almost no tendency to lose a proton.



The abovementioned factors combine to give ATP a $\Delta G^{\circ\prime}$ of −30.5 kJ/mol, twice the phosphate transfer potential of phosphate esters such as AMP. On the other hand, several important metabolites have $\Delta G^{\circ\prime}$ values much more negative than that of ATP. Examples include phosphoenolpyruvate, 1,3-bisphosphoglycerate, and creatine phosphate (see Figure 3.7, p. 81), with $\Delta G^{\circ\prime}$ values of −61.9, −49.4, and −43.1 kJ/mol, respectively. This means that ATP is actually

intermediate on the scale of "energy wealth." This is important as well because it means that the breakdown of a compound such as phosphoenolpyruvate can be coupled to drive the synthesis of ATP itself from ADP and P_i. In fact, such coupled reactions, called **substrate-level phosphorylation** reactions, represent the process by which ATP is synthesized in glycolysis, as we discuss in Chapter 13.

The Important Differences Between ΔG and $\Delta G^{\circ\prime}$

But what provides the energy for synthesis of compounds with a much higher phosphate transfer potential than that of ATP itself? Much of the answer lies in the fact that ΔG values under intracellular conditions are quite different from standard ($\Delta G^{\circ\prime}$) values. This is mainly because intracellular concentrations are far different from the 1 M concentrations used to compute standard free energies. If you work Problem 5 at the end of this chapter, you will see that ATP hydrolysis has a considerably more negative ΔG value at intracellular concentrations of ATP, ADP, AMP, and P_i than it has under standard conditions. In fact, the physiological ratio of [ATP]/[ADP] is ~10^8 times higher than the equilibrium ratio. This value is not coincidental—if the physiological [ATP]/[ADP] ratio is 10^8 times larger than its equilibrium ratio, then the equilibrium ratio of any other reaction that is coupled to ATP hydrolysis will be altered by this same order of magnitude (see p. 519). As we shall see, maintenance of the physiological ratio of [ATP]/[ADP] so far away from equilibrium is accomplished by *kinetic* control, i.e. by regulation of enzymes. The key point is that maintaining the physiological ratio so far from equilibrium provides the thermodynamic driving force for nearly every biochemical event in the cell.

Kinetic Control of Substrate Cycles

Substrate cycles provide a beautiful illustration of the advantages of ATP as a coupling agent and the role of kinetic control over the direction, as well as the rate, of these opposing pathways. Let's return once again to the fructose-6-phosphate/fructose-1,6-bisphosphate substrate cycle:

	$\Delta G^{\circ\prime}$	K	ATP coupling coefficient
Fructose-6-phosphate + ATP ⟶ fructose-1,6-bisphosphate + ADP (PFK)	−14.2	308	−1
Fructose-1,6-bisphosphate + H_2O ⟶ fructose-6-bisphosphate + P_i (FBPase)	−16.3	719	0
Net: ATP + H_2O ⟶ ADP + P_i			

For the phosphofructokinase (PFK) reaction, under physiological conditions where [ATP] ≈ [ADP], we calculated a value of 308 for the equilibrium ratio of [fructose-1,6-bisphosphate]/[fructose-6-phosphate] (equation 12.10). Thus, this reaction is favorable in the direction of fructose-1,6-bisphosphate until its concentration approaches a level 310 times that of fructose-6-phosphate. For the fructose-1,6-bisphosphatase (FBPase) reaction, using the physiological concentration of P_i ($\sim 10^{-3}$ M), we obtain a value of 719,000 for the equilibrium ratio of [fructose-6-phosphate]/[fructose-1,6-bisphosphate]. Thus, under virtually any likely cellular condition, the FBPase reaction is thermodynamically favorable in the direction of fructose-6-phosphate. It follows, then, that *both* the PFK and FBPase reactions are thermodynamically favorable in their respective directions as long as [fructose-1,6-bisphosphate]/[fructose-6-phosphate] ratio is between 0.0000014 (1/719,000) and 310. In fact, the ratio will *always* be in this wide range in a healthy cell, and thus both reactions are *always* favorable. What then prevents these two reactions from occurring simultaneously, accomplishing nothing more than the net hydrolysis of ATP (i.e., a futile cycle)? The answer, of course, is the independent, but coordinated, kinetic control of the two opposed enzymes. As we

will see in the next chapter, enzymes in substrate cycles are kinetically regulated by the levels of allosteric effectors.

Thus, substrate cycles illustrate the important metabolic design feature mentioned previously: ATP-coupling coefficients of opposing reactions or pathways always differ. This difference allows both sequences to be thermodynamically favorable at all times. The choice of which pathway operates, however, is determined entirely by the metabolic needs of the cell (via allosteric effectors), not by thermodynamics (because both pathways are favorable). Why is it so important for both pathways to be thermodynamically favorable at all times? *Because regulation can be imposed only on reactions that are displaced far from equilibrium.* Consider the following analogy:

A dam separates two bodies of water. If the water level is the same on both sides of the dam, we can say the system is at equilibrium. If we now open the floodgate, what happens? Water may move back and forth through the floodgate, but there will be no net movement of water or change in water levels. Thus, regulation (opening the floodgate) has no impact on a system at equilibrium. Now imagine a different system.

Here, the water level on the left side of the dam is much higher than that on the right side of the dam. We would say this system is far from equilibrium. If we now open the floodgate, water will rush from the left side to the right side and will continue flowing until it reaches equilibrium, or until we impose regulation (close the floodgate). This characteristic, that each pathway of an opposing pair is thermodynamically favorable (made possible by the different ATP-coupling coefficients for each), results in each pathway being unidirectional (because the reverse direction is highly thermodynamically unfavorable). These opposing pathways (e.g, glycolysis vs. gluconeogenesis) are like dams holding back high waters, each poised to flow downhill (thermodynamics), just waiting for the signal to open the floodgates (kinetics). The signal (e.g., allosteric effectors) will logically be different for each floodgate (regulatory enzyme) so that both pathways never flow at same time (but both could if allowed).

The theoretical basis of this concept, which was developed in Chapter 3, is illustrated in Figure 12.10. It shows a plot of G (Gibbs free energy) vs. the log of the ratio of Q (the mass action ratio; p. 76) to K (the equilibrium constant) for the reaction A $\rightleftharpoons$ B. This plot gives a parabola and the slope at any point on the curve corresponds to ΔG. The free energy is at a minimum when the Q/K ratio $= 1$ (i.e., when the actual concentrations of reactants and products is equal to their equilibrium concentrations). In other words, when the system is at equilibrium, $\Delta G = 0$ (verified by the slope $= 0$ at this point). The magnitude of ΔG is greatest when the mass action ratio is far from equilibrium concentrations, and the further the reaction is from equilibrium, the greater the driving force. ΔG will be large and negative when [A] is high and [B] is low (left side of parabola; $Q/K < 1$), and the reaction will proceed to the right due to the large driving force. Conversely, when [B] is high and [A] is low (right side of parabola; $Q/K > 1$), the reaction will proceed to the left due to an equally large driving force.

Earlier we estimated a ΔG of approximately -1300 kJ/mol for the oxidation of one mole of glucose during respiration (p. 518). We can calculate the corresponding Q/K ratio at 298 K using Equation 3.33 from Chapter 3:

$$\Delta G = RT \ln\left(\frac{Q}{K}\right)$$

$$\frac{Q}{K} = e^{\left(\frac{\Delta G}{RT}\right)} = e^{\left(\frac{-1300 \times 10^3 \text{J/mol}}{(8.315 \text{ J/mol}) \cdot (298K)}\right)} \approx 10^{-228}$$

This Q/K ratio, 10^{-228}, is based on approximations of ΔG and might be off by a few orders of magnitude. Whatever the actual value, however, it lies far, far up the left arm of the parabola in Figure 12.10, and thus represents an immense driving force.

Regulation can be imposed only on reactions that are displaced far from equilibrium.

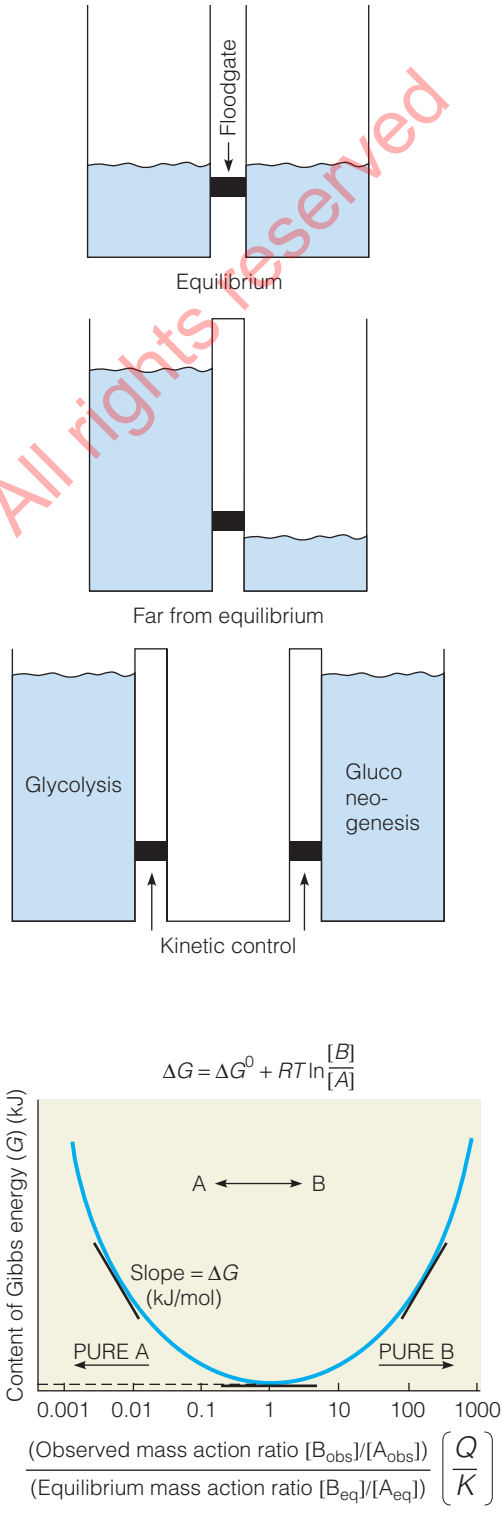
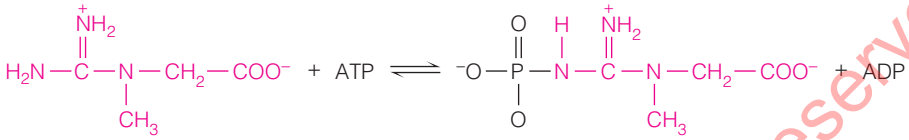


FIGURE 12.10

Gibbs free energy of a reaction as a function of its displacement from equilibrium. Modified from Fig. 3.2 (p. 35) of Bioenergetics 3, by DG Nicholls and SJ Ferguson, Academic Press (2002).

Other High-Energy Phosphate Compounds

This same principle allows ATP to drive the synthesis of compounds of even higher phosphate transfer potential, such as creatine phosphate. This compound shuttles phosphate bond energy from ATP in mitochondria to myofibrils, where that bond energy is transduced to the mechanical energy of muscle contraction. Creatine phosphate (CrP) is produced from creatine by the enzyme **creatine kinase:**



Creatine **Creatine phosphate**

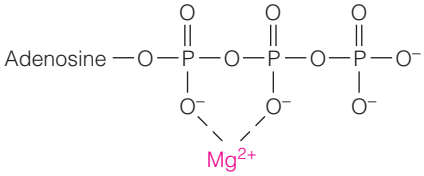
Mammalian cells have several different creatine kinase isozymes, one of which is localized to the intermembrane space of mitochondria (mCK; see margin). From the respective $\Delta G^{\circ\prime}$ values for creatine phosphate and ATP (Figure 3.7, p. 81), you can calculate that this reaction is endergonic under standard conditions, with a $\Delta G^{\circ\prime}$ value of +12.6 kJ/mol. However, because ATP levels are high within mitochondria, and creatine phosphate levels are relatively low ($Q/K << 1$), the reaction is exergonic as written and proceeds to the right in the mitochondrial intermembrane space. Creatine phosphate then diffuses from mitochondria to the myofibrils, where it provides the energy for muscle contraction. However, the direct energy source for that contraction is ATP hydrolysis once again. High levels of ADP formed during contraction favor the reverse reaction, catalyzed by a myofibril creatine kinase isozyme (myoCK). ATP is resynthesized at the expense of creatine phosphate cleavage to creatine, which can then return to mitochondria for resynthesis of creatine phosphate. The popularity of creatine as a dietary supplement for athletes, to increase muscle strength, suggests that the biosynthesis of creatine itself (see Chapter 21 for the pathway) may be a limiting factor in operating this intracellular energy shuttle.

ATP can drive the synthesis of higher-energy compounds, if nonequilibrium intracellular concentrations make such reactions exergonic.

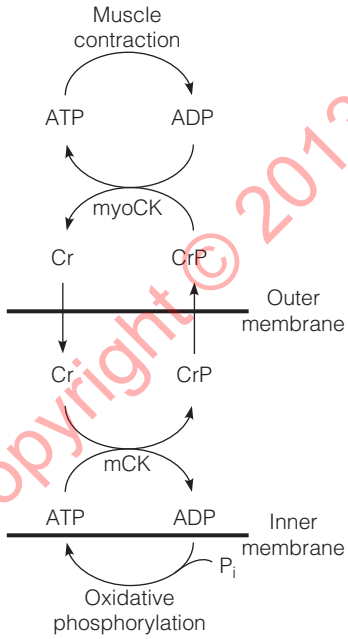
In some invertebrate animals **arginine phosphate**, instead of creatine phosphate, plays a similar role in storing high-energy phosphate for rapid production, as needed, of ATP. In both instances, the varying concentrations of adenine nucleotides in the two environments—mitochondria and myofibrils—both at levels far from standard values, are critical in understanding how these high-energy compounds can be synthesized and utilized.

But factors in addition to concentration combine to make physiological ΔG values quite different from standard values. For example, as pH increases, the negative charge on the ATP molecule increases. This in turn increases the electrostatic repulsion between oxygen atoms linked to adjacent phosphorus atoms, and this in turn promotes hydrolysis, thus making ΔG more negative.

Also significant is the fact that most ATP is chelated within cells, as a complex with Mg^{2+}:



ADP is also complexed with Mg^{2+}, but ADP has a different affinity for Mg^{2+} from that of ATP. Varying levels of Mg^{2+} will change ΔG in complicated ways, depending upon relative affinities of reactants and products for the magnesium ion. (For a detailed discussion of the effects of pH, magnesium, and other ionic conditions, see the article by R. Alberty cited in the Chapter 3 references.)



Creatine-creatine phosphate shuttle in muscle contraction

Other High-Energy Nucleotides

As discussed earlier, there is nothing unique about the properties endowing ATP with its special role as energy currency. All other nucleoside triphosphates, as well as more complex nucleotides, such as NAD^+, have $\Delta G^{\circ\prime}$ values close to −31 kJ/mol and could have been selected for the role played by ATP. However, evolution has created an array of enzymes that preferentially bind ATP and use its free energy of hydrolysis to drive endergonic reactions. There are exceptions, such as the use of GTP as the primary energy-providing nucleotide in protein synthesis. But phosphate-bond energy is created almost exclusively at the adenine nucleotide level, through oxidative phosphorylation in aerobic cells, photosynthesis in plants, and substrate-level phosphorylation during glycolysis in virtually all organisms. As a result, ATP is usually the most abundant nucleotide.

In most cells, ATP levels, at 2–8 mM, are severalfold higher than those of the other nucleoside triphosphates and also severalfold higher than the levels of ADP or AMP. These factors give ATP a strong tendency to distribute its γ (outermost) phosphate in the synthesis of other nucleoside triphosphates. This is accomplished through the action of **nucleoside diphosphate kinase**, which synthesizes CTP from CDP in the following example.

$$\text{ATP} + \text{CDP} \rightleftharpoons \text{ADP} + \text{CTP}$$

Nucleoside diphosphate kinase is active with a wide variety of phosphate donors and acceptors. Because its equilibrium constant is close to unity and because ATP is the most abundant nucleotide within cells, the enzyme normally uses ATP to drive the synthesis of the other common ribo- and deoxyribonucleoside triphosphates from their respective diphosphates.

Some metabolic reactions, such as the activation of amino acids for protein synthesis, cleave ATP, not to ADP and P_i, but to AMP and PP_i. The conversion of AMP to ATP, allowing reuse of the nucleotide, involves another enzyme, **adenylate kinase** (also called myokinase because of its abundance in muscle).

$$\text{AMP} + \text{ATP} \rightleftharpoons 2\text{ADP}$$

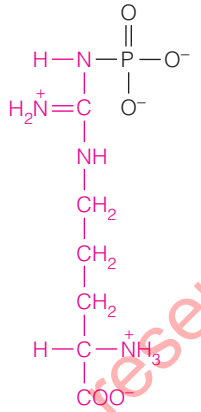
ADP is reconverted to ATP by substrate-level phosphorylation, oxidative phosphorylation, or (in plants) photosynthetic energy. Because the reaction is readily reversible, it can also be used for resynthesis of ATP when ADP levels rise, for example, after a burst of energy consumption. This function is particularly important in muscle metabolism.

Adenylate Energy Charge

As noted above, ATP levels are normally severalfold higher than the levels of ADP and AMP in well-nourished, energy-sufficient cells. Many enzymes that participate in regulating energy-generating or storage pathways are acutely sensitive to concentrations of adenine nucleotides. In general, energy-generating pathways, such as glycolysis and the citric acid cycle, are activated at low energy states, when levels of ATP are relatively low and those of ADP and AMP are relatively high. It is useful to be able to describe the energy status of a cell in quantitative terms. In his excellent book (cited in the references at the end of this chapter), Daniel Atkinson has likened the cell to a battery. When the cellular battery is fully charged, all of the adenine ribonucleotides are present in the form of ATP. When fully discharged, all of the ATP has been broken down to AMP. Atkinson has proposed the term **adenylate energy charge**, which is defined in terms of the intracellular concentrations of ATP, ADP, and AMP.

$$\text{Adenylate energy charge} = \frac{[\text{ATP}] + 0.5[\text{ADP}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}]} \qquad (12.14)$$

The term is the proportion of total energy-rich bonds that could be present in the adenine nucleotides in a cell to those that are actually present. Note that ADP has one energy-rich bond compared with two in ATP, so it carries one-half the weight of ATP in the numerator of equation 12.14. The value of the adenylate energy charge is probably never far from 1 in well-nourished aerobic cells.



Arginine phosphate

Major Metabolic Control Mechanisms

The living cell uses a marvelous array of regulatory devices to control its functions. These mechanisms include those that act primarily to control enzyme *activity*, such as substrate concentration and allosteric control, as discussed in Chapter 11. Control of enzyme *concentration*, through regulation of enzyme synthesis and degradation, is the major focus of Chapters 27–29 and 20, respectively. In eukaryotic cells, *compartmentation* represents another regulatory mechanism, with the fate of a metabolite being controlled by the flow of that metabolite through a membrane. Overlying all of these mechanisms are the actions of *hormones*, chemical messengers that act at all levels of regulation.

Control of Enzyme Levels

If you were to prepare a cell-free extract of a particular tissue and determine intracellular concentrations of several different enzymes, you would find tremendous variations. Enzymes of the central energy-generating pathways are present at many thousands of molecules per cell, whereas enzymes that have limited or specialized functions might be present at fewer than a dozen molecules per cell. Two-dimensional gel electrophoresis of a cell extract (see Figure 1.11, p. 21) gives an impression, from the varying spot intensities, of the wide variations in amounts of individual proteins in a particular cell.

Enzyme levels in a cell may change in response to changes in metabolic needs.

The level of a single enzyme can also vary widely under different environmental conditions, in large part because of variation in the enzyme's rate of synthesis. For example, when a usable substrate is added to a bacterial culture, the abundance of the enzymes needed to process the substrate may increase, through synthesis of new protein, from less than one molecule per cell to many thousands of molecules per cell. This phenomenon is called enzyme **induction**. Similarly, the presence of the end product of a pathway may turn off the synthesis of enzymes needed to generate that end product, a process called **repression**.

For some time it was thought that controlling the intracellular level of a protein was primarily a matter of controlling the *synthesis* of that protein—in other words, through genetic regulation. We now know that intracellular protein *degradation* is also important in determining enzyme levels (see Chapter 20).

Control of Enzyme Activity

Enzyme activity is regulated by interaction with substrates, products, and allosteric effectors and by covalent modification of enzyme protein.

The *catalytic activity* of an enzyme molecule can be controlled in two ways: by reversible interaction with ligands (substrates, products, or allosteric modifiers) and by covalent modification of the protein molecule.

Enzyme activity is most commonly controlled by low-molecular-weight ligands, principally substrates and allosteric effectors. Substrates are usually present within cells at concentrations lower than the K_M values for the enzymes that act on them, but within an order of magnitude of these values. In other words, substrate concentrations usually lie within the first-order ranges of substrate concentration–velocity curves for the enzymes that act on them. Therefore, reaction velocities respond to small changes in substrate concentration. Ligands that control enzyme activity can also be polymers. For example, protein–protein interactions can affect enzyme activity, and several enzymes of nucleic acid metabolism are activated by binding to DNA.

We saw in Chapter 11 that allosteric activation or inhibition usually acts on committed steps of a metabolic pathway, often initial reactions. The effectors function by binding at specific regulatory sites, thereby affecting subunit–subunit interactions in the enzyme protein. This effect in turn either facilitates or hinders the binding of substrates. Such a mechanism operates in an obvious way to control product formation if a pathway is unidirectional and unbranched. However, some substrates are involved in numerous pathways, so many branch points exist. Therefore, some of the allosteric enzymes that we will describe display somewhat more complicated regulation than the examples presented in Chapter 11.

Covalent modification of enzyme structure represents another efficient way to control enzyme activity. Chapter 11 introduced several types of covalent modification that are used to regulate enzyme activity, including **phosphorylation**, **acetylation**, **methylation**, **adenylylation** (the transfer of an adenylate moiety from ATP), and **ADP-ribosylation** (the transfer of an ADP-ribosyl moiety from NAD^+). Many other less common covalent modifications are now known. However, phosphorylation is by far the most widespread covalent modification used to control enzyme activity.

Control through covalent modification is often associated with regulatory cascades. Modification activates an enzyme, which in turn acts on a second enzyme, which may activate yet a third enzyme, which finally acts on the substrate. Because enzymes act catalytically, this cascading provides an efficient way to *amplify* the original biological signal. Suppose that the original signal modifying enzyme A activates it 10-fold, that modified enzyme A then activates enzyme B 100-fold, and that B activates enzyme C by 1000-fold. Thus, with the involvement of relatively few molecules of enzyme, a pathway can be activated by a million-fold ($10^1 \times 10^2 \times 10^3$).

The first well-understood regulatory cascade was the one controlling glycogen breakdown in animal cells, a critical process that provides carbohydrate substrates for energy generation. This regulatory cascade, involving enzyme phosphorylation and dephosphorylation, is described in detail in Chapter 13. Blood clotting, described in Chapter 11, is another well-understood regulatory cascade.

Compartmentation

We have already described the physical division of labor that exists in a eukaryotic cell, in the sense that enzymes participating in the same process are localized to a particular *compartment* within the cell. For example, RNA polymerases are found in the nucleus and nucleolus, where DNA transcription occurs, and the enzymes of the citric acid cycle are all found in mitochondria. Figure 12.11 presents the locations of a number of metabolic pathways within eukaryotic cells.

Compartmentation creates a division of labor within a cell, which increases the efficiency of cell function. The creatine–creatine phosphate shuttle (p. 524) is a good example of this. In addition, compartmentation has an important regula-

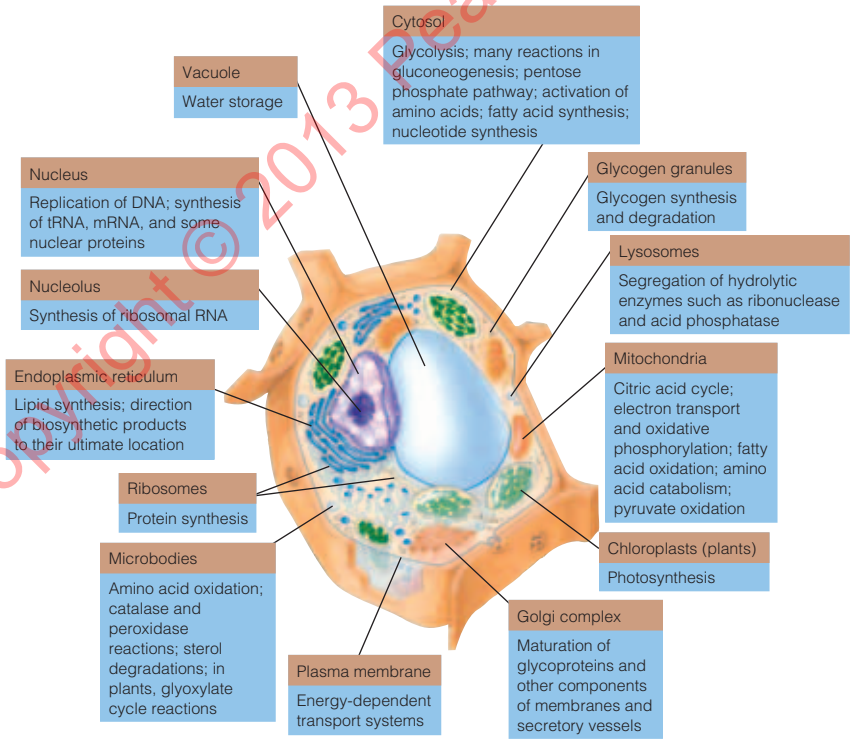


FIGURE 12.11

Locations of major metabolic pathways within a eukaryotic cell. This hypothetical cell combines features of a plant cell and an animal cell.

From Neil Campbell, Jane Reece, and Larry Mitchell, *Biology,* 5th ed. (Menlo Park, CA: Addison Wesley Longman, 1999). © Addison Wesley Longman, Inc.

tory function. This function derives largely from the selective permeability of membranes to different metabolites, thereby controlling the passage of intermediates from one compartment into another. Typically, intermediates of a pathway remain trapped within an organelle, while specific carriers allow substrates to enter and products to exit. The flux through a pathway, therefore, can be regulated by controlling the rate at which a substrate enters the compartment. For example, one of the ways in which the hormone insulin stimulates carbohydrate utilization is by moving glucose transporters into the plasma membrane so that glucose is more readily taken into cells for catabolism or for synthesis of glycogen.

Compartmentation is more than a matter of tucking enzymes into the proper organelles. Juxtaposition of enzymes that catalyze sequential reactions localizes substrates even in the absence of membrane-bound organelles. Opportunities for diffusion are reduced because the product of one reaction is released close to the active site of the next enzyme in a pathway. The enzymes may be bound to one another in a membrane, as are the enzymes of mitochondrial electron transport. Alternatively, they may be part of a highly organized multiprotein complex, such as the pyruvate dehydrogenase complex, a major entry point to the citric acid cycle.

Compartmentation can also result from weak interactions among enzymes that do not remain complexed when they are isolated. For example, conversion of glucose to pyruvate by glycolysis is catalyzed by enzymes that interact quite weakly in solution. However, there is evidence that these enzymes interact within the cytosol, forming a supramolecular structure that facilitates the multistep glycolytic pathway. The concept of intracellular interactions among readily solubilized enzymes developed as scientists began to realize that the cytosol is much more highly structured than was formerly thought. High-resolution electron micrographs of mammalian cytosol reveal the outlines of an organized structure that has been termed the **cytomatrix**, a model of which is shown in Figure 12.12. It is likely that such structures form as a result of the extremely high concentrations of proteins inside cells, which decrease the concentration of water and drive weakly interacting proteins to associate. It has been proposed that soluble enzymes are bound within the cell to the structural elements of the cytomatrix.

Enzymes catalyzing sequential reactions are often associated, even in the cytosol, where organized structures are difficult to visualize.

Whether highly structured or loosely associated, multienzyme complexes allow for efficient control of reaction pathways. Enzyme complexes restrict diffusion of intermediates, thereby keeping the average concentrations of intermediates low (but their local concentrations high, at enzyme catalytic sites). This complexing reduces *transient time*, the average time needed for a molecule to traverse a pathway. Thus, the flux through a pathway can change quickly in response to a change in the concentration of the first substrate for that pathway.

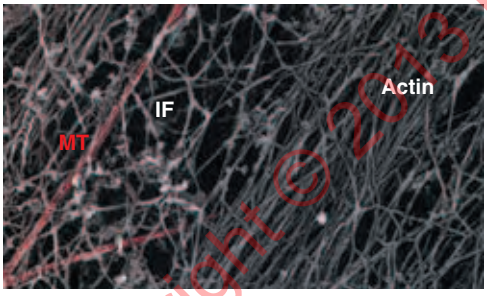


FIGURE 12.12

Model of the structural organization of the cytomatrix. The model is based on high-voltage electron microscopic examination of cultured mammalian fibroblast cells. PM = plasma membrane; C = cortex (part of the cell surface); ER= endoplasmic reticulum; MT = microtubules; R = free ribosomes; M = mitochondrion. Approximate magnification, 150,000.

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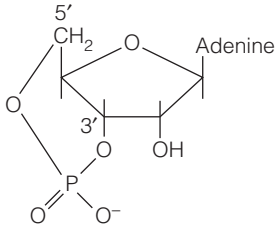
Hormonal Regulation

Overlaid and interspersed with the regulatory mechanisms operating within a eukaryotic cell are messages dispatched from other tissues and organs. The process of transmitting these messages and bringing about metabolic changes is called **signal transduction**. The extracellular messengers include hormones, growth factors, neurotransmitters, and pheromones, which interact with specific receptors, resulting in specific metabolic changes in the target cell.

Metabolic responses to hormones can involve changes in gene expression, leading to changes in enzyme levels. This type of response typically operates on a timescale of hours to days, resulting in a reprogramming of the metabolic capability of the cell. On a shorter timescale (seconds to hours), some hormones stimulate the synthesis of intracellular **second messengers** that control metabolic reactions. One of the most important second messengers is **adenosine 3′, 5′-cyclic monophosphate**, more commonly called **cyclic AMP**, or simply **cAMP**. The hormone (the first messenger) acts extracellularly, by binding to receptors in the plasma membrane. Because the receptor protein traverses the membrane, it can, on the intracellular side, stimulate the formation of a second messenger in response to extracellular

binding of the first messenger. These signal transduction systems can effect precise control over metabolic pathways, often through reversible covalent modification of critical enzymes in the pathway.

Signal transduction systems are modular in nature, allowing for a diversity of metabolic responses based on the same operating principles. Thus, secretion of one hormone can have quite diverse effects in different tissues, depending upon the nature of the receptor and other components and second messenger systems in different target cells. Moreover, a single second messenger may have diverse effects within a single cell. For example, cyclic AMP, which activates glycogen degradation, also activates a cascade that inhibits the synthesis of glycogen. This dual effect is an example of a coordinated metabolic response: Glycogen synthesis is *inhibited* under the same physiological conditions that *promote* glycogen breakdown. These signal transduction systems will be described in great detail in Chapters 13, 18, and 23.



Adenosine-3′, 5′-cyclic monophosphate (cyclic AMP)

Second messengers transmit information from hormones bound at the cell surface, thereby controlling intracellular metabolic processes.

Distributive Control of Metabolism

In recent years, an important principle of metabolic regulation has emerged. With the discovery in the 1950s and 1960s of allosterically regulated enzymes, the concept arose that metabolic pathway flux is regulated primarily through control of the intracellular activity of one or a few key enzymes in that pathway. When it was realized that allosteric enzymes often catalyze committed reactions, i.e., the first reaction in a pathway that leads to an intermediate with no other known function, the concept arose that these enzymes catalyze the "rate-limiting reactions" in metabolic pathways. Taken literally, the term *rate-limiting reaction* implies that the flux rate of the pathway is identical to the intracellular activity of the rate-limiting step. However, because in a metabolic steady state all of the steps along a linear pathway are proceeding at the same rate, it is difficult or impossible to establish that the rate is controlled by a single enzymatic step. In other words, the concept of a rate-limiting enzyme is seriously flawed.

We now realize that metabolic regulation is more complex and that all of the enzymes in a pathway contribute toward control of pathway flux. An approach called **metabolic control analysis** assigns to each enzyme in a pathway a **flux control coefficient**, C^J, a value that can vary between zero and one. Consider a metabolic pathway where substrate A is converted to product D in several steps. The **flux** through the pathway, J, is equal to the rate of the forward process, v_f, minus the rate of the reverse process, v_r:

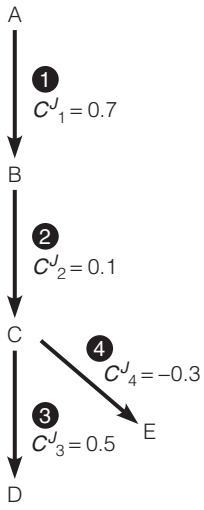
$$J = v_f - v_r$$

For a given enzyme, the flux control coefficient is the relative increase in flux, divided by the relative increase in enzyme activity that brought about that flux increase. For a true rate-limiting enzyme, the flux control coefficient is 1; a 20% increase in the activity of that enzyme would increase that flux rate by 20%. But metabolic control theory predicts that all enzymes in a pathway contribute toward regulation, meaning that all enzymes have flux-control coefficients greater than zero (but none has a value as high as 1, which would be the case if flux were truly controlled solely by one rate-limiting enzyme.) Flux-control coefficients are properties of the pathway or metabolic system, and thus all the flux-control coefficients in the pathway must sum to 1:

$$C_1^J + C_2^J + \cdots + C_n^J = 1$$

In the hypothetical pathway in the margin, intermediate C has two alternative fates. Because reaction 4 draws C away from the pathway A ⟶ D, it decreases flux through the pathway, and the enzyme that catalyzes step 4 has a negative flux control coefficient. The sum of the four flux control coefficients in the pathway still equals 1.

The predictions of metabolic control theory can be tested, for example, by using mutations affecting a specific enzyme to bring about defined changes in the activity of that enzyme in vivo, and then measuring the change in flux rate of a pathway in which that enzyme is involved. Such analyses confirm that enzymes catalyzing committed



reactions do play large roles in regulation (i.e., they have high flux-control coefficients). More important, however, these analyses confirm that *every* enzyme in a pathway contributes toward control of that pathway; in other words, every enzyme has a flux-control coefficient greater than zero. Thus, regulation of a pathway is *distributed* among all of the enzymes involved in the pathway, giving rise to the concept of **distributive control of metabolism**. In retrospect, this concept should have been predicted simply from the long-understood complexity of metabolism. Many intermediates participate in more than one pathway, making different pathways interdependent and interlocking. Regulatory schemes that depend on control of just one or two enzymes in each pathway lack the flexibility and subtlety to account for the ability of cells to maintain homeostasis under widely varying nutritional and energetic conditions. To be sure, for each pathway or process, one or a few regulatory enzymes of primary importance have been identified, and these will be pointed out as we present the individual pathways involved.

Experimental Analysis of Metabolism

Goals of the Study of Metabolism

Given that metabolism consists of all the chemical reactions in living matter, how does a biochemist approach metabolism in the laboratory? To subdivide a particular metabolic process into experimentally attainable goals, the biochemist seeks (1) to identify reactants, products, and cofactors, plus the stoichiometry, for each reaction involved; (2) to understand how the rate of each reaction is controlled in the tissue of origin; and (3) to identify the physiological function of each reaction and control mechanism. These goals necessitate isolating and characterizing the enzyme catalyzing each reaction in a pathway. This latter task—extrapolating from test tube biochemistry to the intact cell—is especially challenging. For instance, given that most enzymes catalyze reactions that can proceed in either direction, what is the direction of an enzymatic reaction in vivo? Many reactions originally found to proceed one way in vitro have been shown to proceed in the opposite direction in vivo. The mitochondrial enzyme that synthesizes ATP from ADP, for example, was originally isolated as an ATPase—an enzyme that hydrolyzes ATP to ADP and P_i. Therefore, it is not enough to isolate an enzyme and to demonstrate that it catalyzes a particular reaction in the test tube. We must show also that the same enzyme catalyzes the same reaction in intact tissue, usually a more difficult task.

To achieve the stated goals, a biochemist must perform analyses at several levels of biological organization, from living organisms and intact cells to broken-cell preparations and ultimately, to purified components. Cell-free, or in vitro, preparations can be manipulated in ways that intact cells cannot—for example, by addition of substrates and cofactors that will not pass through cell membranes. The researcher attempts to duplicate in vitro the process that is known to occur in vivo.

Preparation of cell-free components usually destroys biological organization. Cells can be broken open by sonication, shear forces, or enzymatic digestion of cell walls. These harsh treatments lead to mixing of components that were in separate compartments in the intact cell, making it possible to misinterpret data obtained from in vitro systems. An example comes from studies of protein biosynthesis. The existence of messenger RNA as a template for protein synthesis was predicted in 1961 from the behavior of bacterial mutants altered in genetic regulation. However, it was difficult to demonstrate the existence of messenger RNA in vitro because the putative template was present in very small amounts and was rapidly degraded by enzymes in cell-free extracts. Only when investigators learned how to prevent this degradation could they prove the existence of messenger RNA.

The foregoing discussion reveals the necessity of studying metabolism at various levels of biological organization, from intact organism to purified chemical components. Here we discuss what can be learned at each level.

Levels of Organization at Which Metabolism is Studied

Whole Organism

Biochemists must investigate metabolism in whole organisms because our ultimate aim is to understand chemical processes in intact living systems. Radioisotopic tracers are widely used to characterize metabolic pathways, as described in Tools of Biochemistry 12A. A classic example, described in Chapter 19, is the elucidation of cholesterol synthesis in the 1940s. Konrad Bloch injected ^{14}C-labeled acetate into rats and followed the flow of label into intermediates by sacrificing rats at intervals and analyzing the radioactive compounds in their livers. In designing experiments of this type, the investigator must pay attention to the efficiency of transport of the labeled precursor to the organs of interest, uptake of the precursor into cells, and competition of exogenous precursors with preexisting pools of unlabeled intermediates.

Many diagnostic tests in clinical medicine are in vivo metabolic experiments. Instead of using radioisotopes, we sample tissue at intervals and carry out biochemical assays. In the **glucose tolerance test**, for example, a subject consumes a large oral dose of glucose, and its level in the blood is then determined at intervals over several hours. The glucose tolerance test is used to diagnose diabetes and other disorders of carbohydrate metabolism.

In recent years, *nuclear magnetic resonance* (NMR) spectroscopy has become widely available for noninvasive monitoring of intact cells and organs. As explained in Tools of Biochemistry 6A, compounds containing certain atomic nuclei can be identified from an NMR spectrum, which measures shifts in the frequency of absorbed electromagnetic radiation. A researcher can determine an NMR spectrum of whole cells, or of organs or tissues in an intact plant or animal. NMR has even become a powerful noninvasive diagnostic tool, referred to as magnetic resonance imaging (MRI) in the medical arena. For the most part, macromolecular components do not contribute to the spectrum, nor do compounds that are present at less than about 0.5 mM. The nuclei most commonly used in this in vivo technique are ^{1}H, ^{31}P, and ^{13}C. Figure 12.13 shows ^{31}P NMR spectra that represent components in the human forearm muscle. The five major peaks correspond to the phosphorus nuclei in orthophosphate (P_i), creatine phosphate, and the three phosphates of ATP. Because peak area is proportional to concentration, the energy status of intact cells can be determined. For example, an energy-rich muscle has lots of creatine phosphate, whereas a fatigued muscle uses up most of its creatine phosphate in order to maintain ATP levels (note also the accumulation of AMP—peak 6—in the third scan). NMR is finding wide applicability in monitoring recovery from heart attacks, in which cellular ischemia (insufficient oxygenation) damages cells by reducing ATP content. NMR can also be used to study metabolite compartmentation, flux rates through major metabolic pathways, and intracellular pH.

Isolated or Perfused Organ

Some of the difficulties in transporting a precursor or inhibitor to the desired organ can be circumvented by using an isolated organ. A researcher usually **perfuses** the isolated organ during the experimental manipulations. This procedure involves pumping a buffered isotonic solution containing nutrients, drugs, or hormones through the organ. The solution partly takes the place of the normal circulation, delivering nutrients and removing waste products. The researcher can also perfuse an organ within a living animal, following appropriate surgical procedures. Of course, perfusion is much less efficient than circulation; thus, experiments at this level must be of limited duration.

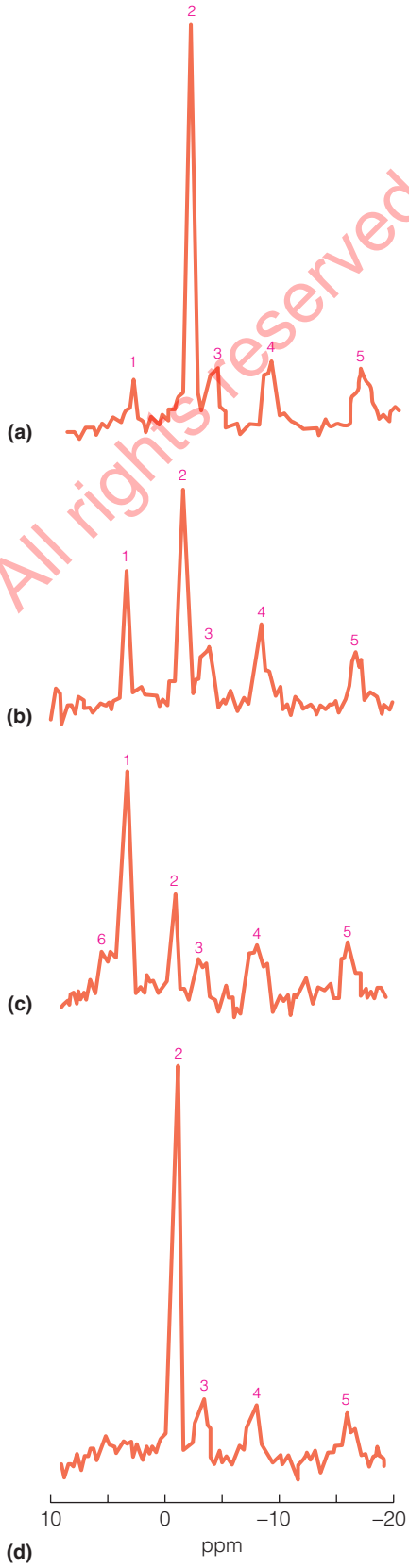


FIGURE 12.13

Effect of anaerobic exercise on ^{31}P NMR spectra of human forearm muscle. (a) Before exercise. **(b)** One minute into a 19-minute exercise period. **(c)** The 19th minute. **(d)** Ten minutes after exercise. Peak areas are proportional to intracellular concentrations. Peak 1 = P_i; 2 = creatine phosphate; 3 = ATPγ-phosphate; 4 = ATP α-phosphate; 5 = ATPβ-phosphate; 6 = phosphomonoesters. See Tools of Biochemistry 6A for interpretation of NMR spectra.

The circulation problem can be partly overcome by cutting the tissue into thin slices before the experimental manipulations begin. The structural integrity of the organ is lost, but most of the cells remain intact, and they are in better contact with the fluid that bathes the tissue. The cells may be better oxygenated and supplied with substrates than in a whole organ. The elucidation of the citric acid cycle resulted largely from experiments with slices of liver and heart.

Whole Cells

Tissue slices are not now widely used, partly because methods are now available for disaggregating an organ or a tissue into its component cells. Liver, kidney, and heart cells can be prepared by treatment of the organ with trypsin or collagenase, to break down the extracellular matrix that holds the organ together. For plant cells, enzymes such as cellulase or pectinase, which attack the cell wall, can be used to make comparable preparations.

Any plant or animal organ contains a complex mixture of different cell types. Several means of fractionating the cells after disaggregation of an organ are used to obtain preparations enriched in one cell type. One method is centrifugation to separate cells on the basis of size. In recent years the **fluorescence-activated cell sorter** has come into widespread use. In a typical application a cell suspension is treated with a fluorescent-tagged antibody to a cell surface antigen, which is present in varying amounts among different cell types. Cells pass in single file through a laser beam and are physically separated according to the amount of fluorescence recorded from each cell. Such machines, which can sort several thousand cells per second, result in fractionation based on the abundance of the selected surface antigen.

Uniformity in a cell population is often achieved by growth of cells in **tissue culture**. Disaggregated cells of an organ or a tissue can, with special care, be induced to grow in a medium containing cell nutrients and protein growth factors. The cells grow and divide independently of one another, much like the cells in a bacterial culture. Although animal cells usually cease growth after a certain number of divisions in culture, variant lines arise that are capable of indefinite growth, as long as they are adequately nourished. In such cultures, **clonal** cell lines can be generated in which all of the cells in a line are derived from a single cell so that they are genetically and metabolically uniform. This uniformity is a boon for many biochemical investigations. For example, much of our understanding of virus replication depends upon the ability to infect a large number of identical cells in culture simultaneously and then follow the metabolic changes by sampling the cell culture at various times after infection.

One problem with tissue culture is that cells adapted to long-term growth in culture take on characteristics different from those of their parent cells, which were originally embedded in plant or animal tissue. Maintaining specialized cell characteristics in culture always presents a challenge.

Cell-Free Systems

Problems of transport through membranes are obviated by working with broken-cell preparations. Animal cells are easily lysed (ruptured) by mild shear forces, suspension in hypotonic medium, or freezing and thawing. Bacterial cells have a rigid cell wall that requires vigorous treatment such as sonication (using ultrasound energy to agitate particles). Enzymatic digestion with lysozyme is often used to open bacterial cells under relatively mild conditions. Breaking the especially tough cell walls of yeasts and plants usually requires combinations of enzymatic and mechanical treatments.

Initial metabolic experiments are usually carried out in unfractionated cell-free homogenates. However, localizing a metabolic pathway within a particular cell compartment requires fractionating the homogenate to separate the organelles, usually by **differential centrifugation**. Lysis is carried out in isotonic sucrose solutions and generally yields morphologically intact organelles. These components can be pelleted by centrifugation at different speeds and for different lengths of time. Typically, nuclei, mitochondria, chloroplasts, lysosomes, and **microsomes** (artifactual membrane vesicles formed from disrupted endoplasmic reticulum) can all be at

least partially separated from each other. The contents of the cytosol remain in the supernatant after the final centrifugation step. Much of our understanding of DNA replication and transcription in eukaryotic cells comes from investigations with isolated nuclei, whereas purified mitochondria have yielded much of our understanding of respiratory electron transport and oxidative phosphorylation.

Purified Components

To understand a biological process at the molecular level, the investigator must purify to homogeneity all of the factors thought to be involved and determine their interactions. Often, as with the citric acid cycle, this process is simply a matter of purifying the individual enzymes involved, determining the substrate and cofactor requirements of each, recombining the purified enzymes, and showing that the entire process can be catalyzed by purified components. This process is called **reconstitution**. Some pathways require cell constituents other than enzymes, such as the ribosomes and transfer RNAs needed for protein synthesis.

In purifying individual components, the biochemist continually risks losing factors that are essential for normal control or for some other aspect of the process under study. Avoiding such pitfalls requires painstaking experiments in which the researcher defines criteria for biological activity and continually examines fractions to ensure that each activity is retained through each fractionation step. A good example of this approach is presented in Chapter 25, in which we discuss the several enzymes and proteins that must function at a DNA replication fork.

Metabolic Probes

By inactivating individual enzymes, mutations and enzyme inhibitors help identify the metabolic roles of enzymes.

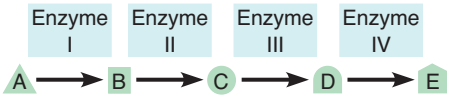
An invaluable biochemical aid is the use of metabolic probes, agents that allow a researcher to interfere specifically with one or a small number of reactions in a pathway. The consequences of such interference can be extremely informative. Two kinds of probes are most widely used—*metabolic inhibitors* and *mutations*. By blocking a specific reaction in vivo and determining the results of the blockade, such probes help identify the metabolic role of a reaction. For example, respiratory poisons such as carbon monoxide and cyanide block specific steps in respiration, and metabolic inhibitors helped identify the order of electron carriers in the respiratory electron transport chain (see Chapter 15).

Inhibitors can present difficulties for the researcher, such as poor transport into cells or multiple sites of action. It is often easier to interfere with a pathway by selecting mutant strains deficient in the enzyme of interest. In the 1940s George Beadle and Edward Tatum were the first to use mutations as biochemical probes, in their work with the bread mold *Neurospora crassa.* Beadle and Tatum isolated a number of X-ray-induced mutants that required arginine for growth, in addition to the constituents of minimal medium. Furthermore, different mutations affected different enzymes in the arginine biosynthetic pathway, with each mutant accumulating in the culture medium the intermediate that was the substrate for the deficient enzyme. This observation allowed Beadle and Tatum to order the enzymes according to the reactions they controlled, by the rationale illustrated in Figure 12.14. If a culture filtrate (containing an accumulated intermediate from one mutant allowed a second mutant to grow without arginine, the researchers concluded that the first mutation blocked an enzymatic step *later* in the pathway than the step blocked by the second mutation. Ultimately, the accumulating intermediates were identified and the pathway was elucidated. The impact of this "biochemical genetics" approach was much wider than simply working out the details of a metabolic pathway. Beadle and Tatum recognized that there was a one-to-one correspondence between a genetic mutation and the loss of a specific enzyme, leading them to propose the **one gene–one enzyme hypothesis**, years before the chemical nature of the gene was known.

In addition to identifying pathways, mutants have been used to elucidate regulatory mechanisms. The earliest successes came from studies in Paris by François Jacob and Jacques Monod, who isolated dozens of *Escherichia coli* mutants with

A mutant defective in enzyme	Accumulates metabolite in culture medium	Requires an external source of	Culture filtrate allows the growth of another mutant, de-fective in enzyme
I	A	B, C, D, or E	—
II	B	C, D, or E	I
III	C	D, or E	I or II
IV	D	E	I, II, or III

Analysis of mutants



Pathway

FIGURE 12.14

Using mutations as biochemical probes. The steps of a hypothetical metabolic pathway are identified by analysis of mutants defective in individual steps of the pathway. For example, we can identify metabolite C as the substrate for enzyme III by the absence of this enzyme in mutants that accumulate C. We know that D and E follow C in the pathway because feeding either D or E to mutants defective in enzyme III bypasses the genetic block and allows the cells to grow.

defects in regulation of lactose catabolism or with abnormalities in virus–host relationships. These data led ultimately to the discovery of mRNA and the repressor–operator mechanism of genetic regulation, which we discuss in Chapter 27. Recombinant DNA technologies (see Tools of Biochemistry 4B) have allowed even more sophisticated perturbations of metabolic regulatory systems. For example, a researcher might use site-directed mutagenesis to disable the allosteric regulatory sites on an enzyme in a particular substrate cycle. The mutated enzyme is then introduced back into the cell, replacing the wild-type version, and the effects of loss of regulation can be examined in vivo.

A single investigation can use both metabolic inhibitors and mutations. This combined approach helped to elucidate the function of DNA gyrase, one of the DNA topoisomerases mentioned in Chapter 4. This enzyme is inhibited by nalidixic acid. When nalidixic acid is administered to bacteria, DNA replication is inhibited, suggesting that DNA gyrase plays an essential role in DNA replication. However, because nalidixic acid *could* inhibit DNA replication by blocking some other enzyme, stronger evidence is needed. Such evidence was obtained when mutants resistant to nalidixic acid were found to contain an altered form of DNA gyrase that was resistant to nalidixic acid. Thus, a single mutation abolished nalidixic acid sensitivity for both the enzyme and the ability of the cells to replicate their DNA, strongly supporting an essential role for DNA gyrase in DNA replication.

Finally, **systems biology** approaches are now being applied to the study of metabolism. Systems biologists try to catalogue the full set of components in a system, and then study the interactions between these components and how these interactions give rise to the specific function and behavior of that system. Metabolic profiling (see Tools of Biochemistry 12B) is an example of this kind of approach.

In this chapter we have described the general strategy of metabolism, identified the major pathways, outlined how pathways are regulated, and identified experimental approaches to understanding metabolism. We are now prepared for detailed descriptions of metabolic pathways, which we begin in Chapter 13 with carbohydrates.

SUMMARY

Metabolism is the totality of chemical reactions occurring within a cell. Catabolic pathways break down substrates to provide energy, largely through oxidative reactions, whereas anabolic pathways synthesize complex biomolecules from small molecules, often from intermediates in catabolic pathways. Catabolic and anabolic pathways with the same end points are actually different pathways, not simple reversals of each other so that both pathways are thermodynamically favorable. Regulation can be imposed only on reactions that are displaced far from equilibrium. Most metabolic energy comes from oxidation of substrates, with energy release coming in a series of small steps as the electrons released are transferred ultimately to oxygen. The more highly reduced a substrate, the more energy is released through its catabolism.

Flux through metabolic pathways is controlled by regulation of enzyme concentration (through control of enzyme synthesis and degradation), enzyme activity (through concentrations of substrates, products, and allosteric effectors, and covalent modification of enzyme proteins), compartmentation, and hormonal control. Hormonal regulation may involve control of enzyme synthesis at the genetic level or regulation of enzyme activity. In the latter case, intracellular second messengers are formed in response to hormonal signals.

The understanding of metabolic processes requires identification of each reaction in a pathway and a knowledge of the reaction's function and control. This understanding requires experimentation at all levels of biological organization, from living organism to purified enzyme. The ability to block specific enzymes, either with inhibitors or mutations, is of great value in identifying the functions of those enzymes.

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PROBLEMS

1. Write a balanced equation for the complete oxidation of each of the following, and calculate the respiratory quotient for each substance.
   (a) Ethanol
   (b) Acetic acid
   (c) Stearic acid
   (d) Oleic acid
   (e) Linoleic acid

2. Given what you know about the involvement of nicotinamide nucleotides in oxidative and reductive metabolic reactions, predict whether the following intracellular concentration ratios should be (1) unity, (2) greater than unity, or (3) less than unity. Explain your answers.
   (a) $[NAD^+]/[NADH]$
   (b) $[NADP^+]/[NADPH]$

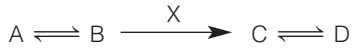
   Because NAD^+ and $NADP^+$ are essentially equivalent in their tendency to attract electrons, discuss how the two concentration ratios might be maintained inside cells at greatly differing values.

3. (a) NAD^+ kinase catalyzes the ATP-dependent conversion of NAD^+ to $NADP^+$. How many reducing equivalents are involved in this reaction?
   (b) How many reducing equivalents are involved in the conversion of ferric to ferrous ion?
   (c) How many reducing equivalents are involved in reducing one molecule of oxygen gas to water?

4. On page 516 we showed that the oxidation of glucose and palmitic acid yields 15.64 kJ/g and 38.90 kJ/g, respectively. Calculate these values in terms of kJ/mol and kJ per carbon atom oxidized for both glucose and palmitic acid.

5. Free energy changes under intracellular conditions differ markedly from those determined under standard conditions. $\Delta G^{\circ\prime}$ for ATP hydrolysis to ADP and P_i is -30.5 kJ/mol. Calculate ΔG for ATP hydrolysis in a cell at 37 °C that contains ATP at 3 mM, ADP at 1 mM, and P_i at 1 mM.

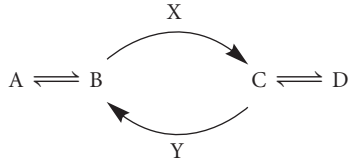
6. Consider the following hypothetical metabolic pathway:



   (a) Under intracellular conditions, the activity of enzyme X is 100 pmol/10^6 cells/sec. Calculate the effect on metabolic flux rate (pmol/10^6 cells/sec) of B ⟶ C of the following treatments. Calculate as % change (increase or decrease).

Treatment	% Change in flux
Inhibitor that reduces activity of X by 10%	
Activator that increases activity of X by 10%	

   (b) Now consider a substrate cycle operating with enzymes X and Y in the same hypothetical metabolic pathway:



Under intracellular conditions, the activity of enzyme X is 100 pmol/10^6 cells/sec, and that of enzyme Y is 80 pmol/10^6cells/sec, What are the direction and rate (pmol/10^6cells/sec) of metabolic flux between B and C?

(c) Calculate the effect on direction and metabolic flux rate of the following treatments. Calculate as % change (increase or decrease).

Treatment	Direction of flux (B → C or C → B)	% Change in flux
Inhibitor that reduces activity of X by 10%		
Activator that increases activity of X by 10%		
Doubling the activity of enzyme Y		

(d) Briefly summarize the regulatory advantage(s) of a substrate cycle in a pathway.

7. The glucose/glucose-6-phosphate substrate cycle involves distinct reactions of glycolysis and gluconeogenesis that interconvert these two metabolites. Assume that under physiological conditions, [ATP] = [ADP]; [P_i] = 1 mM.

Consider the glycolytic reaction catalyzed by hexokinase:

$$\text{ATP} + \text{glucose} \rightleftharpoons \text{ADP} + \text{glucose-6-phosphate}$$
$$\Delta G^{\circ\prime} = -16.7\ \text{kJ/mol}$$

(a) Calculate the equilibrium constant (K) for this reaction at 298°K, and from that, calculate the maximum [glucose-6-phosphate]/[glucose] ratio that would exist under conditions where the reaction is still thermodynamically favorable.

(b) Reversal of this interconversion in gluconeogenesis is catalyzed by glucose-6-phosphatase:

$$\text{glucose-6-phosphate} + H_2O \rightleftharpoons \text{glucose} + P_i$$
$$\Delta G^{\circ\prime} = -13.8\ \text{kJ/mol}$$

K for this reaction is 262. Calculate the maximum ratio of [glucose]/[glucose-6-phosphate] that would exist under conditions where the reaction is still thermodynamically favorable.

(c) Under what cellular conditions would both directions in the substrate cycle be strongly favored?

(d) What ultimately controls the direction of net conversion of a substrate cycle such as this in the cell?

Read Tools of Biochemistry 12A before attempting to work Problems 8–10.

*8. Two-dimensional gel electrophoresis of proteins in a cell extract provides a qualitative way to compare proteins with respect to intracellular abundance. Describe a quantitative approach to the determination of number of molecules of an enzyme per cell.

*9. Mammalian cells growing in culture were labeled for a long time in [^{3}H]thymidine to estimate the rate of DNA synthesis. The thymidine administered had a specific activity of 3000 cpm/pmol. At intervals, samples of culture were taken and acidified to precipitate nucleic acids. The rate of incorporation of isotope into DNA was 1500 cpm/10^6 cells/min. A portion of culture was taken for determination of specific activity of the intracellular dTTP pool, which was found to be 600 cpm/pmol.

(a) What fraction of the intracellular dTTP is synthesized from the exogenous precursor?

(b) What is the rate of DNA synthesis, in molecules per minute per cell of thymine nucleotides incorporated into DNA?

(c) How might you determine the specific activity of the dTTP pool?

*10. Suppliers of radioisotopically labeled compounds usually provide each product as a mixture of labeled and unlabeled material. Unlabeled material is added deliberately as a **carrier,** partly because the specific activity of the carrier-free product is too high to be useful and partly because the product is more stable at lower specific activities. Using the radioactive decay law, calculate the following.

(a) The specific activity of carrier-free [^{32}P]orthophosphate, in mCi/mmol.

(b) In a preparation of uniform-label [^{3}H]leucine, provided at 10 mCi/mmol, the fraction of H atoms that are radioactive.

TOOLS OF BIOCHEMISTRY 12A

RADIOISOTOPES AND THE LIQUID SCINTILLATION COUNTER

Radioisotopes revolutionized biochemistry when they became available to investigators shortly after World War II. Radioisotopes extend by orders of magnitude the sensitivity with which chemical species can be detected. Traditional chemical analysis can detect and quantify molecules in the micromole or nanomole range (i.e., 10^{-6} to 10^{-9} mole). A compound that is "labeled," containing one or more atoms of a radioisotope, can be detected in picomole or even femtomole amounts (i.e., 10^{-12} or 10^{-15} mole). Radiolabeled compounds are called **tracers** because they allow an investigator to follow specific chemical or biochemical transformations in the presence of a huge excess of nonradioactive material.

Recall that isotopes are different forms of the same element. They have different atomic weights but the same atomic number. Thus, the chemical properties of the different isotopes of a particular element are virtually identical. Isotopic forms of an element exist naturally, and substances enriched for rare isotopes can be isolated and purified from natural sources. Most of the isotopes used in biochemistry, however, are produced in nuclear reactors. Simple chemical compounds produced in such

reactors are then converted to radiolabeled biochemicals by chemical and enzymatic synthesis.

Stable Isotopes

Although radioisotopes are widely used in biochemistry, stable isotopes are also used as tracers, particularly when convenient radioisotopes are not available. For example, the two rare isotopes of hydrogen include a stable isotope ($^2H_1$, or **deuterium**) and a radioactive isotope ($^3H_1$, or **tritium**). Of the many uses of stable isotopes in biochemical research, we mention three applications here. First, incorporation of a stable isotope often increases the density of a material because the rare isotopes usually have higher atomic weights than their more abundant counterparts. This difference presents a way to separate labeled from nonlabeled compounds physically, as in the Meselson–Stahl experiment on DNA replication (see Chapter 4). Second, compounds labeled with stable isotopes, particularly ^{13}C, are widely used in nuclear magnetic resonance studies of molecular structure and dynamics (see Tools of Biochemistry 6A). Third, stable isotopes are used to study reaction mechanisms. The "isotope rate effect" refers to the effect on reaction rate of substitution of an atom by a heavy isotope. As discussed in Chapter 11, such an effect helps to identify rate-limiting steps in enzyme-catalyzed reactions. Table 12A.1 gives information about the isotopes, both stable and radioactive, that have found the greatest use in biochemistry.

The Nature of Radioactive Decay

The atomic nucleus of an unstable element can decay, giving rise to one or more of the three types of ionizing radiation: α- β-, or γ-rays. Only β- and γ-emitting radioisotopes are used in biochemical research; the most useful are shown in Table 12.1. A β-ray is an emitted electron, and a γ-ray is a high-energy photon.

TABLE 12A.1 Some useful isotopes in biochemistry

Isotope	Stable or Radioactive	Emission	Half-Life	Maximum Energy (MeV[a])
^2H	Stable			
^3H	Radioactive	β	12.1 years	0.018
^{13}C	Stable			
^{14}C	Radioactive	β	5568 years	0.155
^{15}N	Stable			
^{18}O	Stable			
^{24}Na	Radioactive	β (and γ)	15 hours	1.39
^{32}P	Radioactive	β	14.2 days	1.71
^{35}S	Radioactive	β	87 days	0.167
^{45}Ca	Radioactive	β	164 days	0.254
^{59}Fe	Radioactive	β (and γ)	45 days	0.46, 0.27
^{131}I	Radioactive	β (and γ)	8.1 days	0.335, 0.608

[a]MeV = million electron volts.

γ-Ray detectors have found wide use in immunological research because γ-emitting isotopes of iodine are available, and antibodies, like many proteins, can easily be iodinated without substantial changes in their biological properties. Most biochemical uses of radioisotopes, however, involve β-emitters.

Radioactive decay is a first-order kinetic process. The probability that a given atomic nucleus will decay is affected neither by the number of preceding decay events that have occurred nor by interaction with other radioactive nuclei. Rather, it is an intrinsic property of that nucleus. Thus, the number of decay events occurring in a given time interval is related only to the number of radioactive atoms present. This phenomenon gives rise to the **law of radioactive decay**:

$$N = N_0 e^{-\lambda t}$$

where N_0 is the number of radioactive atoms at time zero, N is the number remaining at time t, and λ is a radioactive decay constant for a particular isotope, related to the intrinsic instability of that isotope. This equation states that the *fraction* of nuclei in a population that decays within a given time interval is constant. For this reason, a more convenient parameter than the decay constant λ is the **half-life**, $t_{1/2}$, the time required for half of the nuclei in a sample to decay. The half-life is equal to $-\ln 0.5/\lambda$, or $+ 0.693/\lambda$. The half-life, like λ, is an intrinsic property of a given radioisotope (see Table 12.1).

The basic unit of radioactive decay is the **curie** (Ci). This unit is defined as an amount of radioactivity equivalent to that in 1 g of radium, namely 2.22×10^{12} disintegrations per minute (dpm). Biochemists usually work with far smaller amounts of radioactive material—the **millicurie** (mCi) and the **microcurie** (μCi), which correspond, respectively, to 2.2×10^9 and 2.2×10^6 dpm. Because detectors of radioactivity rarely record every decay event in a sample—that is, they are not 100% efficient—we often speak of radioactivity in terms of the decay events actually recorded: counts per minute, or cpm. Counting efficiency is the percentage of decay events actually recorded, as determined, for example, by reference to standards. A counter that is 50% efficient for a given isotope would show a count rate of 1.1×10^5 cpm for a 0.1 $-$ μCi sample.

Detection of Radioactivity: The Liquid Scintillation Counter

In scintillation counting, the most widely used method for measuring β-emissions, the sample is dissolved or suspended in an organic solvent, although aqueous mixtures are available as well. Also present are one or two fluorescent organic compounds, or **fluors**. A β-particle emitted from the sample has a high probability of hitting a molecule of the solvent; this contact excites the solvent molecule, driving an electron into a higher-energy orbital. When that electron returns to the ground state, a photon of light is emitted. The photon is absorbed by a molecule of the fluor, which in turn becomes excited. Fluorescence involves the absorption of light at a given energy, followed by emission of that light at lower energy, or longer wavelength. A photomultiplier detects the fluorescence and for each disintegration converts it to an electrical signal, which is recorded and counted.

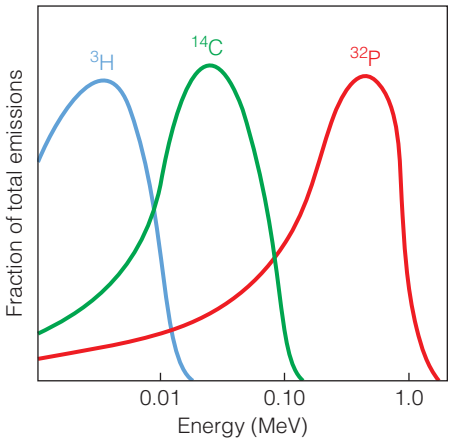


FIGURE 12A.1

Energy spectra for some β-emitting isotopes. Shown here are spectra for the three β-emitters most widely used in biochemistry—^{3}H, ^{14}C, and ^{32}P.

Whereas γ-rays are emitted at distinct and characteristic energy values, a β-emitting isotope will display a range of energy values among its emissions. Each β-emitter shows a characteristic **energy spectrum**—that is, a plot of energy (in millions of electron volts, or MeV) against the probability of an individual emission having that energy. Figure 12A.1 shows the energy spectra for three widely used radioisotopes—^{3}H, ^{14}C, and ^{32}P.

These differences in emission energies are exploited in the liquid scintillation counter so that two isotopes in the same sample can be simultaneously quantified (a dual-label experiment). A strong β-emission excites more fluor molecules than a weak emission does, so a brighter light flash is produced. This flash level can be detected by setting electronic discriminators so that energy values falling only within a desired range are recorded—that is, setting a "window" that is optimized for detection of a particular isotope. This restriction reduces counting efficiency because only a fraction of the emissions from the higher-energy isotope are counted, but it does permit great selectivity. Most scintillation counters have three different sets of discriminators, or **counting channels**. Thus, isotopes can be counted simultaneously in three different windows.

Because radioactive decay is a random process, the more counts observed during the analysis of a sample, the more closely the measured radioactivity will approach the true decay rate. In practice, therefore, a researcher wants to count a sample for enough time to accumulate several thousand counts. Also, the researcher must consider the signal-to-noise ratio and design an experiment so that the measured radioactivity of the samples of interest will be many times higher than **background radioactivity**, or the counts recorded in the absence of a radioactive sample.

Some Uses of Radioisotopes in Biochemistry

Radioisotopes have many uses in metabolic investigations. Here we describe a few examples. Tracer experiments can be categorized in terms of the time of exposure of the biological system to the radioisotope and include (1) equilibrium labeling, (2) pulse labeling, and (3) pulse-chase labeling.

In **equilibrium labeling** the exposure to the tracer is relatively long so that each labeled species reaches a constant **specific radioactivity**, or **specific activity**. The specific activity is a measure of the relative abundance of radioactive molecules in a labeled sample and is reported as radioactivity per unit mass—for example, cpm/mol. Experiments to identify metabolic precursors often involve equilibrium labeling conditions. The investigator administers a radiolabeled precursor so as to maximize the chance of detecting label in the product. A good example, mentioned in Chapter 19, is Konrad Bloch's study of cholesterol biosynthesis, in which ^{14}C-labeled acetate was administered to rats. Bloch then isolated cholesterol from the liver and determined, by chemical degradation, the specific carbon atoms in cholesterol that had incorporated radioactivity.

Another use of equilibrium labeling is to measure rates of biological processes from the rates of labeling by low-molecular-weight precursors. An example is the widespread use of radiolabeled thymidine to follow rates of DNA synthesis, either in cell cultures or in intact organisms. Incorporation of thymidine into macromolecules other than DNA is negligible, so the investigator merely samples the population with time and observes the incorporation into acid-insoluble material. However, this measurement alone does not give the true rate of DNA replication. The investigator must also consider the metabolism of the labeled precursor en route to its ultimate destination. If intracellular metabolism is producing dTTP, that nucleotide will be nonradioactive, and its mixing with the labeled pool will dilute the specific radioactivity incorporated into DNA. The labeling experiment gives only a rate of DNA labeling, in cpm incorporated per cell per unit time. To get a true rate, in molecules incorporated per cell per unit time, the initial result must be divided by the specific activity of the immediate precursor, in this case, dTTP. Calculating that specific activity requires isolation of dTTP in sufficient amount and purity to allow determination of both its mass and radioactivity.

Pulse labeling involves administration of an isotopic precursor for an interval that is short relative to the process under study. Radiolabel accumulates preferentially in the shortest-lived species—that is, the earliest intermediates in a metabolic pathway—because the shorter the labeling interval is, the less time is available for loss of radioactivity from a labeled pool through breakdown of rapidly labeled and short-lived metabolites. Melvin Calvin identified the pathway of photosynthetic carbon fixation by labeling green algae with $^{14}CO_2$ for just a few seconds. If labeling was carried out for 10 s, a dozen or more radioactive compounds could be detected. After a 5-s pulse of radioactivity, only a single compound was labeled, namely 3-phosphoglycerate. This finding led ultimately to the discovery of ribulose-1,5-bisphosphate carboxylase as the first enzyme in the photosynthetic carbon fixation pathway (see p. 729 in Chapter 16).

In a **pulse-chase** experiment, the investigator administers label for a short time (the pulse) and then rapidly reduces the specific activity of the isotopic precursor to prevent further incorporation. This reduction can be done by adding unlabeled precursor at a molar excess of about 1000-fold, which greatly

dilutes the radioisotope still present (the chase). The investigator samples at various times afterward, to determine the metabolic fate of the material labeled during the pulse. Messenger RNA was originally detected by pulse-labeling bacterial cultures (see Chapter 27). Traditional analytical methods could not detect mRNA because of its low abundance and its metabolic instability in bacterial cells. When label incorporated into mRNA by a pulse of [^{32}P]orthophosphate or labeled uridine was chased out, the label was ultimately found to be distributed uniformly in all cellular RNA species. This finding showed that the metabolic instability of mRNA involves its degradation to nucleotides, which can then be used for synthesis of other RNA species.

A final application of radioisotopes is **radioautography**, in which the investigator incorporates an isotopic precursor into a biomolecule and prepares an image of the radiolabeled molecule on a sheet of photographic film. Chapter 25 shows several radioautographs of individual DNA molecules made radioactive by growth of *E. coli* in the presence of [^{3}H]thymidine. The two-dimensional gel shown in Figure 1.11 (p. 21) is a radioautograph because the radiolabeled proteins were detected by their ability to darken photosensitive film. Film radioautography has been largely replaced by **phosphorimaging**, in which the photographic film is replaced by an imaging plate coated with photostimulable phosphors. Phosphorimaging is much more accurate than film radioautography in quantifying the amount of radioactivity in the sample because its response to radioactivity is far more linear than that of X-ray film. Moreover, the imaging plate can be erased and reused indefinitely.

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TOOLS OF BIOCHEMISTRY 12B

METABOLOMICS

Virtually all the facts in this textbook came from experiments in which a biochemist measured something in a cell extract: the level of a particular mRNA or protein, the activity of a particular enzyme, or the concentration of a particular metabolite. Indeed, a large part of experimental biochemistry involves the development of specific and sensitive *assays* for a particular cellular component. For example, Hans Krebs's elucidation of the citric acid cycle (see Chapter 14) depended on his ability to accurately measure the concentrations of potential substrates such as pyruvate, citrate, succinate, or oxaloacetate in muscle or liver slices. A specific assay had to be developed to measure each metabolite. To fully analyze a pathway such as the citric acid cycle or glycolysis might require 10 or more separate metabolite assays on each sample. Furthermore, these assays could only measure already known metabolites—they were not very useful for discovering new or missing metabolic intermediates.

In recent years, however, new technologies have made it possible to move beyond the measurement of single mRNAs, single proteins, or single metabolites. The development of these new technologies has driven the "-omics" revolution—genomics, transcriptomics, proteomics, and metabolomics—where hundreds or even thousands of specific components are measured simultaneously in a biological sample. Thus, it is now feasible to measure the full set of transcripts (transcriptome), proteins (proteome), or metabolites (metabolome) in a particular cell or tissue. The metabolome represents the ultimate molecular phenotype of a cell under a given set of conditions because all the changes in gene expression and enzyme activity eventually lead to changes in cellular metabolite levels (the metabolic state or profile). The metabolic state of a cell or a whole organism is a sensitive indicator of the physiological status of the organism. Changes in metabolic state can be used to understand and diagnose disease, study effects of drugs, and even predict the effectiveness of a drug in a particular patient. There are many analytical approaches to determining the metabolic state of a cell (metabolic profiling), but they all follow the same basic process (Figure 12B.1): sample extraction; metabolite identification and quantitation; and data analysis (informatics).

Metabolic Profiling

Sample collection and extraction

Samples of interest are collected—for example drug-treated vs. untreated cells, serum from a patient, or a tissue biopsy from a tumor. The small molecules are extracted from the samples by methods that are matched to the analytical technique to be used. No single method will extract all metabolites from a sample. Different extraction procedures can be used to select specific subsets of metabolome (e.g., lipophilic metabolites, amino acids, or carbohydrates).

Metabolite identification and quantitation

While the number of distinct metabolites in a cell is not known, it is certainly on the order of a few thousand. Therefore, the analytical method must have both a separation component and a detection

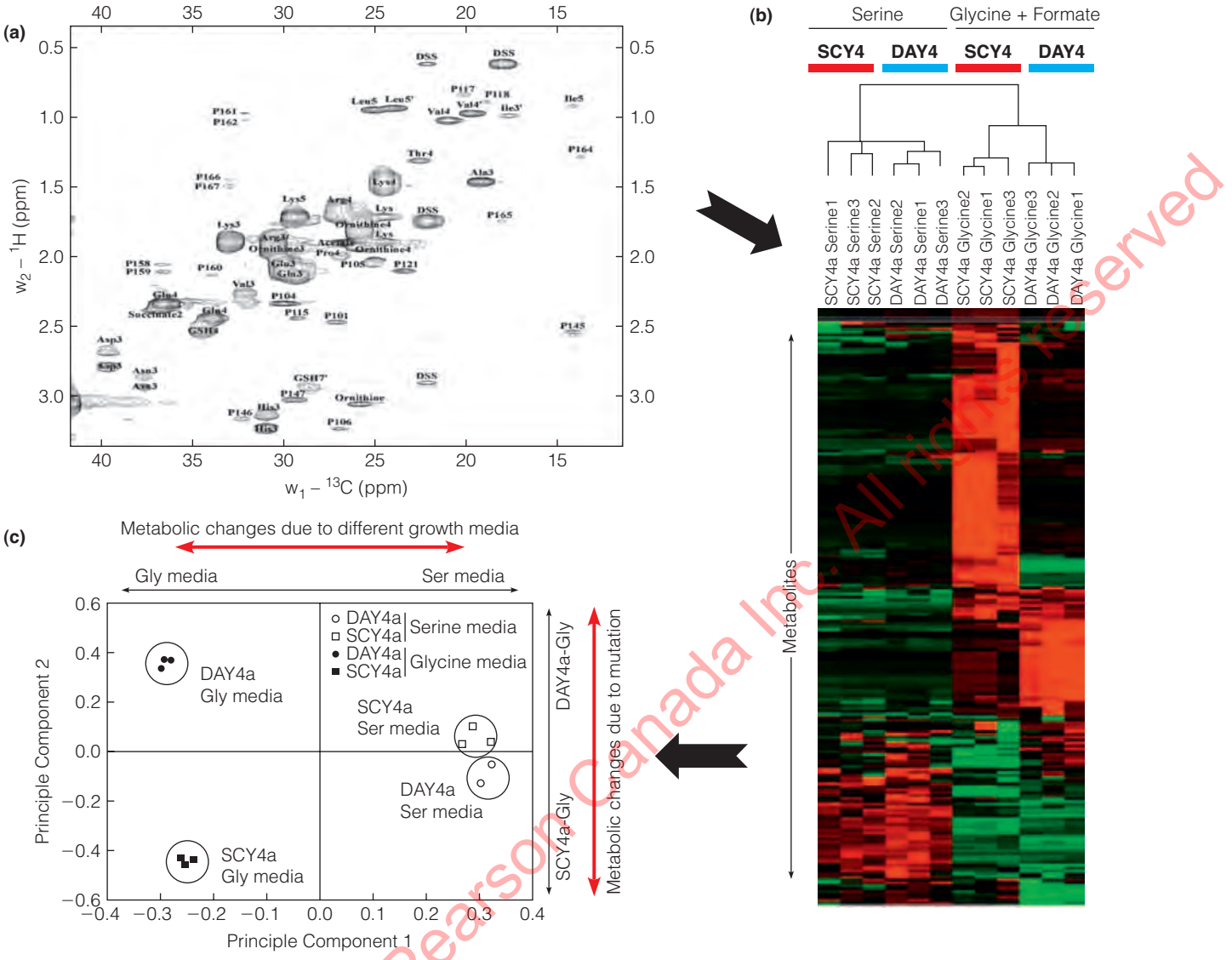

FIGURE 12B.1

Basic process of metabolic profiling. Metabolites are identified and quantified by an analytical method. Panel **a** shows a section of a $^1H - {}^{13}C$ 2D-NMR spectrum. Data are collected and visualized by informatics approaches. In the "heat map" shown in panel **b**, each row corresponds to a single 2D-NMR metabolite peak, with columns representing different experimental conditions or cell type. The normalized magnitude of each 2D-NMR peak is indicated by color, with red indicating an increase in metabolite abundance relative to the control, green indicating a decrease in abundance, and black for the median level of abundance. Informatics approaches are then used to reveal relationships and patterns among the samples (panel **c**).

Modified from Lu, P., Rangan, A., Chan, S. Y., Appling, D. R., Hoffman, D. W., and Marcotte, E. M. (2007) *Metab Eng* **9,** 8–20.

component. Mass spectrometry (MS) and nuclear magnetic resonance (NMR) are the most widely used detection methods. The MS-based techniques use liquid chromatography (LC), gas chromatography (GC), or capillary electrophoresis (CE) to separate the metabolites based on some chemical or physical property (size, charge, hydrophobicity, etc.). Effluent from the LC, GC, or CE is introduced into the mass spectrometer, where the metabolites are detected and quantified (see Tools of Biochemistry 5B). NMR (see Tools of Biochemistry 6A) can be applied directly to samples without a separation step by obtaining spectra in two dimensions (e.g., ^1H vs. ^{13}C), termed 2D-NMR (Figure 12B.1a). Both MS and NMR methods are capable of detecting and quantifying hundreds of distinct metabolites in a single run, and both have advantages and disadvantages. NMR can detect many different classes of metabolites and offers great power in metabolite identification and quantitation. MS-based methods are much more sensitive than NMR, but quantitation is not as straightforward. Note, however, that both methods can detect and quantify unidentified metabolites, which can facilitate the discovery of new intermediates or even new pathways.

Data analysis (informatics)

The third critical step in metabolic profiling is data analysis. Because the datasets are generally quite large, and sophisticated statistical methods are required to visualize and compare metabolic profiles, data analysis uses modern informatics tools. These informatics algorithms attempt to identify patterns in the data that are reproducibly characteristic of a particular metabolic state. These patterns, or fingerprints, can then be compared between samples. The most common informatics methods for analyzing and comparing metabolome data are hierarchical clustering (the partitioning of a data set into subsets or clusters) (Figure 12B.1b) and principal component analysis (PCA) (Figure 12B.1c). PCA is used to reduce multidimensional data sets to lower dimensions (principal components). PCA can often reveal the internal structure of the data in a way which best explains the variance in the data.

Applications

Biomarker discovery

The identification of new biomarkers to predict or diagnose disease is a major goal of biomedical research. Although there are some useful single molecule biomarkers known, for many diseases a pattern of several metabolites will be a more informative biomarker. The following example illustrates the power of metabolic profiling to discover new biomarkers. Acetaminophen (e.g., Tylenol) is a widely used over-the-counter analgesic. Excess acetaminophen is toxic to the liver because it causes oxidative stress, but individuals vary in their

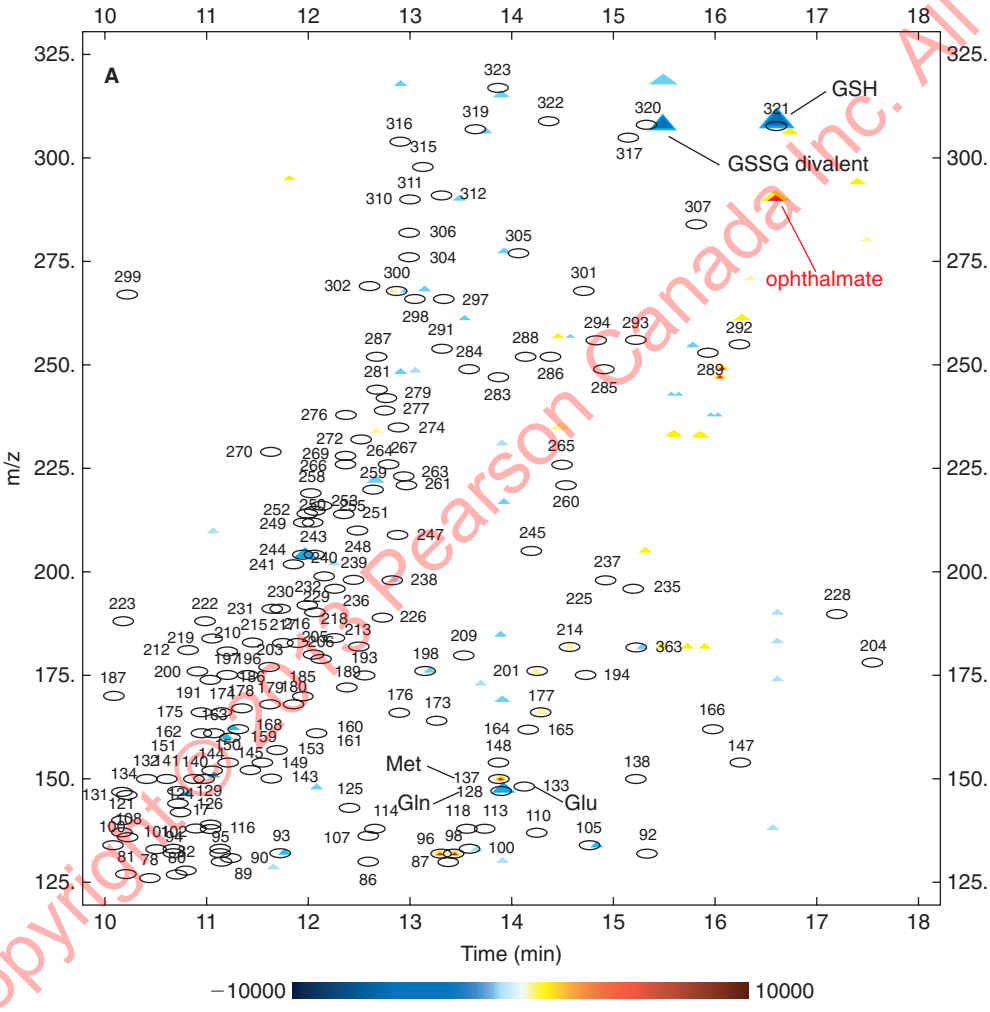

FIGURE 12B.2

Differential metabolic profile from livers of control mice and livers from mice 2 h after treatment with acetaminophen. The 2D plot (m/z vs. elution time) shows cations detected by CE-MS analysis. The arrow points to the metabolite subsequently identified as ophthalmate whose level significantly increased in treated mice. The color bar indicates the increase (red) or the decrease (blue) of metabolite level after drug treatment.

Courtesy of V.K. Mootha and O. Shaham.

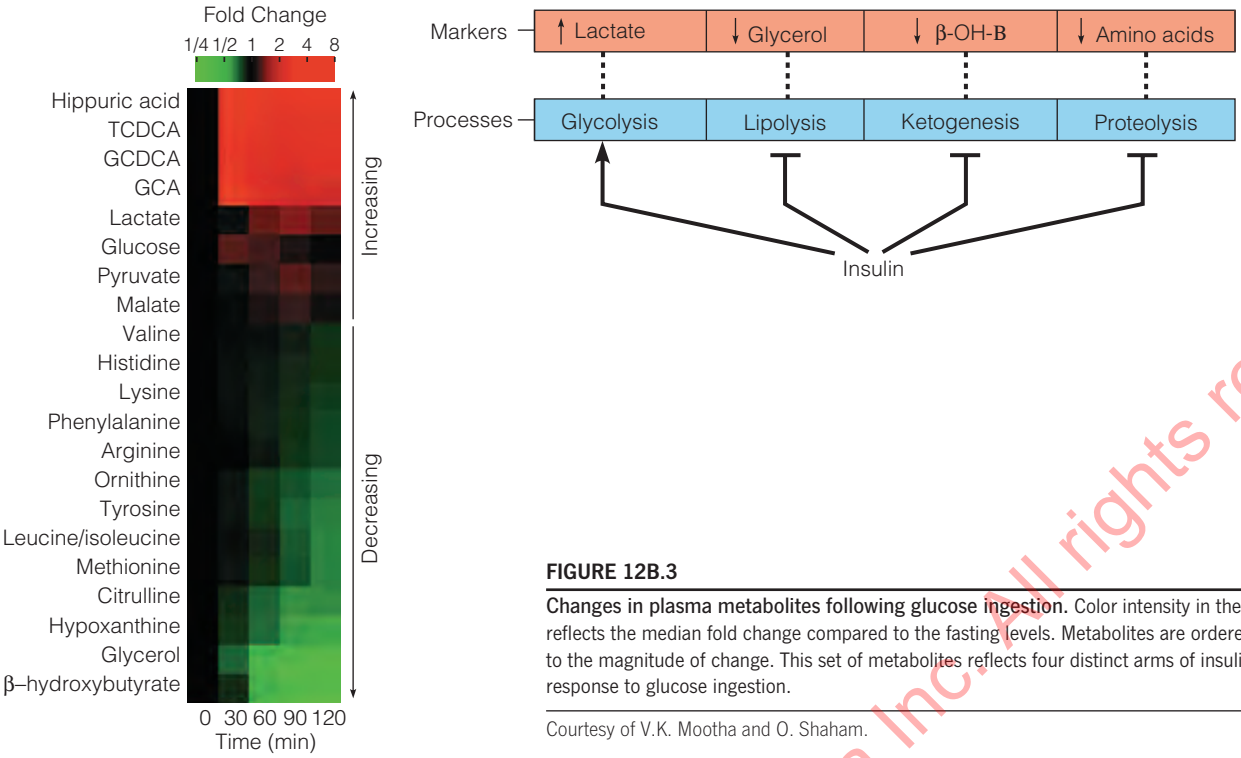

FIGURE 12B.3

Changes in plasma metabolites following glucose ingestion. Color intensity in the heat map reflects the median fold change compared to the fasting levels. Metabolites are ordered according to the magnitude of change. This set of metabolites reflects four distinct arms of insulin action in response to glucose ingestion.

Courtesy of V.K. Mootha and O. Shaham.

susceptibility to liver damage. To identify potential biomarkers for the oxidative stress caused by acetaminophen, CE-MS was used to study changes in liver metabolites in mice treated with excess acetaminophen. Metabolic profiling identified 132 compounds, including a new metabolite (ophthalmate) (Figure 12B.2). This compound is not a metabolite of acetaminophen, but its levels rise in response to the drug. This new metabolite may serve as a useful biomarker for acetaminophen-induced hepatotoxicity in humans.

Disease diagnosis

The incidence of diabetes has increased so rapidly in the United States that it is now considered an epidemic. Today, more than 21 million Americans have diabetes, and it is estimated that 54 million have prediabetes—they have elevated blood sugar levels, but not high enough to be classified as diabetes. Prediabetics are at high risk of developing type 2 diabetes. If detected early enough, diabetes is a manageable disease. Insulin resistance, traditionally defined as the reduced ability of insulin to promote glucose uptake, is a characteristic feature of type 2 diabetes. However, insulin regulates many other metabolic processes, and metabolic profiling can be used to look beyond glucose metabolism. LC-MS has been used to quantify changes in 191 metabolites in human plasma following glucose ingestion. Eighteen of these were observed to change reproducibly in healthy individuals, reflecting four distinct arms of insulin function (Figure 12B.3). In prediabetic patients, however, a subset of these metabolites showed blunted responses to glucose ingestion, providing a strong indicator of loss of insulin sensitivity. This application of metabolic profiling holds great promise in early diagnosis of diabetes.

Predicting drug response

One of the most serious problems with therapeutic drugs is that patients can vary considerably in their response to the drug and in their susceptibility to side effects. For example, the tyrosine kinase inhibitor imatinib is a highly effective drug for treatment of chronic myeloid leukemia (CML). Unfortunately, some patients develop resistance to this drug, and treatment fails. To try to understand the mechanisms underlying this individual variation in drug response, blood plasma metabolites were profiled in CML patients previously determined to be either sensitive or resistant to imatinib. Principal component analysis (PCA) revealed metabolic profiles that differed between those patients who were sensitive to drug treatment from those who were resistant (Figure 12B.4). Thus metabolic profiling can be used to predict whether a drug will be effective in a particular patient.

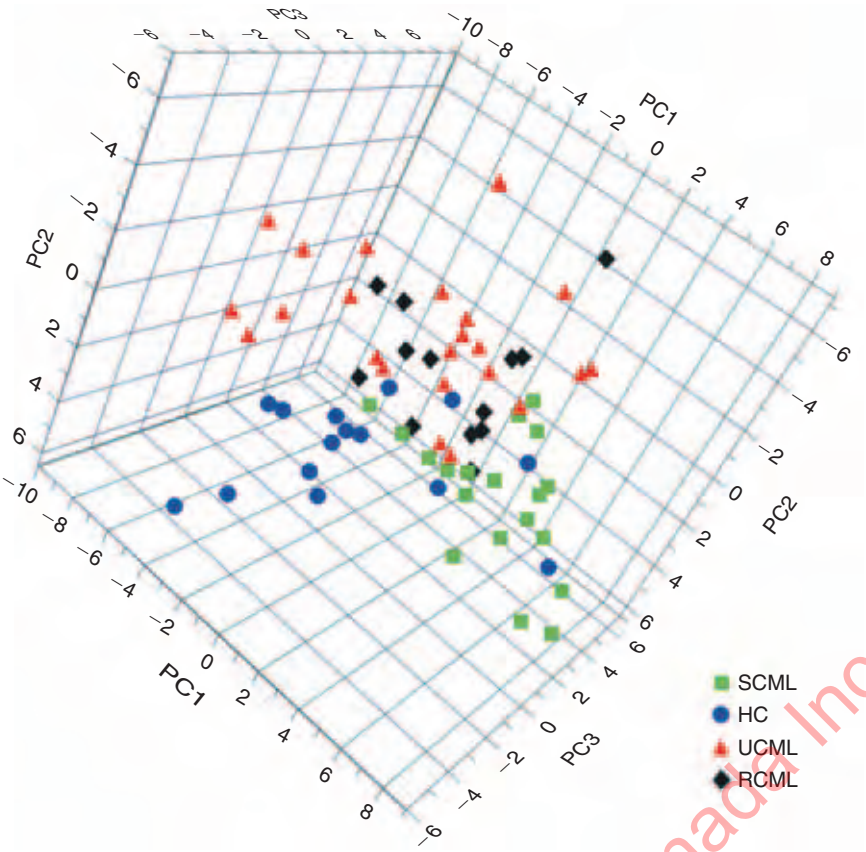

FIGURE 12B.4

Principal component analysis of blood plasma metabolites in chronic myeloid leukemia (CML) patients. 3D scatter plot of the first three principal components (PC1 vs. PC2 vs. PC3). Green squares are patients who are sensitive to drug treatment (SCML). Black diamonds are patients who are resistant to drug treatment (RCML). Red triangles are untreated CML patients (UCML). Blue circles are healthy controls (HC).

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