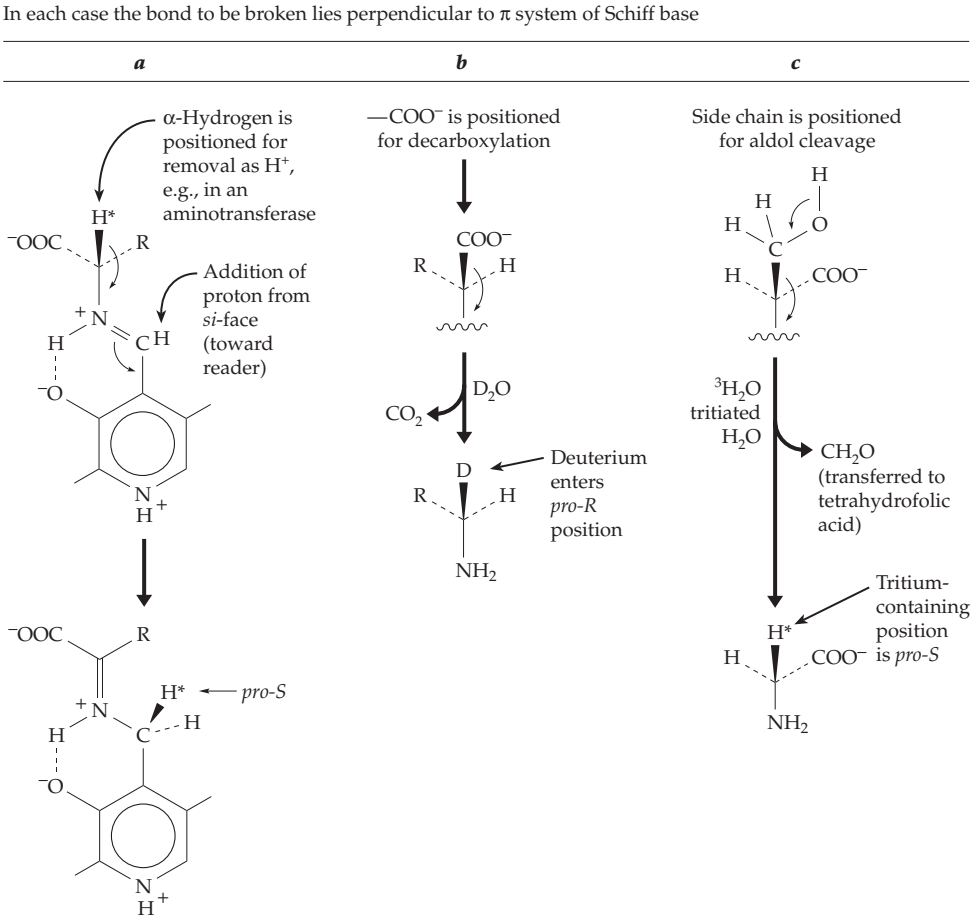
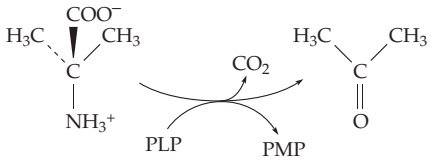



**Figure 14-8** Some stereochemical aspects of catalysis of PLP-requiring enzymes.

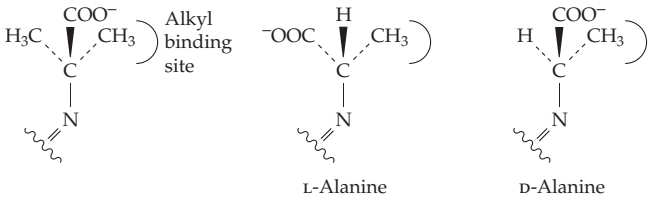
obtained by rotating the amino acid through 180°.

Dunathan suggested that this stereoelectronic requirement explains certain side reactions observed with PLP-requiring enzymes. The idea also received support from experiments with a bacterial α-dialkylglycinedecarboxylase.[231,232] The enzyme ordinarily catalyzes, as one half-reaction, the combination decarboxylation–transamination reaction shown in Eq. 14-37. It also acts on both D- and L-alanine, decarboxylating the former but catalyzing only removal of the α-H




(14-37)

from L-alanine. The results can be rationalized by assuming that the enzyme possesses a definite site for one alkyl group but that the position of the second alkyl group can be occupied by –H or –COO⁻ and that the group labilized lies perpendicular to the π system:

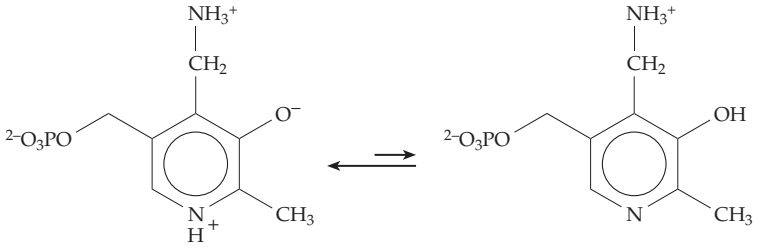



Glycine is unreactive, suggesting that occupation of the alkyl binding site is required for catalysis.

According to Dunathan's postulate, there are only two possible orientations of the amino acid substrate in an aminotransferase. One is shown in Fig. 14-8. In the other, the amino acid is rotated 180° so that the α-hydrogen protrudes *behind* the plane of the paper. Dunathan studied **pyridoxamine:pyruvate aminotransferase**, an enzyme closely related to PLP-requiring aminotransferases and which catalyzes the transamination of pyridoxal with L-alanine to form pyridoxamine and pyruvate. The same reaction is catalyzed by the apoenzyme of aspartate aminotransferase. In both cases, when the alanine contained $^2H$ in the α position the $^2H$ was transferred stereospecifically into

the *pro-S* position at C-4' of the pyridoxamine (indicated by asterisks in Fig. 14-8).[233] The results suggested that a group from the protein abstracts a proton from the α position and transfers it on the same side of the π system (**suprafacial transfer**), adding it to the *si* face of the C=N group as shown in Fig. 14-8. Later, the same stereospecific proton transfer was demonstrated for the PLP present in the holoenzyme.[234] Not surprisingly, the D-amino acid aminotransferase adds the proton to the *re* face of the C=N group.[235]

When a decarboxylase acts on an amino acid in $^2H_2O$, an atom of $^2H$ is incorporated in the *pro-R* position, the position originally occupied by the carboxyl group (Fig. 14-8). Cleavage of serine by serine hydroxymethyltransferase in $^3H$-containing water leads to incorporation of $^3H$ in the *pro-S* position. Stereospecific introduction of $^2H$ or $^3H$ has been observed in the β position of 2-oxobutyrate formed in β or γ elimination reactions. Conversion of serine to tryptophan by tryptophan synthetase occurs without inversion at C-3.[236] These and many other observations on PLP-dependent enzymes[189,237,238] can be generalized by saying that enzymatic reactions of PLP Schiff bases usually take place on only one face of the relatively planar structure. This is the *si* face at C-4' of the coenzyme (see Fig. 14-8). This result is expected if a single acid–base group serves as proton acceptor in one step and as proton donor in a later step. This leads naturally to the observed retention of configuration in steps involving replacement and the suprafacial transfer of protons from one position on that face to another.




Dipolar ionic form
I 327 nm
II 253 nm
III 217 nm

Minor non-dipolar ionic tautomer
I 283 nm

(14-38)

## 6. Seeing Changes in the Optical Properties of the Coenzyme

The absorption of light in the ultraviolet and visible regions is a striking characteristic of many coenzymes. It can be measured accurately and displayed as an absorption spectrum and may also give rise to circular dichroism and to fluorescence (Chapter 23). The optical properties of the vitamin $B_6$ coenzymes are sensitive to changes both in environment and in the state of protonation of groups in the molecule. For example, PMP in the neutral dipolar ionic form, which exists at pH 7, has three strong light absorption bands centered at 327, 253, and 217 nm.[239] The other ionic forms of PMP and other derivatives of vitamin $B_6$ also each have three absorption bands spaced at roughly similar intervals, but with varying positions and intensities. The minor tautomer of PMP containing an uncharged ring (Eq. 14-38) has its low-energy (long-wavelength) band at 283 nm. When both the ring nitrogen and phenolic oxygen are protonated, the band shifts again to 294 nm and if both groups are deprotonated the resulting anion absorbs at 312 nm. Thus, observation of the absorption spectrum of the coenzyme bound to an enzyme surface can tell us whether particular groups are protonated or unprotonated. The peak of bound PMP at 330 nm in aspartate aminotransferase (Fig. 14-9) is indicative of the dipolar ionic form (Eq. 14-38). However, the 5-nm shift from the position of free PMP suggests a distinct change in environment.

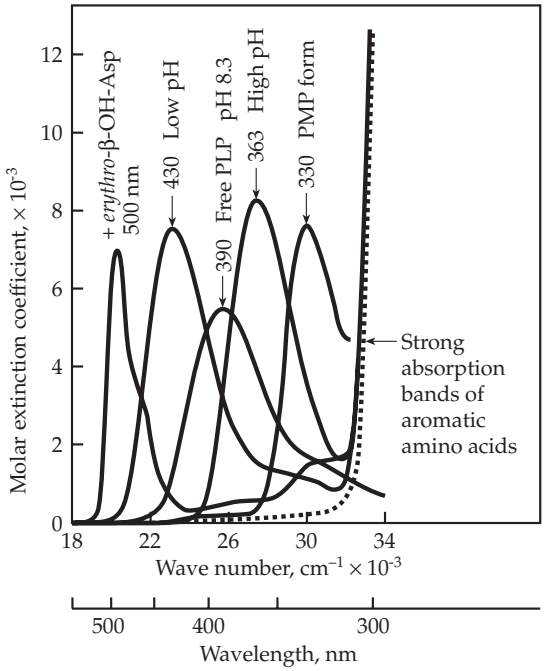
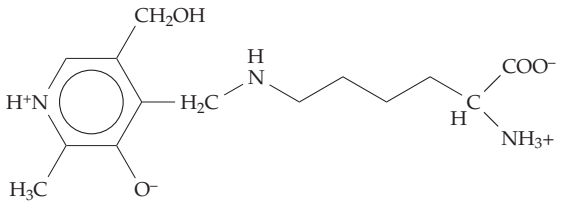



**Figure 14-9** Absorption spectra of various forms of aspartate aminotransferase compared with that of free pyridoxal phosphate. The low pH form of the enzyme observed at pH <5 is converted to the high pH form with $pK_a \sim 6.3$. Addition of *erythro*-3-hydroxyaspartate produces a quinonoid form whose spectrum here is shown only 1/3 its true height. The spectrum of free PLP at pH 8.3 is also shown . The spectrum of the apoenzyme (**- - -**) contains a small amount of residual absorption of uncertain origin in the 300- to 400-nm region.

Pyridoxal phosphate exists in an equilibrium between the aldehyde and its covalent hydrate (as in Eq. 13-1). The aldehyde has a yellow color and absorbs at 390 nm (Fig. 14-9), while the hydrate absorbs at nearly the same position as does PMP. The absorption bands of Schiff bases of PLP are shifted even further to longer wavelengths, with N-protonated forms absorbing at 415–430 nm. Forms with an unprotonated C=N group absorb at shorter wavelengths.[149,240]

***Imine groups in free enzymes.*** When, in 1957, W. T. Jenkins examined one of the first highly purified aspartate aminotransferase preparations he noted a surprising fact: The bound coenzyme, at pH 5, absorbed not at 390 nm, as does PLP, but at 430 nm, like a Schiff base (Fig. 14-9). When the pH was raised, the absorption band shifted to 363 nm. The result suggests dissociation of a proton (with a $pK_a$ of ~ 6.3) from the hydrogen-bonded position in a Schiff base of the type shown in Fig. 14-6. It was quickly demonstrated for this enzyme and for many other PLP-dependent enzymes that reduction with sodium borohydride caused the spectrum to revert to one similar to that of PMP and fixed the coenzyme to the protein. After complete HCl digestion of such borohydride-reduced proteins a fluorescent amino acid containing the reduced pyridoxyl group was obtained and in every case was identified as ε-pyridoxyl-lysine. Thus, PLP-containing enzymes in the absence of substrates usually exist as Schiff bases with lysine side chains of the proteins. Even the PLP in glycogen phosphorylase is joined in this way. However, its absorption maximum at 330 nm shows




ε-Pyridoxyllysine

that in phosphorylase it is present as the nondipolar ionic tautomer with a 3-OH group on the ring as in Eq. 14-38.

***Absorption bands at 500 nm.*** With many PLP enzymes certain substrates and inhibitors cause the appearance of intense and unusually narrow bands at ~500 nm. Such a band is observed with aspartate aminotransferases acting on *erythro*-3-hydroxyaspartate (Fig. 14-9). This substrate undergoes transamination very slowly, and the 500-nm absorbing form which accumulates is probably an intermediate in the normal reaction sequence. A similar spectrum is produced by tryptophan indole-lyase acting on the competitive inhibitor L-alanine. Under the same conditions the enzyme promotes a rapid exchange of the α-hydrogen of the alanine with $^2H$ of $^2H_2O$. Serine hydroxymethyltransferase gives a 495- to 500-nm band with both D-alanine and the normal product glycine.[241] Similar spectra have been produced in nonenzymatic model reactions[242] and probably represent the postulated quinonoid–carbanionic intermediates.

## 7. Atomic Structures

The three-dimensional structures of aspartate aminotransferases from *E. coli* to humans are very similar.[163–167a] The folding pattern (Fig. 2-6) and active site structure (Figs. 14-6 and 14-10) are completely conserved. The major domain of the protein contains a central β sheet surrounded by helices with coenzyme attached to a lysine at the C terminus of one of the β strands. The protein is a dimer with the two major domains held together by both polar and nonpolar interactions. The two active sites are located at the interface between the subunits and residues from both subunits participate in forming the active site (Fig. 14-10). The internal Schiff base is formed with Lys 258. The protonated ring nitrogen of the dipolar ionic PLP forms an ion pair with the carboxylate of Asp 222 which protrudes from a central seven-stranded β sheet. The phenolic $-O^-$ forms a hydrogen bond with the –OH of Tyr 225. The interactions of Asp 222 and Tyr 225 fix the ring as the dipolar ionic tautomer.

The phosphate group of the coenzyme, which $^{31}P$ NMR shows to be predominantly dianionic,[243] forms an ion pair with the side chain of Arg 266 and hydrogen bonds to a backbone N–H at the N terminus of a long helix where it can interact with the positive end of the helix dipole. In addition, the phosphate forms hydrogen bonds to four OH groups of Ser, Thr, and Tyr side chains. In front of the coenzyme ring are two guanidinium groups from the side chains of Arg 386 and of Arg 292* (from the second subunit). These have been shown by X-ray crystallography to bind the two carboxylate groups of a substrate such as glutamate, 2-oxoglutarate, aspartate, oxaloacetate, or cysteine sulfinate; of quasi-substrates such as 2-methylaspartate and *erythro*-β-hydroxyaspartate; or of dicarboxylic inhibitors (Fig. 14-10).

Comparison of amino acid sequences suggests that many other PLP-dependent enzymes have folding patterns similar to those of aspartate aminotransferase but that there are four or more additional different folding patterns.[205,243a,b] Among the enzymes resembling aspartate aminotransferase are ω-amino acid: pyruvate aminotransferase,[244] 2,3-dialkylglycine decarboxylase,[232] tyrosine phenol-lyase,[177] a bacterial ornithine decarboxylase,[182,204] and cystathionine β-lyase.[182] The tryptophan synthase β subunit has a second folding pattern,[184] while alanine racemase[153] and eukaryotic

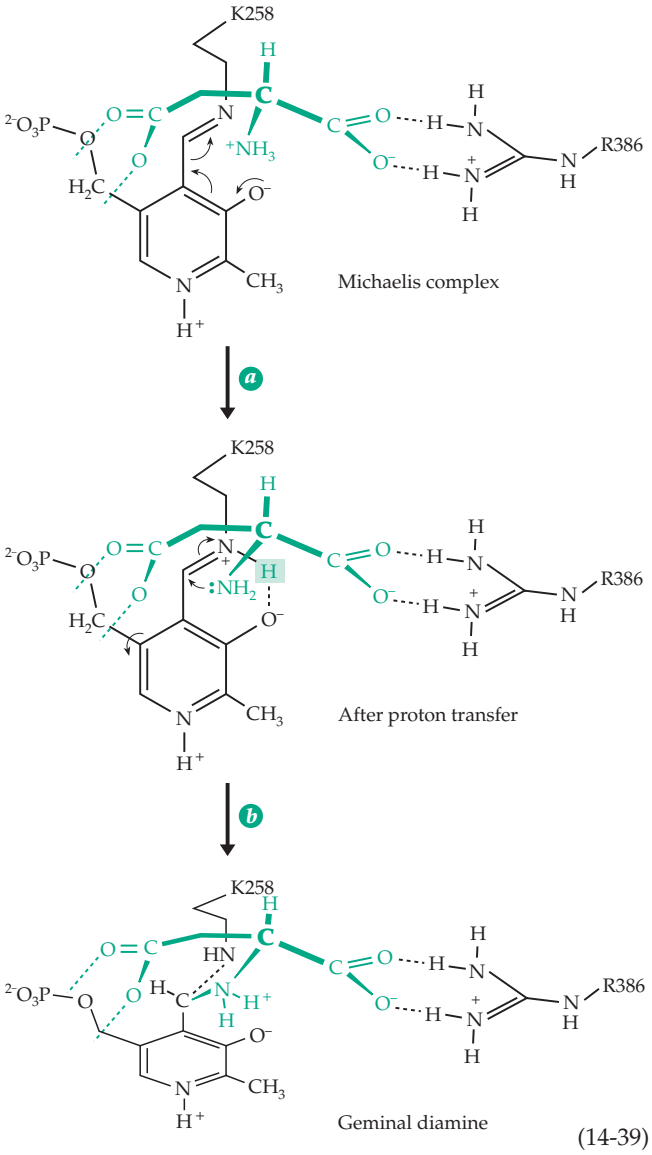
ornithine decarboxylases[205] have $(\alpha\beta)_8$-barrel structures resembling that in Fig. 2-28. A fourth structural pattern is that of D-amino acid aminotransferase.[235] It is anticipated that the branched-chain aminotransferase[245] will have a similar stucture. Glycogen phosphorylase (Fig 11-5) has a fifth folding pattern.

## 8. Constructing a Detailed Picture of the Action of a PLP Enzyme

Consider the number of different steps that must occur in about one-thousanths of a second during the action of an aminotransferase. First, the substrate binds to form the "Michaelis complex." Then the transimination (Eq. 14-26) takes place in two steps and is followed by the removal of the α-hydrogen to form the quinonoid intermediate. An additional four steps are needed to form the ketimine, to hydrolyze it, and to release the oxoacid product to give the PMP form of the enzyme. The reaction sequences in some of the other enzymes are even more complex. How can one enzyme do all this?

The first step in the sequence is the binding of the substrate to form the "Michaelis complex." The positive charges on Arg 386 and Arg 292 doubtless attract the carboxylate groups of the substrate and aid in guiding it toward a correct fit. In a similar manner the $-O^-$ of the coenzyme, which is distributed by resonance into the $-C=N$ of the Schiff base linkage, attracts the $-NH_3^+$ of the substrate. When a substrate or inhibitor binds to the two guanidinium groups a small structural domain of the enzyme moves and closes around the substrate which now has very little contact with the external solvent. In the initial "Michaelis complex" the $-NH_3^+$ group of the substrate lies directly in front of the C-4' carbon of the coenzyme (Fig. 14-10), where it can initiate the transimination reaction of Eq. 14-26. However, before this can happen a proton must be removed to convert the $-NH_3^+$ to $-NH_2$.

Long before the three-dimensional structures were known, Ivanov and Karpeisky[246] suggested that in the free enzyme the positively charged group (Arg 386) that binds the α-carboxylate of the substrate interacts electrostatically with the $-O^-$ of the coenzyme. This is one of the factors that keeps the $pK_a$ of the $-CH=N^+H-$ that is conjugated with this $-O^-$ at a low value of ~6.3 (at 0.1 M anion concentration). However, in the Michaelis complex this interaction of the + charge of Arg 386 with the imine group must be weakened because of the pairing of the $\alpha-COO^-$ of the substrate with the + charge. This will increase the basicity of the imine nitrogen and will also cause a decrease in the $pK_a$ of the substrate $-NH_3^+$, making is easier for a proton to jump from the $-NH_3^+$ to the imine group. This proton transfer is shown in Eq. 14-39, step *a*. Thus, the nucleophilic $-NH_2$ group is generated by a




(14-39)

process that at the same time increases the electrophilic properties of the carbon atom of the imine group. This favors the immediate addition of $-NH_2$ to $-C=N^+H-$ to give the adduct a **geminal diamine** (Eq. 14-39, step *b*), which is shown in three dimensions in Fig. 14-10B.

Notice that each step in the overall sequence changes the electronic or steric characteristics of the complex in a way that facilitates the next step.[246] This is an important principle that is applicable throughout enzymology: *For an enzyme to be an efficient catalyst each step must lead to a change that sets the stage for the next*. These consecutive steps often require proton transfers, and each such transfer will influence the subsequent step in the sequence. Some steps also require alterations in the conformation of substrate, coenzyme, and enzyme. One of these is the transimination sequence (Eqs. 14-26, 14-39). On the basis of the observed loss of circular dichroism in the external aldimine, Ivanov and Karpeisky suggested that a

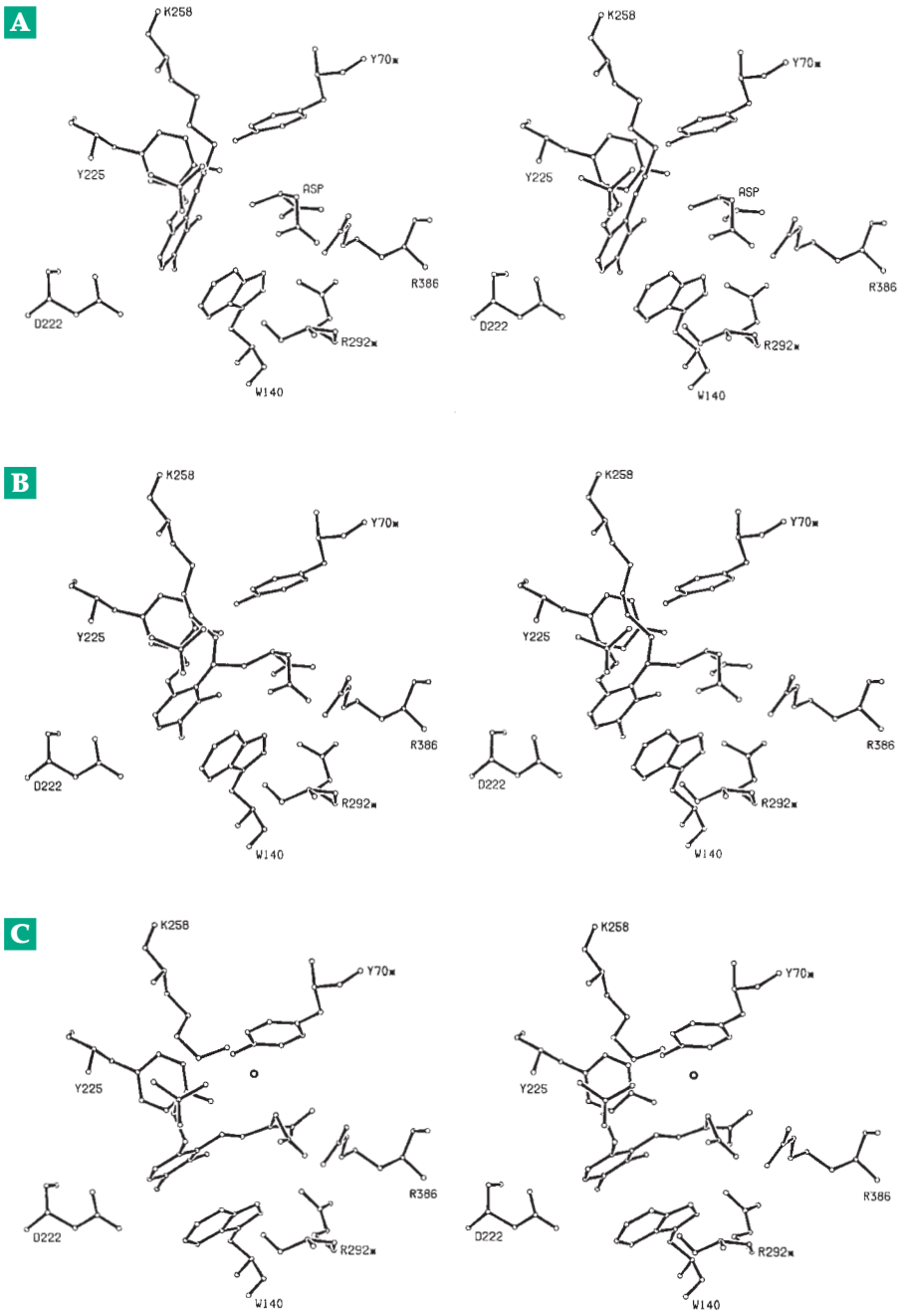



**Figure 14-10** Models of catalytic intermediates for aspartate aminotransferase in a half-transamination reaction from aspartate to oxalocetate. For clarity, only a selection of the active site groups are shown. (A) Michaelis complex of PLP enzyme with aspartate. (B) Geminal diamine. (C) Ketimine intermediate. The circle indicates a bound water molecule. See Jansonius and Vincent in Jurnak and McPherson.[163] Courtesy of J.N. Jansonius.

rotation of the coenzyme occurs as the $-NH_2$ of the substrate adds to the C = N bond during transimination (Eq. 14-39, step *b*). This accomplishes the essential shortening by ~0.15 nm of the distance between C-4' and the N atom from a van der Waals contact distance to a covalent bond distance while the carboxylate groups of the substrate remain bound in their initial positions.

That the coenzyme really does change its orientation was suggested by a dramatic change in the absorption spectrum of a crystal recorded with plane polarized light (linear dichroism) when 2-methylaspartate was soaked into a crystal (Fig. 23-9).[247] X-ray crystallography confirmed rotation of the ring by ~30°.[163,242,247] To complete the transimination sequence, which is shown only partially by Eq. 14-39, another proton transfer is needed to move the positive charge on the substrate $-N^+H_2-$ to that of lysine 258, whose amino group is then eliminated. This requires additional tilting of the ring. The crystal structure of the external aldimine with α-methylaspartate has been determined[248] as have those of ketimines with glutamate and aspartate,[249,250] a carbinolamine,[251] and quinonoid complexes of related enzymes.[212a]

The ε-amino group, which is eliminated in Eq. 14-26, is basic and functions in the next several steps of catalysis, including the abstraction of the α-hydrogen and in its transfer to the 4'-carbon (Eq. 14-28). This amino group can be seen in Fig. 14-10C, where it is positioned beside a water molecule that is needed to hydrolyze the ketimine. From this figure it can be seen that the group is able to move from site to site on one side of the planar external aldimine, quinonoid–carbanionic, and nearly planar ketimine forms. If the Lys 258 amino group is the catalytic base for these reactions it must be present at pH 7. What is its $pK_a$? And why doesn't this $pK_a$ show up in the plot of $V_{max}$ vs pH as in Fig 9-9? In fact, the maximum velocity of aspartate aminotransferase and many other PLP-dependent enzymes is independent of pH over a broad range. The answer is probably that the $pK_a$'s of both the $-NH_2$ groups of Lys 258 and the amino acid in the Michalis complex are high when there is no positive charge on the adjacent Schiff base $-C=N-$ but

very low when the Schiff base is protonated (–C=$NH^+$–). This is a result of the large electrostatic effect of a closely adjacent charge in a medium of low dielectric constant (see Chapter 7, Section A). When the Schiff base is unprotonated, as in the first structure of Eq. 14-39, the adjacent amino group has a *high* p$K_a$ and is mostly protonated. However, the proton can jump as in step *a* of that equation to give a protonated Schiff base. Now the unprotonated $-NH_2$ group has a very *low* p$K_a$, but it is still strongly nucleophilic and can readily add to the Schiff base double bond in step *b* of Eq. 14-39. In a similar fashion the amino group of Lys 258 will alternately be unprotonated and then protonated, its microscopic p$K_a$ alternating between low and high, as it catalyzes the steps of abstracting the α-hydrogen and forming and hydrolyzing the ketimine. Only two microscopic p$K_a$ values, one very high and one very low appear in the $V_{max}$ vs pH profile. This alternation of microscopic p$K_a$ values may be a common characteristic of enzymes that bind ionized substrates and can make good use of the strong electrostatic effects that arise in the active sites to facilitate essential proton transfers.

Aspartate aminotransferases are distinguished from most other PLP-dependent enzymes including transaminases by the relatively low p$K_a$ of ~6.3 for the *free* enzyme. The unprotonated Schiff base in the free enzyme can then react with the protonated amino group of the substrate. How can other PLP enzymes react with amino acids when they have protonated Schiff bases even at relatively high pH? A logical answer is that some basic group with a low p$K_a$ is close to the Schiff base and acts to deprotonate the substrate $-NH_3^+$ so that transimination can occur. Clausen *et al*. suggested that in cystathionine β-lyase this is probably tyrosine (Y111), which is adjacent to the Schiff base –C=$NH^+$– of the external aldimine and is thought to be ionized in the active enzyme.[182] NMR evidence suggests that protonation of an adjacent catalytic base occurs upon substrate binding in D-serine dehydratase as well.[252] Many other variations in active site environments are seen among PLP-dependent enzymes. As with aspartate aminotransferases, there is often an essential carboxylate group that holds a proton onto the pyridine ring of the coenzyme. However, in alanine racemase a guanidinium group from arginine is hydrogen bonded to the coenzyme ring.[153]

Below the active site of aspartate aminotransferase, as shown in Fig. 14-6, is a cluster of three buried histidine side chains in close contact with each other. The imidazole of H143 is hydrogen bonded to the D222 carboxylate, the same carboxylate that forms an ion pair with the coenzyme. This system looks somewhat like the catalytic triad of the serine proteases in reverse. As with the serine proteases, the proton-labeled $H_b$ in Fig. 14-6 can be "seen" by NMR spectroscopy (Fig. 3-30). So can the proton $H_a$ on the PLP ring. These protons act as built-in sensors able to detect small changes in the electronic environment. For example, when the Schiff base proton dissociates around the p$K_a$ of ~6.2 the NMR resonance of $H_a$ shifts upfield from 17.2 to 15.2 ppm as a result of donation of electons into the ring from the $-O^-$ of the coenzyme.[169] This shift illustrates the reality of the strong electrostatic forces that operate across heterocyclic aromatic rings within active sites of proteins. In alanine racemase a different histidine cluster is present beneath the active site and constitutes part of the "solvent" in which the catalyzed reaction takes place. At least in the case of aspartate aminotransferase, none of the histidines are absolutely essential for activity but the hydrogen-bonded network, which can be altered in mutant forms, may be important.

The detailed description of a reaction sequence given here has to be altered for each specific enzyme. A vast amount of work, only a little of which is cited here, has been done on PLP enzymes.[253–254a] These studies involve calorimetry,[255] kinetics,[210,256–259] crystallography, optical spectroscopy,[188,260,261] NMR,[243,262–264] and genetic engineering and chemical modification.[178,185,186,265]

## F. Pyruvoyl Groups and Other Unusual Electrophilic Centers

A few enzymes that might be expected to have PLP at their active sites have instead a prosthetic group consisting of pyruvic acid bound by an amide linkage, a **pyruvoyl group** (Table 14-4). These and several apparently related enzymes are the subject of this section.

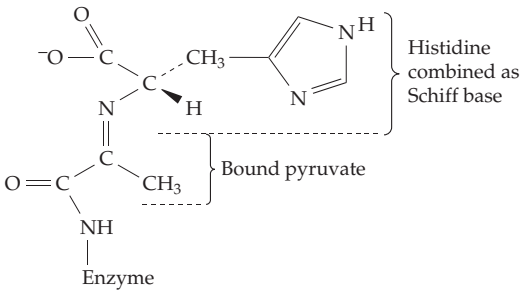
**TABLE 14-4**
**Some Pyruvoyl Enzymes**

| Decarboxylases | Product |
|---|---|
| Histidine (bacterial) | |
| *S*-Adenosylmethionine | |
| Aspartate α- decarboxylase | β-Alanine |
| Phosphatidylserine | Phosphatidyl-ethanolamine |
| 4' - Phosphopantothenylcysteine | 4' - Phosphopan-tetheine |
| **Reductases (clostridial)** | |
| Proline | |
| Glycine | |

Adapted from van Peolje and Snell.[267]

## 1. Decarboxylases

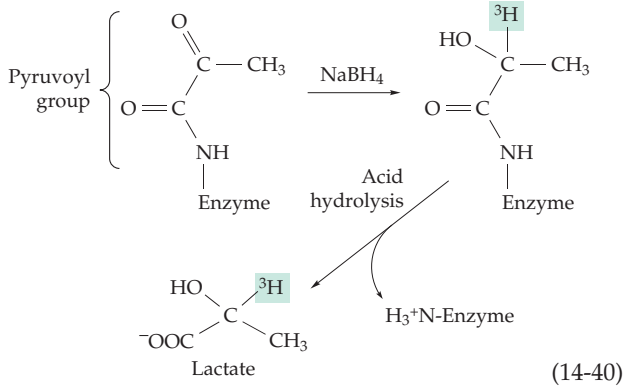
Mammalian **histidine decarboxylase** contains PLP but the enzyme from many bacteria contains a pyruvoyl group, as do a few other decarboxylases both of bacterial and eukaryotic origin.[266,267] These enzymes are inhibited by carbonyl reagents and by borohydride. When $^3$H-containing borohydride was used to reduce the histidine decarboxylase of *Lactobacillus*, $^3$H was incorporated and was recovered in lactic acid following hydrolysis. This suggested the presence of a pyruvoyl group attached by an amide linkage and undergoing the chemical reactions that are shown in Eq. 14-40. Reduction in the presence of the substrate histidine resulted in covalent binding of the histidine to the bound pyruvate. Thus, as with the PLP-containing decarboxylases, a Schiff base is formed with the substrate. Decarboxylation is presumably accomplished by using the electron-attracting properties of the carbonyl group of the amide:



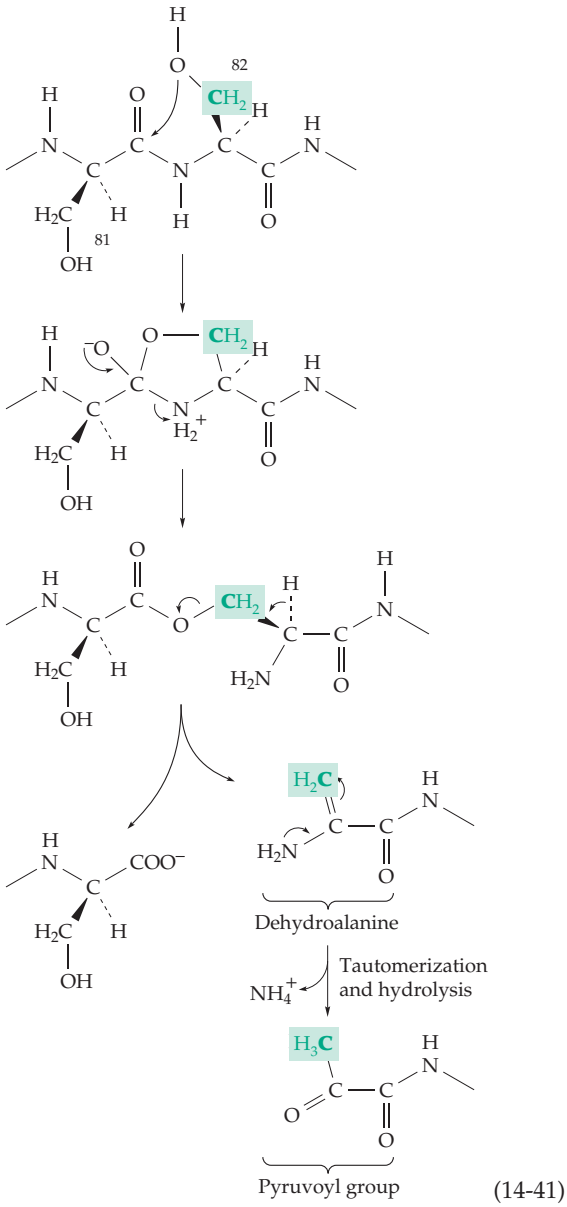

When $^{14}$C-labeled serine was fed to organisms producing histidine decarboxylase, $^{14}$C was incorporated into the bound pyruvoyl group (Fig. 14-11). Thus, serine is a precursor of the bound pyruvate. The enzyme is manufactured in the cell as a longer 307-residue proenzyme which associates as hexamers (designated $\pi_6$). The active enzyme was found to be formed by cleavage of the π chains between Ser 81 and Ser 82 to form 226-residue α chains and 81-residue β chains which associate as $(\alpha\beta)_6$.[270,271] The α chains carry the N-terminal pyruvoyl group, which was formed from Ser 82. The activation occurs spontaneously by incubations of the proenzyme for 24–48 h at pH ~7 in the presence of divalent metal ions.

Substituted serine residues under mildly alkaline conditions readily undergo α,β elimination to form **dehydroalanine** residues. When prohistidine decarboxylase containing $^{18}$O in its serine side chains was activated $^{18}$O was found in the carboxylate group of Ser 81 of the β chains. It was shown that it had been transferred from the side chain of Ser 82. This suggested the formation of an intermediate oxygen ester of Ser 81 during formation of the pyruvoyl group (Eq. 14-41).[272]

***S*-Adenosylmethionine decarboxylase** is the first enzyme in the biosynthetic pathway to spermidine (Chapter 24). Whether isolated from bacteria, yeast, animals, or other eukaryotes, this enzyme always contains a bound pyruvoyl group.[273–274b] Both the




(14-40)




(14-41)

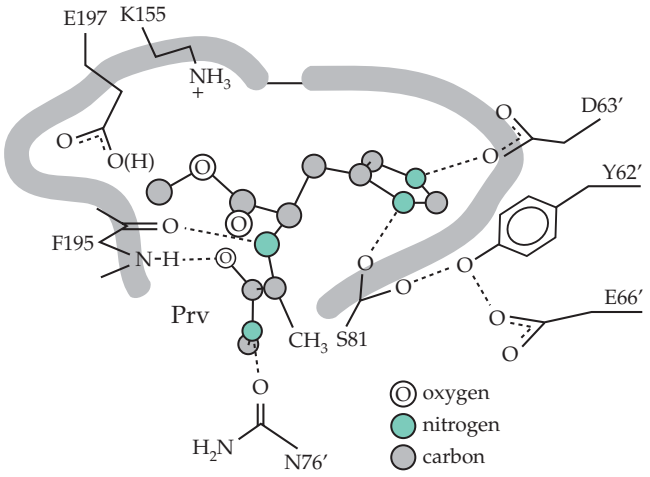
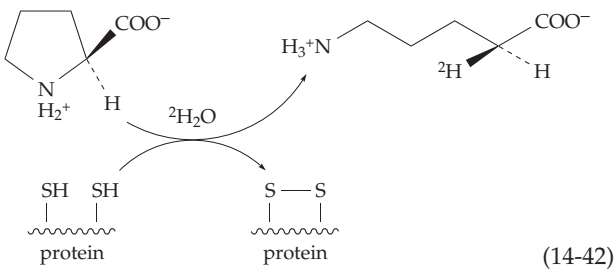



**Figure 14-11** Schematic diagram of the active site of the pyruvoyl enzyme histidine decarboxylase showing key polar interactions between the pyruvoyl group and groups of the inhibitor *O*-methylhistidine and surrounding enzyme groups. Aspartate 63 appears to form an ion pair with the imidazolium group of the substrate.[268] Hydrogen bonds are indicated by dotted lines. See Gallagher *et al.*[269]

mammalian enzyme and that from *E. coli* are $(\alpha\beta)_4$ tetramers formed in a manner similar to that of bacterial histidine decarboxylase. Other pyruvoyl decarboxylases are **phosphatidylserine decarboxylase**,[275,276] an intrinsic membrane protein used to form phosphatidylethanolamine, **aspartate α-decarboxylase**,[277] which forms β alanine needed for biosynthesis of coenzyme A, and **4'-phosphopantethenoylcysteine decarboxylase**,[278] the second of two decarboxylases required in the synthesis of coenzyme A. Because of the lack of a primary amino group, its mechanism must be somewhat different from that of other enzymes in this group.[267]

## 2. Proline and Glycine Reductases

An enzyme required in the anaerobic breakdown of proline by clostridia utilizes a dithiol-containing protein to reductively open the ring (Eq. 14-42).[279,280]




(14-42)

This enzyme also contains an N-terminal pyruvoyl residue as does one subunit of a selenium-containing glycine reductase which utilizes a dithiol to convert glycine into acetate with coupled formation of ATP:

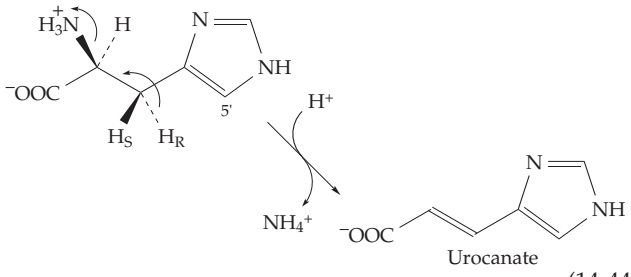
$$\text{Glycine} + \text{ADP} + P_i + 2\,e^- \rightarrow \text{acetate}^- + NH_4^+ + \text{ATP} \qquad (14\text{-}43)$$

In both cases it has been proposed that the pyruvoyl group forms a positively charged Schiff base with the substrate. The bond-breaking mechanisms are not obvious but some ideas have been proposed.[281–283a] The ATP may arise from reaction with an intermediate acetyl phosphate.[282] See Eq. 15-61.

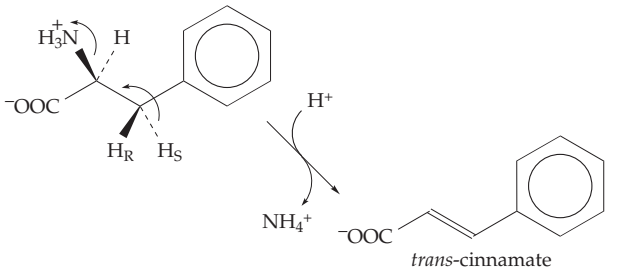
## 3. Dehydroalanine and Histidine and Phenylalanine Ammonia-Lyases

Catabolism of histidine in most organisms proceeds via an initial elimination of $NH_3$ to form **urocanic acid** (Eq. 14-44). The absence of the enzyme **L-histidine ammonia-lyase** (histidase) causes the genetic disease histidinemia.[284,285] A similar reaction is catalyzed by the important plant enzyme **L-phenylalanine ammonia-lyase**. It eliminates $-NH_3^+$ along with the *pro-S* hydrogen in the β position of phenylalanine to form *trans*-cinnamate (Eq. 14-45). Tyrosine is converted to *p*-coumarate by the same enzyme. Cinnamate and coumarate are formed in higher plants and are converted into a vast array of derivatives (Box 21-E, Fig. 25-8).

The reactions of Eqs. 14-44 and 14-45 are unusual because the nucleophilic substituent eliminated is on the α-carbon atom rather than the β. There is nothing in the structures of the substrates that would permit an




(14-44)




(14-45)

easy elimination of the α-amino group. Thus, it should not be surprising that both enzymes contain a special active center. When $^2H$ is introduced into the β position of phenylalanine, no isotope effect on the rate is observed. Rather, the rate-limiting step appears to be the release of ammonia from the coenzyme group. It appears that the enzyme must in some way make the amino group a much better leaving group than it would be otherwise.

Both enzymes are inhibited by sodium borohydride and also by nitromethane. After reduction with $NaB^3H_4$ and hydrolysis, $^3H$-containing alanine was isolated. This suggested that they contain **dehydroalanine**, which could arise by dehydration of a specific serine residue.[286,287] For phenylalanine ammonia-lyase from *Pseudomonas putida* this active site residue has been identified as S143. Replacement by cysteine in the S143C mutant also gave active enzyme while S143A

## BOX 14-D DIETARY REQUIREMENTS FOR B VITAMINS

It is difficult to determine the amounts of vitamins needed for good health and they may differ considerably from one individual to another. The quantities listed below are probably adequate for most young adults but must be increased during pregnancy and lactation and after very strenuous exercise.

**Pantothenic acid:** 10–15 mg/day. Deficiency causes apathy, depression, impaired adrenal function, and muscular weakness. ω-Methylpantothenic acid is a specific antagonist. The calcium salt, calcium pantothenate, is the usual commercial form.

**Biotin:** 0.15–0.3 mg/day. The discovery that biotin deficiency in young chickens can lead to sudden death resulted in a recommendation to supplement infant formulations with biotin.[a] **Desthiobiotin**, in which the sulfur has been removed and replaced by two hydrogen atoms, can replace biotin in some organisms and appears to lie on one pathway of biosynthesis.[b,c] **Oxybiotin,** in which the sulfur has been replaced by oxygen, is active for many organisms and partially active for others. No evidence for conversion to biotin itself has been reported, and oxybiotin may function satisfactorily in at least some enzymes.

**Thiamin:** 0.23 mg or more per 1000 kcal of food consumed and a minimum total of 0.8 mg/day. Replacement of the methyl group on the pyrimidine ring by ethyl, propyl, or isopropyl gives compounds with some vitamin activity, but replacement by hydrogen cuts activity to 5% of the original. The butyl analog is a competitive inhibitor.

**Vitamin $B_6$:** 1.5–2 mg/day; 0.4 mg/day for infants. Vitamin $B_6$ is widely distributed in foods, and symptoms of severe deficiency are seldom observed. However, a number of cases of convulsions have been attributed to partial destruction of vitamin $B_6$ in infant liquid milk formulas. Convulsions occurred when the vitamin $B_6$ content was reduced to about one-half that normally present in human milk.

Several cases of children with an abnormally high vitamin $B_6$ requirement (2–10 mg/day) have been reported, and rare metabolic diseases are known[d–f] in which specific enzymes, such as cystathionine synthase, have a reduced affinity for PLP. Patients with these diseases also benefit from a higher than normal intake of the vitamin. Excessive excretion of the vitamin may also occur, an example being provided by a strain of laboratory mice that require twice the normal amount of vitamin $B_6$ and which die in convulsions after a brief period of vitamin $B_6$ depletion.[d] Dietary supplementation with large amounts of vitamin $B_6$ for treatment of medical conditions such as carpal tunnel syndrome has been controversial.[g] Amounts of pyridoxine over 50 mg per day may damage peripheral nerves, probably because of the chemical reactivity of pyridoxal and PLP.

**Nicotinamide:** About 7.5 mg/day. Tryptophan can substitute to some extent. See also Box 15-A.

**Riboflavin:** About 1.5 mg/day. See also Box 15-B.

**Folic acid:** About 0.2–0.4 mg/day. See also Box 15-D.

**Vitamin $B_{12}$:** About 2 μg/day. See also Box 16-B.

**Vitamin C (ascorbic acid):** 50–200 mg/day. See also Box 18-D.

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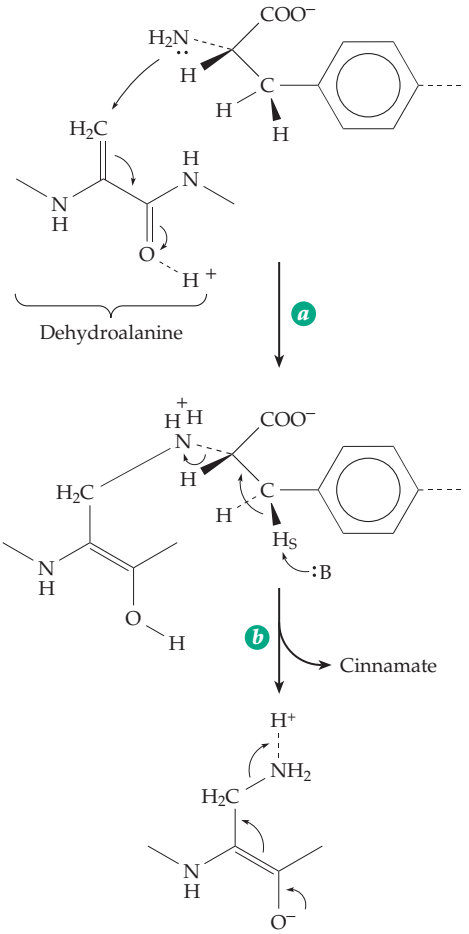
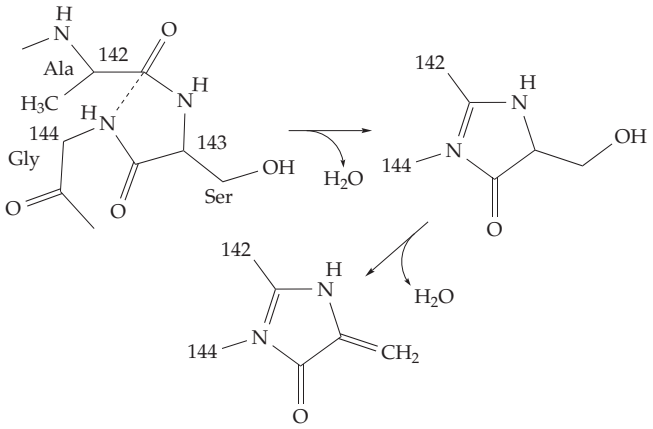
was inactive.[288,289] These results support the formation of dehydroalanine. One proposed mechanism of action of the enzymes involved addition of the substrate amino group to the C = C bond of the dehydroalanine followed by elimination (Eq. 14-46). Dissociation of the C – H bond in this step would be assisted by the electron-accepting properties of the adjacent aromatic ring. However, the proposed chemistry was not convincing. Noting that a 5-nitro substituent on the histidine greatly enhances the rate of reaction, Langer *et al.* proposed that the histidine reacts with the electrophilic carbon in the pyruvoyl center as in Eq. 14-47.[290,291] However, this chemistry, too, was unprecedented in enzymology. Determination of the three-dimensional structure of histidine ammonia-lyase[292] led to the discovery of a new prosthetic group and a solution to the problem. Two dehydration steps, (Eq. 14-47) convert an Ala-Gly-Ser sequence within the protein into **4-methylidine-imidazole-5-one (MIO)**, a modified dehydroalanine with enhanced electron-accepting properties. The proposed mechanism of action is portrayed in Eq. 14-48.

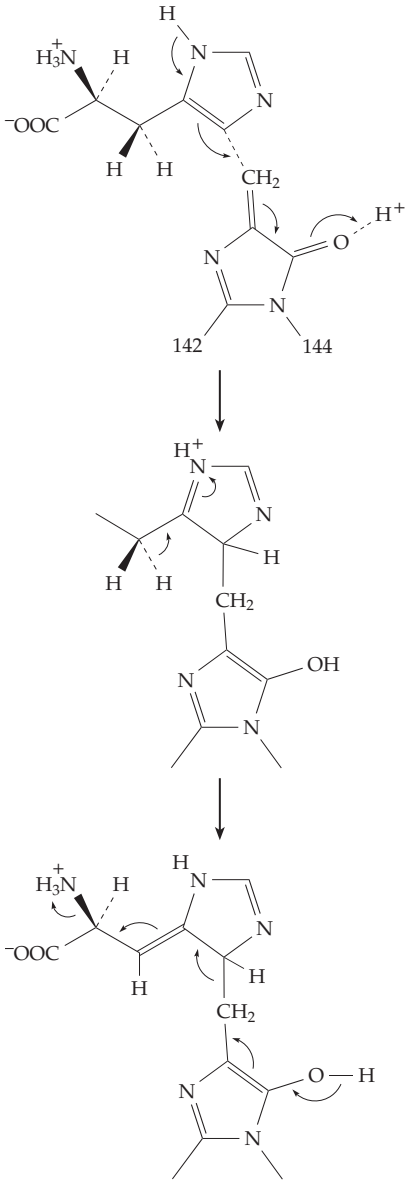



(14-46)




4-Methylidene-imidazole-5-one (MIO)

(14-47)



(14-48)

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## Study Questions

1. Discuss the role of biotin in metabolism and the chemical mechanism of its action. Illustrate with examples.

2. Biotin has been shown to be an essential component of a bacterial oxaloacetate *decarboxylase* that pumps two sodium ions out of a cell for each oxaloacetate molecule decarboxylated. Propose a chemical mechanism for the functioning of biotin and also any ideas that you may have for the operation of the sodium pump.

3. Write out the equation by which **acetyl-CoA** and **pyruvate** can be converted into **L-glutamate**, which can be observed in living animals by $^{13}C$ NMR. If [2-$^{13}C$] sodium acetate were injected into the animal what labeling pattern could be anticipated in the L-glutamate?

4. Compare mechanisms and coenzyme requirements for biological decarboxylation of the following three groups of compounds:

   β-oxoacids
   α-oxoacids
   α-amino acids

5. Illustrate, using structural equations, the chemical mechanisms of the following biochemical reactions.

   a) Pyruvate → acetaldehyde + $CO_2$
   b) 2-Pyruvate → α-acetolactate + $CO_2$
   c) Pyruvate + $NAD^+$ + CoA-SH → acetyl-CoA + NADH + $H^+$ + $CO_2$
   d) Fructose 6-*P* + glyceraldehyde-3-*P* → xylulose-5-*P* + erythrose-4-*P*

6. 3-Fluoropyruvate is converted quantitatively by pyruvate decarboxylase from wheat germ into acetate, fluoride ($F^-$), and carbon dioxide. Propose a reaction mechanism. See Gish, G., Smyth, T., and Kluger, R. (1988) *J. Am. Chem. Soc.* **110**, 6230-6234.

7. In *E. coli* **L-cysteine** is formed from L-serine and the sulfide ion $S^{2-}$ in a reaction that also requires acetyl-CoA and is catalyzed by the consecutive action of an acyl transferase and cysteine synthase. Outline the mechanism of this conversion indicating participation of any essential coenzymes.

8. Illustrate, using structural equations, the chemical mechanisms of the following biochemical reactions.

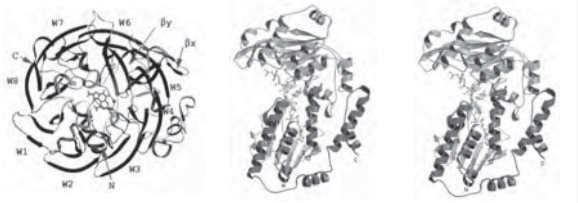
   a) L-Glutamate + oxaloacetate → 2-oxoglutarate + L-aspartate
   b) L-Serine → pyruvate + $NH_4^+$
   c) L-Serine + indole (from cleavage of indole-3-glycerol phosphate) → L-tryptophan
   d) L-Serine + tetrahydrofolate → glycine + $N^5$, $N^{10}$-methylene-tetrahydrofolate
   e) L-Selenocysteine → L-alanine + $Se^0$

9. Threonine is formed by *E. coli* from homoserine via the intermediate γ-phosphohomoserine. Write out an abbreviated reaction sequence for its conversion to L-threonine by the action of threonine synthase.

10. Write out a plausible step-by-step mechanism by which 1-aminocyclopropane-1-carboxylate synthase (ACC synthase) of plant tissues can form ACC from *S*-adenosylmethionine. This reaction requires a specific cofactor


11. Tissues of the mammalian central nervous system contain a pyridoxal phosphate-dependent glutamate decarboxylase that catalyzes conversion of Glu to γ-aminobutyrate (GABA), an inhibitory synaptic transmitter. GABA is degraded by transimination with α-oxoglutarate as the acceptor to yield succinic semialdehyde, which then is oxidized to succinate by an NAD-linked dehydrogenase.

    a) Show how these reactions can operate as a shunt pathway that allows the citric acid cycle to function without the enzymes α-oxoglutarate dehydrogenase and succinate thiokinase.
    b) Is the shunt more or less efficient than the normal cycle from the standpoint of energy recovery? Explain.



Biological oxidation-reduction reactions rely upon many organic coenzymes and transition metal ions. Left, a molecule of the hydrogen carrier pyrroloquinoline quinone (**PQQ**, Fig. 15-23) is seen at the bottom of a 7-bladed β propeller of a bacterial methanol dehydrogenase. In adrenodoxin reductase (right) the reducing power of **NADPH** is passed to **FAD** and then to the small redox protein adrenodoxin. In this stereoscopic view the pyridine ring of the oxidized coenzyme NADP+ and the tricyclic flavin ring of FAD are seen stacked against each other in the center; the adenylate ends stretching toward the top and bottom of the complex. From Ziegler and Schulz, (2000) *Biochemistry* **39**, 10986-10995.

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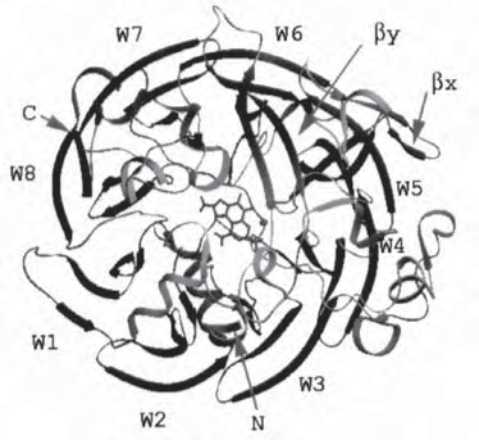
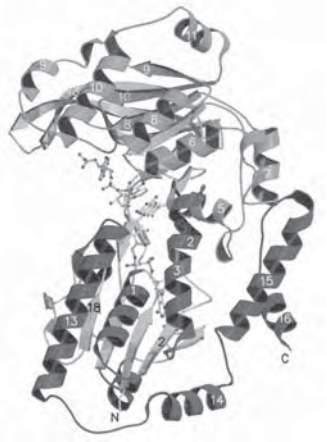
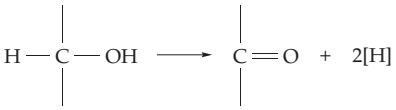
# Coenzymes of Oxidation–Reduction Reactions

# 15



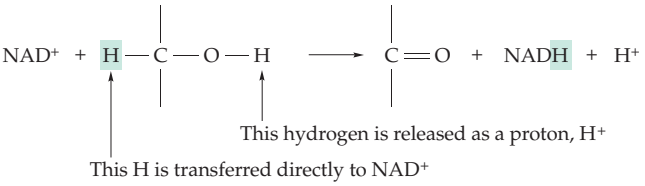
The **dehydrogenation** of an alcohol to a ketone or aldehyde (Eq. 15-1) is one of the most frequent biological oxidation reactions. Although the hydrogen atoms removed from the substrate are often indicated simply as 2[H], it was recognized early in the twentieth century that they are actually transferred to hydrogen-carrying coenzymes such as $NAD^+$, $NADP^+$, FAD, and **riboflavin**



(15-1)

**5'-phosphate** (FMN). This chapter deals with these coenzymes and also with a number of other organic oxidation–reduction coenzymes. They may be considered either as carriers of hydrogen or as carriers of electrons ($H = H^+ + e^-$) in metabolic reactions.

When $NAD^+$ becomes reduced by dehydrogenation of an alcohol, one of the hydrogen atoms removed from the alcohol becomes firmly attached to the $NAD^+$, converting it to NADH. The other is released as a proton (Eq. 15-2). Study of $^2H$-labeled alcohols and their oxidation by $NAD^+$ has shown that *dehydrogenases catalyze direct transfer to $NAD^+$ of the hydrogen that is attached to carbon in the alcohol*. There is never any exchange of this hydrogen atom with protons of the medium. At the same time, the hydrogen attached to the oxygen of the alcohol is released into the medium as $H^+$:




(15-2)

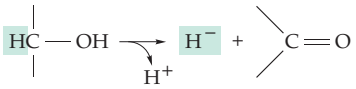
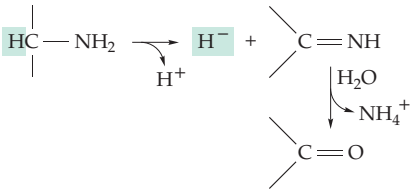
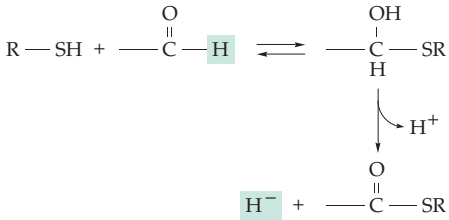
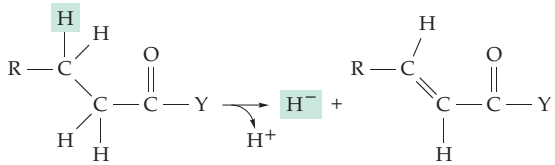
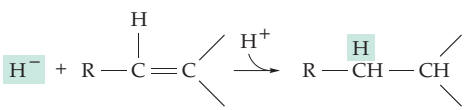
The foregoing observations suggested that these biological dehydrogenations may be viewed as removal of a hydride ion ($H^-$) together with a proton ($H^+$) rather than as removal of two hydrogen atoms. $NAD^+$ and $NADP^+$ are regarded as hydride ion-accepting coenzymes. However, it has been impossible to establish conclusively that the hydrogen atom and electron are transferred simultaneously as $H^-$. Transfer of the hydrogen atom to or from these coenzymes may conceivably be followed by or preceded by transfer of an electron. The situation is even less clear for the flavin coenzymes FAD and riboflavin phosphate for which intermediate **free radical** oxidation states are known to exist. However, regardless of the actual mechanism, it is convenient to classify most hydrogen transfer reactions of metabolism as if they occurred by transfer of a hydride ion. *The hydride ion can be regarded as a nucleophile which can add to double bonds or can be eliminated from substrates in reactions of types that we have already considered.* Some of these reactions are listed in Table 15-1.

Why are there *four* major hydrogen transfer coenzymes, $NAD^+$, $NADP^+$, FAD, and riboflavin phosphate (FMN), instead of just one? Part of the answer is that the **reduced pyridine nucleotides** NADPH and NADH are more powerful reducing agents than are reduced **flavins** (Table 6-7). Conversely, flavin coenzymes are more powerful oxidizing agents than are

$NAD^+$ and $NADP^+$. This difference reflects the chemical difference between the vitamins **riboflavin** and **nicotinamide** which form the oxidation–reduction centers of the coenzymes. Another difference is that $NAD^+$ and $NADP^+$ tend to be present in free forms within cells, diffusing from a site on one enzyme to a site on another. These coenzymes are *sometimes* tightly bound but flavin coenzymes are *usually* firmly bound to proteins, fixed, and unable to move. Thus, they tend to accept hydrogen atoms from one substrate and to pass them to a second substrate while attached to a single enzyme.

The oxidation–reduction potential of a pyridine nucleotide coenzyme system is determined by the standard redox potential for the free coenzyme (Table 6-8) together with the ratio of concentrations of oxidized to reduced coenzyme ([$NAD^+$] / [NADH], Eq. 6-64). If these concentrations are known, a redox

**TABLE 15-1**
**Some Biochemical Hydrogen Transfer Reactions[a]**

| Reaction | Example (oxidant) |
|---|---|
| **A** Dehydrogenation of an alcohol  | Alcohol dehydrogenase ($NAD^+$) |
| **B** Dehydrogenation of an amine  | Amino acid dehydrogenases, amine oxidases ($NAD^+$ or flavin) |
| **C** Dehydrogenation of adduct of thiol and aldehyde  | Glyceraldehyde 3-phosphate dehydrogenase ($NAD^+$ or $NADP^+$) |
| **D** Dehydrogenation of acyl-CoA, acyl-ACP, or carboxylic acid  | Acyl-CoA dehydrogenases (Flavin) |
| | Succinic dehydrogenase (Flavin) |
| (Y = —S—CoA or —OH) | Opposite: enoyl reductase (NADPH) |
| **E** Reduction of desmosterol to cholesterol by NADPH  | Reduction of desmosterol to cholesterol by NADPH |

[a] Reaction type 9 A,B of Table on the inside cover at the end of the book: A hypothetical hydride ion $H^-$ is transferred from the substrate to a coenzyme of suitable reduction potential such as $NAD^+$, $NADP^+$, FAD, or riboflavin 5′-phosphate. The reverse of hydrogenation is shown for E. Many of the reactions are reversible and often go spontaneously in the reverse direction from that shown here.

potential can be defined for the $NAD^+$ system within a cell. This potential may vary in different parts of the cell because of differences in the $[NAD^+]$ / [NADH] ratio, but within a given region of the cell it is constant. On the other hand, the redox potentials of flavoproteins vary. Since the flavin coenzymes are not dissociable, two flavoproteins may operate at very different potentials even when they are physically close together.

Why are there two pyridine nucleotides, $NAD^+$ and $NADP^+$, differing only in the presence or absence of an extra phosphate group? One important answer is that they are members of two different oxidation–reduction systems, both based on nicotinamide but functionally independent. The experimentally measured ratio $[NAD^+]$ / [NADH] is much higher than the ratio $[NADP^+]$ / [NADPH]. Thus, these two coenzyme systems also can operate within a cell at different redox potentials. A related generalization that holds much of the time is that *$NAD^+$ is usually involved in pathways of catabolism, where it functions as an oxidant, while NADPH is more often used as a reducing agent in biosynthetic processes.* See Chapter 17, Section I for further discussion.

## A. Pyridine Nucleotide Coenzymes and Dehydrogenases

In 1897, Buchner discovered that "yeast juice," prepared by grinding yeast with sand and filtering, catalyzed fermentation of sugar. This was a major discovery which excited the interest of many other biochemists.[1] Among them were Harden and Young, who, in 1904, showed that Buchner's cell-free yeast juice lost its ability to ferment glucose to alcohol and carbon dioxide when it was dialyzed. Apparently, fermentation depended upon a low-molecular-weight substance that passed out through the pores of the dialysis membrane. Fermentation could be restored by adding back to the yeast juice either the concentrated dialysate or boiled yeast juice (in which the enzyme proteins had been destroyed). The heat-stable material, which Harden and Young called **cozymase**, was eventually found to be a mixture of inorganic phosphate ions, thiamin diphosphate, and $NAD^+$. However, characterization of $NAD^+$ was not accomplished until 1935.

Pure $NADP^+$ was isolated from red blood cells in 1934 by Otto Warburg and W. Christian, who had been studying the oxidation of glucose 6-phosphate by erythrocytes.[1a] They demonstrated a requirement for a dialyzable coenzyme which they characterized and named **triphosphopyridine nucleotide** ($TPN^+$, but now officially $NADP^+$; Fig. 15-1). Thus, even before its recognition as an important vitamin in human nutrition, nicotinamide was identified as a component of $NADP^+$.

Warburg and Christian recognized the relationship of $NADP^+$ and $NAD^+$ (then called $DPN^+$) and proposed that both of these compounds act as hydrogen carriers through alternate reduction and oxidation of the pyridine ring. They showed that the coenzymes could be reduced either enzymatically or with sodium dithionite $Na_2S_2O_4$.

$$S_2O_4^{2-} + NAD^+ + H^+ \rightarrow NADH + 2\,SO_2 \qquad (15\text{-}3)$$

The reduced coenzymes NADH and NADPH were characterized by a new light-absorption band at 340 nm. This is not present in the oxidized forms, which absorb maximally at 260 nm (Fig. 15-2). The reduced forms are stable in air, but their reoxidation was found

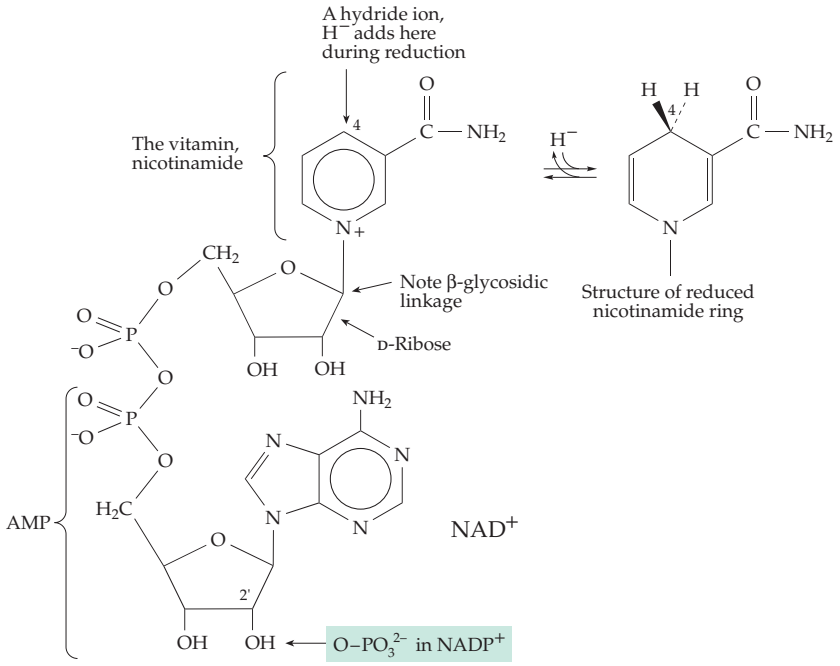



**Figure 15-1** The hydrogen-carrying coenzymes $NAD^+$ (nicotinanide adenine dinucleotide) and $NADP^+$ (nicotinamide adenine dinucleotide phosphate). We use the abbreviations $NAD^+$ and $NADP^+$, even though the net charge on the entire molecule at pH 7 is negative because of the charges on oxygen atoms of the phospho groups.

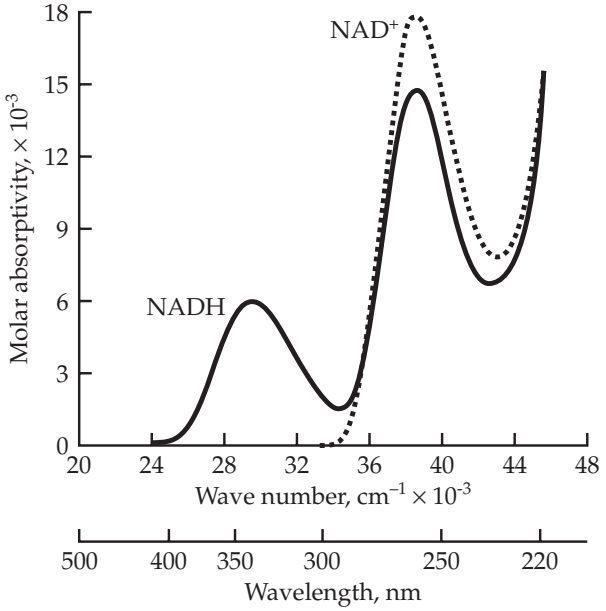



**Figure 15-2** Absorption spectra of $NAD^+$ and NADH. Spectra of $NADP^+$ and NADPH are nearly the same as these. The difference in absorbance between oxidized and reduced forms at 340 nm is the basis for what is probably the single most often used spectral measurement in biochemistry. Reduction of $NAD^+$ or $NADP^+$ or oxidation of NADH or NADPH is measured by changes in absorbance at 340 nm in many methods of enzyme assay. If a pyridine nucleotide is not a reactant for the enzyme being studied, a **coupled assay** is often possible. For example, the rate of enzymatic formation of ATP in a process can be measured by adding to the reaction mixture the following enzymes and substrates: hexokinase + glucose + glucose-6-phosphate dehydrogenase + $NADP^+$. As ATP is formed, it phosphorylates glucose via the action of hexokinase. $NADP^+$ then oxidizes the glucose 6-phosphate that is formed with production of NADPH, whose rate of appearance is monitored at 340 nm.

to be catalyzed by certain yellow enzymes which were later identified as flavoproteins.

## 1. Three-Dimensional Structures of Dehydrogenases

Most $NAD^+$- or $NADP^+$- dependent dehydrogenases are dimers or trimers of 20- to 40-kDa subunits. Among them are some of the first enzymes for which complete structures were determined by X-ray diffraction methods. The structure of the 329-residue per subunit muscle ($M_4$) isoenzyme of **lactate dehydrogenases** (see Chapter 11) from the dogfish was determined to 0.25 nm resolution by Rossmann and associates in 1971.[2–4] More recently, structures have been determined for mammaliam muscle and heart type ($H_4$) isoenzymes,[5] for the testicular ($C_4$) isoenzyme from the mouse,[6] and for bacterial lactate dehydrogenases.[6–8] In all of these the polypeptide is folded nearly identically. The structures of the homologous cytosolic and mitochondrial isoenzymes of **malate dehydrogenase** are also similar,[9–11] as are those of the bacterial enzyme.[12,13] All of these proteins consist of two structural domains and the $NAD^+$ is bound to the nucleotide-binding domain in a similar manner as is shown in Fig. 2-13 for glyceraldehyde phosphate dehydrogenase. The coenzyme-binding domains of the dehydrogenases of known structures all have this nearly constant structural feature (often called the Rossmann fold) consisting of a six-stranded parallel sheet together with several α helical coils[14] (Figs. 2-13 and 2-27).

The coenzyme molecule curls around one end of the nucleotide-binding domains in a "C" conformation with the nicotinamide ring lying in a pocket (Figs. 2-13, 15-3). Even before the crystal structure of lactate dehydrogenase was known, the lack of pH dependence of coenzyme binding from pH 5 to 10 together with observed inactivation by butanedione suggested that the pyrophosphate group of $NAD^+$ binds to a guanidinium group of an arginine residue. This was identified by X-ray diffraction studies as Arg 101. This ion pairing interaction, as well as the hydrogen bond between Asp 53 and the 2' oxygen atom of a ribose ring (Fig. 15-3), is present in all of the lactate and malate dehydrogenases.

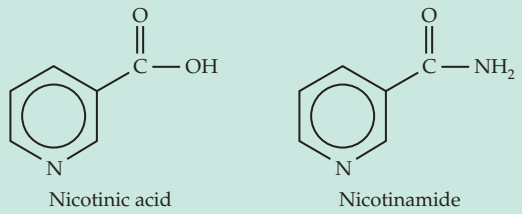
The adenine ring of the coenzyme is bound in a hydrophobic pocket with its amino group pointed out into the solvent. A second structural domain holds additional catalytic groups needed to form the active site.

## 2. Stereospecificity and Mechanism

When $NAD^+$ is reduced in $^2H_2O$ by dithionite (Eq. 15-3) an atom of $^2H$ is introduced into the reduced pyridine. Chemical degradation showed the $^2H$ to be present at the 4 position of the ring para to the nitrogen atom[15] (see Fig. 15-1). As shown by Westheimer and associates, during enzymatic reduction of $NAD^+$ by deuterium-containing ethanol, $CH_3–C^2H_2OH$, one of the $^2H$ atoms is transferred into the NADH formed, thus establishing the *direct transfer of a hydrogen atom*.[16,17] When the $NAD^2H$ formed in this way is reoxidized enzymatically with acetaldehyde, with regeneration of $NAD^+$ and ethanol, the $^2H$ is completely removed.

This was one of the first recognized examples of the ability of an enzyme to choose between two identical atoms at a *pro*-chiral center (Chapter 9). The two sides of the nicotinamide ring of NAD were designated A and B and the two hydrogen atoms at the 4 position of NADH as $H_A$ (now known as *pro-R*) and $H_B$ (*pro-S*). Alcohol dehydrogenase always removes the *pro-R* hydrogen. Malate, isocitrate, lactate, and D-glycerate dehydrogenases select the same hydrogen. However,

## BOX 15-A NICOTINIC ACID AND NICOTINAMIDE




Nicotinic acid Nicotinamide

Nicotinic acid was prepared in 1867 by oxidation of nicotine. Although it was later isolated by Funk and independently by Suzuki in 1911–1912 from yeast and rice polishings, it was not recognized as a vitamin. Its biological significance was established in 1935 when nicotinamide was identified as a component of $NAD^+$ by von Euler and associates and of $NADP^+$ by Warburg and Christian.[a] Both forms of the vitamin are stable, colorless compounds highly soluble in water.

In 1937, Elvehjem and coworkers demonstrated that nicotinic acid cured canine "blacktongue." In the same year it was found to cure human **pellagra**, a terrible disease characterized by weakness, indigestion, and loss of appetite followed by dermatitis, diarrhea, mental disorders, and eventual death. At that time pellagra was common in the United States, especially in the south. The U.S. Public Health Service estimated that during 1912–1916 there were 100,000 victims and 10,000 deaths a year.[b,c]

The daily requirement for an adult is about 7.5 mg. The amount is decreased by the presence in the diet of tryptophan, which can be converted partially to nicotinic acid (Chapter 25).[d,e] Tryptophan is about 1/60 as active as nicotinic acid itself. The one-time prevalence of pellagra in the southern United States was a direct consequence of a diet high in maize whose proteins have an unusually low tryptophan content.

[a] Schlenk, F. (1984) *Trends Biochem. Sci.* **9**, 286–288
[b] Wagner, A. F., and Folkers, K. (1964) *Vitamins and Coenzymes*, Wiley (Interscience), New York (p. 73)
[c] Rosenkrantz, B. G. (1974) *Science* **183**, 949–950
[d] Teply, L. J. (1993) *FASEB J.* **7**, 1300
[e] Sauberlich, H. E. (1987) in *Pyridine Nucleotide Coenzymes: Chemical, Biochemical and Medical Aspects*, Vol. B (Dolphin, D., Avramović, O., and Poulson, R., eds), pp. 599–626, Wiley (Interscience), New York

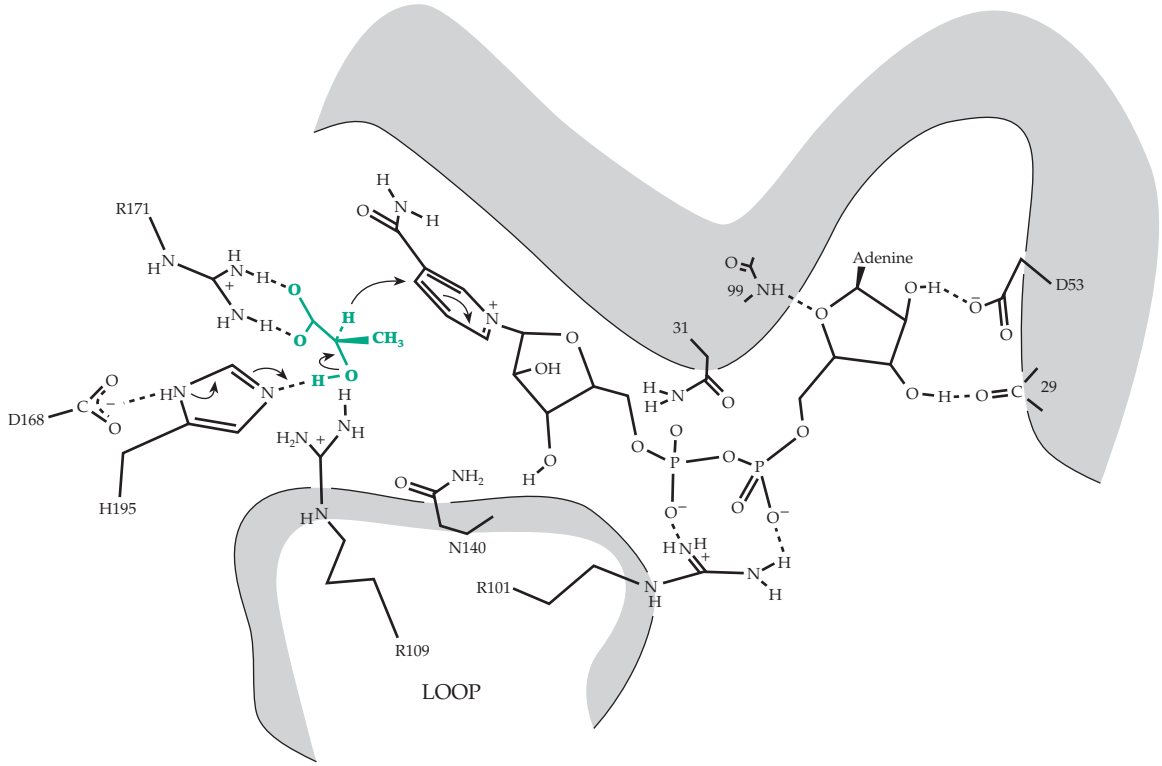
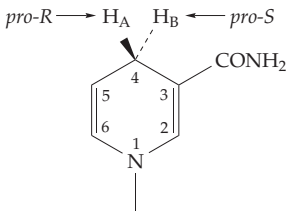



**Figure 15-3** Diagrammatic structure of $NAD^+$ and L-lactate bound into the active site of lactate dehydrogenase. See Eventhoff *et al.*[5]

dehydrogenases acting on glucose 6-phosphate, glutamate, 6-phosphogluconate, and 3-phosphoglyceraldehyde remove the *pro-S* hydrogen. By 1979 this stereospecificity had been determined for 127 dehydrogenases, about half of which were found A-specific and half B-specific.[18] Isotopically labeled substrates




have usually been used for this purpose but a simple NMR method has been devised.[19,20]

How complete is the stereospecificity? Does the enzyme sometimes make a mistake? *Lactate dehydrogenase displays nearly absolute specificity*, transferring a proton into the "incorrect" *pro-S* position no more than once in $5 \times 10^7$ catalytic cycles.[21] This suggests a difference in transition-state energies of about 40 kJ / mol for the two isomers. X-ray structural studies suggested that a major factor determining this stereoselectivity might be the location of hydrogen-bonding groups that hold the $-CONH_2$ group of the nicotinamide. If these are located in such a way that the nicotinamide ring has a *syn* orientation with respect to the ribose ring to which it is attached (Fig. 15-4), the A face of the coenzyme will be against the substrate undergoing oxidation or reduction. If the ring has an *anti* conformation, the B side will be against the substrate.[18,22] However, the explanation cannot be this simple. High specificity is still retained when NADH is modified, for example, by replacement of the $-CONH_2$ on the pyridine ring with $-COCH_3$ or $-CHO$ or by use of α-NADH, in which the ribose–nicotinamide linkage is α instead of β as in normal NADH.[23]

An intriguing idea is that the enzyme selectively stabilizes one of the boat conformations of NADH or NADPH. According to stereoelectronic principles the axially oriented hydrogen in such a boat structure (*pro-R* in Fig. 15-4) will be the most readily transferred. On the basis of this principle, together with the idea that enzymes adjust the Gibbs energies of intermediate states to achieve optimum catalytic rates, the following prediction was made by Benner and associates:[24] *The thermodynamically most easily reduced carbonyl compounds will react by enzymatic transfer of the hydride ion from the pro-R position of NADH.* Conversely, the most difficultly reduced carbonyl compounds will receive the hydride ion from the *pro-S* position of NADH. The proposal has been controversial and it has been argued that evolutionary relationships play a dominant role in determining stereoselectivity.[23] Theoretical computations suggest that the boatlike puckering of the ring is flexible, raising some doubts about the rigidity of the reactants in the active site.[12,25] Nevertheless, it is likely that the coenzyme as well as bound substrates must assume very specific conformations before the enzyme is able to move to the transition state for the reaction. A great deal of effort has been expended in trying to understand other factors that may explain the high stereospecificity of dehydrogenases.[12,22,26–28]

When a hydrogen atom is transferred by an enzyme from the 4 position of NADH or NADPH to an aldehyde or ketone to form an alcohol, the placement of the hydrogen atom on the alcohol is also stereospecific. Thus, alcohol dehydrogenase acting on $NAD^2H$ converts acetaldehyde to (*R*)-mono-[$^2$H]ethanol (Eq. 9-73). Pyruvate is reduced by lactate dehydrogenase to L-lactate, and so on. Even mutations that disrupt the binding of the carboxylate of lactate, e.g., substitution of arginine 171 (see Fig. 15-3) by tryptophan or phenylalanine and introduction of a new arginine on the other side of the active site, do not alter the L-stereospecificity. However, the specificity for lactate is lost.[29]

***One step or two-step transfer?*** Another major question about dehydrogenases is whether the hydrogen atom that is transferred moves as a hydride ion, as is generally accepted, or as a hydrogen atom with separate transfer of an electron and with an intermediate NAD or NADPH free radical. In one study para-substituted benzaldehydes were reduced with NADH and $NAD^2H$ using yeast alcohol dehydrogenase as a catalyst.[30] This permitted the application of the Hammett equation (Box 6-C) to the rate data. For a series of benzaldehydes for which $\sigma^+$ varied widely, a value

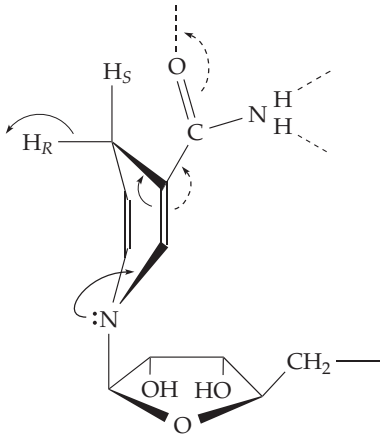



**Figure 15-4** The nicotinamide ring of NADH in a *syn* boat conformation suitable for transfer of an axially oriented *pro-R* hydrogen atom from its A face as $H^-$. The flow of electrons is shown by the solid arrows. The dashed arrows indicate competing resonance which favors planarity of the ring and opposes the $H^-$ transfer. Hydrogen bonds from the protein to the carboxamide group (dashed lines) affect both this tendency and the conformation of the nucleotide.

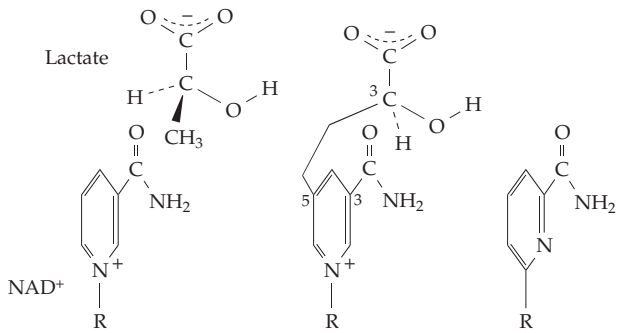
of ρ = +2.2 was observed for the rate constant with both NADH and NAD²H. Thus, electron-accepting substituents in the para position hasten the reaction. While the significance of this observation is not immediately obvious, the relatively low value of ρ is probably incompatible with a mechanism that requires complete transfer of a single electron from NADH to acetaldehyde in the first step. A primary isotope effect on the rates was $k_H / k_D = 3.6$, indicating that the C–H bond in NADH is broken in the rate-limiting step. The fact that the isotope effect is the same for all of the substituted benzaldehydes also argued in favor of a hydride ion transfer.

Studies of the kinetics of nonenzymatic model reactions of NADH with quinones[31,32] have also been interpreted to favor a single-step hydride ion transfer. Application of Marcus theory (Chapter 9, Section D,4) to data from model systems also supports the hydride transfer mechanism.[33,34] Quantum mechanical tunneling may be involved in enzymatic transfer of protons, hydride ions, and electrons.[35–36] Tunneling is often recognized by unusually large primary or secondary kinetic isotope effects. According to semi-classical theory (Chapter 12), the maximum effects for deuterium and tritium are given by the following ratios:

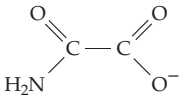
$$(k_{2_H} / k_{1_H}) = (k_{3_H} / k_{2_H})^{3.3} \approx 7.$$

Higher ratios, which suggest tunneling, are frequently observed for dehydrogenases.[36a,b] Tunneling is apparently coupled to fluctuations in motion within the enzyme-substrate complex. Study of effects of pressure on reactions provides a new approach that can aid interpretation.[36c,36d]

***Coenzyme and substrate analogs.*** The structures of enzyme•NAD⁺•substrate complexes (Fig. 15-3) may be studied by X-ray crystallography under certain conditions or can be inferred from those of various stable enzyme-inhibitor complexes or from enzyme reconstituted with NAD⁺ that has been covalently linked to the substrate. In the following diagram,[27] NAD⁺ (I) is shown with L-lactate lying next to its A face, ready to transfer a hydride ion to the *pro-R* position in NADPH. Also shown (II) is 5-(*S*)-lac-NAD⁺, with the covalently linked lactate portion in nearly the same position as in diagram I. This NAD derivative was used to obtain the first 0.27-nm structure with a bound substrate-like molecule in lactate dehydrogenase.[37] Since then the structures of many complexes with a variety of dehydrogenases have been studied. In coenzyme analog III the ring is bound to ribose with a C–C bond and it lacks the positive charge on the nicotinamide ring in NAD⁺. It does not react with substrate. However, it binds to the coenzyme site of alcohol dehydrogenase and forms with ethanol a ternary complex whose structure has been solved.[38]

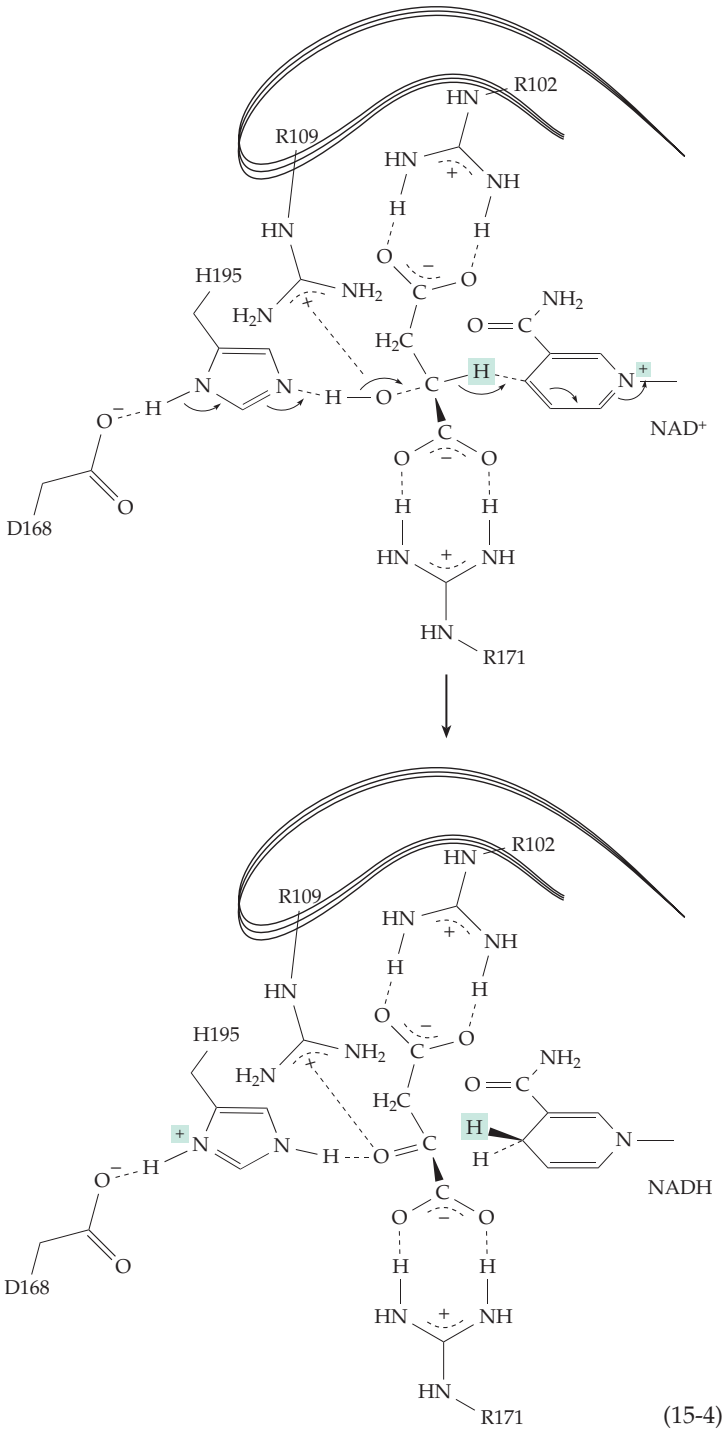



A related approach is to study complexes formed with normal NAD⁺ but with an unreactive second substrate. An example is oxamate, which binds well to lactate dehydrogenase to form stable ternary complexes for which equilibrium isotope effects have been studied.[39]




In the structure of the lactate dehydrogenase active site shown in Fig. 15-3, the lactate carboxylate ion is held and neutralized by the guanidinium group of Arg 171, and the imidazole group of His 195 is in position to serve as a general base catalyst to abstract a proton from the hydroxyl group of the substrate (Eq. 15-4). This imidazole group is also hydrogen bonded to the carboxylate of Asp 168 as in the "charge-relay" system of the serine proteases (Fig. 12-10). The same features are present in the active site of malate dehydrogenases and have been shown essential by study of various mutant forms.[11,40–42] The His:Asp pair of the dehydrogenases is not part of the nucleotide-binding domain but is present in the second structural domain, the "catalytic domain." This is another feature reminiscent of the serine proteases. A bacterial D-glycerate dehydrogenase also has a similar structure and the same catalytic groups. However, the placement of the catalytic histidine and the arginine that binds the α-carboxylate group of the substrate is reversed, allowing the enzyme to act on the D-isomer.[43]

***Conformational changes during dehydrogenase action.*** Dehydrogenases bind coenzyme and substrate in an ordered sequence. The coenzyme binds first, then the oxidizable or reducible substrate. The binding of the coenzyme to lactate dehydrogenase is accompanied by a conformational change by which a loop, involving residues 98–120 and including one helix,




(15-4)

folds over the coenzyme like a lid.[41,44] This conformational change must occur during each catalytic cycle, just as in the previously discussed cases of citrate synthase (Chapter 13) and aspartate aminotransferase (Chapter 14). One effect of folding of the loop is to bring the side chain of Arg 109 into close proximity to His 195 and to the OH group of the bound lactate (Eq. 15-4). The closing of the loop also forces the positively charged nicotinamide ring more deeply into a relatively nonpolar pocket. This may induce a movement of the positive charge toward the 4' carbon of the ring, assisting in the transfer of the hydride ion (Eq. 15-4).[41] At the end of this transfer both Arg 109 and His 196 are positively charged and electrostatic repulsion between them may help to move the loop and release the products. If the reaction proceeds in the opposite direction the presence of two positive charges will assist in the hydride ion transfer. The importance of Arg 109 is demonstrated by the fact that a mutant with glutamine in place of Arg 109 has a value of $k_{cat}$ only 1/400 that of native enzyme.[45]

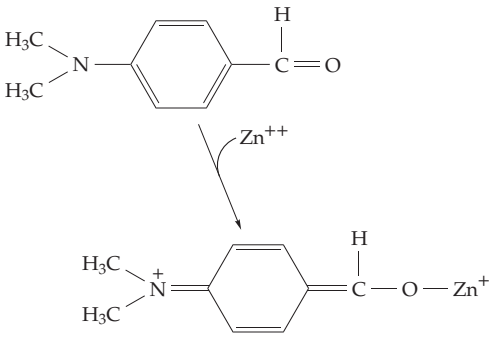
The loop closure also causes significant changes in the Raman spectra of the bound $NAD^+$, especially in vibrational modes that involve the carboxamide group of the nicotinamide ring. These have been interpreted as indicating an increased ridigity of binding of the coenzyme in the closed conformation.[46] As with many other enzymes acting on polar substrates, a characteristic of the pretransition state complex appears to be formation of a complex with a network of hydrogen bonds extending into the protein, exclusion of most water molecules, and tight packing of protein groups around the substrate. It is also significant that an overall net electrical charge on the ES complexes must be correct for tight binding of substrates to occur for lactate or malate dehydrogenase.[44] Positive and negative charges are balanced except for one excess positive charge which may be needed for catalysis.

***Zinc-containing alcohol dehydrogenases.*** Liver alcohol dehydrogenase is a relatively nonspecific enzyme that oxidizes ethanol and many other alcohols. The much studied horse liver enzyme[47–60] is a dimer of 374-residue subunits, each of which contains a "catalytic" $Zn^{2+}$ ion deeply buried in a crevice between the nucleotide-binding and catalytic domains. The enzyme also contains a "structural" $Zn^{2+}$ ion[48] that is bound by four sulfur atoms from cysteine side chains but does not represent a conserved feature of all alcohol dehydrogenases. The catalytic $Zn^{2+}$ is ligated by sulfur atoms from cysteines 46 and 174 and by a nitrogen atom of the imidazole group of His 67 (Fig. 15-5). In the free enzyme a water molecule is thought to occupy the 4th coordination position and its dissociation to form the $Zn^+–HO^-$ complex (Eq. 15-5) may account for a $pK_a$ of 9.2 in the free enzyme and of 7.6 in the $NAD^+$ complex.[48] The apparent $pK_a$ drops further to about 6.4 in the presence of the substrate

$$Zn^{2+}–OH_2 \rightarrow Zn^+–OH + H^+ \qquad (15\text{-}5)$$

ethanol. The assignment of this $pK_a$ value has been controversial.[48,49] Histidine 51 (Fig. 15-5), Glu 68 and Asp 49 have side chains close to the zinc and, as we have seen (Chapter 7), macroscopic $pK_a$ values of proteins can be *shared* by two or more closely placed groups.

Substrate binding also induces a conformational change in this enzyme. When both coenzymes and substrate bind the "closed" conformation of the enzyme is formed by a rotation of the catalytic domains of the two subunits relative to the coenzyme-binding domains.[50–51a] Structures of ternary complexes with inhibitors and with substrates have also been established. For example, liver alcohol dehydrogenase was crystallized as the enzyme•$NAD^+$• *p*-bromobenzyl alcohol complex with saturating concentrations of substrates in an equilibrium mixture[51b] and studied at low resolution. Transient kinetic studies or direct spectroscopic determinations led to the conclusion that the internal equilibrium (E•$NAD^+$•alcohol = E•NADH•aldehyde) favors the $NAD^+$•alcohol complex.[52] Subsequently, the complex was studied at higher resolution, and the basic structural features were confirmed with a




(15-6)

structure of the enzyme complexed with $NAD^+$ and 2,3,4,5,6-pentafluorobenzyl alcohol.[53] From the crystal structures of the $NAD^+$ • *p*-bromobenzyl alcohol complex and the previously mentioned complex with ethanol and analog III it appears that the oxygen of the alcohol substrate coordinates with the $Zn^{2+}$, displacing the bound water. Binding of the chromophoric aldehyde 4-*trans*-(*N*, *N*-dimethylamino) cinnamaldehyde shifts the wavelength of maximum absorbance by 66 nm, suggesting that $Zn^{2+}$ binds directly to the oxygen of this ligand (Eq. 15-6).[54] Resonance-enhanced Raman spectroscopy (Chapter 23) of the complex of dimethylaminobenzaldehyde with alcohol dehydrogenase also supports an intermediate in which the $Zn^{2+}$ becomes coordinated directly with the substrate oxygen (Eq. 15-6).[55] Rapid scanning spectrophotometry of complexes with another chromophoric substrate, 3-hydroxy-4-nitrobenzyl alcohol, also suggested that the alcohol first formed a complex with an undissociated alcoholic – OH group (Eq. 15-7, step *a*) and then lost a proton to form a zinc alcoholate complex (step *b*)[56] which could react by hydride ion transfer (step *c*).

Eklund *et al.* suggested that the side chains of Ser 48 and His 51 act as a proton relay system to remove the proton from the alcohol, in step *b* of Eq. 15-7, leaving the transient zinc-bound alcoholate ion, which can then transfer a hydride ion to $NAD^+$, in step *c*.[52] The shaded hydrogen atom leaves as $H^+$. The role of His 51 as a base is supported by studies of the inactivation of the horse liver enzyme by diethyl pyrocarbonate[57] and by directed mutation of yeast and liver enzymes. When His 51 was substituted by Gln the $pK_a$ of 7 was abolished and the activity was decreased ten-fold.[58]

The functioning of zinc ions in enzymes has been controversial and other mechanisms have been proposed. Makinen *et al.* suggested a transient pentacoordinate $Zn^{2+}$ complex on the basis of EPR measurements on en-

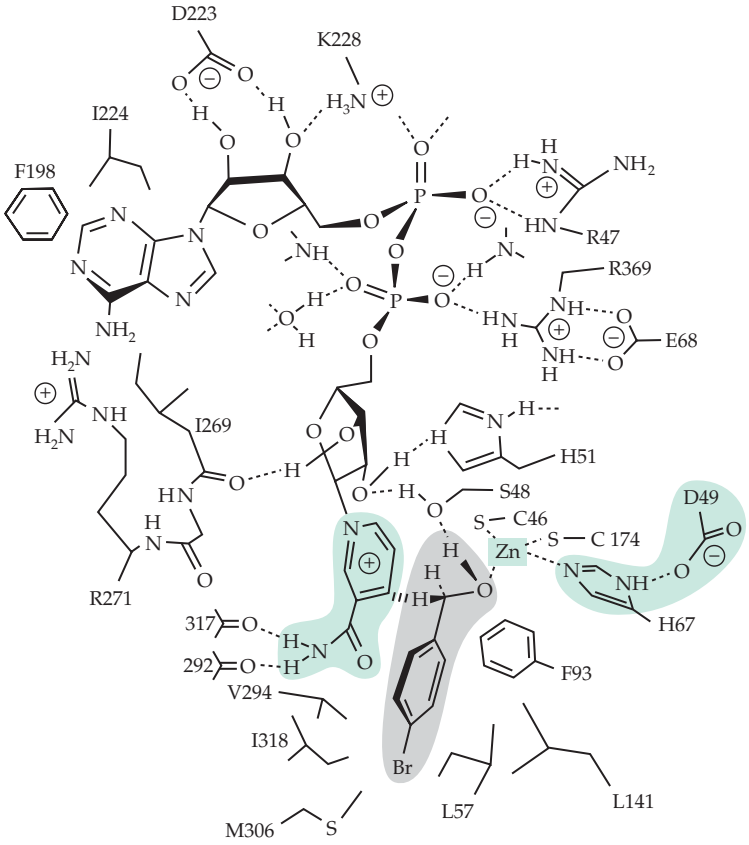
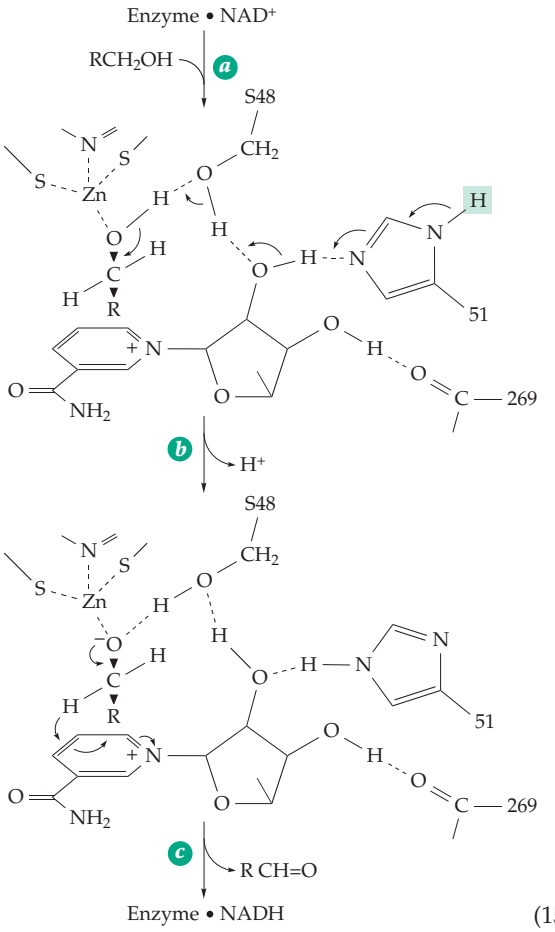



**Figure 15-5** Structure of the complex of horse liver alcohol dehydrogenase with $NAD^+$ and the slow substrate *p*-bromobenzyl alcohol. The zinc atom and the nicotinamide ring of the bound $NAD^+$ are shaded. Adjacent to them is the bound substrate. Courtesy of Bryce Plapp. Based on Ramaswamy *et al.*[53]




(15-7)

zyme containing $Co^{2+}$. Such a complex, in which the side chain of nearby Glu 68 would participate, would allow the coordinated water molecule to act as the base in deprotonation of the alcohol.[59] Molecular dynamics calculations indicate that Glu 68 can coordinate the zinc ion in this fashion, but Ryde suggests that its function may be to assist the exchange of ligands, i.e., the release of an alcohol or aldehyde product.[60] A variety of kinetic and spectroscopic studies have provided additional information that makes alcohol dehydrogenase one of the most investigated of all enzymes.

Liver alcohol dehydrogenase is important to the metabolism of ethanol by drinkers. Human beings exhibit small individual differences in their rates of alcohol metabolism which may reflect the fact that there are several isoenzymes and a number of genetic variants whose distribution differs from one person to the next as well as among tissues.[61–64] Inhibition of these isoenzymes by *uncompetitive* inhibitors, discussed in Chapter 9, is a goal in treatment of poisoning by methanol or ethylene glycol. Inhibition of the dehydrogenases slows the two-step oxidation of these substrates to toxic carboxylic acids.[65]

Yeast contains two cytosolic alcohol dehydrogenase isoenzymes.[66] Alcohol dehydrogenase I, present in large amounts in cells undergoing fermentation, functions to reduce acetaldehyde in the fermentation process. Alcohol dehydrogenase II is synthesized by cells growing on such carbon sources as ethanol itself and needing to oxidize it to obtain energy. A third isoenzyme is present in mitochondria. An alcohol dehydrogenase isoenzyme of green plants is induced by anaerobic stress such as flooding. It permits ethanolic fermentation to provide energy temporarily to submerged roots and other tissues.[67] Some bacteria contain an $NADP^+$-dependent, $Zn^{2+}$-containing alcohol dehydrogenase.[67a]

***Other alcohol dehydrogenases and aldo-keto reductases.*** The oxidation of an alcohol to a carbonyl compound and the reverse reaction of reduction of a carbonyl group are found in so many metabolic pathways that numerous specialized dehydrogenases exist. A large group of "short-chain" dehydrogenases and reductases had at least 57 known members by 1995.[68–69a] Their structures and functions are variable. Most appear to be single-domain proteins with a large β sheet, a nucleotide-binding pocket, and a conserved pair of residues: Tyr 152 and Lys 156. These may function in a manner similar to that of the His-Asp pair in lactate dehydrogenase. A possible role of a cysteine side chain has been suggested for another member of this group, 3-hydroxyisobutyrate dehydrogenase, an enzyme of valine catabolism.[70]

Dehydrogenases often act primarily to reduce a carbonyl compound rather than to dehydrogenate an alcohol. These enzymes may still be called dehydrogenases. For example, in the lactic acid fermentation lactate is formed by reduction of pyruvate but we still call the enzyme lactate dehydrogenase. In our bodies this enzyme functions in both directions. However, some enzymes that act mainly in the direction of reduction are called reductases. An example is **aldose reductase**, a member of a family of **aldo-keto reductases**[71–73] which have $(\alpha/\beta)_8$-barrel structures.[74–76]

The normal physiological function of aldose reductase is uncertain but it can cause a problem in diabetic persons by reducing glucose to sorbitol (glucitol), ribose to ribitol, etc. The resulting sugar alcohols accumulate in the lens and are thought to promote cataract formation and may also be involved in the severe damage to retinas and kidneys that occurs in diabetes mellitus. Inhibitors of aldose reductase delay development of these complications in animals but the compounds tested are too toxic for human use.[76]

The active sites of aldo-keto reductases contain an essential tyrosine (Tyr 48) with a low $pK_a$ value. The nearby His 110, Asp 43, and Lys 77 may also participate in catalysis.[76,77] The kinetics are unusual. Both $NAD^+$ and NADH are bound tightly and the overall rate of reduction of a substrate is limited by the rate of dissociation of $NAD^+$.[78] Citrate is a natural uncompetitive inhibitor of aldose reductase.[79]

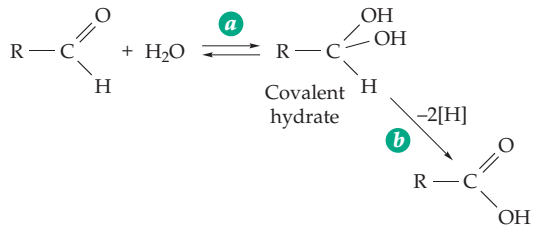
### 3. Dehydrogenation of Amino Acids and Amines

The dehydrogenation of an amine or the reverse reaction, the reduction of a Schiff base (Table 15-1, reaction type B), is another important pyridine nucleotide-dependent process. **Glutamate dehydrogenase,** a large oligomeric protein whose subunits contain 450 or more residues, is the best known enzyme catalyzing this reaction. An intermediate Schiff base of 2-oxoglutarate and $NH_3$ is a presumed intermediate.[80,81] Similar reactions are catalyzed by dehydrogenases for alanine,[82,83] leucine,[84] phenylalanine,[85] and other amino acids.[83,86,87]

### 4. Glyceraldehyde-3-Phosphate Dehydrogenase and the Generation of ATP in Fermentation Reactions

The oxidation of an aldehyde to a carboxylic acid, a highly exergonic process, often proceeds through a thioester intermediate whose cleavage can then be coupled to synthesis of ATP. This sequence is of central importance to the energy metabolism of cells (Chapters 10 and 17) and is shown in Fig. 15-6.

The best known enzyme catalyzing the first step of this reaction sequence is glyceraldehyde 3-phosphate dehydrogenase which functions in the glycolytic sequence (steps *a* and *b* of Fig. 10-3). It is present in both prokaryotes and eukaryotes as a tetramer of identical 36- to 37-kDa subunits. Three-dimensional structures have been determined for enzyme from several species, including lobster,[47,88] *E. coli*,[89] the thermophilic bacterium *Bacillus stearothermophilus* (Fig. 2-13),[90–90a] and trypanosomes.[91] Recall that aldehydes are in equilibrium with their covalent hydrates (Eq. 15-8, step *a*). Dehydrogenation of such a hydrate yields an acid (Eq. 15-8, step *b*) but such a mechanism offers no possibility of conserving the energy available from the reaction. However, during catalysis by glyceraldehyde-phosphate dehydrogenase the SH group of Cys 149, in the first step (step *a*, Fig. 15-6), adds to the substrate carbonyl group to form an adduct, a thiohemiacetal. This adduct is oxidized by $NAD^+$ to a thioester, an *S*-acyl enzyme (step *b*), which is then cleaved by the same enzyme through a displacement on carbon by an oxygen atom of $P_i$ (phosphorolysis; step *c*). The sulfhydryl group of the enzyme is released simultaneously and the product, the acyl phosphate **1,3-bisphosphoglycerate**, is formed. The imidazole group of His 176 may catalyze both steps *a* and *b*.[89,92] A separate enzyme then transfers the phospho group from the 1 position of 1,3-bisphosphoglycerate to ADP to form ATP and 3-phosphoglycerate (step *d*). The overall sequence of Fig. 15-6 is the synthesis of one mole of ATP coupled to the oxidation of an aldehyde




(15-8)

to a carboxylic acid and the conversion of $NAD^+$ to NADH.

In green plants and in some bacteria an $NADP^+$-dependent cytoplasmic glyceraldehyde 3-phosphate dehydrogenase *does not* use inorganic phosphate to form an acyl phosphate intermediate but gives 3-phosphoglycerate with a free carboxylate as in Eq. 15-8.[93,93a] Because it doesn't couple ATP cleavage to the dehydrogenation, it drives the [NADPH] / [$NADP^+$] ratio to a high value favorable to biosynthetic processes (see Chapter 17).

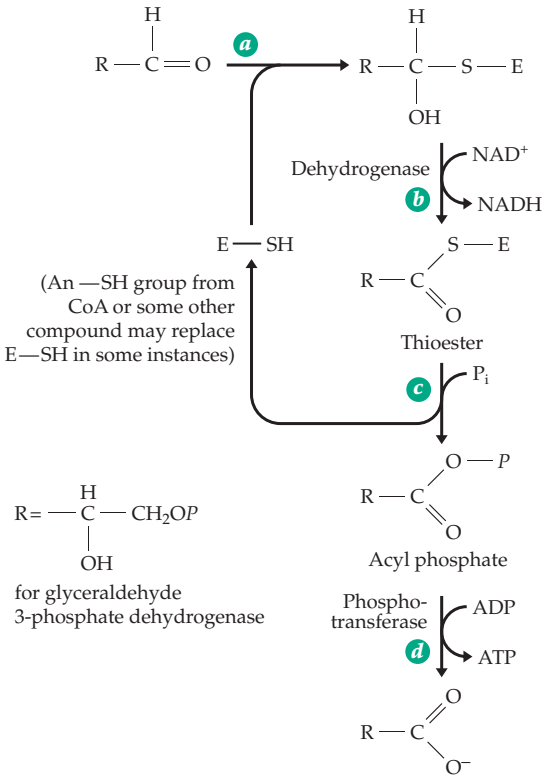



**Figure 15-6** Generation of ATP coupled to oxidation of an aldehyde to a carboxylic acid. The most important known example of this sequence is the oxidation of glyceraldehyde 3-phosphate to 3-phosphoglycerate (Fig. 10-3, steps *a* and *b*). Other important sequences for "substrate-level" phosphorylation are shown in Eq. 14-23 and in Fig. 15-16.

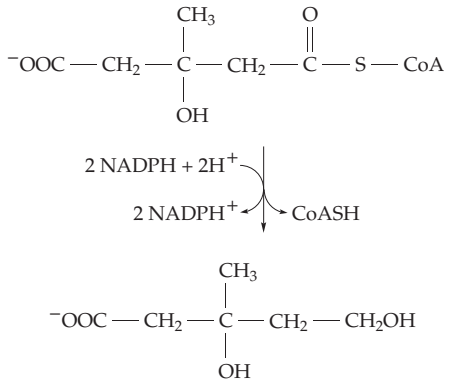
Animal tissues also contain aldehyde dehydrogenases of a nonspecific type which are thought to act to remove toxic aldehydes from tissues.[94,95] Like glyceraldehyde 3-phosphate, these enzymes form thioester intermediates as in Fig. 15-6 but which are hydrolyzed rather than being converted to acyl phosphates. A mutation (E487K) in the mitochondrial enzyme occurs in about 50% of the Asian population. Although the structural alteration is not at the active site, the enzyme activity is low. Individuals carrying the mutation are healthy but have an aversion to alcohol, whose consumption causes an elevated blood level of acetaldehyde, facial flushing, dizziness, and other symptoms. A similar effect is exerted by the drug disulfiram (Antabuse), which has been used to discourage drinking and whose metabolites are thought to inhibit aldehyde dehydrogenase.[95]

Alcohol dehydrogenases also oxidize aldehydes, probably most often as the geminal diol forms, according to Eq. 15-8. No ATP is formed. The same enzymes can catalyze the dismutation of aldehydes, with equal numbers of aldehyde molecules going to carboxylic acid and to the alcohol.[96–98]

## 5. Reduction of Carboxyl Groups

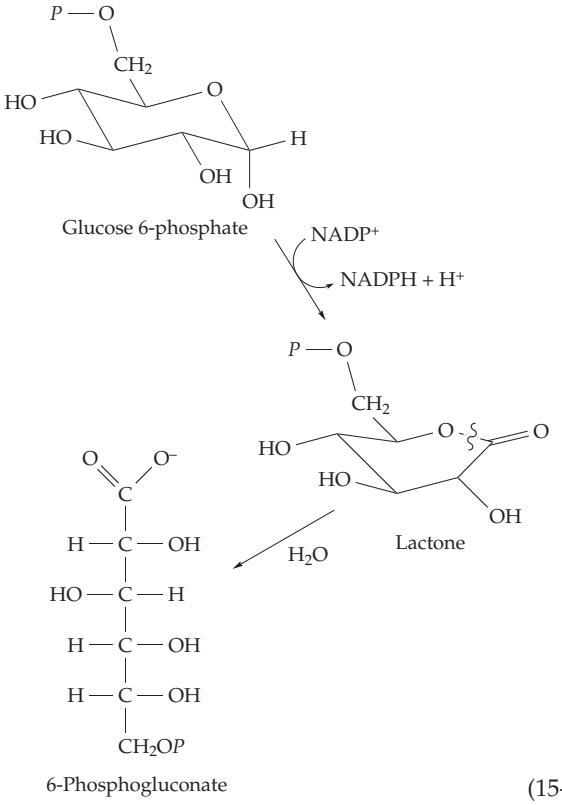
The last two reaction steps in Fig. 15-6, steps *c* and *d*, are in essence the reverse of the sequence used for synthesis of a thioester such as a fatty acyl coenzyme A. Thus, the chemistry by which ATP is generated during glycolysis and that by which it is utilized in biosynthesis is nearly the same. Furthermore, a standard biochemical method for reduction of carboxyl groups to aldehyde groups is conversion, in an ATP-requiring process, to a thioester followed by reduction of the thioester (Table 15-1, reaction type C). For example, the sequence of Fig. 15-6 is reversed during gluconeogenesis (see Fig. 17-17). The carboxyl group of the side chain of aspartate can be reduced in two steps to form the alcohol homoserine (Figs. 11-3; 24-13).

The aldehyde generated by reduction of a thioester is not always released from the enzyme but may be converted on to the alcohol in a second reduction step.[99] This is the case for **3-hydroxy-3-methylglutaryl-CoA reductase** (HMG-CoA reductase), a large 887-residue protein that synthesizes mevalonate (Eq. 15-9).[100–102] This highly regulated enzyme controls the rate of synthesis of cholesterol and is a major target of drugs designed to block cholesterol synthesis. The structure of a smaller 428-residue bacterial enzyme is known.[103,104] Aspartate 766 is a probable proton donor in both reduction steps and Glu 558 and His 865 may act as a catalytic pair that protonates the sulfur of coenzyme A.[100]




(15-9)

A related oxidation reaction is catalyzed by **glucose-6-phosphate dehydrogenase**, the enzyme that originally attracted Warburg's attention and led to the discovery of $NADP^+$. The substrate, the hemiacetal ring form of glucose, is oxidized to a lactone which is then hydrolyzed to 6-phosphogluconate (Eq. 15-10).[104a] This oxidation of an aldehyde to a carboxylic acid is not linked directly to ATP synthesis as in Fig. 15-6. The ring-opening step ensures that the reaction goes to completion. This reaction is a major supplier of NADPH for reductive biosynthesis and the large Gibbs energy decrease for the overall reaction ensures that the ratio [NADPH] / [$NADP^+$] is kept high within cells. This is the only source of NADPH for mature erythrocytes and a deficiency of glucose 6-phosphate dehydrogenase is a common cause of drug- and food-

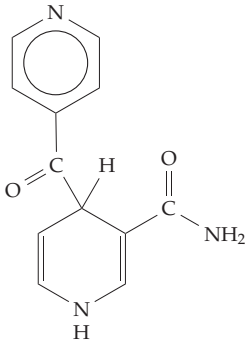



(15-10)

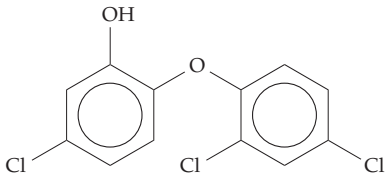
induced hemolytic anemia in human beings. About 400 variant forms of this enzyme are known.[105] Like the sickle cell trait (Box 7-B) some mutant forms of glucose-6-phosphate dehydrogenase appear to confer resistance to malaria.

## 6. Reduction of Carbon–Carbon Double Bonds

Neither $NADP^+$ nor $NAD^+$ is a strong enough oxidant to carry out the dehydrogenation of an acyl-CoA (reaction type D of Table 15-1). However, NADPH or NADH can participate in the opposite reaction. Thus, NADPH transfers a hydride ion to the β-carbon of an unsaturated acyl group during the biosynthesis of fatty acids (Chapter 17) and during elongation of shorter fatty acids (Chapter 21).[106–107b] A discovery of medical importance is that isonicotinyl hydrazide (INH), the most widely used antituberculosis drug, forms an adduct (of an INH anion or radical) with $NAD^+$ of long-chain **enoyl-acyl carrier protein reductase** (enoyl-ACP reductase).[133a,b] This enzyme utilizes NADH in reduction of a C=C double bond during synthesis of mycolic acids. The same enzyme is blocked by **triclosan**, an antibacterial compound used widely in household products such as antiseptic soaps, toothpastes, cosmetics, fabrics, and toys.[133c–e]

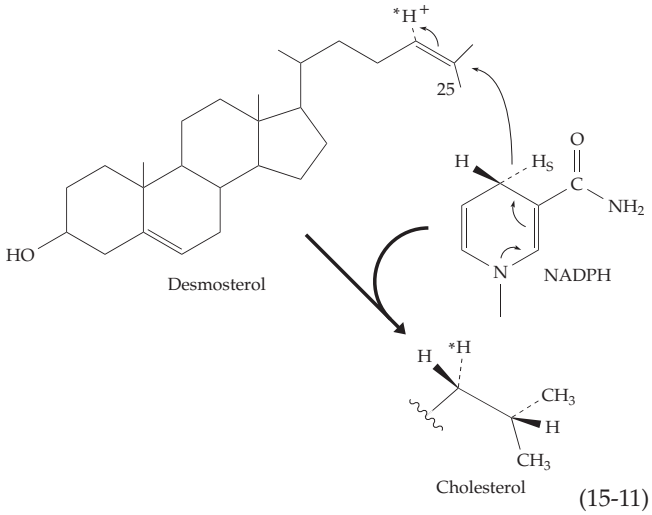



Adduct of isonicotinyl hydrazide anion with $NAD^+$




Triclosan

Less frequently NADPH is used to reduce an *isolated double bond*. An example is the hydrogenation of **desmosterol** by NADPH (Eq. 15-11), the final step in one of the pathways of biosynthesis of cholesterol (Fig. 22-7). In this and in other reactions of the same



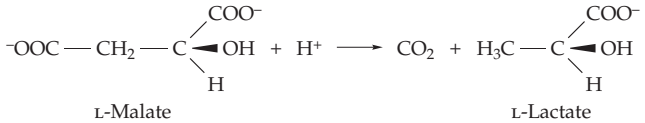

(15-11)

type, hydrogen transfer has been shown to be from the *pro-S* position in NADPH directly to C-25 of the sterol. The proton introduced from the medium (designated by the asterisks in Eq. 15-11) enters *trans* to the $H^-$ ion from NADPH. The proton always adds to the more electron-rich terminus of the double bond, i.e., it follows the Markovnikov rule. This result suggests that protonation of the double bond may precede $H^-$ transfer.[108]

Additional pyridine nucleotide-dependent dehydrogenases include **glutathione reductase** (Figs. 15-10, 15-12), **dihydrofolate reductase** (Fig. 15-19), isocitrate dehydrogenase, ***sn*-glycerol-3-phosphate dehydrogenase** (Chapter 21), L-3-hydroxyacyl-CoA dehydrogenase (Chapter 21), **retinol dehydrogenase** (Chapter 23), and a bacterial quinone oxidoreductase.[109] Some of these also contain a flavin coenzyme.

## 7. Transient Carbonyl Groups in Catalysis

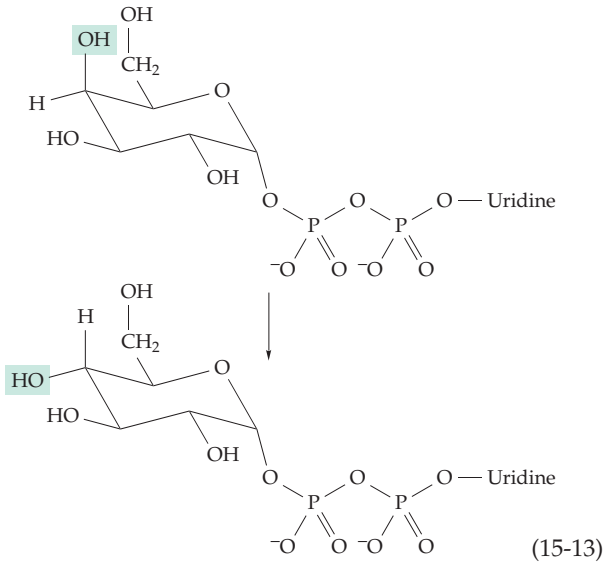
Some enzymes contain bound $NAD^+$ which oxidizes a substrate alcohol to facilitate a reaction step and is then regenerated. For example, the **malolactic enzyme** found in some lactic acid bacteria and also in *Ascaris* decarboxylates L-malate to lactate (Eq. 15-12). This reaction is similar to those of isocitrate dehydrogenase,[110–112] 6-phosphogluconate dehydrogenase,[113] and the malic enzyme (Eq. 13-45)[114] which utilize free $NAD^+$ to first dehydrogenate the substrate to a bound oxoacid whose β carbonyl group facilitates decarboxylation. Likewise, the bound $NAD^+$ of the malolactic




(15-12)

enzyme apparently dehydrogenates the malate to a bound oxaloacetate which is decarboxylated to pyruvate.[115] The latter remains in the active site and is reduced by the bound NADH to lactate which is released from the enzyme.

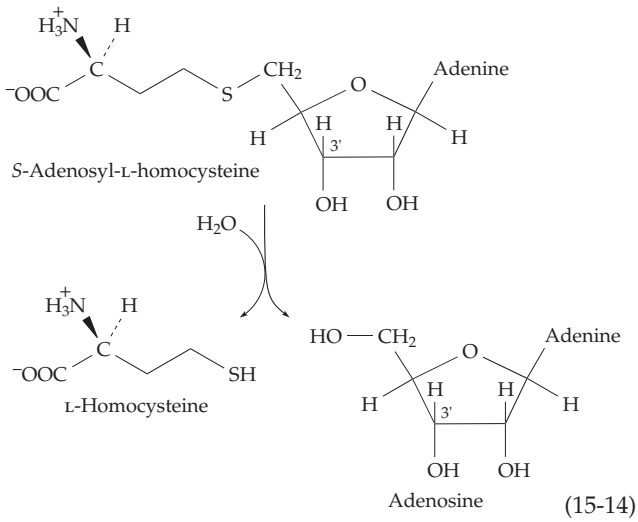
Another reason for introducing a carbonyl group is to form a symmetric intermediate in a reaction that inverts the configuration about a chiral center. An example is **UDP-galactose 4-epimerase**, an enzyme that converts UDP-galactose to UDP-glucose (Eq. 15-13; Chapter 20) and is essential in the metabolism of galactose in our bodies. The enzyme contains bound $NAD^+$ and forms a transient 4-oxo intermediate and bound NADH. Rotation of the intermediate allows nonstereospecific reduction by the NADH, leading to epimerization.[116–117a]



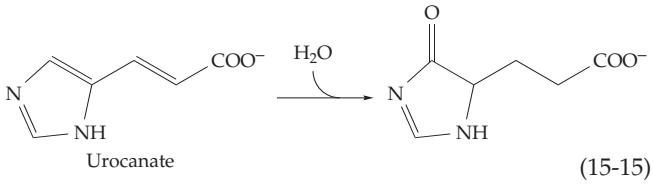

(15-13)

The enzyme is a member of the short-chain dehydrogenase group with a catalytic Tyr–Lys pair in the active site.[118] Another way that formation of an oxo group can assist in epimerization of a sugar is through enolization with nonstereospecific return of a proton to the intermediate enediol. A third possible mechanism of epimerization is through aldol cleavage followed by aldol condensation, with inversion of configuration. In each case the initial creation of an oxo group by dehydrogenation is essential.

Bound $NAD^+$ is also present in **S-adenosylhomocysteine hydrolase**,[119,120] which catalyzes the irreversible reaction of Eq. 15-14. Transient oxidation at the 3 position of the ribose ring facilitates the reaction. The reader can doubtless deduce the function that has been established for the bound $NAD^+$ in this enzyme. However, the role of NAD in the **urocanase** reaction (Eq. 15-15) is puzzling. This reaction, which is the second step in the catabolism of histidine, following Eq. 14-44, appears simple. However, there is no obvious




(15-14)

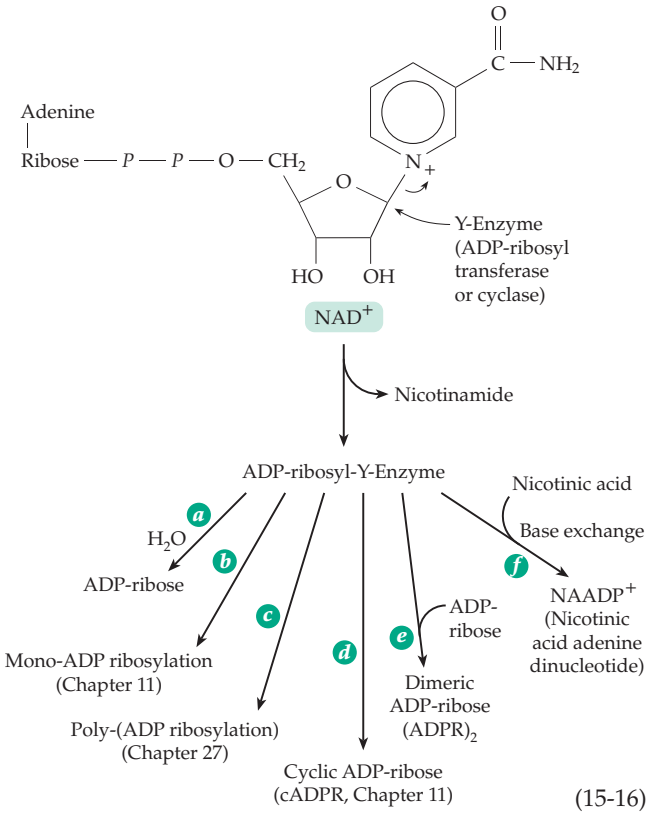



(15-15)

mechanism and no obvious role for $NAD^+$. See Frey for a discussion.[117]

## 8. ADP Ribosylation and Related Reactions of $NAD^+$ and $NADP^+$

The linkage of nicotinamide to ribose in $NAD^+$ and $NADP^+$ is easily broken by nucleophilic attack on C-1 of ribose. In Chapter 11 enzyme-catalyzed ADP ribosylation, which can be shown as in Eq. 15-16, is discussed briefly. The nucleophilic group –Y from an ADP-ribosyltransferase carries the ADP-ribosyl group which can then be transferred by a second displacement onto a suitable nucleophilic acceptor group.[121,122] Hydrolysis (Eq. 15-16, step *a*) gives free ADP-ribose.[123] Other known products of enzymatic action are indicated in steps *c*–*f*. Poly-(ADP ribosylation) is discussed in Chapter 27. The structure of cyclic ADP-ribose (cADPR)[124] is shown in Chapter 11, Section E,2. The acceptor nucleophile is N-1 of the adenine ring which is made more nucleophilic by electron donation from the amino group. A similar reaction with ADP ribose (Eq. 15-16, step *e*) produces a dimeric ADP-ribose $(ADPR)_2$[125] while reaction with free nicotinic acid (step *f*) yields, in an overall base exchange, nicotinic acid adenine dinucleotide ($NAADP^+$).[126,127] Some of these compounds, e.g., cADPR, $NAADP^+$, and $(ADPR)_2$, are involved in signaling with calcium ions.[124]

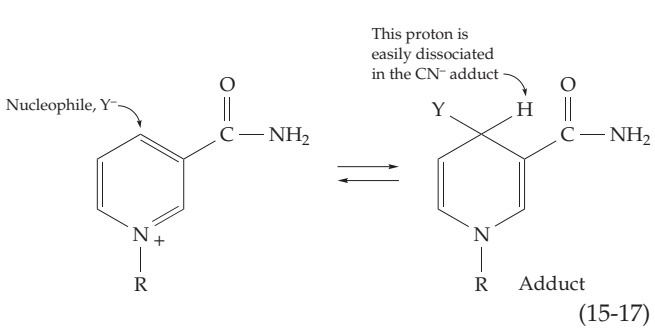



(15-16)

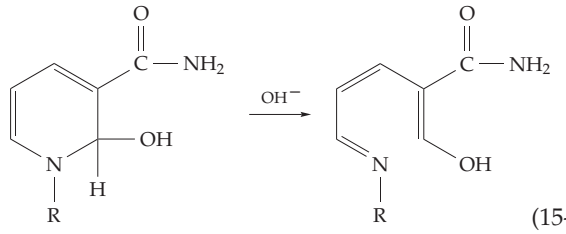
## 9. The Varied Chemistry of the Pyridine Nucleotides

Despite the apparent simplicity of their structures, the chemistry of the nicotinamide ring in $NAD^+$ and $NADP^+$ is surprisingly complex.[128,129] $NAD^+$ is extremely unstable in basic solutions, whereas NADH is just as unstable in slightly acidic media. These properties, together with the ability of $NAD^+$ to undergo condensation reactions with other compounds, have sometimes caused serious errors in interpretation of experiments and may be of significance to biological function.

***Addition to $NAD^+$ and $NADP^+$.*** Many nucleophilic reagents add reversibly at the para (or 4) position (Eq. 15-17) to form adducts having structures resembling those of the reduced coenzymes. Formation of the cyanide adduct, whose absorption maximum is at 327 nm, has been used to introduce deuterium into the para position of the pyridine nucleotides. In the adduct, the hydrogen adjacent to the highly polarized C – N bond is easily dissociated as a proton. Thiolate anions and bisulfite also add. Dithionite ion, $S_2O_4^{2-}$, can lose $SO_2$ and acquire a proton to form the sulfoxylate ion $HSO_2^-$ which also adds to the 4 position of the $NAD^+$ ring.[130] The resulting adduct is unstable and loses $SO_2$ to give NADH + $H^+$. Addition can also occur at the two ortho positions. The adducts of $HO^-$ to the 4 position of $NAD^+$ are stable but those to the 2 position undergo ring opening (Eq. 15-18) in base-catalyzed reactions which are followed by further degradation.[128,131]

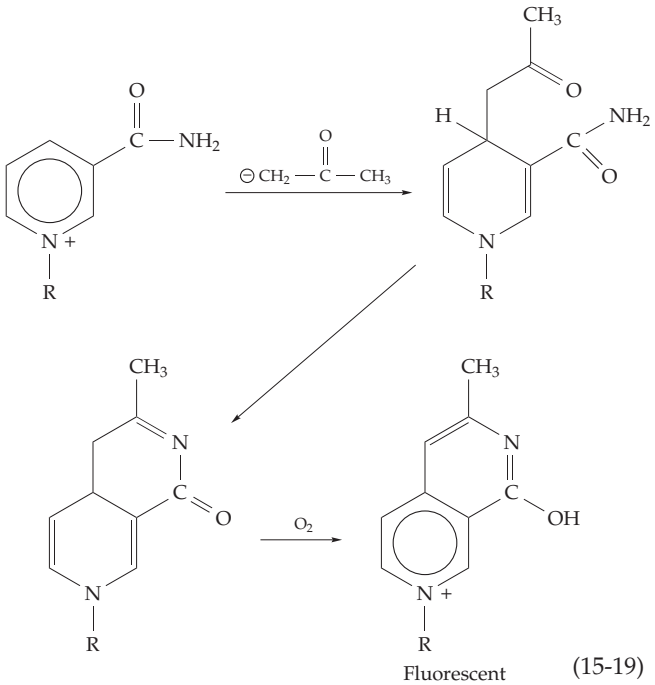



(15-17)




(15-18)

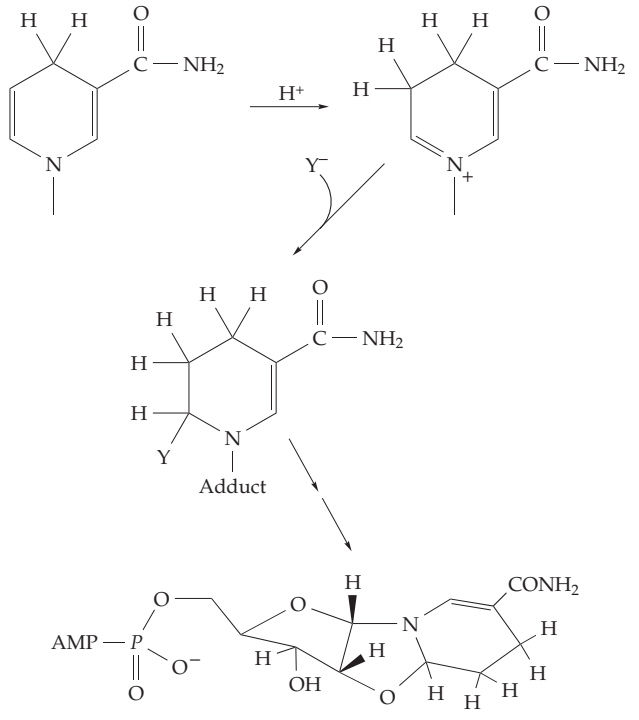
Another base-catalyzed reaction is the addition of enolate anions derived from ketones to the 4 position of the pyridine nucleotides (Eq. 15-19). The adducts undergo ring closure and in the presence of oxygen are converted slowly to fluorescent materials. While forming the basis for a useful analytical method for determination of $NAD^+$ (using 2-butanone), these reactions also have created a troublesome enzyme inhibitor from traces of acetone present in commercial NADH.[132]




(15-19)

The reactions of Eq. 15-19 occur nonenzymatically only under the influence of strong base but dehydrogenases often catalyze similar condensations relatively rapidly and reversibly. Pyruvate inhibits lactate dehydrogenase, 2-oxoglutarate inhibits glutamate dehydrogenase, and ketones inhibit a short-chain alcohol dehydrogenase in this manner.[133,69a]

***Modification of NADH in acid.*** Reduced pyridine nucleotides are destroyed rapidly in dilute HCl and more slowly at pH 7 in reactions catalyzed by buffer acids.[128,131,134] Apparently the reduced nicotinamide ring is first protonated at C-5, after which a nucleophile $Y^-$ adds at the 6 position (Eq. 15-20). The nucleophile may be $OH^-$, and the adduct may undergo further reactions. For example, water may add to the other double bond and the ring may open on either side of the nitrogen. The glycosidic linkage can be isomerized from β to α or can be hydrolytically cleaved. The early steps in the modification reaction are partially reversible, but the overall sequence is irreversible. One of the products, which has been characterized by crystal structure determination, is shown in Eq. 15-20.[128,135] It can arise if the group Y of Eq. 15-20 is the C-2' hydroxyl of the ribose ring and if the configuration of the glycosidic linkage is inverted (anomerized).

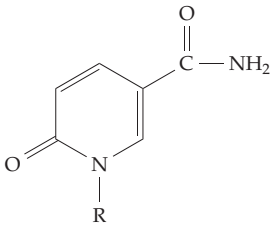



An acid modification product from NADH (15-20)

The foregoing reactions have attracted interest because glyceraldehyde-3-phosphate dehydrogenase, in a side reaction, converts NADH to a substance referred to as NADH-X which has been shown to be the 6(*R*) adduct of Eq. 15-20, where Y is –OH. In an ATP-dependent reaction an enzyme from yeast reconverts NADH-X to NADH.[136]

Mercury (II) ions can add in place of $H^+$ in the first step of Eq. 15-20 and subsequent reactions similar to those promoted by acid can occur.[137]

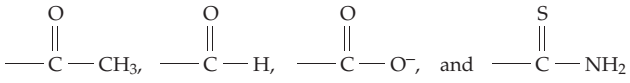
***Other reactions of pyridine nucleotides.*** Alkaline hexacyanoferrate (III) oxidizes $NAD^+$ and $NADP^+$ to 2-,4-, and 6-pyridones. The 6-pyridone of *N*-methylnicotinamide is a well-known excretion product of nicotinic acid in mammals. Reoxidation of NADH and NADPH to $NAD^+$ and $NADP^+$ can be accomplished with hexacyanoferrate (III), quinones, and riboflavin



6-Pyridone

but not by $H_2O_2$ or $O_2$. However, $O_2$ does react at neutral pH with uptake of a proton to form a peroxide derivative of NADH.[138] When heated in 0.1 N alkali at 100°C for 5 min, $NAD^+$ is hydrolyzed to nicotinamide and adenosine-diphosphate-ribose.

Treatment of $NAD^+$ with nitrous acid deaminates the adenine ring. The resulting deamino $NAD^+$ as well as synthetic analogs containing the following groups in place of the carboxyamide have been used




widely in enzyme studies. In fact, almost every part of the coenzyme molecule has been varied systematically and the effects on the chemical and enzymatic properties have been investigated.[139–141] "Caged" $NAD^+$ and $NADP^+$ have also been made.[142] These compounds do not react as substrates until they are released ("uncaged") by photolytic action of a laser beam (see Chapter 23).

## B. The Flavin Coenzymes

**Flavin adenine diphosphate** (**FAD**, flavin adenine dinucleotide) and **riboflavin 5'-monophosphate** (**FMN**, flavin mononucleotide), whose structures are shown in Fig. 15-7, are perhaps the most versatile of all

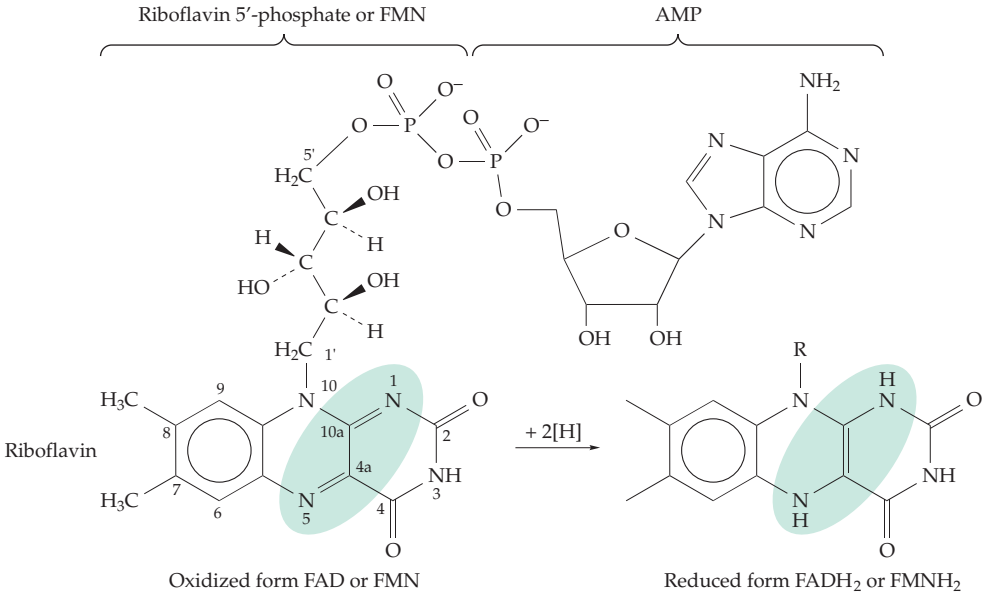



**Figure 15-7** The flavin coenzymes flavin adenine dinucleotide (FAD) and riboflavin 5'-phosphate (FMN). Dotted lines enclose the region that is altered upon reduction.

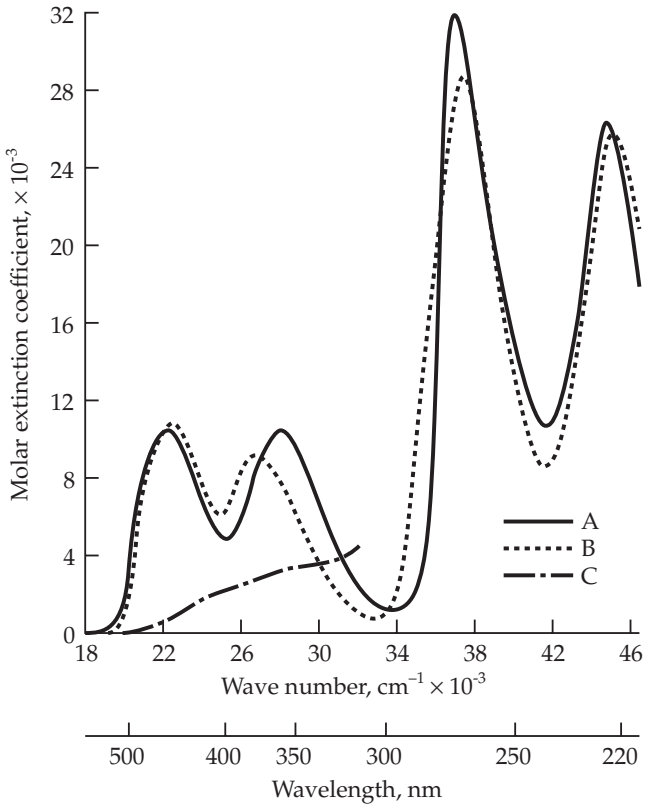



**Figure 15-8** Absorption spectrum of neutral, uncharged riboflavin (A), the riboflavin anion (B), and reduced to the dihydro form (Fig. 15-7) by the action of light in the presence of EDTA (C). A solution of 1.1 x $10^{-4}$ M riboflavin containing 0.01 M EDTA was placed 11.5 cm from a 40-W incandescent lamp for 30 min.

the oxidation coenzymes. The name flavin adenine dinucleotide is not entirely appropriate because the D-ribityl group is not linked to the riboflavin in a glycosidic linkage. Hemmerich suggested that FAD be called flavin adenine diphosphate.[143]

The attention of biochemists was first attracted to flavins as a result of their color and fluorescence. The study of spectral properties of flavins (Fig. 15-8) has been of importance in understanding these coenzymes. The biochemical role of the flavin coenzymes was first recognized through studies of the "old yellow enzyme"[144,145] which was shown by Theorell to contain riboflavin 5'-phosphate. By 1938, FAD was recognized as the coenzyme of a different yellow protein, **D-amino acid oxidase** of kidney tissue. Like the pyridine nucleotides, the new flavin coenzymes were reduced by dithionite to nearly colorless dihydro forms (Figs. 15-7 and 15-8) revealing the chemical basis for their function as hydrogen carriers.

Flavins are also among the natural light receptors and display an interesting and much studied photochemistry (Fig. 3-5).[143] Flavins may function in some photoresponses of plants, and they serve as light emitters in bacterial bioluminescence (Chapter 23).[146,147]

Three facts account for the need of cells for both the flavin and pyridine nucleotide coenzymes: (1) Flavins are usually stronger oxidizing agents than is $NAD^+$. This property fits them for a role in the electron transport chains of mitochondria where a sequence of increasingly more powerful oxidants is needed and makes them ideal oxidants in a variety of other dehydrogenations. (2) Flavins can be reduced either by one- or two-electron processes. This enables them to participate in oxidation reactions involving free radicals and in reactions with metal ions. (3) Reduced flavins

are "autooxidizable," i.e., they can be reoxidized directly and rapidly by $O_2$, a property shared with relatively few other organic substances. For example, NADH and NADPH are not spontaneously reoxidized by oxygen. Autooxidizability allows flavins of some enzymes to pass electrons directly to $O_2$ and also provides a basis for the functioning of flavins in hydroxylation reactions.

## 1. Flavoproteins and Their Reduction Potentials

Flavin coenzymes are usually bound tightly to proteins and cycle between reduced and oxidized states while attached to the same protein molecule. In a free unbound coenzyme the redox potential is determined by the structures of the oxidized and reduced forms of the couple. Both riboflavin and the pyridine nucleotides contain aromatic ring systems that are stabilized by resonance. Part of this resonance stabilization is lost upon reduction. The value of $E°'$ depends in part upon the varying amounts of resonance in the oxidized and reduced forms. The structures of the coenzymes have apparently evolved to provide values of $E°'$ appropriate for their biological functions.

The relative strengths of binding of oxidized and reduced flavin coenzymes to a protein also have strong effects upon the reduction potential of the coenzyme.[148] If the oxidized form is bound weakly, but the reduced form is bound tightly, a bound flavin will have a greater tendency to stay in the reduced form than it did when free. The reduction potential $E°'$ will be less negative than it is for the free flavin-dihydroflavin couple. On the other hand, if the oxidized form of the flavin is bound more tightly by the protein than is the reduced form, $E°'$ will be more negative and the flavoenzyme will be a less powerful oxidizing agent. In fact, the values of $E°'$ at pH 7 for flavoproteins span a remarkably wide range from – 0.49 to +0.19 V. The state of protonation of the reduced flavin when bound to the enzyme will also have a major effect on the oxidation–reduction potential. For example, acyl-CoA dehydrogenases are thought to form an anionic species of reduced FAD ($FADH^-$) which is tightly bound to the protein.[149]

Every flavoprotein accepts electrons from the substrate that it oxidizes and passes these electrons on to another substrate, an oxidant. In the following sections we will consider for several enzymes how the electrons may get into the flavin from the oxidizable substrate and how they may flow out of the flavin into the final electron acceptor.

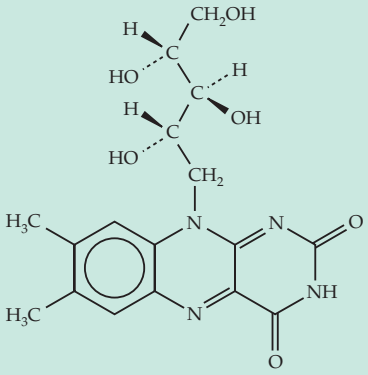
## 2. Typical Dehydrogenation Reactions Catalyzed by Flavoproteins

The functions of flavoprotein enzymes are numerous and diversified.[151–153a] A few of them are shown in Table 15-2 and are classified there as follows: (A) oxidation of hemiacetals to lactones, (B) oxidation of alcohols to aldehydes or ketones, (C) oxidation of amines to imines, (D) oxidation of carbonyl compounds or carboxylic acids to α,β-unsaturated compounds, (E) oxidation of NADH and NADPH in electron transport chains, and (F) oxidation of dithiols to disulfides or the reverse reaction. Three-dimensional structures are known for enzymes of each of these types.

Reactions of types A–C could equally well be catalyzed by pyridine nucleotide-requiring dehydrogenases. Recall that D-glucose-6-phosphate dehydrogenase uses $NADP^+$ as the oxidant (Eq. 15-10). The first product is the lactone which is hydrolyzed to 6-phosphogluconic acid. The similar reaction of free glucose (Table 15-2, reaction type A) is catalyzed by fungal **glucose oxidase**, a 580-residue FAD-containing enzyme.[154–155] A bacterial cholesterol oxidase has a similar structure.[156] The important plant enzyme **glycolate oxidase** is a dimer containing riboflavin 5'-phosphate.[157–159] It catalyzes a reaction of type B, which plays an important role in photorespiration (Chapter 23). **Amino acid oxidases** (reaction type C) are well-known. The peroxisomal D-amino acid oxidase from kidney was the source from which Warburg first isolated FAD and has been the subject of much investigation of mechanism and structure.[153,160–163] Many snake venoms contain an active 140-kDa L-amino-acid oxidase which contains FAD. Flavin-dependent **amine oxidases**, important in the human body, catalyze the related reaction with primary, secondary, or tertiary amines and in which a carboxyl group need not be present.[164–166a] Reduced flavin produced by all of these oxidases is reoxidized with molecular oxygen and hydrogen peroxide is the product. Nature has chosen to forego the use of an electron transport chain (Fig. 10-5), giving up the possible gain of ATP in favor of simplicity and a more direct reaction with oxygen. In some cases there is specific value to the organism in forming $H_2O_2$ (see Chapter 18).

In contrast to the flavin oxidases, flavin dehydrogenases pass electrons to carriers within electron transport chains and the flavin does *not* react with $O_2$. Examples include a bacterial **trimethylamine dehydrogenase** (Fig. 15-9) which contains an iron–sulfur cluster that serves as the immediate electron acceptor[167–169] and yeast **flavocytochrome $b_2$**, a lactate dehydrogenase that passes electrons to a built-in heme group which can then pass the electrons to an external acceptor, another heme in cytochrome *c*.[170–173] Like glycolate oxidase, these enzymes bind their flavin coenzyme at the ends of 8-stranded αβ barrels similar

## BOX 15-B RIBOFLAVIN



The bright orange-yellow color and brilliant greenish fluorescence of riboflavin first attracted the attention of chemists. Blyth isolated the vitamin from whey in 1879 and others later obtained the same fluorescent, yellow compound from eggs, muscle, and urine. All of these substances, referred to as **flavins** because of their yellow color, were eventually recognized as identical. The structure of riboflavin was established in 1933 by R. Kuhn and associates, who had isolated 30 mg of the pure material from 30 kg of dried albumin from 10,000 eggs. The intense fluorescence assisted in the final stages of purification. The vitamin was synthesized in 1935 by P. Karrer.[a]

Riboflavin, a yellow solid, has a low solubility of ~100 mg / l at 25°C. Three crystalline forms are known. One of these, the "readily soluble form," is ten times more soluble than the others and can be used to prepare metastable solutions of higher concentration. One crystalline form is platelike and occurs naturally in the tapetum (Box 13-C) of the nocturnal lemur.

Discovery of the role of riboflavin in biological oxidation was an outgrowth of biochemists' interest in respiration. In the 1920s Warburg provided evidence that oxygen reacted with an iron-containing respiration catalyst and it was shown that the dye **methylene blue** could often substitute for oxygen as an oxidant (Box 18-A). Oxidation of glucose 6-phosphate by methylene blue within red blood cells required both a "ferment" (enzyme) and a "coferment," later identified as $NADP^+$. A yellow protein, isolated from yeast, was found to have the remarkable property of being decolorized by the reducing system of glucose 6-phosphate plus the protein and coferment from red blood cells.

Warburg and Christian showed that the color of this **old yellow enzyme** came from a flavin and proposed that its cyclic reduction and reoxidation played a role in cellular oxidation. When $NADP^+$ was isolated the proposal was extended to encompass a **respiratory chain**. The two hydrogen carriers $NADP^+$ and flavin would work in sequence to link dehydrogenation of glucose to the iron-containing catalyst that interacted with oxygen. While we still do not know the physiological function of the old yellow enzyme,[b] the concept of respiratory chain was correct.

Human beings require about 1.5 mg of riboflavin per day. Because of its wide distribution in food, a deficiency, which affects skin and eyes, is rarely seen. Riboflavin is produced commercially in large quantities by fungi such as *Eremothecium asbyii* which, apparently because of some metabolic anomaly, produce the vitamin in such copious amounts that it crystallizes in the culture medium.

When taken up by the body, riboflavin is converted into its coenzyme forms (Chapter 25) and any excess is quickly excreted in the urine. Urine also contains smaller amounts of metabolites. The ribityl group may be cut by the action of intestinal bacteria acting on riboflavin before it is absorbed. The resulting 10-hydroxyethyl flavin may sometimes be a major urinary product.[c,d] The related 10-formylmethyl flavin is also excreted,[c] as are small amounts of 7α- and 8α- hydroxyriboflavins, apparently formed in the body by hydroxylation. These may be degraded farther to the 7α- and 8α- carboxylic acids of lumichrome (riboflavin from which the ribityl side chain is totally missing).[e] A riboflavin glucoside has also been found in rat urine.[f]

The choroid layer of the eye (behind the retina) in many animals contains a high concentration of free riboflavin. Cats' eyes also contain a large amount of 7 α-hydroxyriboflavin (nekoflavin), as do their livers.[g] Nekoflavin is also present in human blood.[h] Hen egg white contains a 219-residue riboflavin-binding protein whose functions are thought to be storage of the vitamin and delivery to the developing embryo.[i–k] Most of the riboflavin in human blood is bound to proteins such as albumin and immunoglobulins. However, during pregnancy a riboflavin-binding protein similar to that of the chicken appears, apparently to carry riboflavin to the fetus.[j]

Riboflavin is stable to heat but is extremely sensitive to light, a fact of some nutritional significance. Do not leave bottled milk in the sunshine (see Fig. 15-8)! Many products of photolysis are formed (Fig. 3-5). Among them is lumichrome.

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[a] Yagi, K. (1990) in *Flavins and Flavoproteins* (Curti, B., Ronchi, S., and Zanetti, G., eds), pp. 3 –16, Walter de Gruyter, Berlin

[b] Kohli, R. M., and Massey, V. (1998) *J. Biol. Chem.* **273**, 32763–32770

[c] Owen, E. C., West, D. W., and Coates, M. E. (1970) *Br. J. Nutr.* **24**, 259–267

[d] Roughead, Z. K., and McCormick, D. B. (1991) *European Journal of Clinical Nutrition* **45**, 299–307

[e] Ohkawa, H., Ohishi, N., and Yagi, K. (1983) *J. Biol. Chem.* **258**, 5623–5628

[f] Ohkawa, H., Ohishi, N., and Yagi, K. (1983) *J. Nutr. Sci. Vitaminol.* **29**, 515–522

[g] Matsui, K., and Kasai, S. (1996) *J. Biochem.* **119**, 441–447

[h] Zempleni, J., Galloway, J. R., and McCormick, D. B. (1995) *Int. J. Vitamins Nutr. Res.* **66**, 151–157

[i] Matsui, K., Sugimoto, K., and Kasai, S. (1982) *J. Biochem.* **91**, 469–475

[j] Miura, R., Tojo, H., Fujii, S., Yamano, T., Miyake, Y., (1984) *J. Biochem.* **96**, 197–206

[k] Monaco, H. L. (1997) *EMBO J.* **16**, 1475–1483

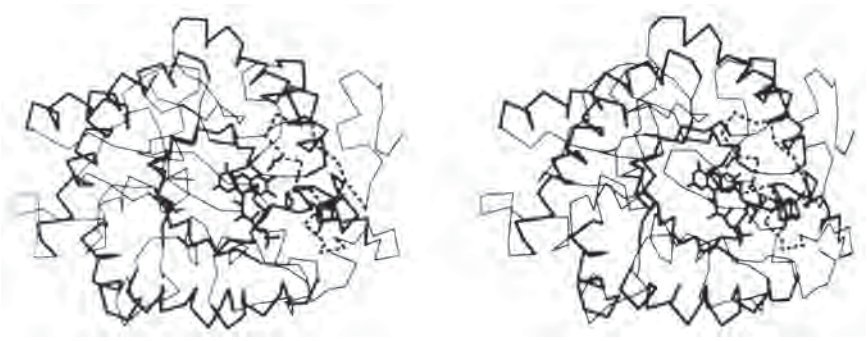


**Figure 15-9** Stereoscopic view of the large domain (residues 1–383) of trimethylamine dehydrogenase from a methylotrophic bacterium. The helices and β strands of the $(\alpha\beta)_8$ barrel are drawn in heavy lines as are the FMN (center) and the $Fe_4S_4$ iron–sulfur cluster at the lower right edge. The α/β loop to which it is bound is drawn with dashed lines. The 733-residue protein also contains two other structural domains. From Lim *et al.*[150] Courtesy of F. S. Mathews.

to that of triose phosphate isomerase (Fig. 2-28). Flavocytochrome $b_2$ has an additional domain which carries the bound heme. Two additional domains of trimethylamine dehydrogenase have a topology resembling that of the FAD- and NADH-binding domains of glutathione reductase shown in Fig. 15-10. A bacterial **mandelate dehydrogenase** is structurally and mechanistically closely related to the glycolate oxidase family.[173a]

Reaction type D of Table 15-2, the dehydrogenation of an acyl-coenzyme A (CoA), could not be accomplished by a pyridine nucleotide system because the reduction potential ($E°'$, pH 7 = −0.32 V) is inappropriate. The more powerfully oxidizing flavin system is needed. (However, the reverse reaction, hydrogenation of a C=C bond, is often carried out biologically with a reduced pyridine nucleotide.) Dehydrogenation reactions of this type are important in the energy metabolism of aerobic cells. For example, the first oxidative step in the β oxidation of fatty acids (Fig. 17-1) is the α,β dehydrogenation of fatty acyl-CoA derivatives. The *pro-R* hydrogen atoms are removed from both the α- and β-carbon atoms to create the double bond (Table 15-2, type C).

## TABLE 15-2
## Some Dehydrogenation Reactions Catalyzed by Flavoproteins[a]

**A** D-Glucose $\xrightarrow[\text{Glucose oxidase}]{-2[H]}$ Gluconolactone $\xrightarrow{H_2O}$ D-Gluconic acid

(See Eq. 17-12 for structures in a closely related reaction.)

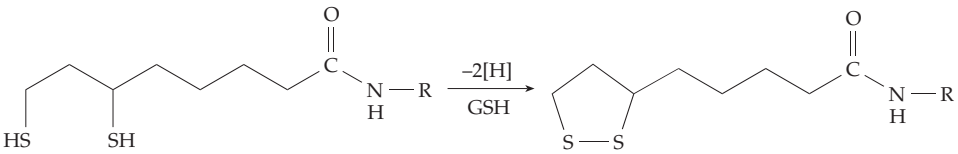
**B** $HOOC-CH_2-OH \xrightarrow[\text{Glycolate oxidase}]{-2[H]} HOOC-CH=O$

**C** $R-\underset{NH_2}{\overset{H}{C}}-COOH \xrightarrow{-2[H]} R-\underset{NH}{C}-COOH \xrightarrow{H_2O} R-\underset{O}{C}-COOH + NH_3$

Amino acid oxidases

**D** $R-CH_2-CH_2-\overset{O}{\overset{\|}{C}}-S-CoA \xrightarrow[\text{Acyl-CoA dehydrogenases}]{-2[H]} R-CH=CH-\overset{O}{\overset{\|}{C}}-S-CoA$

**E** NADH (or NADPH) + $H^+ \xrightarrow{-2[H]}$ $NAD^+$ ($NADP^+$)

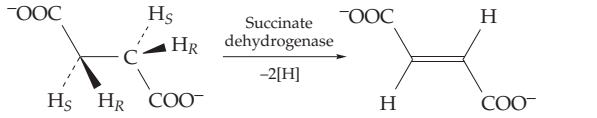
**F** 

Dihydrolipoic acid amide

[a] These are shown as removal of two H atoms [H] and may occur by transfer of $H^-$, $H^+ + e^-$, or $2H^+ + 2e^-$. They represent reaction type 9C of the table inside the back cover.


**Figure 15-10** The three-dimensional structure of glutathione reductase. Bound FAD is shown. $NAD^+$ binds to a separate domain below the FAD. The two cysteine residues forming the reducible disulfide loop are indicated by dots. From Thieme *et al.*[182]

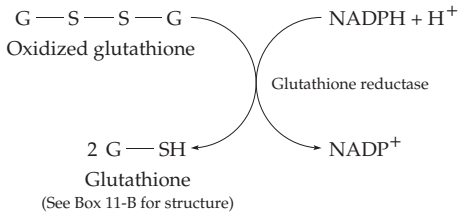
Animal mitochondria contain several different **acyl-CoA dehydrogenases** with differing preferences for chain length or branching pattern.[174–177] A related reaction that occurs in the citric acid cycle is dehydrogenation of succinate to fumarate by **succinate dehydrogenase** (Eq. 15-21).[177a] The dehydrogenation also involves *trans* removal of one of the two hydrogens, one *pro-S* hydrogen and one *pro-R*.[178] The enzyme has a large 621-residue flavoprotein subunit and a smaller 27-kDa iron–sulfur protein subunit.[179–181]




(15-21)

Neither the acyl-CoA dehydrogenases nor succinate dehydrogenase react with $O_2$. Acyl-CoA dehydrogenases pass the electrons removed from substrates to another flavoprotein, a soluble electron transferring flavoprotein (p. 794), which carries the electrons to an iron–sulfur protein embedded in the inner mitochondrial membrane where they enter the electron-transport chain. Succinate dehydrogenase as well as NADH dehydrogenase (Table 15-2, reaction E)[183] are embedded in the same membrane and also pass their electrons to iron–sulfur clusters and eventually to oxygen through the electron transport chain of the mitochondria (Chapter 18). **Fumarate reductase**[184] has properties similar to those of succinate dehydrogenase but catalyzes the opposite reaction in "anaerobic respiration" (Chapter 18),[184] as do similar reductases of bacteria[185] and of some eukaryotes.[186]

**Dihydrolipoyl dehydrogenase** (lipoamide dehydrogenase), **glutathione reductase**, and human **thioredoxin reductase**[187–190] belong to a subclass of flavoproteins that act on dithiols or disulfides. The reaction catalyzed by the first of these is illustrated in Table 15-2 (reaction type F). The other two enzymes usually promote the reverse type of reaction, the reduction of a disulfide to two SH groups by NADPH (Eq. 15-22). Glutathione reductase splits its substrate into two halves while reduction of the small 12-kDa protein **thioredoxin** (Box 15-C) simply opens a loop in its peptide chain. The reduction of lipoic acid opens the small disulfide-containing 5-membered ring in that molecule. Each of these flavoproteins also contains within its structure a reducible disulfide group that participates in catalysis.

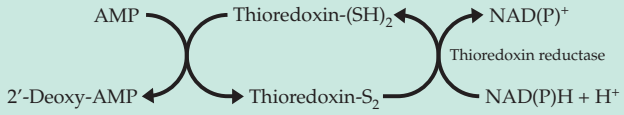



(15-22)

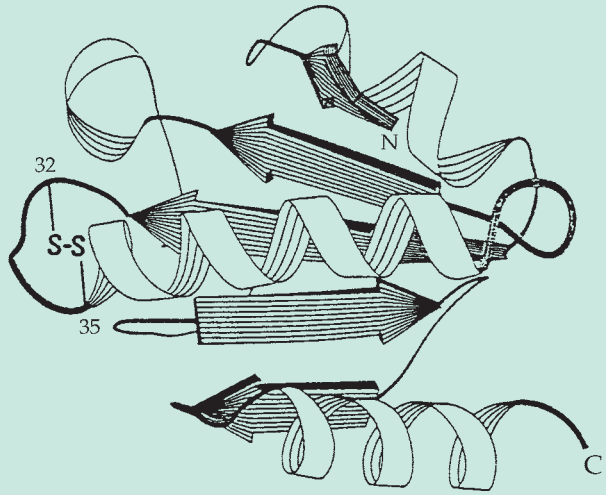
Each 50-kDa chain of the dimeric glutathione reductase is organized into three structural domains (Fig. 15-10).[191–193] Two of the domains each contain a nucleotide-binding motif resembling those of the $NAD^+$-dependent dehydrogenases. One of these domains binds NADPH and the other FAD. The latter domain also contains the reducible disulfide which is formed from Cys 58 and Cys 63. It serves as an intermediate hydrogen carrier which can in turn reduce oxidized glutathione. A **trypanothione reductase** from trypanosomes and related flagellated protozoa has a similar structure and acts on trypanothione, which replaces glutathione in these organisms.[188,194-196] Because it is unique to trypanosomes, this enzyme is a target for design of drugs against these organisms

## BOX 15-C THIOREDOXIN AND GLUTAREDOXIN

The small proteins thioredoxin and glutaredoxin are present in relatively high concentrations in bacteria, plants, and animals. For example, thioredoxin has a concentration of 15 μM in *E. coli*. Both proteins were discovered by their role as reducing agents in conversion of the ribonucleotides AMP, GMP, CMP, and UMP to the corresponding 2-deoxyribonucleotides which are needed for synthesis of DNA:[a,b]




The redox group in thioredoxin is a **disulfide loop** located on a protrusion at one end of the molecule. In the 108-residue *E. coli* thioredoxin, as well as in the 105-residue human thioredoxin,[c] it is formed by cysteines 32 and 35 which are present in the conserved sequence CGPC. The –SH groups of these two interacting cysteines have p$K_a$ values of ~6.9 and 7.5, the former belonging predominantly to the more exposed Cys 32.[d] The buried Asp 26 carboxyl group, which may be a proton donor to Cys 35 during reduction of the disulfide form,[e] forms a salt bridge with the Lys 57 $-NH_3^+$ and has a high p$K_a$ of ~7.4.[e–g]

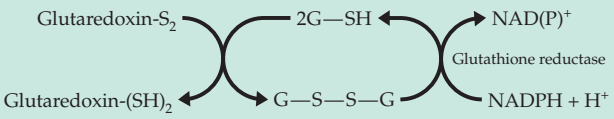



Oxidized *E. coli* thioredoxin. From Langsetmo *et al.*[f]

The p$K_a$ assignments, which have been controversial, are discussed in Chapter 7. This disulfide loop is reduced by NADPH through the action of the flavoprotein enzyme **thioredoxin reductase**. The resulting thiol pair of the reduced thioredoxin is the reductant used for ribonucleotide reductase (Chapter 16). The standard redox potential $E°'$ of *E. coli* thioredoxin is –0.27 V, appropriately low for coupling to the NADPH / $NADP^+$ system.

Reduced thioredoxin has a variety of functions.[h] It is the reductant for conversion of methionine sulfoxide to methionine in bacteria, for reduction of sulfate in yeast, and for additional specific enzymatic reactions.[b,h] However, its major function may be to reduce disulfide linkages in various proteins.[i] Several photosynthetic enzymes are activated by reduction of disulfide linkages via photosynthetically generated reduced ferredoxin and thioredoxin[j,k] (Chapter 23). Reduced thioredoxin may also play a similar role in nonphotosynthetic cells. It may reduce mixed disulfides such as those formed between glutathione and proteins (Box 11-B).[l–n] Thioredoxin may participate in regulation of the level of nitric oxide (NO) in tissues[m] and it is needed in the assembly of filamentous bacteriophages.[b,o] For reasons that are not clear, thioredoxin is also an essential subunit for a virus-induced DNA polymerase formed in *E. coli* following infection by bacteriophage T7 (see Chapter 27).

It was a surprise to discover that a mutant of *E. coli* lacking thioredoxin can still reduce ribonucleotides. In the mutant cells thioredoxin is replaced by glutaredoxin, whose active site disulfide linkage is reduced by glutathione rather than directly by NADPH. Oxidized glutathione is, in turn, reduced by NADPH and glutathione reductase. Thus, the end result is the same with respect to ribonucleotide reduction.




However, the two proteins have significantly different specificities and functions. The disulfide loop in glutaredoxin, whose eukaryotic forms are often called **thioltransferases**,[p] has the sequence CPYC. Although glutaredoxins are weaker reductants of mixed disulfides of proteins with glutathione than are thioredoxins,[q–s] they are more specific.

Both thioredoxin and glutaredoxin are members of a larger group of thiol:disulfide oxidoreductases which are found in all known organisms. In *E. coli* there are one thioredoxin, three different glutaredoxins,[h,t] and the periplasmic protein disulfide

**BOX 15-C (continued)**

isomerases DsbA and DsbC, which were discussed in Chapter 10.[u–w] Similar enzymes are found in the endoplasmic reticulum of eukaryotic cells.[u,x] Like thioredoxins, they contain disulfide loops which contain the sequences CGHC and CGYC as well as CPHC. Thioredoxin reductase itself also can keep cytoplasmic bacterial proteins reduced.[y] Redox potentials vary within this family of proteins:[n,s,z,aa]

| | $E^{\circ\prime}$, pH 7 |
|---|---|
| Thioredoxins | –.27 V |
| Glutaredoxins | –.20 to –.23 V |
| Protein disulfide isomerase | –.18 V |
| DsbA | –.09 to –.12 V |

These differences are correlated with differing functions. The intracellular reduced thioredoxins are thermodynamically the best reductants of disulfide linkages in proteins and they help keep intracellular proteins reduced. Glutaredoxin can be reduced efficiently by reduced glutathione or by NADPH and glutathione reductase and can, in turn, reduce cysteine and the oxidized form of vitamin C, **dehydroascorbic acid** (Box 18-D).[bb,cc] The periplasmic bacterial proteins DsbA and DsbC have the highest redox potentials and an unusually low first p$K_a$ for the dithiol pair in their thioredoxinlike domains.[aa] The basis for these properties has been hard to understand but is consistent with their role in assisting formation of disulfide bridges in extracellular proteins and with the role of the related protein disulfide isomerase in the ER.[u,dd–ff] These disulfide exchanges are nucleophilic displacement reactions (Chapter 12).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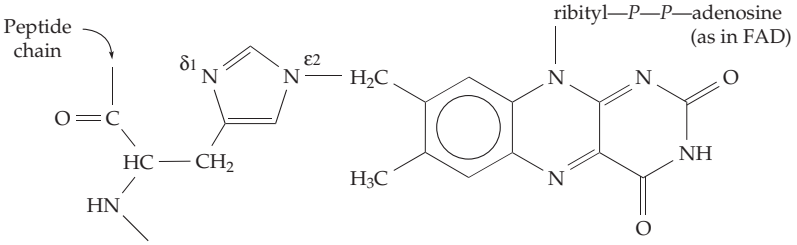
which cause such terrible diseases as African sleeping sickness and Chagas disease.[195,197]

Another flavoprotein constructed on the glutathione reductase pattern is the bacterial plasmid-encoded **mercuric reductase** which reduces the highly toxic $Hg^{2+}$ to volatile elemental mercury, $Hg^0$. A reducible disulfide loop corresponding to that in glutathione reductase is present in this enzyme but there is also a second pair of cysteines nearby. All of these may participate in binding and reduction of $Hg^{2+}$.[198–199a]

## 3. More Flavoproteins

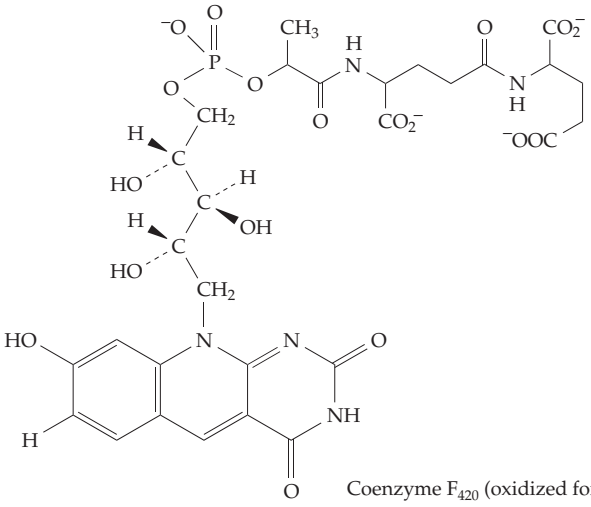
Flavoproteins function in virtually every area of metabolism and we have considered only a small fraction of the total number. Here are a few more. Flavin-dependent reductases use hydrogen atoms from NADH or NADPH to reduce many specific substances or classes of compounds. The FAD-containing ferredoxin: $NADP^+$ oxidoreductase catalyzes the reduction of free $NADP^+$ by reduced ferredoxin generated in the chloroplasts of green leaves.[200,201] Similar enzymes, some of which utilize reduced flavodoxins, are found in bacteria.[202,203] The FMN-containing subunit of NADH: ubiquinone oxidoreductase is an essential link in the mitochondrial electron transport chain for oxidation of NADH in plants and animals[183,204,205] and for related processes in bacteria. **Glutamate synthase**, a key enzyme in the nitrogen metabolism of plants and microorganisms, uses electrons from NADPH to reduce 2-oxoglutarate to glutamate in a complex glutamine-dependent process (see Fig. 24-5). The enzyme contains both FMN and FAD and three different iron–sulfur clusters.[205a] Flavin reductases use NADH or NADPH to reduce free riboflavin, FMN, or FAD needed for various purposes[206,206a] including emission of light by luminous bacteria.[207] They provide electrons to many enzymes that react with $O_2$ such as the cytochromes P450[208,209] and nitric oxide synthase (Chapter 18). An example is adrenodoxin reductase (see chapter banner, p. 764), which passes electrons from NADPH to cytochrome P450 via the small redox protein adrenodoxin. This system functions in steroid biosynthesis as is indicated in Fig. 22-7.[209a,b] Other flavin-dependent reductases have protective functions catalyzing the reduction of ascorbic acid radicals,[210,211] toxic quinones,[212–214] and peroxides.[215–218]




Covalently bound modified FAD of succinate dehydrogenase

has been isolated from a bacterial electron-transferring flavoprotein.[225] Commercial FAD may contain some riboflavin 5'-pyrophosphate which activates some flavoproteins and inhibits others.[226]

Methanogenic bacteria contain a series of unique coenzymes (Section F) among which is **coenzyme $F_{420}$**, a 5-deazaflavin substituted by H at position 7 and –OH at position 8 (8-hydroxy-7,8-didemethyl-5-deazariboflavin).[227,228]



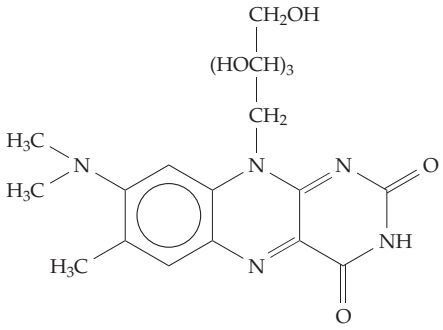
Coenzyme $F_{420}$ (oxidized form)

## 4. Modified Flavin Coenzymes

Mitochondrial succinate dehydrogenase, which catalyzes the reaction of Eq. 15-21, contains a flavin prosthetic group that is covalently attached to a histidine side chain. This modified FAD was isolated and identified as 8α-($N^{\varepsilon 2}$-histidyl)-FAD.[219] The same prosthetic group has also been found in several other dehydrogenases.[220] It was the first identified member of a series of modified FAD or riboflavin 5'-phosphate derivatives that are attached by covalent bonds to the active sites of more than 20 different enzymes.[219]

These include 8α-($N^{\varepsilon 2}$-histidyl)-FMN,[221] 8α-($N^{\delta 1}$-histidyl)-FAD,[222] 8α-(*O*-tyrosyl-FAD),[223] and 6-(*S*-cysteinyl)-riboflavin 5'-phosphate, which is found in trimethylamine dehydrogenase (Fig. 15-9).[224] An 8-hydroxy analog of FAD (–OH in place of the 8-$CH_3$)

This unique redox catalyst links the oxidation of $H_2$ or of formate to the reduction of $NADP^+$[229] and also serves as the reductant in the final step of methane biosynthesis (see Section E).[228] It resembles $NAD^+$ in having a redox potential of about –0.345 volts and the tendency to be only a two-electron donor. More recently free 8-hydroxy-7,8-didemethyl-5-deazariboflavin has been identified as an essential light-absorbing chromophore in DNA photolyase of *Methanobacterium*, other bacteria, and eukaryotic algae.[230] **Roseoflavin** is not a coenzyme but an antibiotic from *Streptomyces davawensis*.[231] Many synthetic flavins have been used in studies of mechanisms and for NMR[232] and other forms of spectroscopy.

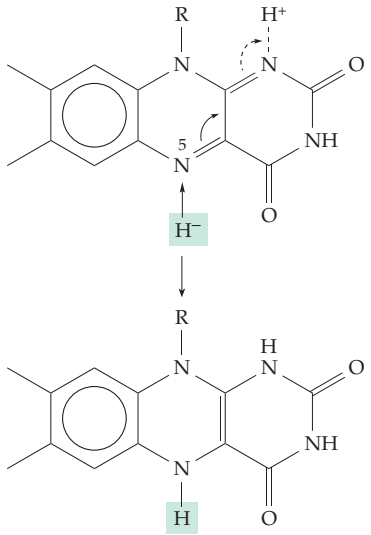


Roseoflavin

## 5. Mechanisms of Flavin Dehydrogenase Action

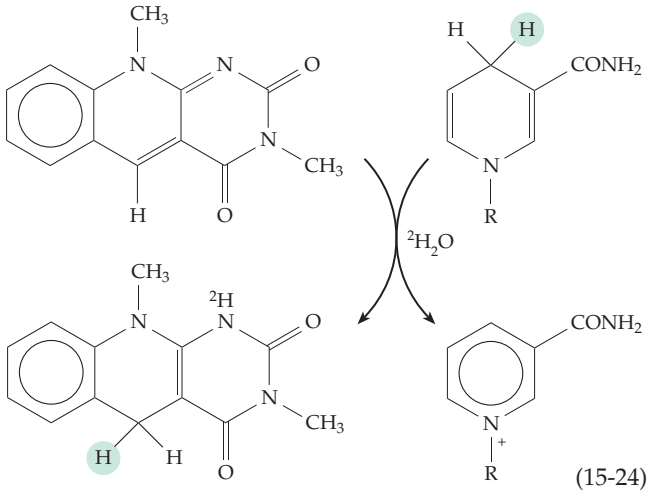
The chemistry of flavins is complex, a fact that is reflected in the uncertainity that has accompanied efforts to understand mechanisms. For flavoproteins at least four mechanistic possibilities must be considered.[153a,233] (a) A reasonable **hydride-transfer** mechanism can be written for flavoprotein dehydrogenases (Eq. 15-23). The hydride ion is donated at N-5 and a proton is accepted at N-1. The oxidation of alcohols, amines, ketones, and reduced pyridine nucleotides can all be visualized in this way. Support for such a mechanism came from study of the nonenzymatic oxidation of NADH by flavins, a reaction that occurs at moderate speed in water at room temperature. A variety of flavins and dihydropyridine derivatives have been studied, and the electronic effects observed for the reaction are compatible with the hydride ion mechanism.[234–236]

According to the mechanism of Eq. 15-23, a hydride ion is transferred directly from a carbon atom in a substrate to the flavin. However, a labeled hydrogen atom transferred to N-5 or N-1 would immediately exchange with the medium, rapid exchange being characteristic of hydrogens attached to nitrogen.



(15-23)

To avoid this problem, Brustlein and Bruice used a **5-deazaflavin** to oxidize NADH nonenzymatically.[237] When this reaction was carried out in $^2H_2O$, no $^2H$ entered the product at C-5, indicating that a hydrogen atom (circled in Eq. 15-24) had been transferred directly from NADH to the C-5 position. Similar direct transfer of hydrogen to C-5 of 5-deazariboflavin 5'-phosphate is catalyzed by flavoproteins such as *N*-methylglutamate synthase[238] and acyl-CoA dehydrogenase.[237–239]



(15-24)

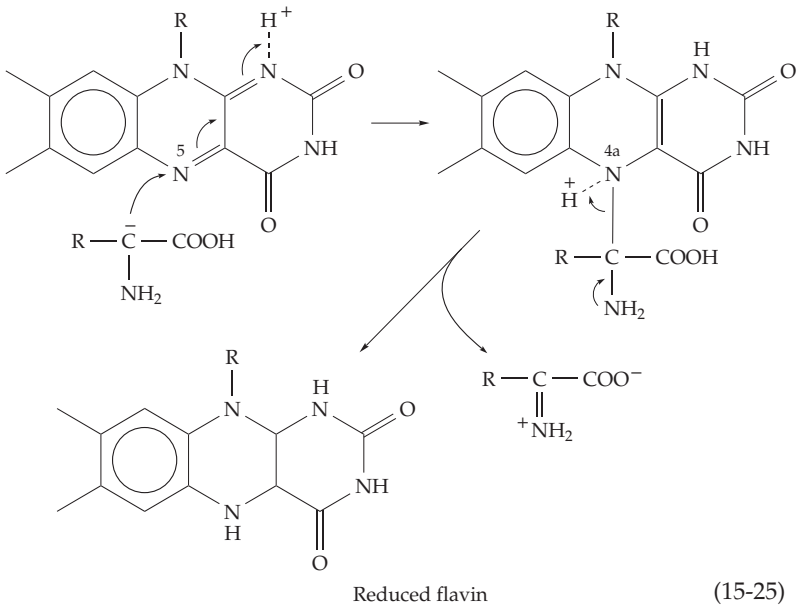
However, these experiments may not have established a mechanism for natural flavoprotein catalysis because the properties of 5-deazaflavins resemble those of $NAD^+$ more than of flavins.[239] Their oxidation–reduction potentials are low, they do not form stable free radicals, and their reduced forms don't react readily with $O_2$. Nevertheless, for an acyl-CoA dehydrogenase the rate of reaction of the deazaflavin is almost as fast as that of natural FAD.[238] For these enzymes a hydride ion transfer from the β CH (reaction type D of Table 15-1) is made easy by removal of the α-H of the acyl-CoA to form an enolate anion intermediate.

The three-dimensional structure of the medium chain acyl-CoA dehydrogenases with bound substrates and inhibitors is known.[174,175,240] A conserved glutamate side chain is positioned to pull the *pro-R* proton from the α carbon to create the initial enolate anion.[174,175,241] The *pro-R* β C–H lies by N-5 of the flavin ring seemingly ready to donate a hydride ion as in Eq. 15-23. NMR spectroscopy has been carried out with $^{13}C$ or $^{15}N$ in each of the atoms of the redox active part of the FAD. The results show directly the effects of strong hydrogen bonding to the protein at N-1, N-3, and N-5 and also suggest that the bound $FADH_2$ is really $FADH^-$ with the negative charge localized on N-1 by strong hydrogen bonding.[149] Many mutants have been made,[242] substrate analogs have been tested,[176,243] kinetic isotope effects have been measured,[242a] and potentiometric titrations have been done.[243] All of the results are compatible with the enolate anion hydride-transfer

mechanism. Questions about the acidity of the α-H and the mechanism of its removal to form the enolate anion[242a] are similar to those discussed in Chapter 13, Section B.

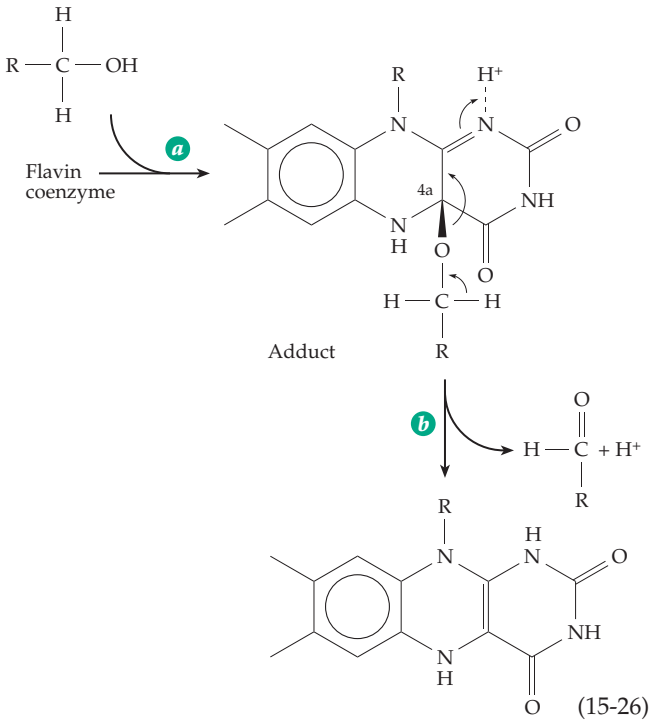
A peculiarity of several acyl-CoA dehydrogenases is a bright green color with an absorption maximum at 710 nm. This was found to result from tightly bound coenzyme A persulfide (CoA–S–S$^-$).[244,245]

(b) A second possible mechanism of flavin reduction is suggested by the occurrence of addition reactions involving the isoalloxazine ring of flavins. Sulfite adds to flavins by forming an N–S bond at the 5 position and nitroethane, which is readily dissociated to the carbanion $H_3C-CH^- - NO_2$, acts as a substrate for D-amino acid oxidase.[246,247] This fact suggested a **carbanion mechanism** according to which normal D-amino acid substrates form carbanions by dissociation of the αH (Eq. 15-25). Ionization would be facilitated by binding of the substrate carboxylate to an adjacent arginine side chain and the carbanion could react at N-5 of the flavin as in Eq. 15-25. Similar mechanisms have been suggested for other flavin enzymes.[248,249]




(15-25)

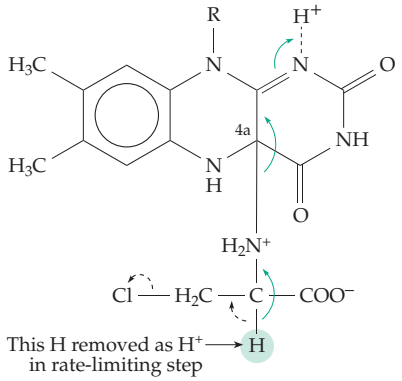
(c) The adducts with nitroethane and other compounds[250] pointed to reaction at N-5, but Hamilton[251] suggested that a better position for addition of nucleophiles is carbon 4a, which together with N-5 forms a cyclic Schiff base. He argued that other electrophilic centers in the flavin molecule, such as carbons 2, 4, and 10a, would be unreactive because of their involvement in amide or amidine-type resonance but an amine, alcohol, or other substrate could add to a flavin at position 4a (Eq. 15-26, step *a*). Cleavage of the newly formed C–O bond could then occur by movement of electrons from the alcohol part of the adduct into the flavin as indicated in step *b*. The products of this




(15-26)

**4a adduct mechanism** are the reduced flavin and an aldehyde, the same as would be obtained by the hydride ion mechanism. However, in Eq. 15-26, both hydrogens in the original substrate (that on oxygen and that on carbon) have dissociated as protons, the electrons having moved as a pair during the cleavage of the adduct. Hamilton argued that an isolated hydride ion has a large diameter while a proton is small and mobile; for this reason dehydrogenation may often take place by proton transfer mechanisms.

Experimental support for the mechanism of Eq. 15-26 has been obtained using D-chloroalanine as a substrate for D-amino acid oxidase.[252–254] Chloropyruvate is the expected product, but under anaerobic conditions pyruvate was formed. Kinetic data obtained with α-$^2$H and α-$^3$H substrates suggested a common intermediate for formation of both pyruvate and chloro-pyruvate. This intermediate could be an anion formed by loss of H$^+$ either from alanine or from a C-4a adduct. The anion could eliminate chloride ion as indicated by the dashed arrows in the following structure. This would lead to formation of pyruvate without reduction of the flavin. Alternatively, the electrons from the carbanion could flow into the flavin (green arrows), reducing it as in Eq. 15-26. A similar mechanism has been suggested for other flavoenzymes.[249,255] Objections to the carbanion mechanism are the expected




very high p$K_a$ for loss of the α-H to form the carbanion[256] and the observed formation of only chloroalanine and no pyruvate in the reverse reaction of chloropyruvate, ammonia, and reduced flavoprotein.

A long-known characteristic of D-amino acid oxidase is its tendency to form charge-transfer complexes with amines, complexes in which a nonbonding electron has been transferred partially to the flavin. Complete electron transfer would yield a flavin radical and a substrate radical which could be intermediates in a **free radical mechanism**, as discussed in the next section.[256]

The three-dimensional structure of the complex of D-amino acid oxidase with the substrate analog benzoate has been determined. The carboxyl group of the inhibitor is bound by an arginine side chain (Fig. 15-11) that probably also holds the amino acid substrate. There is no basic group nearby in the enzyme that could serve to remove the α-H atom in Eq. 15-26 but the position is appropriate for a direct transfer of the hydrogen to the flavin as a hydride ion as in Eq. 15-23.[161,162,257] In spite of all arguments to the contrary the hydride ion mechanism could be correct! However, an adduct mechanism is still possible.

Experimental evidence supports a 4a adduct mechanism for glutathione reductase and related enzymes[191,258,258a] (Fig. 15-12). In this figure the reaction sequence is opposite to that in Eq. 15-26. The enzyme presumably functions as follows. NADPH binds next to the bound FAD and reduces it by transfer of the 4-*pro*-*S* hydrogen of the NADPH (Fig. 15-12, step *a*). The sulfur atom of Cys 63 is in van der Waals contact with the bound FAD at or near carbon atom 4a. In step *b* the nucleophilic center on atom C-4a of $FADH_2$ attacks a sulfur atom of the disulfide loop between cysteines 58 and 63 in the protein to create a C-4a adduct of a thiolate ion with oxidized FAD and to cleave the –S–S– linkage in the loop. In step *c* the thiol group of cysteine 63 is eliminated, after which the thiol of Cys 58 attacks the nearer sulfur atom of the oxidized glutathione in a nucleophilic displacement (step *d*) to give one reduced glutathione (GSH) and a mixed disulfide of glutathione and the enzyme (G-S-S-Cys 58). The thiolate anion of Cys 63, which is stabilized by interaction with the adjacent flavin ring, then attacks this disulfide (step *e*) to regenerate the internal disulfide and to free the second molecule of reduced glutathione. The imidazole group of the nearby His 467 of the second subunit presumably participates in catalysis as may some other side chains.[191] The disulfide exchange reactions are similar to those discussed in Chapter 12.

A variation is observed for *E. coli* thioredoxin reductase. The reducible disulfide and the NADPH binding site are both on the same side of the flavin rather than on opposite sides as in Fig. 15-12.[190,259] Mercuric reductase also uses NADPH as the reductant transferring the 4*S* hydrogen. The $Hg^{2+}$ presumably binds to a sulfur atom of the reduced disulfide loop and there undergoes reduction. The observed geometry of the active site is correct for this mechanism.

## 6. Half-Reduced Flavins

A possible mechanism of flavin dehydrogenation consists of consecutive transfer of a hydrogen atom and of an electron with intermediate radicals being formed both on the flavin and on the substrate. Such

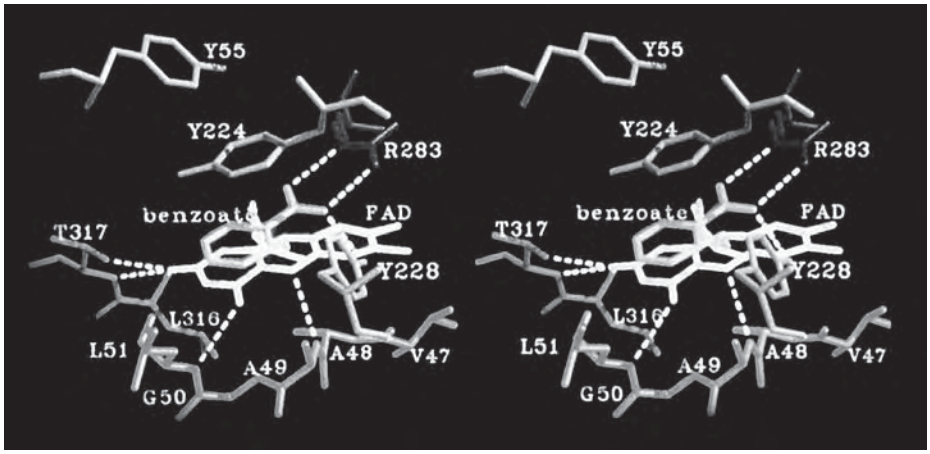



**Figure 15-11** Stereoscopic view of the benzoate ion in its complex with D-amino acid oxidase. A pair of hydrogen bonds binds the carboxylate of the ligand to the guanidinium group of R283. Several hydrogen bonds to the flavin ring of the FAD are also indicated. Courtesy of Retsu Miura.[161]

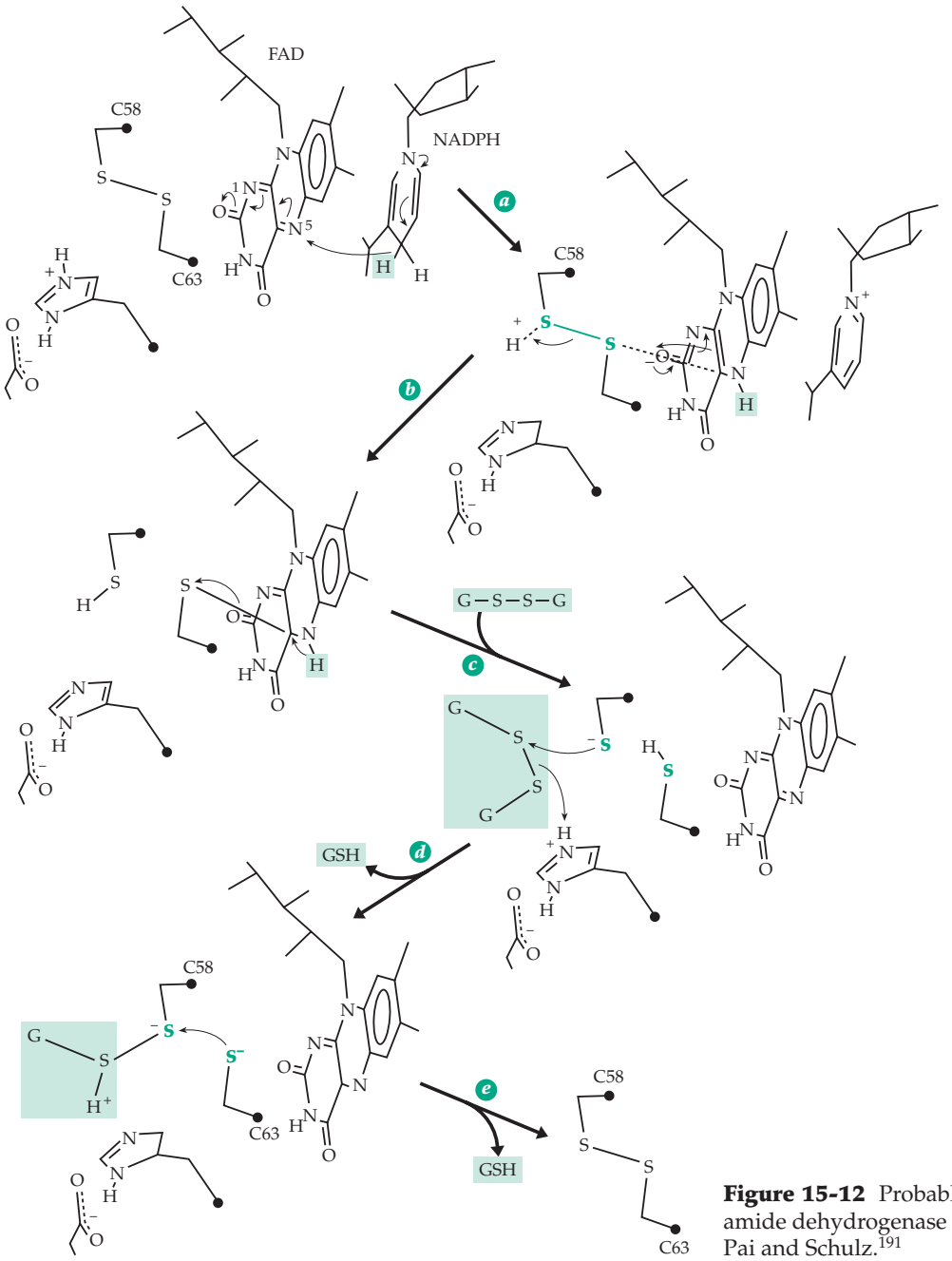


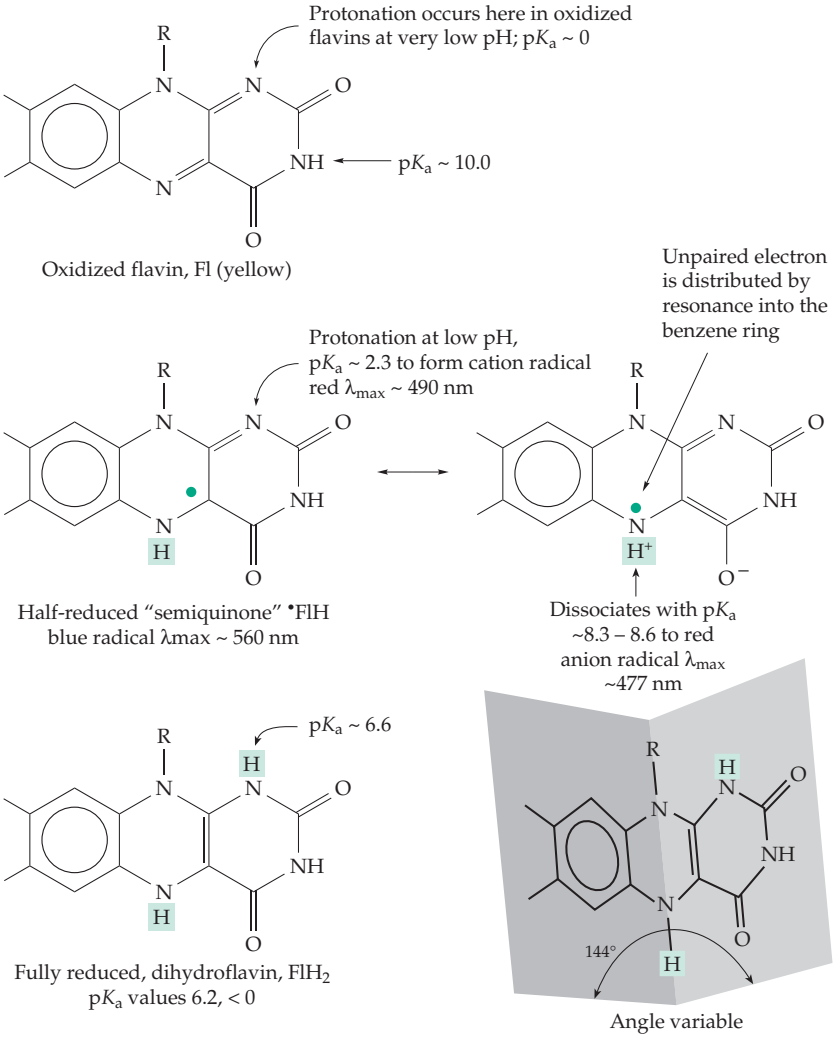

**Figure 15-12** Probable reaction mechanism for lipoamide dehydrogenase and glutathione reductase. See Pai and Schulz.[191]

a mechanism takes full advantage of one of the most characteristic properties of flavins, their ability to accept single electrons to form **semiquinone** radicals. If the oxidized form Fl of a flavin is mixed with the reduced form $FlH_2$, a single hydrogen atom is transferred from $FlH_2$ to Fl to form two $^{\bullet}$F1H radicals (Eq. 15-27).

$$Fl + FlH_2 \underset{}{\overset{K_f}{\rightleftharpoons}} 2\ {}^{\bullet}FlH \qquad (15\text{-}27)$$

The equilibrium represented by this equation is independent of pH, but because all three forms of the flavin have different p$K_a$ values (Fig. 15-13) the apparent equilibrium constants relating total concentrations of oxidized, reduced, and radical forms vary with pH.[143,260–262] The fraction of radicals present is greater at low pH and at high pH than at neutrality. For a 3-alkylated flavin the formation constant $K_f$ has been estimated as $2.3 \times 10^{-2}$ and for riboflavin[260] as $1.5 \times 10^{-2}$. From these values and the p$K_a$ values in Fig. 15-13, it is possible to estimate the amount of radical present at any pH.

Neutral flavin radicals have a blue color (the wavelength of the absorption maximum, $\lambda_{max}$, is ~560 nm) but either protonation at N-1 or dissociation of a proton from N-5 leads to red cation or anion radicals with $\lambda_{max}$ at ~477 nm. Both blue and red radicals are



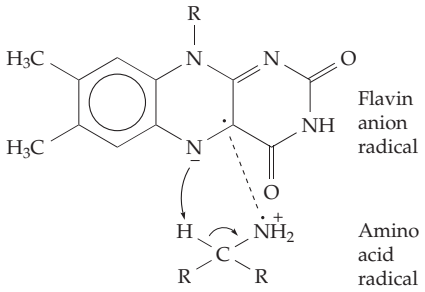




**Figure 15-13** Properties of oxidized, half-reduced, and fully reduced flavins. See Müller *et al.*[263,264]

observed in enzymes, with some enzymes favoring one and some the other. Hemmerich suggested that enzymes forming red radicals make a strong hydrogen bond to the proton in the 5 position of the flavin. This increases the basicity of N-1 leading to its protonation and formation of the red cation radicals.

It is possible that many flavoprotein oxygenases and dehydrogenases react via free radicals. For example, instead of the mechanism of Eq. 15-26, an electron could be transferred to the flavin, leaving a radical pair (at right). The crystallographic structure and modeling of the substrate complex support this possibility.[265,265a] In this pair the flavin radical would be more basic than in the fully oxidized form and the amino acid radical would be more acidic than in the uncharged form. A proton transfer as indicated together with coupling of the radical pair would yield the same product as the mechanism of Eq. 15-26.

Another alternative to a radical pair is a hydrogen atom transfer followed by a second electron transfer.[265]

If an enzyme binds a flavin radical much more tightly than the fully oxidized or reduced forms, reduction of the flavoprotein will take place in two one-electron steps. In such proteins the values of $E°'$ for the two steps may be widely separated. The best known examples are the small, low-potential electron-carrying proteins known as **flavodoxins**.[266–269a] These proteins, which carry electrons between pairs of other redox proteins, have a variety of functions in anaerobic and photosynthetic bacteria, cyanobacteria, and green algae. Their functions are similar to those of the **ferredoxins**, iron–sulfur proteins that are considered in Chapter 16. In some bacteria ferredoxin and flavodoxin are interchangeable and the synthesis of flavodoxin is induced if the bacteria become deficient in iron. Flavodoxins all contain riboflavin monophosphate, which functions by cycling between the fully reduced anionic form and a blue semiquinone radical.[270] The two reduction steps, from oxidized flavin to semiquinone and from semiquinone to dihydroflavin, are well separated. For example, the values of $E°'$ (pH 7) for the flavodoxin from *Megasphaera elsdenii* are −0.115 and −0.373 V, while those of the *Azotobacter vinlandii* flavodoxin (azotoflavin) are + 0.050 and – 0.495 V. The latter is the lowest known for any flavoprotein.

Flavodoxins are small proteins with an α/β structure resembling that of the nucleotide binding domain of dehydrogenases. According to $^{31}P$ NMR data, the phosphate group of the coenzyme bound to flavodoxin is completely ionized,[271] even though it is deeply buried in the protein and is not bound to any positively charged side chain but to the N terminus of an α helix and to four –OH groups of serine and threonine side

chains. The flavin ring is partially buried near the surface of the 138-residue protein. An aromatic side chain, from tryptophan or tyrosine, lies against the flavin on the outside of the molecule. Flavodoxins can be crystallized in all three forms: oxidized, semiquinone, and fully reduced. In the crystals the flavin semiquinone, like the oxidized flavin, is nearly planar.

The **DNA photolyase** of *E. coli*, an enzyme that participates in the photochemical repair of damaged DNA (Chapter 23), contains a blue neutral FAD radical with a 580-nm absorption band and an appropriate ESR signal.[230,272] In contrast, the mitochondrial **electron-transferring flavoprotein** (ETF), a 57-kDa αβ dimer containing one molecule of FAD, functions as a single electron carrier cycling between oxidized FAD and the red anionic semiquinone.[273,274] The reduced forms of the acyl-CoA dehydrogenases transfer their electrons one at a time from their FAD to the FAD of two molecules of electron-transferring flavoprotein. Therefore, an intermediate enzyme-bound radical must be present in the FAD of acyl-CoA dehydrogenase at one stage of its catalytic cycle. A related ETF from a methylotrophic bacterium accepts single electrons from reduced trimethylamine dehydrogenase (Fig. 15-9).[275]

Another flavoprotein that makes use of both one- and two-electron transfer reactions is **ferredoxin-NADP⁺ oxidoreductase** (Eq. 15-28). Its bound FAD accepts electrons one at a time from each of the two

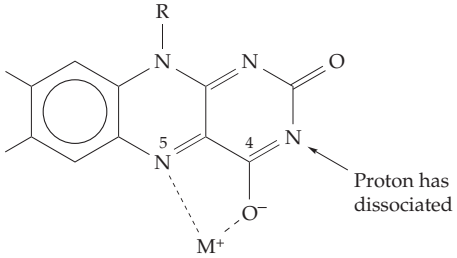
$$2Fd_{red} + NADP^+ + H^+ \rightarrow 2\,Fd_{ox}{}^+ + NADPH \qquad (15\text{-}28)$$

reduced ferredoxins ($Fd_{red}$) in chloroplasts and then presumably transfers a hydride ion to the $NADP^+$. The enzyme is organized into two structural domains,[201] one of which binds FAD and the other $NADP^+$. Similar single-electron transfers through flavoproteins also occur in many other enzymes. Chorismate mutase, an important enzyme in biosynthesis of aromatic rings (Chapter 25), contains bound FMN. Its function is unclear but involves formation of a neutral flavin radical.[276,277]

## 7. Metal Complexes of Flavins and Metalloflavoproteins

The presence of metal ions in many flavoproteins suggested a direct association of metal ions and flavins. Although oxidized flavins do not readily bind most metal ions, they form red complexes with $Ag^+$ and $Cu^+$ with a loss of a proton from N-3.[278] Flavin semiquinone radicals also form strong red complexes with many metals.[264] If the complexed metal ion can exist in more than one oxidation state, electron transfer between the flavin and a substrate could take place through the metal atom. However, *chelation by flavins in nature has not been observed*. Metalloflavoproteins probably function by having the metal centers close enough to the flavin for electron transfer to occur but not in direct contact. This is the case for a bacterial trimethylamine dehydrogenase in which the FeS cluster is bound about 0.4 nm from the alloxazine ring of riboflavin 5'-phosphate as shown in Fig. 15-9.

Some metalloflavoproteins contain heme groups. The previously mentioned **flavocytochrome $b_2$** of yeast is a 230-kDa tetramer, one domain of which carries riboflavin phosphate and another heme. A flavocytochrome from the photosynthetic sulfur bacterium *Chromatium* (cytochrome *c*-552)[279] is a complex of a 21-kDa cytochrome *c* and a 46-kDa flavoprotein containing 8α-(*S*-cysteinyl)-FAD. The 670-kDa **sulfite reductase** of *E. coli* has an $\alpha_8\beta_4$ subunit structure. The eight α chains bind four molecules of FAD and four of riboflavin phosphate, while the β chains bind three or four molecules of **siroheme** (Fig. 16-6) and also contain $Fe_4S_4$ clusters.[280,281] Many nitrate and some nitrite reductases are flavoproteins which also contain Mo or




Fe prosthetic groups.[282,283] A group of **aldehyde oxidases** and **xanthine dehydrogenases** also contain molybdenum as well as iron (Chapter 16). In every case the metal ions are bound independently of the flavin.[283a]

## 8. Reactions of Reduced Flavins with Oxygen

Free dihydroriboflavin reacts nonenzymatically in seconds and reduced flavin oxygenases react even faster with molecular oxygen to form hydrogen peroxide (Eq. 15-29).

$$FlH_2 + O_2 \rightarrow Fl + H_2O_2 \qquad (15\text{-}29)$$

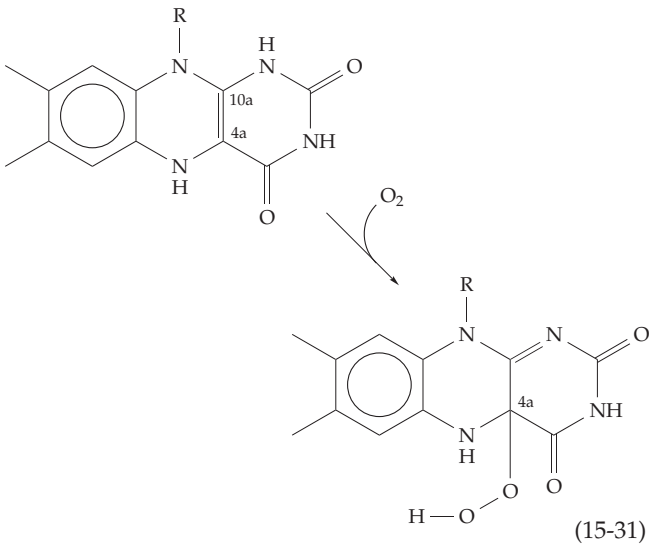
The reaction is more complex than it appears. As soon as a small amount of oxidized flavin is formed, it reacts with reduced flavin to generate flavin radicals $^\bullet$FlH (Eq. 15-27). The latter react rapidly with $O_2$ each donating an electron to form superoxide anion radicals $^\bullet O_2^-$ (Eq. 15-30a) which can then combine with flavin radicals (Eq. 15-30b).[284]

$$^\bullet FlH + O_2 \rightarrow {}^\bullet O_2^- + H^+ + Fl \qquad (15\text{-}30a)$$

$$^\bullet O_2^- + {}^\bullet FlH + H^+ \rightarrow Fl + H_2O_2 \qquad (15\text{-}30b)$$

During the corresponding reactions of reduced flavoproteins with $O_2$, intermediates have been detected. For example, spectrophotometric studies of the FAD-containing bacterial *p*-hydroxybenzoate hydroxylase (Chapter 18) revealed the consecutive appearance of three intermediate forms.[285–287] The first, whose absorption maximum is at 380–390 nm, is thought to be an adduct at position 4a (Eq. 15-31). That such a 4a peroxide really forms with the riboflavin phosphate of the light-emitting bacterial **luciferase** (Chapter 23) was demonstrated using coenzyme enriched with $^{13}C$ at position 4a.[147] A large shift to lower frequency (from 104 to 83 ppm) accompanied formation of the transient adduct. Comparison with reference compounds showed that this change agreed with that predicted.

Other structures for $O_2$ adducts have also been considered, as has the possibility of rearrangements among these structures.[288] Nevertheless, the products observed from many different flavoprotein reactions can be explained on the basis of a 4a peroxide.[289]



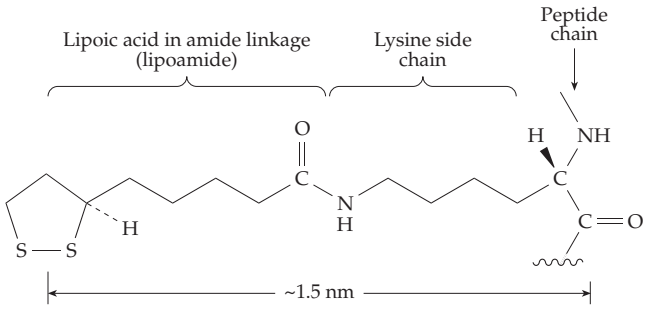

(15-31)

Formation of $H_2O_2$ by flavin oxidases can occur via elimination of a peroxide anion $HOO^-$ from the adduct of Eq. 15-31 with regeneration of the oxidized flavin. In the active site of a hydroxylase, an OH group can be transferred from the peroxide to a suitable substrate (Eq. 18-42). Although radical mechanisms are likely to be involved, such hydroxylation reactions can also be viewed as transfer of $OH^+$ to the substrate together with protonation on the inner oxygen atom of the original peroxide to give a 4a –OH adduct. The latter is a covalent hydrate which can be converted to the oxidized flavin by elimination of $H_2O$. This hydrate is believed to be the third spectral intermediate identified during the action of *p*-hydroxybenzoate hydroxylase.[286,287,290]

## C. Lipoic Acid and the Oxidative Decarboxylation of α-Oxoacids

The isolation of lipoic acid in 1951 followed an earlier discovery that the ciliate protozoan *Tetrahymena geleii* required an unknown factor for growth. In independent experiments acetic acid was observed to promote rapid growth of *Lactobacillus casei*, but it could be replaced by an unknown "acetate replacing factor." Another lactic acid bacterium *Streptococcus faecalis* was unable to oxidize pyruvate without addition of "pyruvate oxidation factor." By 1949, all three unknown substances were recognized as identical.[291,291a] After working up the equivalent of 10 tons of water-soluble residue from liver, Lester Reed and his collaborators isolated 30 mg of a fat-soluble acidic material which was named **lipoic acid** (or 6-thioctic acid).[292–294]

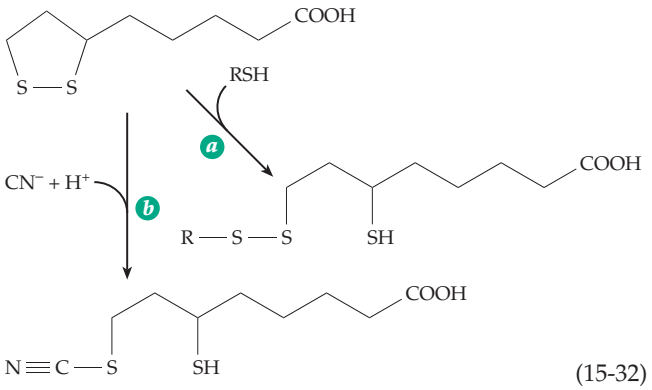
While *Tetrahymena* must have lipoic acid in its diet, we humans can make our own, and it is not considered a vitamin. Lipoic acid is present in tissues in extraordinarily small amounts. Its major function is to participate in the oxidative decarboxylation of α-oxoacids but it also plays an essential role in glycine catabolism in the human body as well as in plants.[295,296] The structure is simple, and the functional group is clearly the cyclic disulfide which swings on the end of a long arm. Like biotin, which is also present in tissues in very small amounts, lipoic acid is bound in covalent amide linkage to lysine side chains in active sites of enzymes:[296a]




### 1. Chemical Reactions of Lipoic Acid

The most striking chemical property of lipoic acid is the presence of ring strain of ~17–25 kJ $mol^{-1}$ in the cyclic disulfide. Because of this, thiol groups and cyanide ions react readily with oxidized lipoic acid to give mixed disulfides (Eq. 15-32a) and isothiocyanates (Eq. 15-32b), respectively.

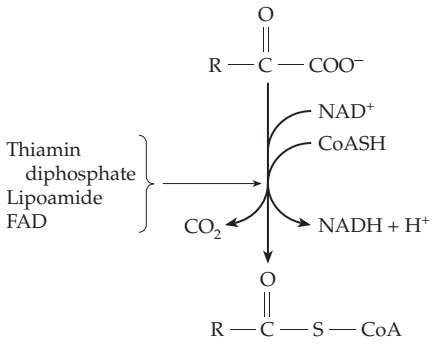
Another result of the ring strain is that the reduction potential $E^{\circ\prime}$ (pH 7, 25°C), is –0.30 V, almost the same as that of reduced NAD (–0.32V). Thus, reoxidation of reduced lipoic acid amide by $NAD^+$ is thermodynamically feasible. Yet another property attributed to the ring strain in lipoic acid is the presence of an absorption maximum at 333 nm.




(15-32)

## 2. Oxidative Decarboxylation of Alpha-Oxoacids

The oxidative cleavage of an α-oxoacid is a major step in the metabolism of carbohydrates and of amino acids and is also a step in the citric acid cycle. In many bacteria and in eukaryotes the process depends upon both thiamin diphosphate and lipoic acid. The oxoacid anion is cleaved to form $CO_2$ and the remaining acyl group is combined with coenzyme A (Eq. 15-33). $NAD^+$ serves as the oxidant. The reaction is catalyzed by a complex of enzymes whose molecular mass varies from ~4 to 10 x $10^6$, depending on the species and exact substrate.[297] Separate oxoacid dehydrogenase systems are known for pyruvate,[298–300] 2-oxoglutarate,[301] and the 2-oxoacids with branched side chains derived metabolically from leucine, isoleucine, and




(15-33)

valine.[302,302a] In eukaryotes these enzymes are located in the mitochondria. The **pyruvate** and **2-oxoglutarate dehydrogenase** complexes of *E. coli* and *Azotobacter vinelandii* have been studied most. In both cases there are three major protein components. The first ($E_1$) is a **decarboxylase** (also referred to as a dehydrogenase) for which the thiamin diphosphate is the dissociable cofactor. The second ($E_2$) is a lipoic acid amide-containing "core" enzyme which is a **dihydrolipoyl transacylase.** The third ($E_3$) is the flavoprotein **dihydrolipoyl dehydrogenase**, a member of the glutathione reductase family with a three-dimensional structure and catalytic mechanism similar to those of glutathione reductase (Figs. 15-10, 15-12).[303 – 305]

Electron microscopy of the core dihydrolipoyl transacylase from *E. coli* reveals a striking octahedral symmetry which has been confirmed by X-ray diffraction.[306–307a] The core from pyruvate dehydrogenase has a mass of ~2390 kDa and contains 24 identical 99.5-kDa $E_2$ subunits. The 2-oxoglutarate dehydrogenase from *E. coli* has a similar but slightly less symmetric structure. Each core subunit is composed of three domains. A lipoyl group is bound in amide linkage to lysine 42 and protrudes from one end of the domain. A second domain is necessary for binding to subunits $E_1$ and $E_3$, while the third major 250-residue domain contains the catalytic acyltransferase center.[308,309] This center closely resembles that of chloramphenicol acetyltransferase (Chapter 12).[310,311] The lipoyl[301,309,312] and catalytic[307] domains of the dihydrolipoyl succinyltransferase from 2-oxoglutarate dehydrogenase resemble those of pyruvate dehydrogenase and also of the branched chain oxoacid dehydrogenase. The three domains of the proteins are joined by long 25- to 30-residue segments rich in alanine, proline, and ionized hydrophilic side chains.[309] This presumably provides flexibility for the lipoyl groups which must move from site to site. The presence of unexpectedly sharp lines in the proton NMR spectrum of the core protein may be a result of this flexibility.[309]

To obtain the X-ray structure of the core protein it was necessary to delete the lipoyl- and $E_1(E_2)$- binding domains. The resulting 24-subunit structure is shown in Fig 15-14A,B.[306] It has been assumed for many years that 12 of the dimeric decarboxylase units ($E_1$) are bound to the 12 edges of the transacetylase cube, while six (50.6 x 2 kDa) flavoprotein ($E_3$) dimers bind on the six faces of the cube. The active centers of all three types of subunits are thought to come close together in the regions where the subunits touch, permitting the sequence of catalytic reactions indicated in Fig. 15-15 to take place. Eukaryotic as well as some bacterial pyruvate decarboxylases have a core of 60 subunits in an icosahedral array with 532 symmetry. This can be seen in the image reconstructions of the enzyme from *Saccharomyces cerevisiae* shown in Fig. 15-14C and D. A surprising discovery is that in this yeast enzyme the $E_3$ units are not on the outside of the 5-fold symmetric faces but protrude into the inner cavity.[299] Each of the 12 $E_3$ subunits is assisted in binding correctly to the $E_2$ core by a molecule of the 47-kDa **$E_3$-binding protein** (BP), also known as protein X.[313,314] Absence of this protein is associated with congenital lactic acidosis.

The unique function of lipoic acid is in the oxidation of the thiamin-bound active aldehyde (Fig. 15-15) in such a way that when the complex with thiamin breaks up, the acyl group formed by the oxidative decarboxylation of the oxoacid is attached to the

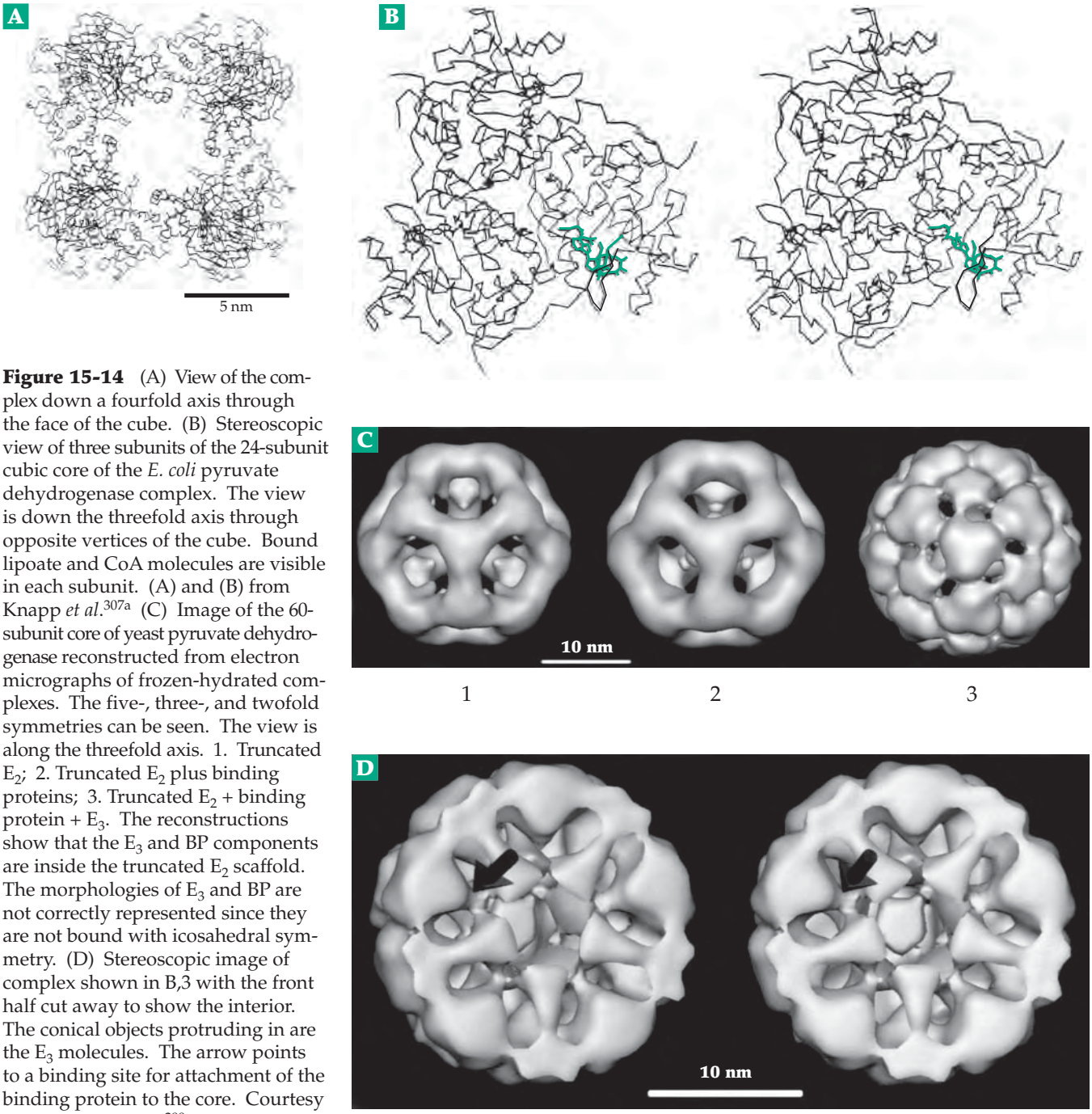



**Figure 15-14** (A) View of the complex down a fourfold axis through the face of the cube. (B) Stereoscopic view of three subunits of the 24-subunit cubic core of the *E. coli* pyruvate dehydrogenase complex. The view is down the threefold axis through opposite vertices of the cube. Bound lipoate and CoA molecules are visible in each subunit. (A) and (B) from Knapp *et al*.[307a] (C) Image of the 60-subunit core of yeast pyruvate dehydrogenase reconstructed from electron micrographs of frozen-hydrated complexes. The five-, three-, and twofold symmetries can be seen. The view is along the threefold axis. 1. Truncated $E_2$; 2. Truncated $E_2$ plus binding proteins; 3. Truncated $E_2$ + binding protein + $E_3$. The reconstructions show that the $E_3$ and BP components are inside the truncated $E_2$ scaffold. The morphologies of $E_3$ and BP are not correctly represented since they are not bound with icosahedral symmetry. (D) Stereoscopic image of complex shown in B,3 with the front half cut away to show the interior. The conical objects protruding in are the $E_3$ molecules. The arrow points to a binding site for attachment of the binding protein to the core. Courtesy of J. K. Stoops *et al*.[299]

dihydrolipoyl group at the S-8 position.[315] The lipoic acid, which is attached to the flexible lipoyl domains of the core enzyme on a 1.5-nm-long arm, apparently first contacts the thiamin diphosphate site on one of the decarboxylase subunits. Bearing the acyl group, it now swings to the catalytic site on the core enzyme where CoA is bound. The acyl group is transferred to CoA producing a dihydrolipoyl group which then swings to the third subunit where the disulfide loop and a bound FAD of dihydrolipoamide dehydrogenase reoxidize the lipoyl group. The reduced flavin-disulfide enzyme is then oxidized by $NAD^+$ (Fig. 15-15) by the reverse of the mechanism depicted in Fig. 15-12.

Although the direct reaction of a lipoyl group with the thiamin-bound enamine (active aldehyde) is generally accepted, and is supported by recent studies,[315a] an alternative must be considered.[315] Hexacyanoferrate (III) can replace $NAD^+$ as an oxidant for pyruvate dehydrogenase and is also able to oxidize nonenzymatically thiamin-bound active acetaldehyde

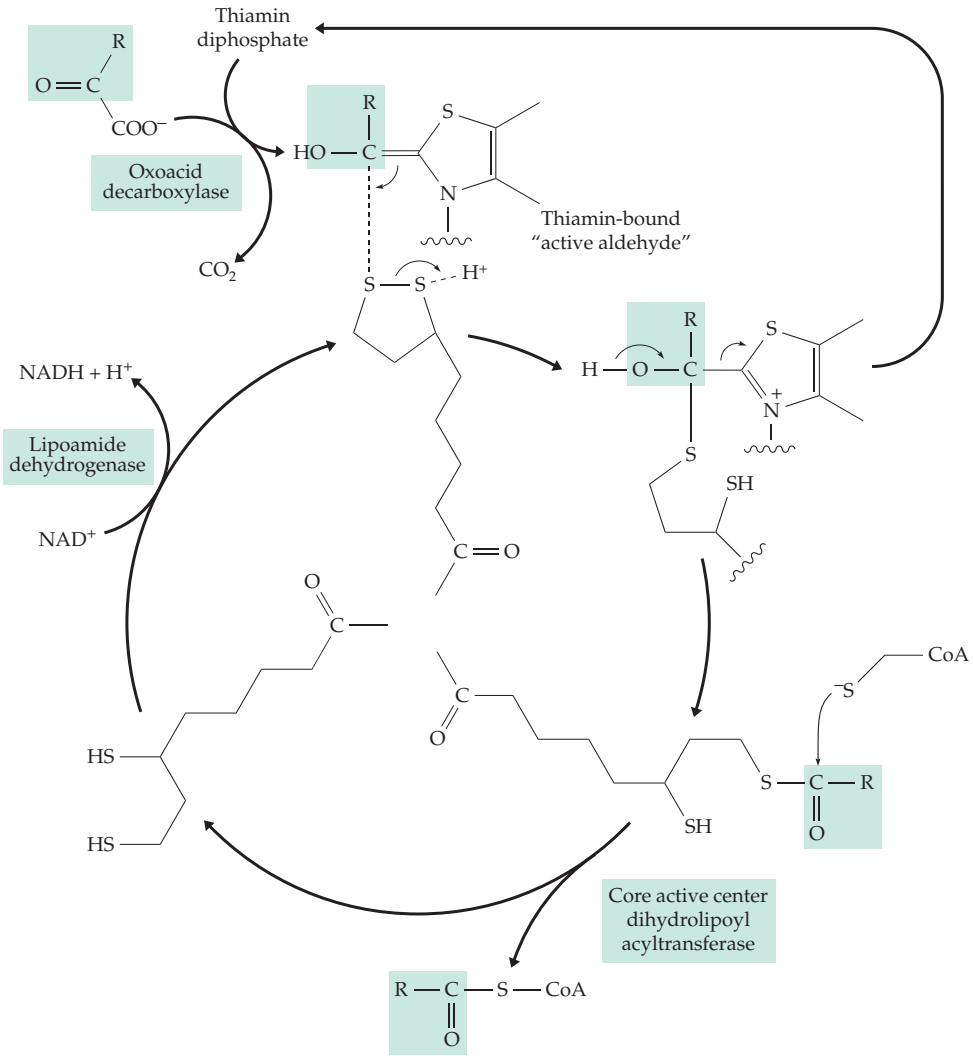



**Figure 15-15** Sequence of reactions catalyzed by α-oxoacid dehydrogenases. The substrate and product are shown in boxes, and the path of the oxidized oxoacid is traced by the heavy arrows. The lipoic acid "head" is shown rotating about the point of attachment to a core subunit. However, a whole flexible domain of the core is also thought to move.

to 2-acetylthiamin, a compound in which the acetyl group has a high group transfer potential (Eq. 14-22). Thus, the lipoyl group could first oxidize the active aldehyde to a thiamin-bound acyl derivative, and then in the second step accept the acyl group by a nucleophilic displacement reaction. This mechanism fails to explain the unique role of lipoic acid in oxidative decarboxylation. However, as we will see in the next section, oxidative decarboxylation does not always require lipoic acid and acetylthiamin is probably an intermediate whenever lipoamide is not utilized.

Within many tissues the enzymatic activities of the pyruvate and branched chain oxoacid dehydrogenases complexes are controlled in part by a phosphorylation–dephosphorylation mechanism (see Eq. 17-9). Phosphorylation of the decarboxylase subunit by an ATP-dependent kinase produces an inactive phosphoenzyme. A phosphatase reactivates the dehydrogenase to complete the regulatory cycle (see Eq. 17-9 and associated discussion). The regulation is apparently accomplished, in part, by controlling the affinity of the protein for thiamin diphosphate.[315b] The lipoamide dehydrogenase component of all three dehydrogenase complexes appears to be the same.

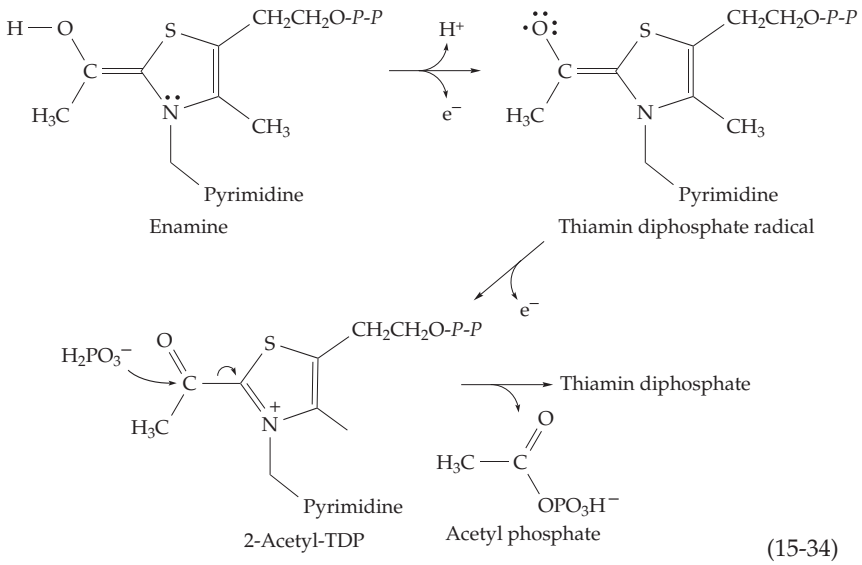
## 3. Other Functions of Lipoic Acid

In addition to its role in the oxidative decarboxylation of 2-oxoacids, lipoic acid functions in the human body as part of the essential mitochondrial glycine-cleavage system described in Section E. It may also participate in bacterial glycine reductase (Eq. 15-61) and other enzyme systems. Dihydrolipoamide dehydrogenase binds to G4-DNA structures of telomeres and may have a biological role in DNA-binding.[315c] Lipoic acid is being utilized as a nutritional supplement and appears to help in maintaining cellular levels of glutathione.[316]

## 4. Additional Mechanisms of Oxidative Decarboxylation

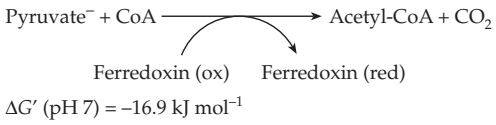
Pyruvate is a metabolite of central importance and a variety of mechanisms exist for its cleavage. The pyruvate dehydrogenase complex is adequate for strict aerobes and is very important in aerobic bacteria and in facultative anaerobes such as *E. coli*. Lactic acid bacteria, which lack cytochromes and other heme proteins, are able to carry out limited oxidation with flavoproteins such as **pyruvate oxidase**.[317] However, *strict* anaerobes have to avoid accumulation of reduced pyridine nucleotides and use a nonoxidative cleavage of pyruvate by **pyruvate formate-lyase**. The pyruvate dehydrogenase complex of *our* bodies generates NADH which can be oxidized in mitochondria to provide energy to our cells. However, NADH is a weak reductant. Some cells, such as those of nitrogen-fixing bacteria, require more powerful reductants such as reduced ferredoxin and utilize **pyruvate: ferredoxin oxidoreductase**. *Escherichia coli*, a facultative anaerobe, is adaptable and makes use of all of these types of pyruvate cleavage.[318]

***Pyruvate oxidase.*** The soluble flavoprotein pyruvate oxidase, which was discussed briefly in Chapter 14 (Fig. 14-2, Eq. 14-22), acts together with a membrane-bound electron transport system to convert pyruvate to acetyl phosphate and $CO_2$.[319] Thiamin diphosphate is needed by this enzyme but lipoic acid is not. The flavin probably dehydrogenates the thiamin-bound intermediate to 2-acetylthiamin as shown in Eq. 15-34. The electron acceptor is the bound FAD and the reaction may occur in two steps as shown with a thiamin diphosphate radical intermediate.[319a] Reaction with inorganic phosphate generates the energy storage metabolite **acetyl phosphate**.




(15-34)

***Pyruvate:ferredoxin oxidoreductase.*** Within clostridia and other strict anaerobes this enzyme catalyzes *reversible* decarboxylation of pyruvate (Eq. 15-35). The oxidant used by clostridia is the low-potential iron–sulfur ferredoxin.[320,320a] Clostridial ferredoxins contain two Fe–S clusters and are therefore two-electron oxidants. Ferredoxin substitutes for $NAD^+$ in Eq. 15-33 but the Gibbs energy decrease is much less (–16.9 vs – 34.9 kJ / mol. for oxidation by $NAD^+$).



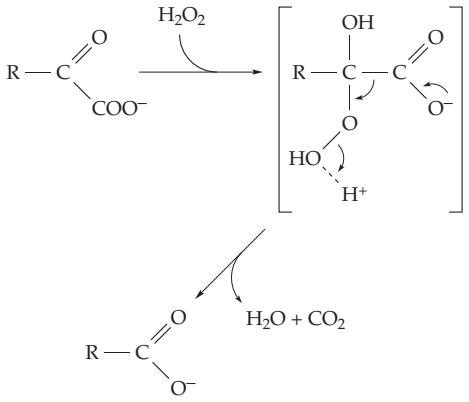

(15-35)

The enzyme does not require lipoic acid. It seems likely that a thiamin-bound enamine is oxidized by an iron–sulfide center in the oxidoreductase to 2-acetylthiamin which then reacts with CoA. A free radical intermediate has been detected[318,321] and the proposed sequence for oxidation of the enamine intermediate is that in Eq. 15-34 but with the Fe–S center as the electron acceptor. Like pyruvate oxidase, this enzyme transfers the acetyl group from acetylthiamin to coenzyme A. Cleavage of the resulting acetyl-CoA is used to generate ATP. An indolepyruvate: ferredoxin oxidoreductase has similar properties.[322]

In methanogenic bacteria[320] the low-potential 5-deazaflavin coenzyme $F_{420}$ serves as the reductant in a reversal of Eq. 15-35. A similar enzyme, **2-oxoglutarate synthase**, apparently functions in synthesis of 2-oxoglutarate from succinyl-CoA and $CO_2$ by photosynthetic bacteria.[323] Either reduced ferredoxin or, in *Azotobacter*, reduced flavodoxin is generated in nitrogen-fixing bacteria (Chapter 24) by cleavage of pyruvate and is used in the $N_2$ fixation process.

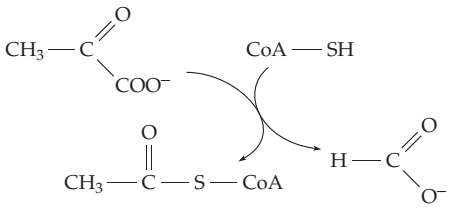
***Oxidative decarboxylation by hydrogen peroxide.*** The nonenzymatic oxidative decarboxylation of α-oxoacids by $H_2O_2$ is well-known. The first step is the formation of an adduct, an organic peroxide, which breaks up as indicated in Eq. 15-36. An enzyme-catalyzed version of this reaction is promoted by **lactate monooxygenase**, a 360-kDa octameric flavoprotein obtained from *Mycobacterium smegmatis*[324] and a member of the glycolate oxidase family. Under anaerobic conditions, the enzyme produces pyruvate by a simple dehydrogenation. However, the pyruvate dissociates slowly and in the presence of

oxygen it forms acetic acid, with one of the oxygen atoms of the carboxyl group coming from $O_2$.[324,325] Hydrogen peroxide is the usual product formed from oxygen by flavoprotein oxidases, and it seems likely that with lactate monooxygenase the hydrogen peroxide formed immediately oxidizes the pyruvate according to Eq. 15-36. The α-oxoacids formed by amino acid oxidases *in vitro* are also oxidized by accumulating hydrogen peroxide. However, if catalase (Chapter 16) is present it destroys the $H_2O_2$ and allows the oxoacid to accumulate.




(15-36)

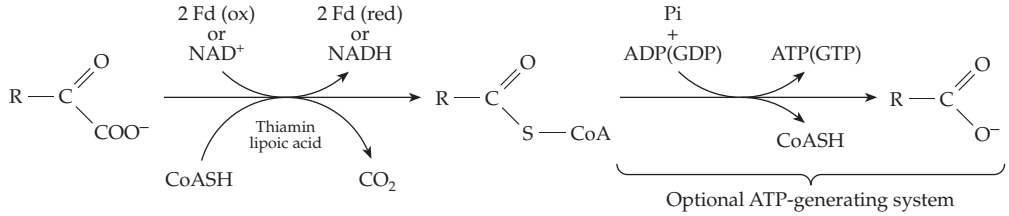
***Pyruvate formate-lyase reaction.*** Anaerobic cleavage of pyruvate to acetyl-CoA and formate (Eq. 15-37) is essential to the energy economy of many cells, including those of *E. coli*. No external oxidant is needed, and the reaction does not require lipoic acid. The reaction has sometimes been called the "phosphoroclastic reaction" because the product acetyl-CoA usually reacts further with inorganic phosphate to form acetyl phosphate (see Fig. 15-16). The latter can then transfer its phospho group to ADP to form ATP.




$\Delta G'$ (pH 7) = –12.9 kJ mol$^{-1}$ (15-37)

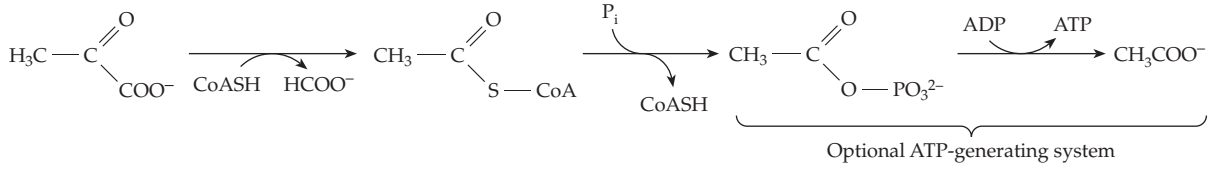
The mechanism of the cleavage of the pyruvate in Eq. 15-37 is not obvious. Thiamin diphosphate is not involved, and free $CO_2$ is not formed. The first identified intermediate is an acetyl-enzyme containing a thioester linkage to a cysteine side chain. This is cleaved by reaction with CoA-SH to give the final product. A clue came when it was found by Knappe and coworkers that the active enzyme, which is rapidly inactivated by oxygen, contains a long-lived free radical.[326] Under anaerobic conditions cells convert the inactive form $E_i$ to the active form $E_a$ by an enzymatic reaction with *S*-adenosylmethionine and reduced flavodoxin Fd(red) as shown in Eq. 15-38.[327–329] A deactivase reverses the process.[330]

**A** Oxidative decarboxylation of an α-oxoacid with thiamin diphosphate



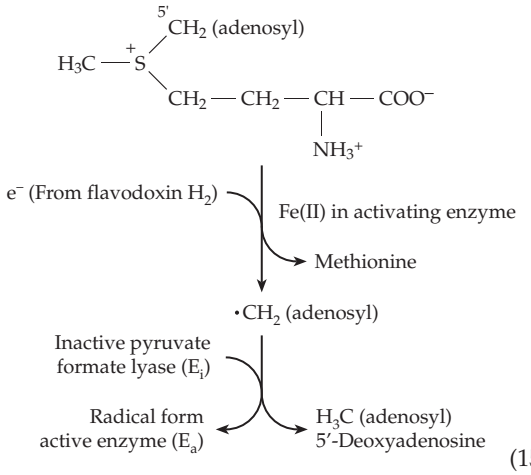

1. with lipoic acid and $NAD^+$ as oxidants — $\Delta G'$ (pH 7, for pyruvate) = –35.5 kJ/mol overall
2. with ferredoxin as oxidant — $\Delta G'$ (pH 7, for pyruvate) = –13.9 kJ/mol overall

**B** The pyruvate formate-lyase reaction



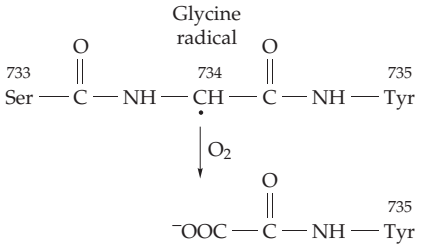

$\Delta G'$ (pH 7) = –13.5 kJ/mol overall

**Figure 15-16** Two systems for oxidative decarboxylation of α-oxoacids and for "substrate-level" phosphorylation. The value of $\Delta G'$ = +34.5 kJ mol$^{-1}$ (Table 6-6) was used for the synthesis of $ATP^{4-}$ from $ADP^{3-}$ and $HPO_4^{2-}$ in computing the values of $\Delta G'$ given.



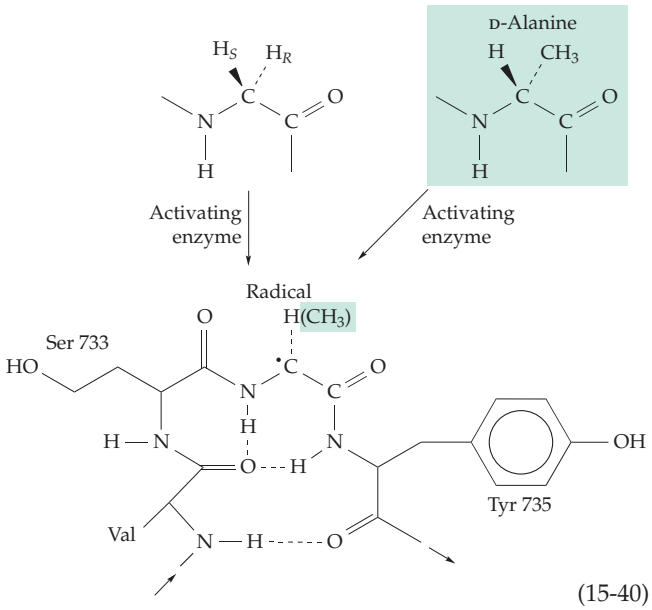

(15-38)

The activating enzyme, which is allosterically activated by pyruvate, is an iron–sulfur ($Fe_4S_4$) protein (Chapter 16).[331,331a] Formation of the observed radical may proceed via a 5'-deoxyadenosyl radical as has been proposed for lysine 2,3-aminomutase (Eq. 16-42).[332] The activation reaction also resembles the free radical-dependent reactions of vitamin $B_{12}$ which are discussed in Chapter 16. When subjected to $O_2$ of air at 25°C pyruvate formate-lyase is destroyed with a half-life of ~10 s. The peptide chain is cleaved and sequence analysis and mass spectrometry of the resulting fragments show that the specific sequence Ser-Gly-Tyr at positions 733–755 is cut with formation of an oxalyl group on the N-terminal tyrosine of one fragment (Eq. 15-39). Various $^{13}C$-containing amino acids were supplied to growing cells of *E. coli* and were incorporated into the proteins of the bacteria. Pyruvate formate-lyase containing $^{13}C$ in carbon-2 of glycine gave an EPR spectrum with hyperfine splitting arising from coupling of the unpaired electron of the radical with the adjacent $^{13}C$ nucleus.[333,333a] This experiment, together with the results described by Eq. 15-39, suggested that the radical is derived from Gly 734.



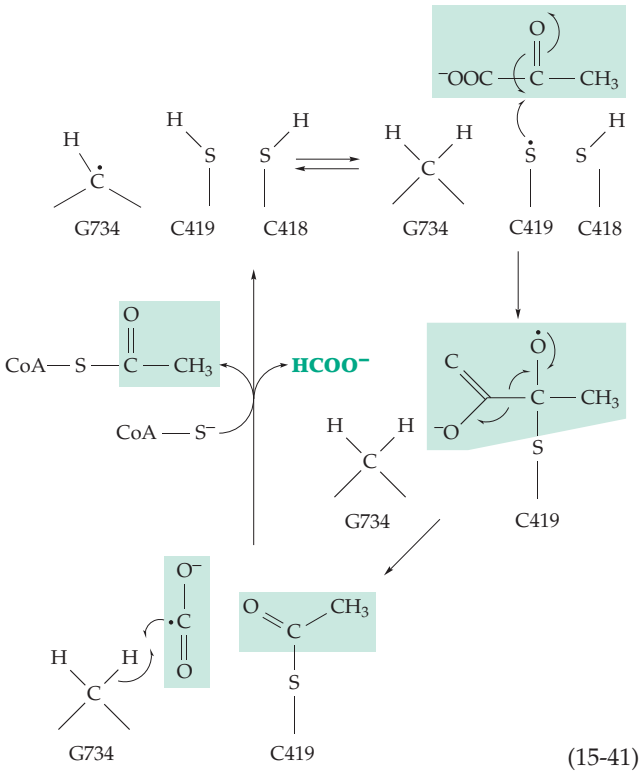

(15-39)

The activating enzyme will also generate radicals from short peptides such as Arg-Val-Ser-Gly-Tyr-Ala-Val, which corresponds to residues 731–737 of the pyruvate formate-lyase active site. If Gly 734 is replaced by L-alanine, no radical is formed, but radical *is* formed if D-alanine is in this position. This suggests that the *pro-S* proton of Gly 734 is removed by the activating enzyme[329] as illustrated in Eq. 15-40. It has been suggested that the peptide exists in the β-bend conformation shown.




(15-40)

How does the enzyme work? The α-CH proton of the glycyl radical of the original *pro-R* proton undergoes an unexpected exchange with the deuterium of $^2H_2O$. The exchange is catalyzed by the thiol group of Cys 419, suggesting that it is close to Gly 734 in the three-dimensional structure.[334] The mutants C419S and C418S are inactive but still allow formation of the Gly 734 radical.[334–335a] The mechanism has been proposed in Eq. 15-41.




(15-41)

### 5. Cleavage of α-Oxoacids and Substrate-Level Phosphorylation

The α-oxoacid dehydrogenases yield CoA derivatives which may enter biosynthetic reactions. Alternatively, the acyl-CoA compounds may be cleaved with generation of ATP. The pyruvate formate-lyase system also operates as part of an ATP-generating system for anaerobic organisms, for example, in the "mixed acid fermentation" of enterobacteria such as *E. coli* (Chapter 17). These two reactions, which are compared in Fig. 15-16, constitute an important pair of processes both of which accomplish substrate-level phosphorylation. They should be compared with the previously considered examples of substrate level phosphorylation depicted in Eq. 14-23 and Fig. 15-16.

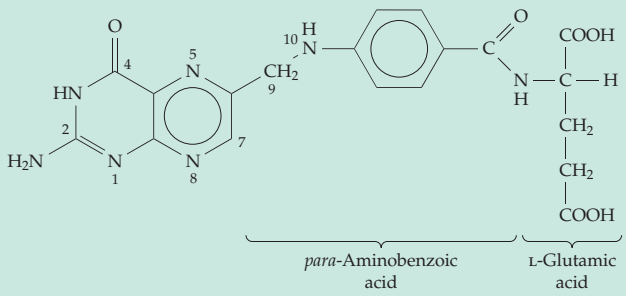
## D. Tetrahydrofolic Acid and Other Pterin Coenzymes

In most organisms reduced forms of the vitamin **folic acid** serve as *carriers for one-carbon groups* at three different oxidation levels corresponding to **formic acid**, **formaldehyde**, and the **methyl group** and also facilitate their interconversion.[336,337] Moreover, folic acid is just one of the derivatives of the **pteridine** ring system that enjoy a widespread natural distribution.[338] One of these derivatives functions in the hydroxylation of aromatic amino acids and in nitric oxide synthase and yet another within several molybdenum-containing enzymes. Pteridines also provide coloring to insect wings and eyes and to the skins of amphibians and fish. They appear to act as protective filters in insect eyes and may function as light receptors. An example is a folic acid derivative found in some forms of the DNA photorepair enzyme **DNA photolyase** (Chapter 23).

### 1. Structure of Pterins

Because of the prevalence of its derivatives, 2-amino-4-hydroxypteridine has been given the trivial name **pterin**. Its structure resembles closely that of guanine, the 5-membered ring of the latter having been expanded to a 6-membered ring. In fact, pterins are derived

## BOX 15-D FOLIC ACID (PTEROYLGLUTAMIC ACID)




In 1931, Lucy Wills, working in India, observed that patients with **tropical macrocytic anemia**, a disease in which the erythrocytes are enlarged but reduced in numbers, were cured by extracts of yeast or liver. The disease could be mimicked in monkeys fed the local diet, and a similar anemia could be induced in chicks. By 1938 it had also been shown that a factor present in yeast, alfalfa, and other materials was required for the growth of chicks. Isolation of the new vitamin came rapidly after it was recognized that it was also an essential nutrient for *Lactobacillus casei* and *Streptococcus faecalis* R.[a,b] Spinach was a rich source of the new compound, and it was named folic acid (from the same root as the word foliage).

The microbiological activity attributed to folic acid in extracts of natural materials was largely that of di- and triglutamyl derivatives, one of the facts that has led to the description of the history of folic acid as "the most complicated chapter in the story of the vitamin B complex."[a] Metabolic functions for folic acid were suggested by the observations that the requirement for *Streptococcus faecalis* could be replaced by thymine plus serine plus a purine base. Folic acid is required for the biosynthesis of all of these substances. A function in the interconversion of serine and glycine was suggested by the observation that certain mutants of *E. coli* required either serine or glycine for growth. Isotopic labeling experiments established that in the rat as well as in the yeast *Torulopsis* serine and glycine could be interconverted. It was also shown that the amount of interconversion decreased in the folate-deficient rat.

Deficiency of folic acid is a common nutritional problem of worldwide importance.[b] A recommended daily intake is 0.2 mg, but because of the association between low folic acid intake and neural tube defects in infants, women of child-bearing age should have 0.4 mg / day.[c–e]

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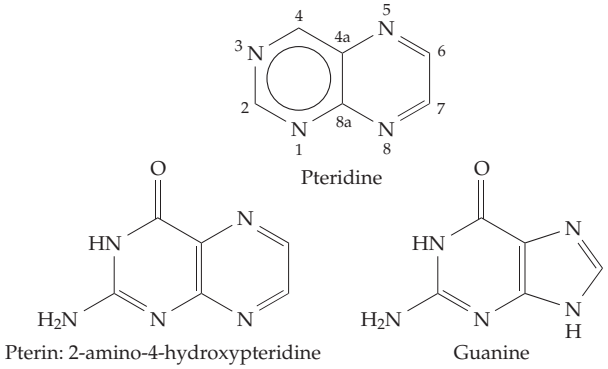
[a] Wagner, A. F., and Folkers, E. (1964) *Vitamins and Coenzymes*, Wiley (Interscience), New York
[b] Jukes, T. H. (1980) *Trends Biochem. Sci.* **5**, 112–113
[c] Cziezel, A. E., and Dudás, I. (1992) *N. Engl. J. Med.* **327**, 1832–1835
[d] Jukes, T. H. (1997) *Protein Sci.* **6**, 254–256
[e] Rosenquist, T. H., Ratashak, S. A., and Selhub, J. (1996) *Proc. Natl. Acad. Sci. U.S.A.* **93**, 15227–15232

biosynthetically from guanine. The two-ring system of pterin is also related structurally and biosynthetically to that of riboflavin (Box 15-B).




Interest in pteridines began with Frederick G. Hopkins, who in 1891 started his investigation of the yellow and white pigments of butterflies. Almost 50 years and a million butterflies later, the structures of the two pigments, **xanthopterin** and **leucopterin** (Fig. 15-17), were established.[339] These pigments are produced in such quantities as to suggest that their synthesis may be a means of deposition of nitrogenous wastes in dry form.

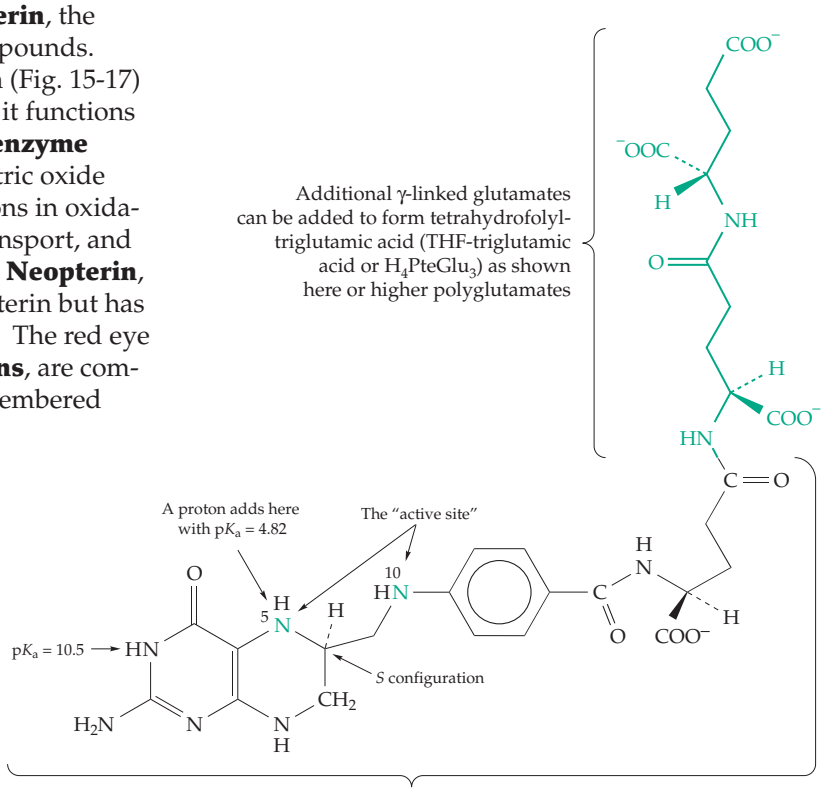
Among the simple pterins isolated from the eyes of *Drosophila*[340] is **sepiapterin** (Fig. 15-17), in which the pyrazine ring has been reduced in the 7,8 position and a short side chain is present at position 6. Reduction of the carbonyl group of sepiapterin with $NaBH_4$ followed by air oxidation produces **biopterin**, the most widely distributed of the pterin compounds. First isolated from human urine, biopterin (Fig. 15-17) is present in liver and other tissues where it functions in a reduced form as a **hydroxylation coenzyme** (see Chapter 18).[338] It is also present in nitric oxide synthase (Chapter 18).[341,342] Other functions in oxidative reactions, in regulation of electron transport, and in photosynthesis have been proposed.[343] **Neopterin**, found in honeybee larvae, resembles biopterin but has a D-*erythro* configuration in the side chain. The red eye pigments of *Drosophila*, called **drosopterins**, are complex dimeric pterins containing fused 7-membered rings (Fig. 15-17).[344,345]

In the pineal gland, as well as in the retina of the eye, light-sensitive pterins may be photochemically cleaved to generate such products as **6-formylpterin**, a compound that could serve as a metabolic regulator.[346] Another pterin acts as a chemical attractant for aggregation of the ameboid cells of *Dictyostelium lacteum*[347](Box 11-C). **Molybdopterin** (Fig. 15-17) is a component of several Mo-containing enzymes discussed in Chapter 16,[348,349] and **methanopterin** is utilized by methanogenic bacteria.[350–353]

## 2. Folate Coenzymes

The coenzymes responsible for carrying single-carbon units in most organisms are derivatives of **5,6,7,8-tetrahydrofolic acid** (abbreviated $H_4$PteGlu, $H_4$folate or THF). However, in methanogenic bacteria the tetrahydro derivative of the structurally unique methanopterin (Fig. 15-17) is the corresponding single-carbon carrier.[353] Naturally occurring tetrahydrofolates contain a chiral center of the *S* configuration.[354,355] They exhibit negative optical rotation at 589 nm. The folate coenzymes are present in extremely low concentrations and the reduced ring is readily oxidized by air.

In addition to the single L-glutamate unit present in tetrahydrofolic acid, the coenzymes occur to the greatest extent as conjugates called **folyl polyglutamates** in which one to eight or more additional molecules of L-glutamic acid have been combined via amide linkages.[356–357a] The first two of the extra glutamates are always joined through the γ (side chain) carboxyl groups but in *E. coli* the rest are joined through their α carboxyls.[358] The distribution of the polyglutamates varies from one organism to the next. Some bacteria contain exclusively the triglutamate derivatives, while in others almost exclusively the tetraglutamate or octaglutamate derivatives predominate.[359] The serum




Tetrahydrofolic acid (THF) or tetrahydropteroylglutamic acid ($H_4$PteGlu)

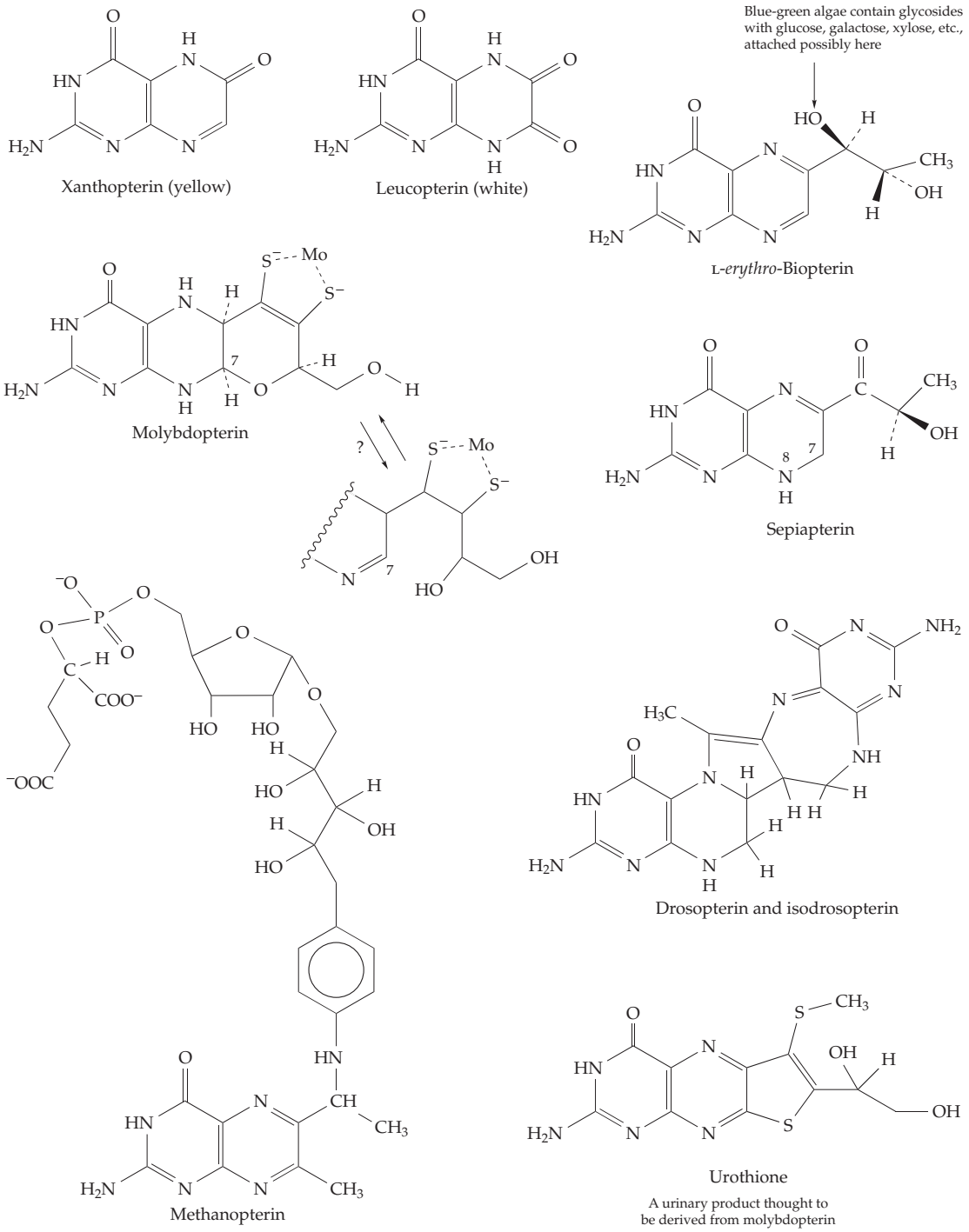



**Figure 15-17** Structures of several biologically important pterins.

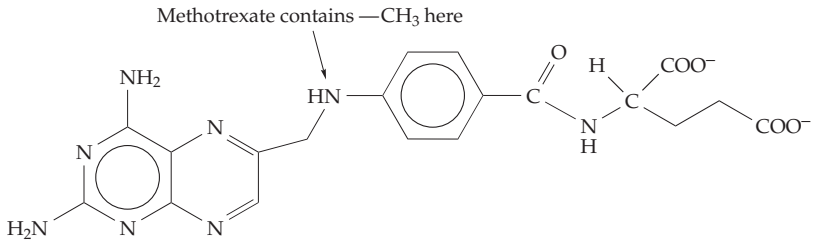
of many species contains only derivatives of folic acid itself but pteroylpentaglutamate is the major folate derivative present in rodent livers.[360,361] A large fraction of these pentaglutamates consists of the 5-methyl-THF derivative, while at the heptaglutamate level most consists of the free THF derivative. Folyl polyglutamate in its oxidized form is a component of some DNA photolyases (Chapter 23).[362]

## 3. Dihydrofolate Reductase

Folic acid and its polyglutamyl derivatives can be reduced to the THF coenzymes in two stages: the first step is a slow reduction with NADPH to 7,8-dihydrofolate (step *a*, Fig. 15-18). The same enzyme that catalyzes this reaction rapidly reduces the dihydrofolates

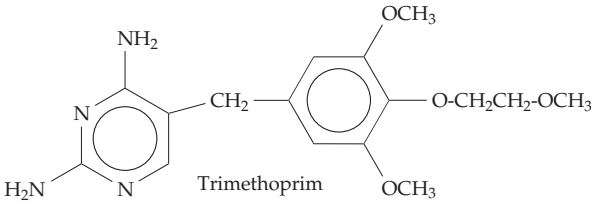
to tetrahydrofolates (step *b*, Fig. 15-18). Again, NADPH is the reducing agent and the enzyme has been given the name **dihydrofolate reductase**. An unusual fact is that bacteriophage T4 not only carries a gene for its own dihydrofolate reductase but also the enzyme is a structural unit in the phage baseplate.[363]

***Inhibitors.*** Aside from its role in providing reduced folate coenzymes for cells, this enzyme has attracted a great deal of attention because it appears to be a site of action of the important anticancer drugs **methotrexate** (amethopterin) and aminopterin.[293,364,365] These compounds inhibit dihydrofolate reductase in concentrations as low as $10^{-8}$ to $10^{-9}$ M. Methotrexate is also widely used as an **immunosuppresant** drug and in the treatment of parasitic infections.

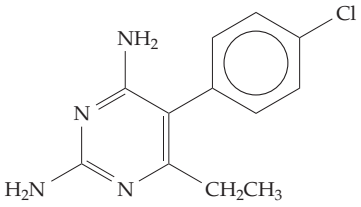


Aminopterin (4-amino-4-deoxyfolic acid)

Another dihydrofolate inhibitor **trimethoprim** is an important antibacterial drug, usually given together with a sulfonamide. Although it is not as close a structural analog of folic acid as is methotrexate, it is



Trimethoprim



Pyrimethamine

a potent inhibitor which binds far more tightly to the enzyme from bacteria than to that from humans.[366–368] The inhibitors **pyrimethamine** and **cycloguanil** are effective antimalarial drugs.[369,370]

An enormous number of synthetic compounds have been prepared in the hope of finding still more effective inhibitors. By 1984, over 1700 different inhibitors of dihydrofolate reductase had been studied.[367] However, methotrexate and trimethoprim remain outstanding. Methotrexate has been in clinical use for nearly 50 years and is very effective against leukemia and some other cancers.[371] Before 1960 persons with acute leukemia lived no more than 3–6 months. However, with antifolate treatment some have lived for 5 years or more and complete cures of the relatively rare choriocarcinoma have been achieved. New methods of chemotherapy use the antifolates in combination with other drugs.

Folate coenzymes are required in the biosynthesis of both purines and thymine. Consequently, rapidly growing cancer cells have a high requirement for activity of this enzyme. However, since all cells require the enzyme the antifolates are toxic and cannot be used for prolonged therapy. An even more serious problem is the development of resistance to the drug by cancer cells, often through "amplification" of the dihydrofolate reductase gene[365,372–374] as discussed in Chapter 27. Cells may also become resistant to methotrexate and other antifolates as a result of mutations that prevent efficient uptake of the drug,[375] cause increased action of effux pumps in the cell,[376] reduce the affinity of dihydrofolate reductase for the drug,[377] or interfere with conversion of the drug to polyglutamate derivatives which are better inhibitors than free methotrexate.[378,379] Cell surface **folate receptors**, present in large numbers in some tumor cells, can be utilized to bring suitably designed antifolate drugs or even unrelated cytotoxic compounds into tumor cells.[379a] Some tumor cells are more active than normal cells in generating folyl polyglutamates, contributing to the effectiveness of methotrexate.[379] Resistance of *E. coli* cells to trimethoprin may result from acquisition by the bacteria of a new form of dihydrofolate reductase carried by a plasmid.[380]

***Structure and mechanism.*** Dihydrofolate reductase from *E. coli* is a small 159-residue protein with a central parallel stranded sheet,[381–383] while that from higher animals is 20% larger. The three-dimensional structures of the enzymes from *Lactobacillus*,[384] chicken, mouse, and human are closely similar. The NADP binds at the C-terminal ends of β strands as in other dehydrogenases. In Fig. 15-19 the reduced nicotinamide end of $NADP^+$ is seen next to a molecule of bound dihydrofolate.[381] The side chain carboxylate of Asp 27 makes a pair of hydrogen bonds to the pterin ring as shown on the right-hand side of Eq. 15-42. As can be seen from Fig. 15-19, the nicotinamide ring of $NADP^+$ is correctly positioned to have donated a hydride ion to C-6 to form THF with the 6*S* configuration. Notice that in Eq. 15-42 the NH hydrogen atom at the 3' position

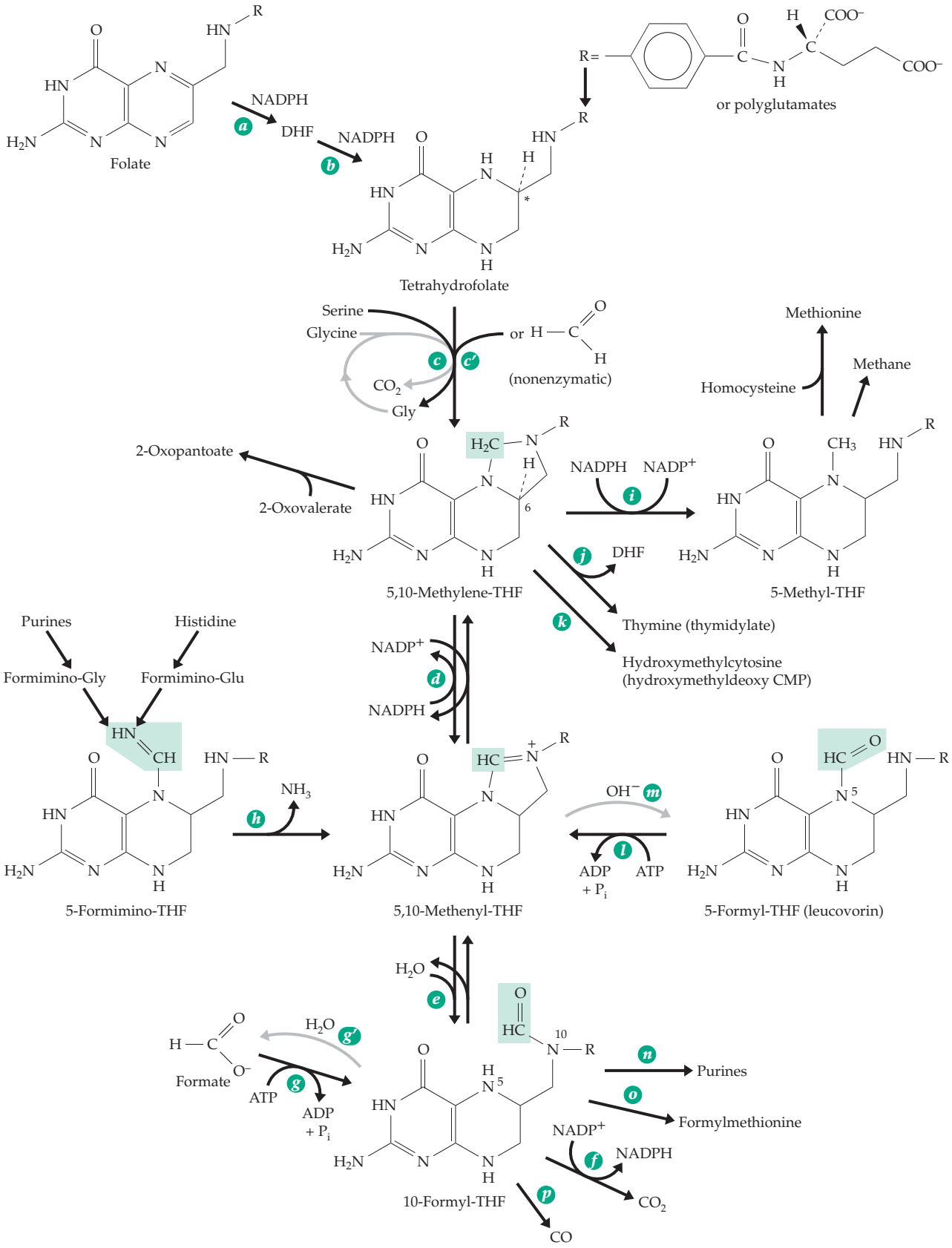
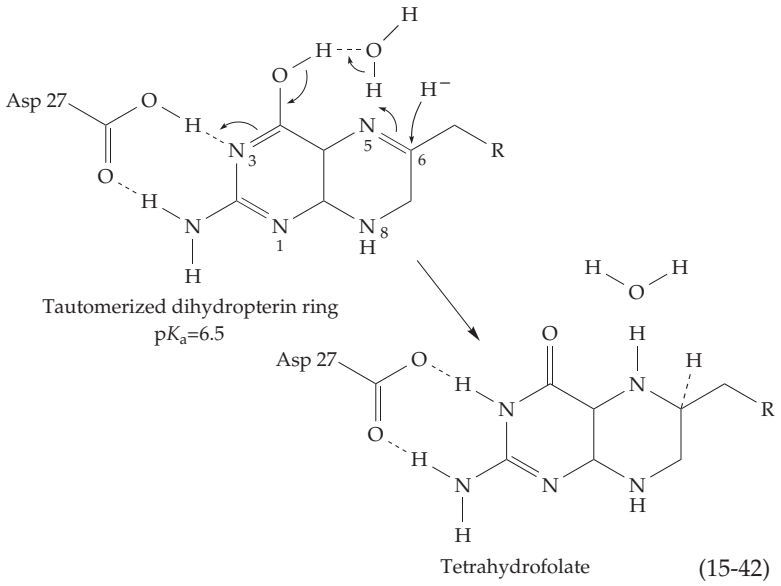



**Figure 15-18** Tetrahydrofolic acid and its one-carbon derivatives.




(15-42)

of the pterin makes a hydrogen bond to Asp 27. However, X-ray studies have shown that the pteridine ring of methotrexate is turned 180° so that its 4-$NH_2$ group forms hydrogen bonds to backbone carbonyls of Ala 97 and Leu 4 at the edge of the central pleated sheet while a protonated N-1 interacts with the side chain carboxylate of Asp 27.

Because of its significance in cancer therapy and its small size, dihydrofolate reductase is one of the most studied of all enzymes. Numerous NMR studies[385–387] and investigations of catalytic mechanism and of other properties[388] have been conducted. Many mutant forms have been created.[389–391] For example, substitution of Asp 27 (Asp 26 in *L. casei*) of the *E. coli*

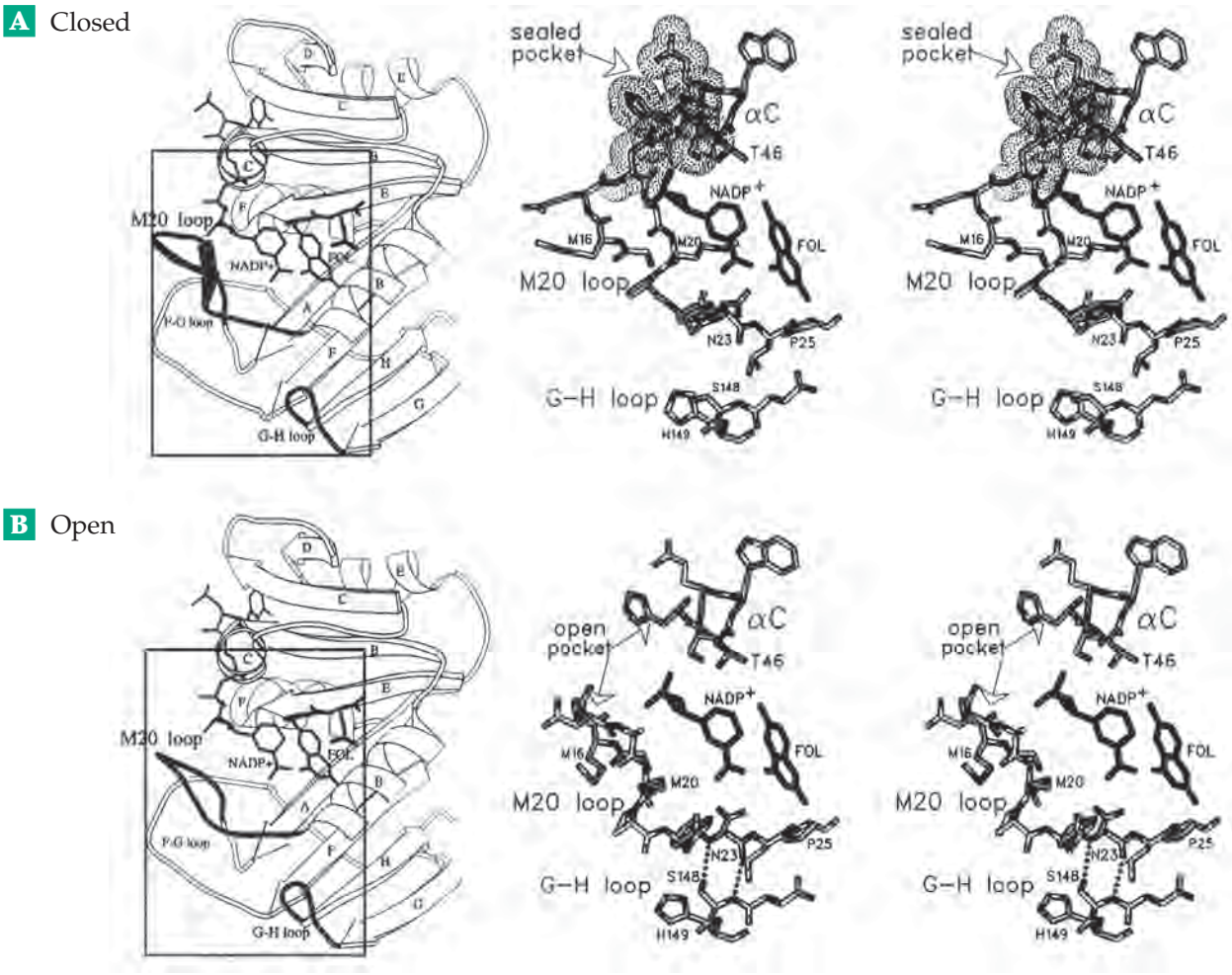



**Figure 15-19** Drawings of the active site of *E. coli* dihydrofolate reductase showing the bound ligands $NADP^+$ and tetrahydrofolate. Several key amino acid side chains are shown in the stereoscopic views on the right. The complete ribbon structures are on the left. (A) Closed form. (B) Open form into which substrates can enter and products can escape. From Sawaya and Kraut.[381] Courtesy of Joseph Kraut. Molscript drawings (Kraulis, 1991).

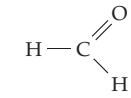
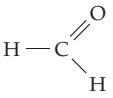
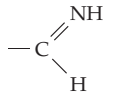
enzyme by Asn reduced the specific catalytic activity to 1/300 that of native enzyme indicating that this residue is important for catalysis.[390] The value of $k_{cat}$ for reduction of dihydrofolate is highest at low pH and varies around a p$K_a$ of ~6.5.[392–394] One interpretation is that this p$K_a$ belongs to the Asp 27 carboxyl group and another is that it belongs to an N-5 protonated species of the coenzyme. As we have seen (Chapter 6) it is often impossible to assign a p$K_a$ to a single group because there may be a mixture of interacting tautomeric species. Despite the enormous amount of study, we still don't know quite how the proton gets to N-5 in this reaction. Only one possibility is illustrated in Eq. 15-42. Asp 27 is protonated, the pterin ring is enolized, and a buried water molecule serves to relay a proton to N-5. The X-ray crystallographic studies on the *E. coli* enzyme show that after the binding of substrates a conformational change closes a lid over the active site (Fig. 15-19). This excludes water from N-5 but it may permit intermittent access, allowing transfer of a proton from a water molecule bound to O-4 as shown in Eq. 15-42.[381] During the reduction of folate to dihydrofolate a different mechanism of proton donation must be followed to allow protonation at C-7.

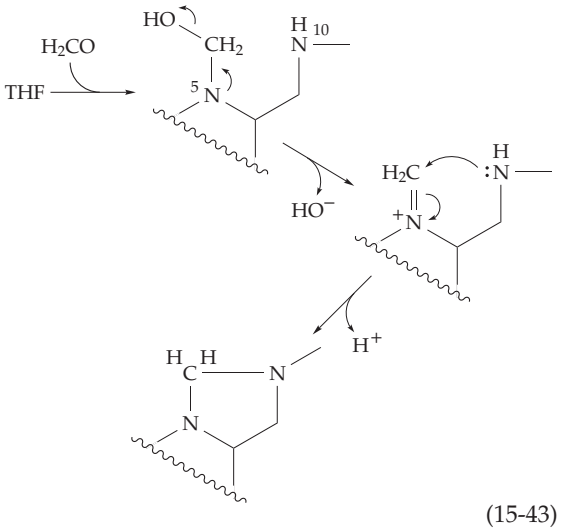
## 4. Single-Carbon Compounds and Groups in Metabolism

Some single-carbon compounds and groups important in metabolism are shown in Table 15-3 in order (from left to right) of increasing oxidation state of the carbon atom. Groups at three different oxidation levels, corresponding to **formic acid**, **formaldehyde**, and the **methyl group**, are carried by tetrahydrofolic acid coenzymes. While the most completely reduced compound, methane, cannot exist in a combined form, its biosynthesis depends upon reduced methanopterin, as does that of carbon monoxide. Figure 15-18 summarizes the metabolic interrelationships of the compounds and groups in this table.

***Serine as a C1 donor.*** For many organisms, from *E. coli* to higher animals, **serine** is a major precursor of C-1 units.[395–397] The β-carbon of serine is removed as formaldehyde through direct transfer to tetrahydrofolate with formation of methylene-THF and glycine (Eq. 14-30, Fig. 15-18, step *c*). This is a stereospecific transfer in which the *pro-S* hydrogen on C-3 of serine enters the *pro-S* position also in methylene-THF.[398] The glycine formed in this reaction can, in turn, yield another single-carbon unit by loss of $CO_2$ under the influence of the THF and the PLP-requiring glycine cleavage system which is discussed in the next paragraph. Free formaldehyde in a low concentration can also combine with THF to form methylene-THF (Fig. 15-18, step *c*′ and Eq. 15-43).[399]

**TABLE 15-3**
**Single-Carbon Compounds in Order of Oxidation State of Carbon**

| | | | | |
|---|---|---|---|---|
| $CH_4$ | $H_3C—Y$ |  |  | $CO_2$ |
| Methane | Methyl groups bound to O, N, S | Formaldehyde | Formic acid | $H_2CO_3$ |
| | | $—CH_2OH$ |  | |
| | | Hydroxymethyl group | Formyl group | |
| | | $—CH_2—$ |  | |
| | | Methylene group | Methenyl group | |
| | | |  | |
| | | | Formimino group | |
| | | | $C{=}O$ | |
| | | | Carbon monoxide | |



(15-43)

***The glycine decarboxylase–synthetase system.*** Glycine is cleaved reversibly within mitochondria of

plants and animals and also by bacteria to $CO_2$, $NH_3$, and a methylene group which is carried by tetrahydrofolic acid[296,400–403] (Fig. 15-20). Four proteins are required. The P-protein consists of two identical 100-kDa subunits, each containing a molecule of PLP. This protein is a **glycine decarboxylase** which, however, replaces the lost $CO_2$ by an electrophilic sulfur of lipoate rather than by a proton. Serine hydroxymethyltransferase can also catalyze this step of the sequence.[404] The lipoate is bound to a second protein, the H-protein. A third protein, the T-protein, carries bound tetrahydrofolate which displaces the aminomethyl group from the dihydrolipoate and converts it to $N^5$,$N^{10}$-methylene tetrahydrofolate with release of ammonia. The dihydrolipoate is then reoxidized by $NAD^+$ and dihydrolipamide dehydrogenase (Fig. 15-20). Glycine can be oxidized completely in liver mitochondria with the methylene group of methylene-THF being converted to $CO_2$ through reaction steps *d*, *e*, and *f* of Fig. 15-18. The glycine cleavage system is reversible and is used by some organisms to synthesize glycine.

Whether it arises from the hydroxymethyl group of serine or from glycine, the single-carbon unit of methylene-THF (which is at the formaldehyde level of oxidation) can either be oxidized further to 5,10-methenyl-THF and 10-formyl-THF (steps *d* and *e*, Fig. 15-18)

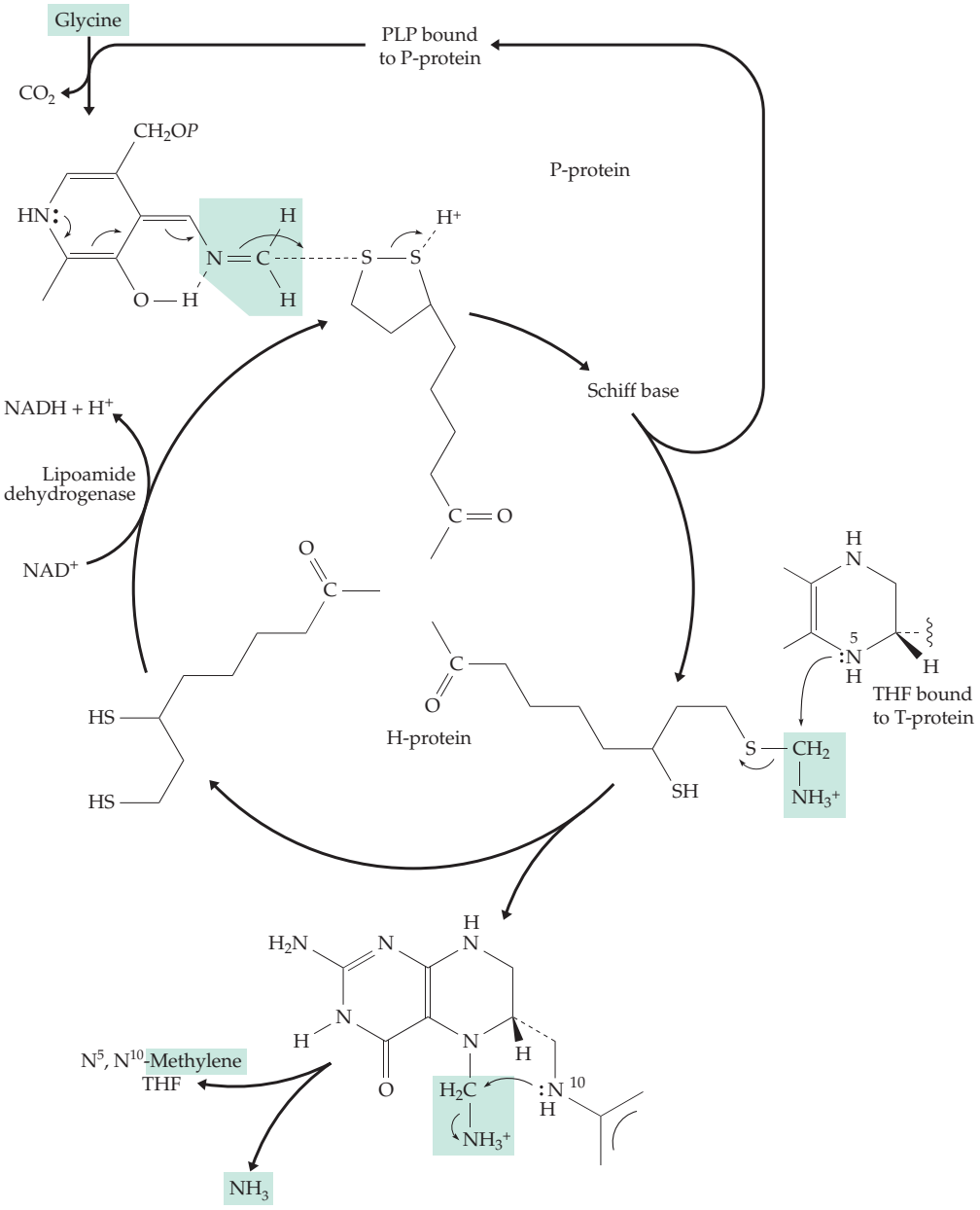



**Figure 15-20** The reversible glycine cleavage system of mitochondria. Compare with Fig. 15-15.

or it can be reduced to methyl-THF (step *i*). The reactions of folate metabolism occurs in both cytoplasm and mitochondria at rates that are affected by the length of the polyglutamate chain. The many complexities of these pathways are not fully understood.[395,397]

***Starting with formate or carbon dioxide.*** Most organisms can, to some extent, also utilize formate as a source of single-carbon units. Human beings have a very limited ability to metabolize formate and the accumulation of formic acid in the body following ingestion of methanol is often fatal. However, many bacteria are able to subsist on formate as a sole carbon source. Archaea may generate the formate by reduction of $CO_2$. Utilization of formate begins with formation of 10-formyl-THF (Fig. 15-18, step *g*, lower left corner). 10-Formyl-THF can be reduced to methylene-THF and the single-carbon unit can be transferred to glycine to form serine. In some bacteria three separate enzymes are required to convert formate to methylene-THF: 10-formyl-THF synthetase (Fig. 15-18, step *g*, presumably via formyl phosphate),[405] methenyl-THF cyclohydrolase (step *e*, reverse), and methylene-THF dehydrogenase (step *d*, reverse). NADH is used by the acetogens but in *E. coli* and in most higher organisms NADPH is the reductant. In some bacteria and in some tumor cells, two of the three enzymes are present as a bifunctional enzyme.[406–407a] In most eukaryotes all three of the enzymatic activities needed for converting formate to methylene-THF are present in a single large (200-kDa) dimeric trifunctional protein called **formyl-THF synthetase**.[407,408]

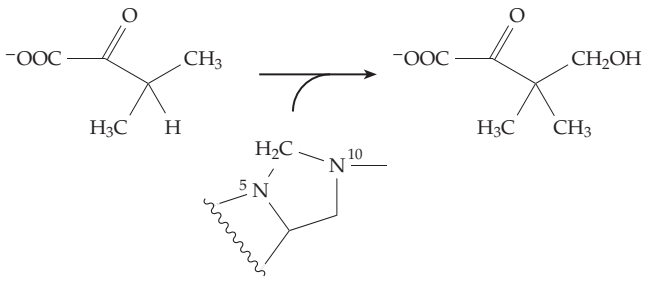
$N^{10}$-Formyl-THF serves as *a biological formylating agent* needed for two steps in the synthesis of purines[409–410b] (Chapter 25) and, in bacteria and in mitochondria and chloroplasts, for synthesis of formyl-methionyl-tRNA[411] which initiates synthesis of all polypeptide chains in bacteria and in these organelles (Chapter 29).

***$N^5$-Formyl-THF (leucovorin).*** The $N^5$-formyl derivative of THF (5-formyl-THF) is a growth factor for *Leuconostoc citrovorum.* Following this discovery in 1949 it was called the "citrovorum factor" or **leucovorin**. Its significance in metabolism is not clear. Perhaps it serves as a storage form of folate in cells that have a dormant stage, e.g., of seeds or spores.[412] It may also have a regulatory function.[413] In some ants and in certain beetles, it is stored and hydrolyzed to formic acid. The carabid beetle *Galerita lecontei* ejects a defensive spray that contains 80% formic acid.[414]

5-Formyl-THF can arise by transfer of a formyl group from formylglutamate and there is an enzyme that converts 5-formyl-THF to 5,10-methenyl-THF with concurrent cleavage of ATP.[412] 5-Formyl-THF is sometimes used in a remarkable way to treat certain highly malignant cancers. Following surgical removal of the tumor the patient is periodically given what would normally be a lethal dose of methotrexate, then, about 36 h later, the patient is rescued by injection of 5-formyl-THF. The mechanism of rescue is thought to depend upon the compound's rapid conversion to 10-formyl-THF within cells. Tumor cells are not rescued, perhaps because they have a higher capacity than normal cells for synthesis of polyglutamates of methotrexate.[379] This observation also suggests that the major anti-cancer effect of methotrexate may not be on dihydrofolate reductase but on enzymes of formyl group transfer that are inhibited by polyglutamate derivatives of methotrexate.

***Catabolism of histidine and purines.*** Another source of single-carbon units in metabolism is the degradation of histidine which occurs both in bacteria and in animals via **formiminoglutamate**. The latter transfers the –CH=NH group to THF forming 5-formimino-THF, which is in turn converted (step *h*, Fig. 15-18) to 5,10-methenyl-THF and ammonia. In bacteria that ferment purines, **formiminoglycine** is an intermediate. Again, the formimino group is transferred to THF and deaminated, the eventual product being 10-formyl-THF. In these organisms, the enzyme 10-formyl-THF synthase also has a very high activity.[415] It probably operates in reverse as a mechanism for synthesis of ATP in this type of fermentation. In other organisms excess 10-formyl-THF may be oxidized to $CO_2$ via an $NADP^+$-dependent dehydrogenase (step *f*, Fig. 15-16), providing a mechanism for detoxifying formic acid. In some organisms excess 10-formyl-THF may simply be hydrolyzed with release of formate (step *q*).[415a]

***Thymidylate synthase.*** Methylene-THF serves as the direct precursor of the 5-methyl group of thymine as well as of the hydroxymethyl groups of **hydroxymethylcytosine**[416] and of **2-oxopantoate**, an intermediate in the formation of pantothenate and coenzyme A.[417] The latter is a simple hydroxymethyl transfer reaction (Eq. 15-44) that is related to an aldol condensation and which may proceed through an imine of the kind shown in Eq. 15-43.




$N^{5,10}$-Methylene-THF (15-44)

During thymine formation the coenzyme is oxidized to dihydrofolate, which must be reduced by dihydrofolate reductase to complete the catalytic cycle. A possible mechanistic sequence for **thymidylate synthase**, an enzyme of known three-dimensional structure,[354,418–421a] is given in Fig. 15-21. In the first step (*a*) a thiolate anion, from the side chain of Cys 198 of the 316-residue *Lactobacillus* enzyme, adds to the 5 position of the substrate 2'-deoxyuridine monophosphate (dUMP). As a consequence the 6 position becomes a nucleophilic center which can combine with methylene-THF as shown in step *b* of Fig. 15-21. After tautomerization (step *c*) this adduct eliminates THF (step *d*) to give a 5-methylene derivative of the dUMP. The latter immediately oxidizes the THF by a hydride ion transfer to form thymidylate and dihydrofolate.[422,422a]

In protozoa thymidylate synthase and dihydrofolate reductase exist as a single bifunctional protein.

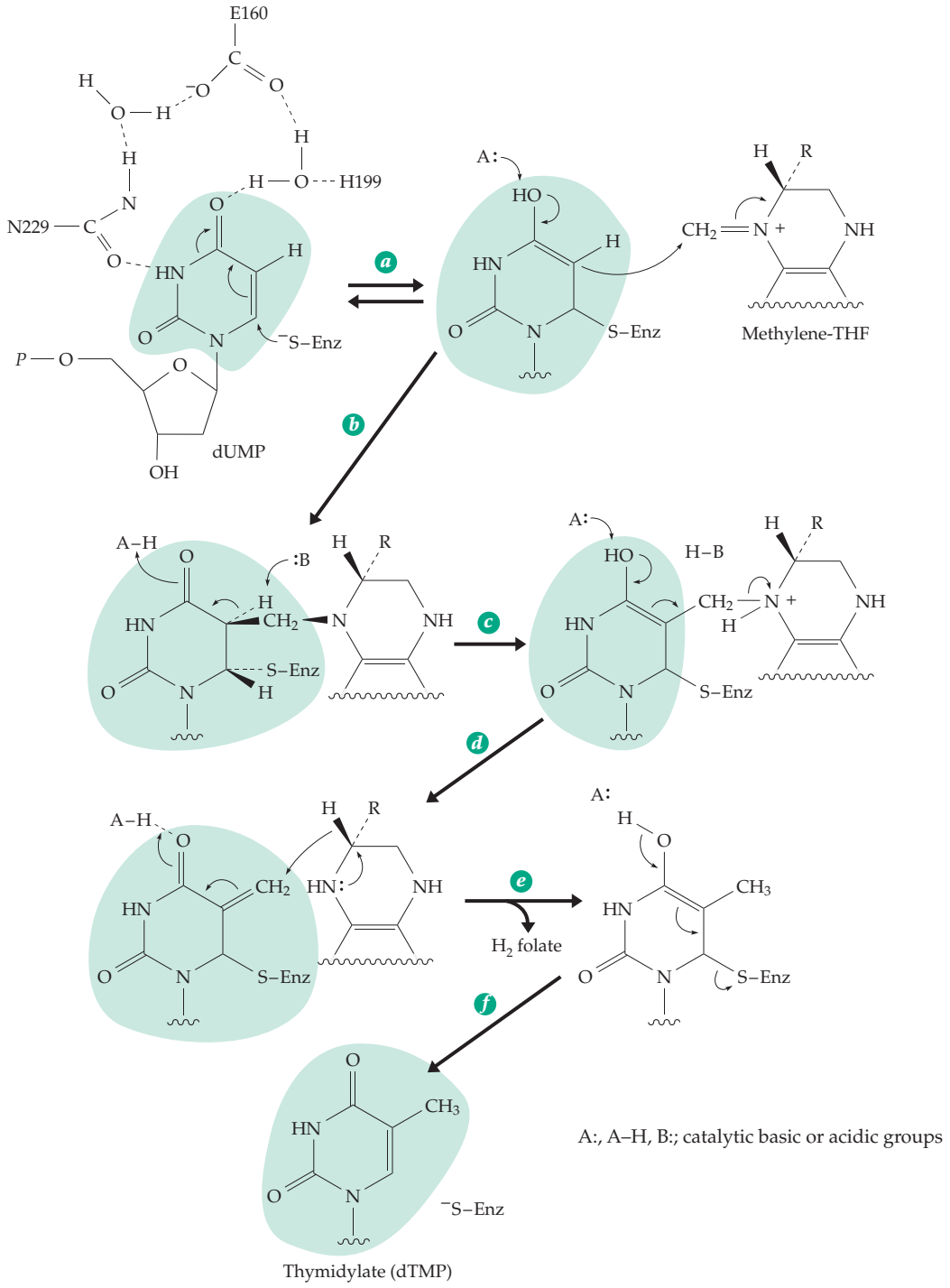
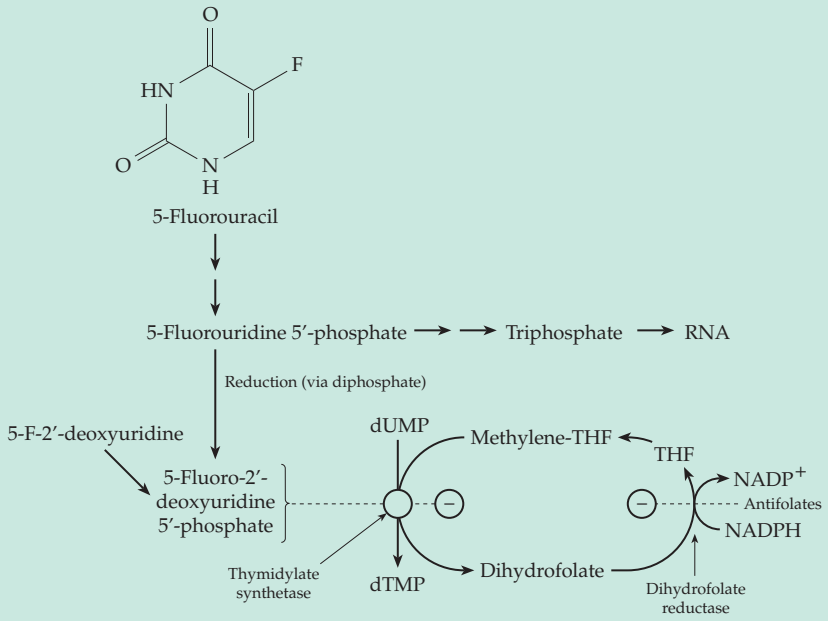



**Figure 15-21** Probable mechanism of action of thymidylate synthase. After Huang and Santi[422] and Hyatt *et al.*[354]

Consequently, in methotrexate-resistant strains of *Leishmania* (and also in cancer cells) both enzyme activities are increased equally by gene amplification.[423] This presents a serious problem in the treatment of protozoal parasitic diseases for which few suitable drugs are known.

Cells of *E. coli*, when infected with T-even bacteriophage, convert dCMP to 5-hydroxymethyl-dCMP[423a]

## BOX 15-E THYMIDYLATE SYNTHASE, A TARGET ENZYME IN CANCER CHEMOTHERAPY[a]




If an animal or bacterial cells are deprived of thymine they can no longer make DNA. However, synthesis of proteins and of RNA continues for some time. This can be demonstrated experimentally with thymine-requiring mutants. However, sooner or later such cells lose their vitality and die. The cause of this **thymineless death**[b] is not entirely clear. Perhaps thymine is needed to repair damage to DNA and if it is not available transcription eventually becomes faulty. Chromosome breakage is also observed.[c] Whatever the cause of death, the phenomenon provides the basis for some of the most effective chemotherapeutic attacks on cancer. Rapidly metabolizing cancer cells are especially vulnerable to thymineless death. Consequently, thymidylate synthase is an important target enzyme for inhibition. One powerful inhibitor is the monophosphate of **5-fluoro-2'-deoxyuridine**. The inhibition was originally discovered when 5-fluorouracil was recognized as a useful cancer chemotherapeutic agent.

Fluorouracil has many effects in cells, including incorporation into RNA,[b] but the inhibition of thymidylate synthase by the reduction product may be the most useful effect in chemotherapy. In fact, 5-fluoro-2'-deoxyuridine is a much less toxic and more potent drug than 5-fluorouracil. It binds into the active site of thymidylate synthase and reacts in the initial steps of catalysis. However, the 5-H of 2'-deoxyuridine, the normal substrate, must be removed as $H^+$ (step *c* of Fig. 15-21) in order for the reaction to continue. The 5-F atom cannot be removed in this way and a stable adduct is formed.[d,e] A crystal structure containing both the bound 5-fluorodeoxyuridine and methylene-THF supports this conclusion.[e]

Thymidylate synthase requires methylene tetrahydrofolate as a reductant and the reduction of dihydrofolate is also an important part of the process. In protozoa dihydrofolate reductase and thymidylate synthase occur as a single-chain bifunctional enzyme.[f] As has been pointed out in the main text, such folic acid analogs as methotrexate are among the most useful anticancer drugs. By inhibiting dihydrofolate reductase they deprive thymidylate synthase of an essential substrate.

Because 5-fluorouracil acts on normal cells as well as cancer cells, its usefulness is limited. Knowledge of the chemistry and three-dimensional structure of thymidylate synthase complexes is being used in an attempt to discover more specific and effective drugs that attack this enzyme.[g,h]

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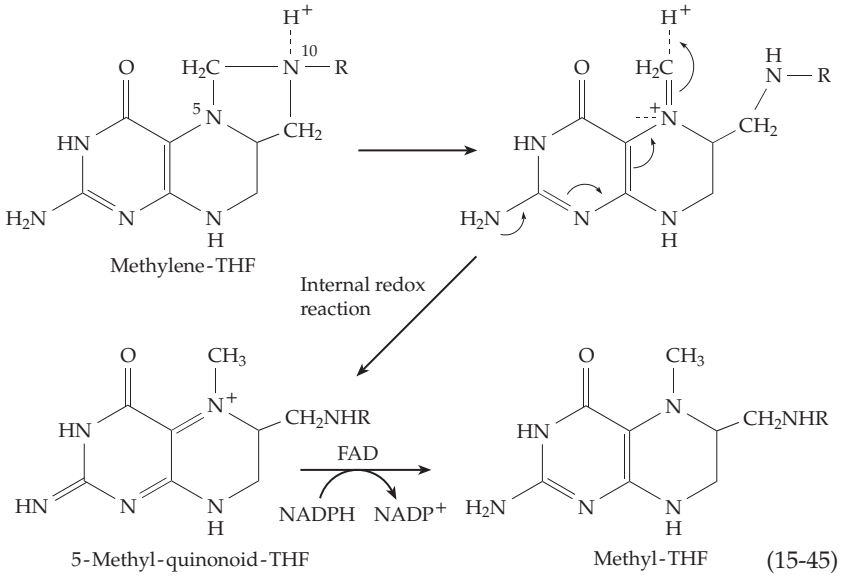
[a] Friedkin, M. (1973) *Adv. Enzymol.* **38**, 235–292
[b] Sahasrabudhe, P. V., and Gmeiner, W. H. (1997) *Biochemistry* **36**, 5981–5991
[c] Ayusawa, D., Shimizu, K., Koyama, H., Takeishi, K., and Seno, T. (1983) *J. Biol. Chem.* **258**, 12448–12454
[d] Huang, X. F., and Arvan, P. (1995) *J. Biol. Chem.* **270**, 20417–20423
[e] Hyatt, D. C., Maley, F., and Montfort, W. R. (1997) *Biochemistry* **36**, 4585–4594
[f] Ivanetich, K. M., and Santi, D. V. (1990) *FASEB J.* **4**, 1591–1597
[g] Shoichet, B. K., Stroud, R. M., Santi, D. V., Kuntz, I. D., and Perry, K. M. (1993) *Science* **259**, 1445–1449
[h] Weichsel, A., Montfort, W. R., Ciesla, J., and Maley, F. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 3493–3497

and suitably infected cells of *Bacillus subtilis* form 5-hydroxymethyl-dUMP.[424,425] These products can be formed by attack of $OH^-$ on quinonoid intermediates of the type postulated for thymidylate synthetase (formed in step *d* of Fig. 15-21).[416] A related reaction is the posttranscriptional conversion of a single uracil residue in tRNA molecules of some bacteria to a thymine ring (a **ribothymidylic acid** residue). In this case, $FADH_2$ is used as a reducing agent to convert the initial adduct of Fig. 15-21 to the ribothymidylic acid and THF.[426]




(15-45)

***Synthesis of methyl groups.*** The reduction of methylene-THF to 5-methyl-THF within all living organisms from bacteria to higher plants and animals provides the methyl groups needed in biosynthesis.[426a] These are required for formation of methionine and, from it, *S*-adenosylmethionine. The latter is used to modify proteins, nucleic acids, and other biochemicals through methylation of specific groups. In the methanogens, reduction of a corresponding methylene derivative of methanopterin gives rise to methane (Section F).

Mammalian methylene-THF reductase is a FAD-containing flavoprotein that utilizes NADPH for the reduction to 5-methyl-THF.[427,428] Matthews[429] suggested that the mechanism of this reaction involves an internal oxidation–reduction reaction that generates a 5-methyl-quinonoid dihydro-THF (Eq. 15-45). Methylene-THF reductase of acetogenic bacteria is also a flavoprotein but it contains Fe–S centers as well. The 237-kDa $\alpha_4\beta_4$ oligomer contains two molecules of FAD and four to six of both Fe and $S^{2-}$ ions.[430,431]

The methyl group of methyl-THF is incorporated into methionine by the vitamin $B_{12}$-dependent methionine synthase which is discussed in Chapter 16. Matthews suggested that methionine synthase may also make use of the 5-methyl-quinonoid-THF of Eq. 15-45. An initial reduction step would precede transfer of the methyl group. Methyl-THF is a precursor to acetate in the acetogenic bacteria.[432] This corrinoid coenzyme-dependent process is also considered in Chapter 16.

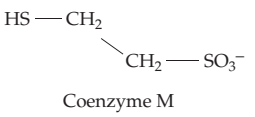
## E. Specialized Coenzymes of Methanogenic Bacteria

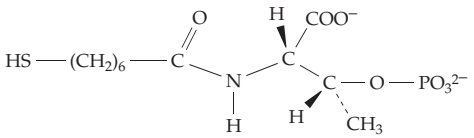
Methane-producing bacteria[433,434] obtain energy by reducing $CO_2$ with molecular hydrogen:

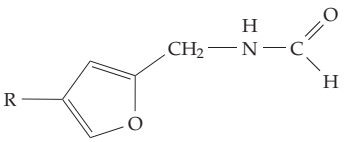
$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \qquad \Delta G^\circ = -131 \text{ kJ / mol} \qquad (15\text{-}46)$$

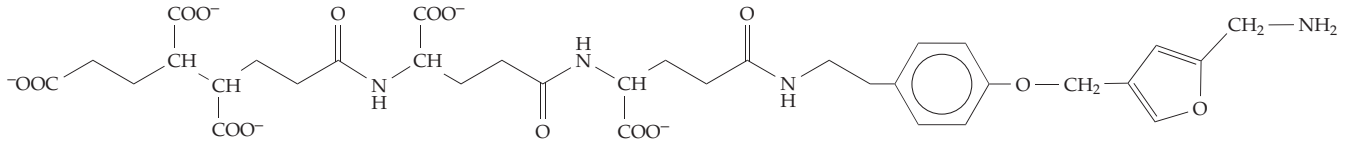
Some species are also able to utilize formate or formaldehyde as reducing agents.[435] These compounds are oxidized to $CO_2$, the reducing equivalents formed being used to reduce $CO_2$ to methane. Carbon monoxide can also be converted to $CO_2$.

$$CO + H_2O \rightarrow CO_2 + H_2 \qquad \Delta G^\circ = -20 \text{ kJ / mol} \qquad (15\text{-}47)$$


Coenzyme M


7-Mercaptoheptanoylthreonine phosphate


Formylmethanofuran


Methanofuran

By using the combined reactions of Eqs. 15-46 and 15-47 the bacteria can subsist on CO alone (Eq. 15-48):

$$4CO + 2\,H_2O \rightarrow 3CO_2 + CH_4 \qquad \Delta G^\circ = -211 \text{ kJ / mol of methane formed} \qquad (15\text{-}48)$$

Some species reduce methanol to methane via Eqs. 15-49 or 15-50.

$$CH_3OH + H_2 \rightarrow CH_4 + H_2O \qquad \Delta G^\circ = -113 \text{ kJ / mol} \qquad (15\text{-}49)$$

$$4CH_3OH \rightarrow 3CH_4 + CO_2 + 2\,H_2O \qquad \Delta G^\circ = -107 \text{ kJ / mol of methane formed} \qquad (15\text{-}50)$$

To accomplish these reactions a surprising variety of specialized cofactors are needed.[351,352,434] The first of these, **coenzyme M**, 2-mercaptoethane sulfonate, was discovered in 1974.[436] It is the simplest known coenzyme. Later, the previously described **5-deazaflavin $F_{420}$** (Section B), a **nickel tetrapyrrole $F_{430}$** (Chapter 16), **methanopterin** (Fig. 15-17),[437] the "carbon dioxide reduction factor" called **methanofuran**,[352,438] and **7-mercaptoheptanoylthreonine phosphate**[439,440] were also identified.

A sketch of the metabolic pathways followed in methane formation is given in Fig. 15-22.[352,435] In the first step (*a*) the amino group of methanofuran is thought to add to $CO_2$ to form a carbamate which is reduced to formylmethanofuran by $H_2$ and an intermediate carrier $H_2X$ in step *b*. The formyl group is then transferred to tetrahydromethanopterin ($H_4$MPT) (step *c*)[440a] and is cyclized and reduced in two stages in steps *d*, *e*, and *f*. The reductant is the deazaflavin $F_{420}$ and the reactions parallel those for conversion of formyl-THF to methyl-THF (Fig. 15-18).[431,440b,441] The methyl group of methyl-$H_4$MPT is then transferred to the sulfur atom of the thiolate anion of coenzyme M, from which it is reduced off as $CH_4$. This is a complex process requiring the nickel-containing $F_{430}$, FAD, and 7-mercaptoheptanoylthreonine phosphate (HS-HTP).

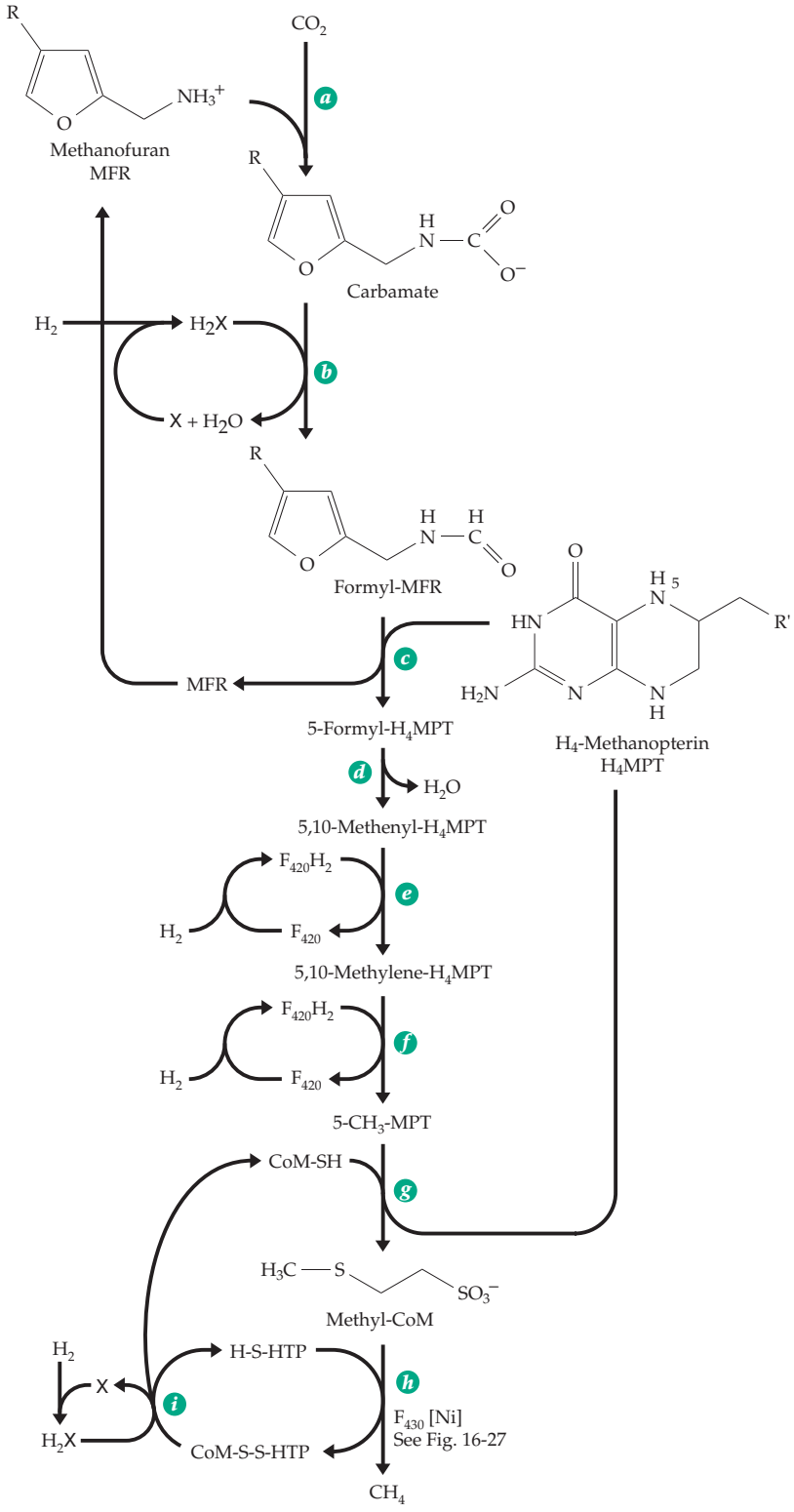



**Figure 15-22** Tentative scheme for reduction of carbon dioxide to methane by methanogens. After Rouvière *et al.*[352] and Thauer *et al.*[435]

The HS-HTP may be the 2-electron donor for the reduction. The mixed-disulfide CoM-S-S-HTP appears to be an intermediate and also an allosteric effector for the first step, the reduction of $CO_2$ to formylmethanofuran. A complex of proteins that is unstable in oxygen is also needed. The principal component of the methyl reductase binds two molecules

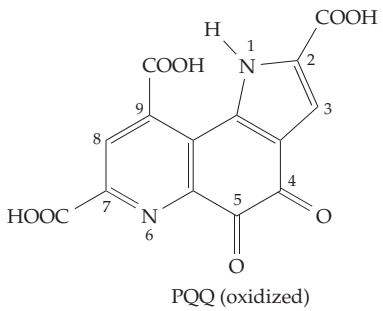
of the nickel-containing $F_{430}$. It also binds two moles of CoM-SH noncovalently. The binding process requires ATP as well as the presence of other proteins, notably those of the hydrogenase system.

Coenzyme M, previously found only in methanogenic archaeobacteria, has recently been discovered in both gram-negative and gram-positive alkene-oxidizing eubacteria. It seems to function in cleavage of epoxy rings and as a carrier of hydroxyalkyl groups.[441a] See also Chapter 17.

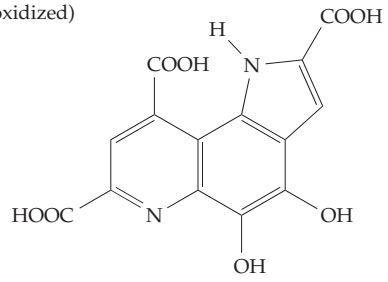
## F. Quinones, Hydroquinones, and Tocopherols

### 1. Pyrroloquinoline Quinone (PQQ)

Bacteria that oxidize methane or methanol (**methylotrophs**) employ a periplasmic methanol dehydrogenase that contains as a bound coenzyme, the pyrroloquinoline quinone designated **PQQ** or methoxatin (Eq. 15-51).[442–444] This fluorescent *ortho*-quinone




PQQ (oxidized)

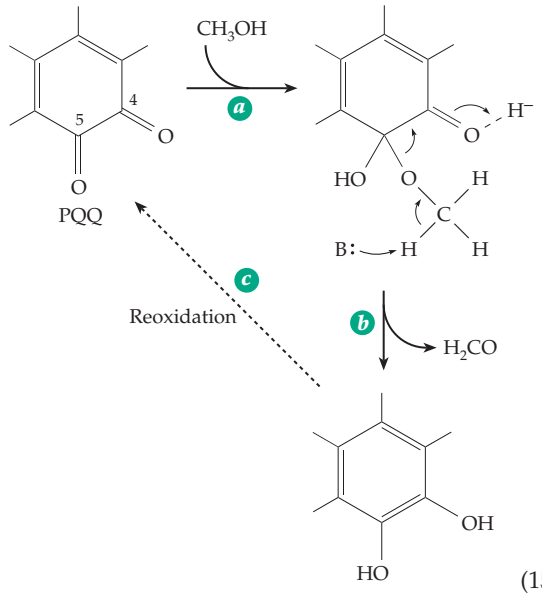



$PQQH_2$ (reduced)

is released when the protein is denatured. Some other bacterial alcohol dehydrogenases[445,445a] and glucose dehydrogenases[446-446d] contain the same cofactor.

PQQ-containing methanol dehydrogenase from *Methylophilus* is a dimer of a 640-residue protein consisting of two disulfide-linked peptide chains with one noncovalently bound PQQ.[447] The large subunit contains a β propellor (Fig. 15-23), which is similar to that in the protein $G_i$ shown in Fig. 11-7. The PQQ binding site lies above the propellor and is formed by a series of loops. The coenzyme interacts with several polar protein side chains and with a bound calcium ion.[448] Bacterial PQQ-dependent glucose dehydrogenases have also been studied intensively. The 450-residue soluble enzyme from *Acinetobacter calcoaceticus* is partially homologous to the methanol dehydrogenase.[446a,446b]

PQQ and the other quinone prosthetic groups described here all function in reactions that would be possible for pyridine nucleotide or flavin coenzymes. All of them, like the flavins, can exist in oxidized, half-reduced semiquinone and fully reduced dihydro forms. The questions to be asked are the same as we asked for flavins. How do the substrates react? How is the reduced cofactor reoxidized? In nonenzymatic reactions alcohols, amines, and enolate anions all add at C-5 of PQQ to give adducts such as that shown for methanol in Eq. 15-51, step *a*.[444,449,449a] Although many additional reactions are possible, this addition is a reasonable first step in the mechanism shown in Eq. 15-51. An enzymatic base could remove a proton as is indicated in step *b* to give $PQQH_2$. The pathway for reoxidation (step *c*) might involve a cytochrome *b*, cytochrome *c*, or bound ubiquinone.[445,446]




(15-51)

Although the soluble PQQ-dependent glucose dehydrogenase forms, with methylhydrazine, an adduct similar to that depicted in Eq. 15-51,[449b] the structure of a glucose complex of the $PPQH_2$- and $Ca^{2+}$-containing enzyme at a resolution of 0.2 nm suggests a direct hydride ion transfer. The only base close to glucose C1 is His 144. The C1-H proton lies directly above the $PQQH_2$ C5 atom. Oubrie *et al.*[446a] propose a deprotonation of the C1-OH by His 144 and $H^-$ transfer to C5 of PQQ followed by tautomerization (Eq. 15-52). Theoretical calculations by Zheng and Bruice[449c] favor a simpler mechanism with hydride transfer to the oxygen atom of the C4 carbonyl (green arrows). In either case, the $Ca^{2+}$ would assist by polarizing the C5 carbonyl group.

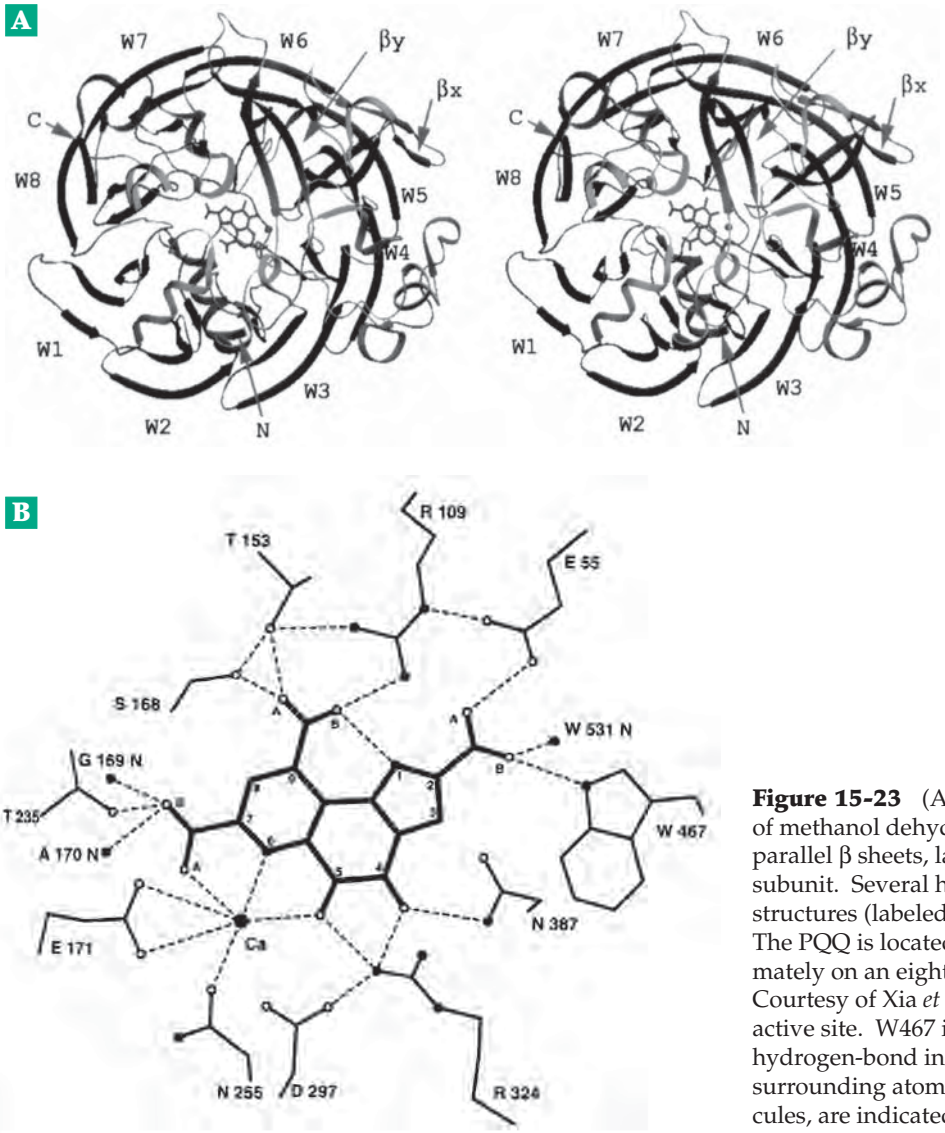
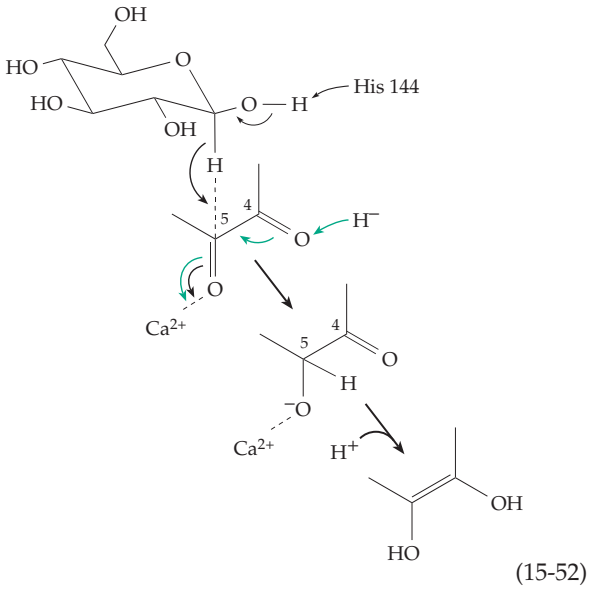



**Figure 15-23** (A) Stereoscopic view of the H subunit of methanol dehydrogenase. Eight four-stranded antiparallel β sheets, labeled W1–W8, form the base of the subunit. Several helices and two additional β-sheet structures (labeled βx and βy) form a cap over the base. The PQQ is located in a funnel within the cap approximately on an eight-fold axis of pseudosymmetry. Courtesy of Xia *et al.*[447] (B) Schematic view of the active site. W467 is parallel to the plane of PQQ. All hydrogen-bond interactions between PQQ and its surrounding atoms, except for the three water molecules, are indicated. Courtesy of White *et al.*[448]



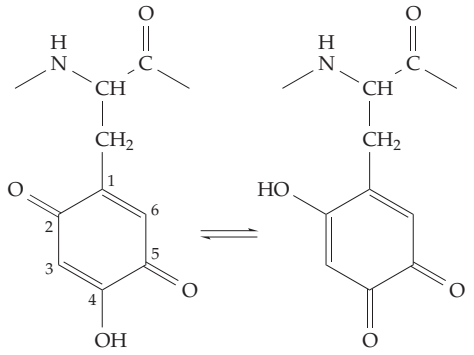

(15-52)

## 2. Copper Amine Oxidases

At first it appeared that PQQ had a broad distribution in enzymes, including eukaryotic amine oxidases. However, it was discovered, after considerable effort, that there are additional quinone cofactors that function in oxidation of amines. These are derivatives of tyrosyl groups of specific enzyme proteins. Together with enzymes containing bound PQQ they are often called **quinoproteins**.[450–454]

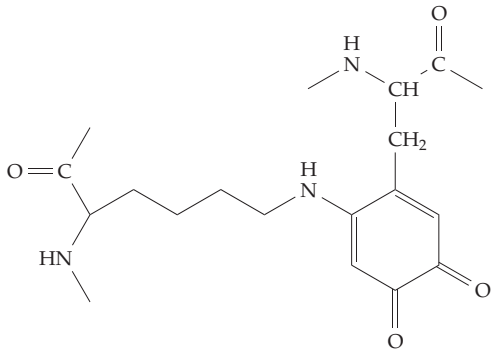
***Topaquinone (TPQ).*** Both bacteria and eukaryotes contain amine oxidases that utilize bound copper ions and $O_2$ as electron acceptors and form an aldehyde, $NH_3$, and $H_2O_2$. The presence of an organic cofactor was suggested by the absorption spectra which was variously attributed to pyridoxal phosphate or PQQ. However, isolation from the active site of bovine serum

amine oxidase established the structure of the reduced form of the cofactor as a trihydroxyphenylalanyl group covalently linked in the peptide chain.[455,456] Its oxidized form is called topaquinone:



Topaquinone (TPQ)

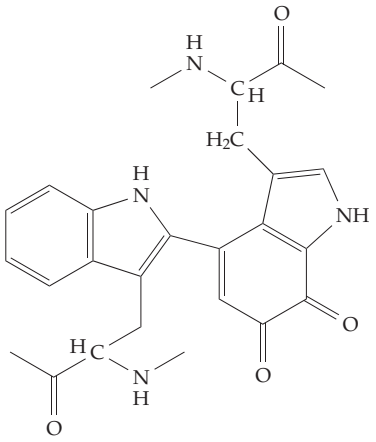
The same cofactor is present in an amine oxidase from *E. coli*[457–460] and in other bacterial[461–463] fungal,[463a] plant,[464] and mammalian[464a] amine oxidases.



Lysine tyrosylquinone (LTQ)

***Lysine tyrosylquinone (LTQ).*** Another copper amine oxidase, **lysyl oxidase**, which oxidizes side chains of lysine in collagen and elastin (Eq. 8-8) contains a cofactor that has been identified as having a lysyl group of a different segment of the protein in place of the –OH in the 2 position of topaquinone.[465] Lysyl oxidase plays an essential role in the crosslinking of collagen and elastin.

***Tryptophan tryptophanylquinone (TTQ).*** This recently discovered quinone cofactor is similar to the lysyl tyrosylquinone but is formed from two tryptophanyl side chains.[466] It has been found in **methylamine dehydrogenase** from methylotrophic gram-negative bacteria[467–469] and also in a bacterial aromatic amine dehydrogenase.[470]

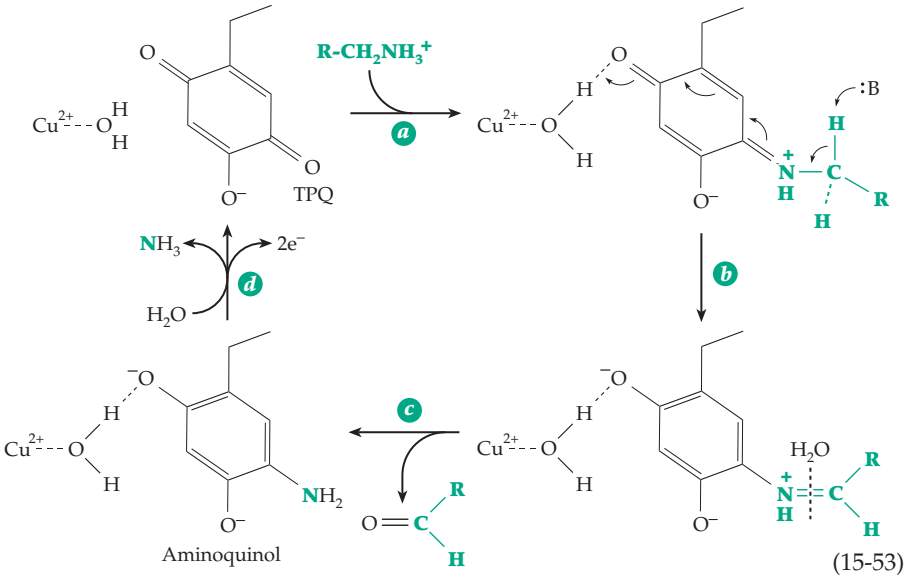


Tryptophan tryptophylquinone (TTQ)

***Three-dimensional structures.*** The TPQ-containing amine oxidase from *E. coli* is a dimer of 727-residue subunits with one molecule of TPQ at position 402 in each subunit.[457,458] Methylamine dehydrogenase is also a large dimeric protein of two large 46.7-kDa subunits and two small 15.5-kDa subunits. Each large subunit contains a TTQ cofactor group.[468,471,471a] Reduced TTQ is reoxidized by the 12.5-kDa blue copper protein **amicyanin**. Crystal structures have been determined for complexes of methylamine dehydrogenase with amicyanin[471] and of these two proteins with a third protein, a small bacterial cytochrome *c*.[472,472a]

***Mechanisms.*** Studies of model reactions[473–476] and of electronic, Raman,[466,477,478] ESR,[479,480] and NMR spectra and kinetics[481] have contributed to an understanding of these enzymes.[459,461,464,482,483] For these copper amine oxidases the experimental evidence suggests an aminotransferase mechanism.[450,453,474,474a-d] The structure of the *E.coli* oxidase shows that a single copper ion is bound by three histidine imidazoles and is located adjacent to the TPQ (Eq. 15-53). Asp 383 is a conserved residue that may be the catalytic base in Eq. 15-53.[474b] A similar mechanism can be invoked for LTQ and TTQ.

How is the reduced cofactor reoxidized? Presumably the copper ion adjacent to the TPQ functions in this process, passing electrons one at a time to the next carrier in a chain. There is no copper in the TTQ-containing subunits. Electrons apparently must jump about 1.6 nm to the copper ion of amicyanin, then another 2.5 nm to the iron ion of the cytochrome *c*.[472] Reoxidation of the aminoquinol formed in Eq. 15-53, step *d*, yields a Schiff base whose hydrolysis will release ammonia and regenerate the TTQ. Intermediate states with $Cu^+$ and a TTQ semiquinone radical have been observed.[483a]




(15-53)

## 3. Ubiquinones, Plastoquinones, Tocopherols, and Vitamin K

In 1955, R. A. Morton and associates in Liverpool announced the isolation of a quinone which they named ubiquinone for its ubiquitous occurrence.[484,485] It was characterized as a derivative of benzoquinone attached to an unsaturated polyprenyl (isoprenoid) side chain (Fig. 15-24). In fact, there is a family of ubiquinones: that from bacteria typically contains six prenyl units in its side chain, while most ubiquinones from mammalian mitochondria contain ten. Ubiquinone was also isolated by F. L. Crane and associates using isooctane extraction of mitochondria. These workers proposed that the new quinone, which they called **coenzyme Q**, might participate in electron transport. As is described in Chapter 18, this function has been fully established. Both the name ubiquinone and the abbreviation Q are in general use. A subscript indicates the number of prenyl units, e.g., $Q_{10}$. Ubiquinones can be reversibly reduced to the hydroquinone forms (Fig. 15-24), providing a basis for their function in electron transport within mitochondria and chloroplasts.[486–490]

While vitamin E (Box 15-G) and vitamin K (Box 15-F) are dietary essentials for humans, ubiquinone is apparently not. Animals are able to make ubiquinones in quantities adequate to meet the need for this essential component of mitochondria. However, an extra dietary supplement may sometimes be of value.[491] A reduced level of ubiquinone has been reported in gum tissues of patients with periodontal disease.[492] Ubiquinones are being tested as possible protectants against heart damage caused by lack of adequate oxygen or by drugs.[491]

A closely related series of **plastoquinones** occur in chloroplasts (Chapter 23). In these compounds the two methoxyl groups of ubiquinone are replaced by methyl groups (Fig. 15-24). The most abundant of these compounds, plastoquinone A, contains nine prenyl units.[493,494]

Addition of the hydroxyl group of the reduced ubiquinone or plastoquinone to the adjacent double bond leads to a chroman-6-ol structure. The compounds derived from ubiquinone in this way are called **ubichromanols** (Fig. 15-24). The corresponding ubichromenol (Fig. 15-24) has been isolated from human kidney. **Plastochromanols** are derived from plastoquinone. That from plastoquinone A, first isolated from tobacco, is also known as solanochromene.

A closely related and important family of chromanols are the **tocopherols** or vitamins E (Fig. 15-24, Box 15-G). Tocopherols are plant products found primarily in plant oils and are essential to proper nutrition of humans and other animals. α-Tocopherol is the most abundant form of the vitamin E family; smaller amounts of the β, δ, and γ forms occur, as do a series of **tocotrienols** which contain unsaturated isoprenoid units.[495] The configuration of α-tocopherol is 2*R*,4'*R*,8'*R* as indicated in Fig. 15-24. When α-tocopherol is oxidized, e.g., with ferric chloride, the ring can be opened by hydrolysis to give **tocopherolquinones** (Fig. 15-24), which can in turn be reduced to tocopherolhydroquinones. Large amounts of the tocopherolquinones have been found in chloroplasts.

Another important family of quinones, related in structure to those already discussed, are the **vitamins K** (Fig. 15-24, Box 15-F). These occur naturally as two families. The vitamins $K_1$ (**phylloquinones**) have only one double bond in the side chain and that is in the prenyl unit closest to the ring. This suggests again the possibility of chromanol formation. In the vitamin $K_2$ (**menaquinone**) series, a double bond is present in each of the prenyl units. A synthetic compound **menadione** completely lacks the polyprenyl side chain and bears a hydrogen in the corresponding position on the ring. Nevertheless, menadione serves as a synthetic vitamin K, apparently because it can be converted in the body to forms containing polyprenyl side chains.

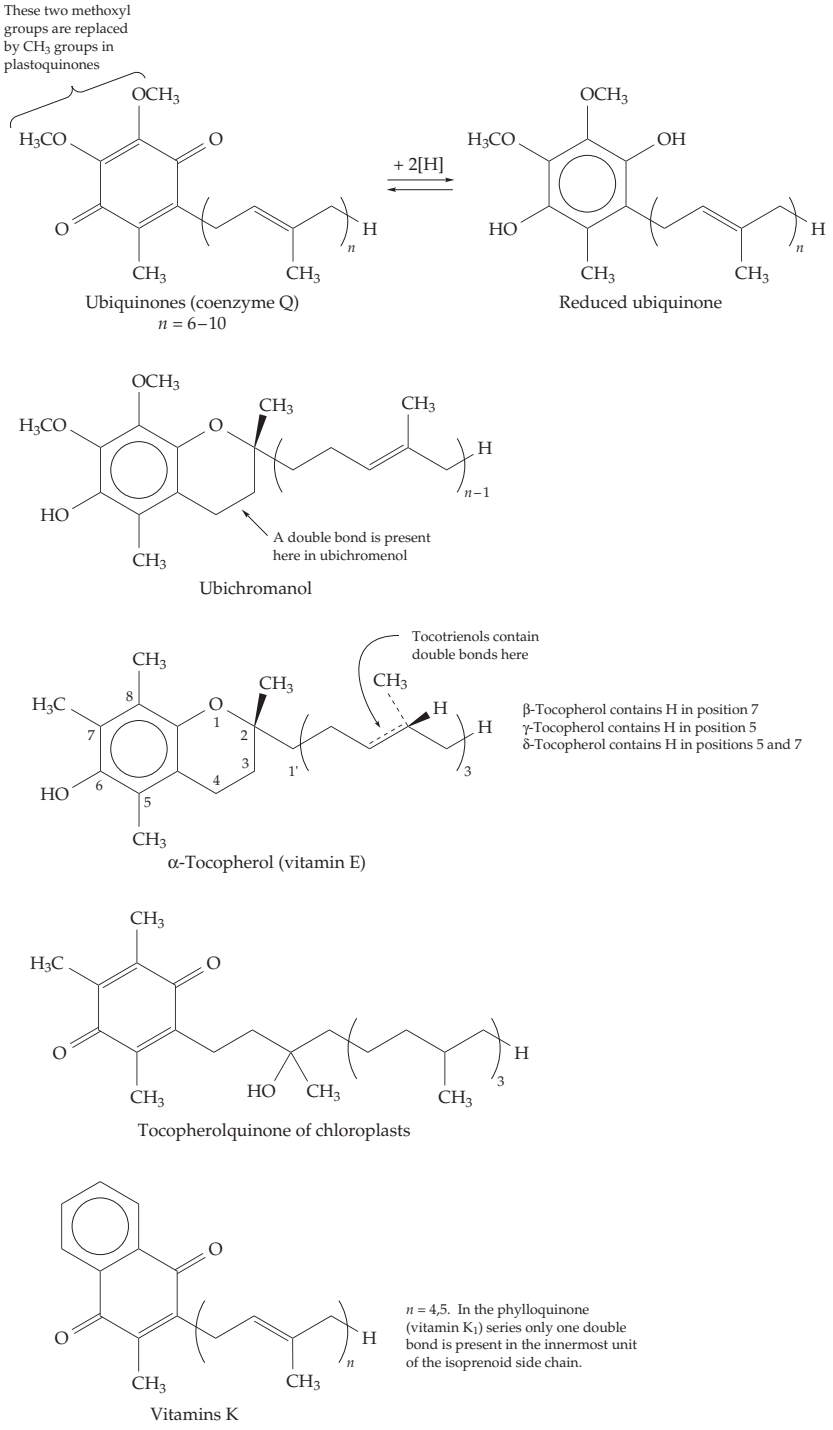
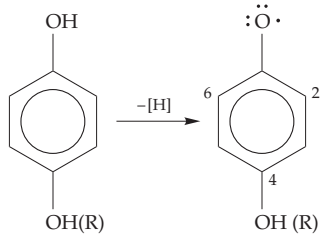



**Figure 15-24** Structures of the isoprenoid quinones and vitamin E.

these membranes. Within some mycobacteria vitamin K apparently participates in electron transport chains in the same way (see Chapter 18). Some bacteria contain both menaquinones and ubiquinones.

The vitamin E derivative **α-tocopherolquinone** (Fig. 15-24) can also serve as an electron carrier, being reversibly reduced to the hydroquinone form α-tocopherolquinol. Such a function has been proposed for the anaerobic rumen bacterium *Butyrovibrio fibrisolvens*.[497]

When a single hydrogen atom is removed from a hydroquinone or from a chromanol such as a tocopherol, a free radical is formed (Eq. 15-54). Phenols substituted in the 2, 4, and 6 positions give especially stable radicals.




(15-54)

Both the presence of methyl substituents in the tocopherols and their chromanol structures increase the ability of these compounds to form relatively stable radicals.[498,499] This ability is doubtless probably important also in the function of ubiquinones and plastoquinones. Ubiquinone radicals (semiquinones) are probably intermediates in mitochondrial electron transport (Chapter 18) and radicals amounting to as much as 40% of the total ubiquinone in the NADH-ubiquinone reductase of heart mitochondria have been detected by EPR measurements.[500,501]

## 4. Quinones as Electron Carriers

Ubiquinones function as electron transport agents within the inner mitochondrial membranes[496] and also within the reaction centers of the photosynthetic membranes of bacteria (Eq. 23-32).[484,488,494] The plastoquinones also function in electron transport within

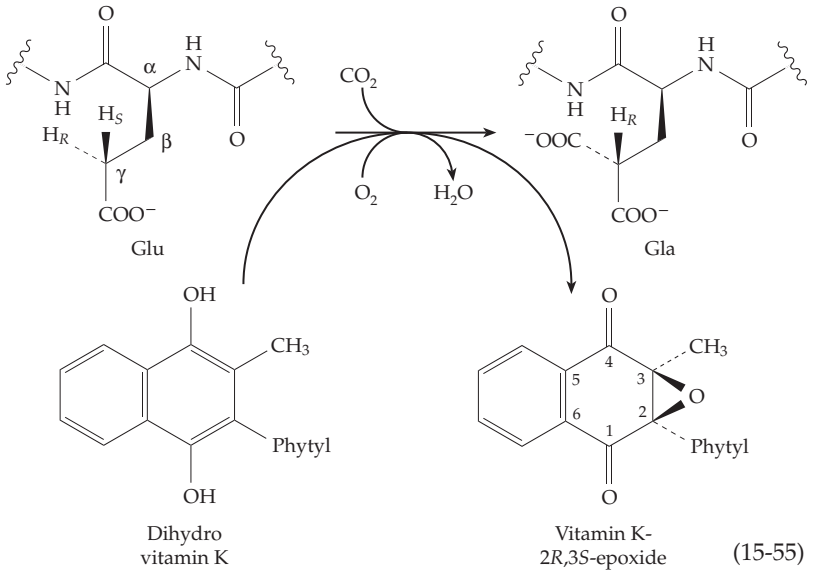
The equilibria governing semiquinone formation from quinones are similar to those for the flavin semiquinones which were discussed in Section B,6. Two consecutive one-electron redox steps can be defined. Their redox potentials will vary with pH because of a p$K_a$ for the semiquinone in the pH 4.5–6.5 region. For ubiquinone this p$K_a$ is about 4.9 in water and 6.45 in methanol. A p$K_a$ of over 13 in the

hydroquinone form[502] will have little effect on redox potentials near pH 7. The potential for the one-electron reaction $Q + e^- \rightarrow Q^-$ is evaluated most readily. For this reaction $E°'$ (pH 7) is −0.074, −0.13, −0.17, and −0.23 for 2,3-dimethylbenzoquinone, plastoquinone, ubiquinone, and phylloquinone, respectively.

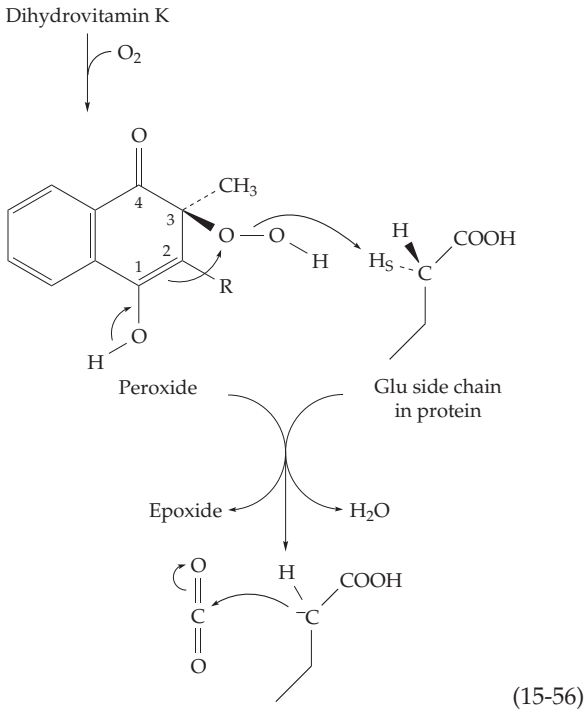
Why does this entire family of compounds have the long polyprenyl side chains? A simple answer is that they serve to anchor the compounds in the lipid portion of the cell membranes where they function. In the case of ubiquinones both the oxidized and the reduced forms may move freely through the lipid phase shuttling electrons between carriers.

## 5. Vitamin K and γ-Carboxyglutamate Formation

In higher animals the only known function of vitamin K is in the synthesis of γ-carboxyglutamate (Gla)-containing proteins, several of which are needed in blood clotting (Box 15-F, Chapter 12). Following the discovery of γ-carboxyglutamate, it was shown that liver microsomes were able to incorporate $^{14}C$-containing bicarbonate or $CO_2$ into the Gla of prothrombin and could also generate Gla in certain simple peptides such as Phe-Leu-Glu-Val. Three enzymes are required. All are probably bound to the microsomal membranes.[503–507a] An NADPH-dependent reductase reduces vitamin K quinone to its hydroquinone form. Conversion of Glu residues to Gla residues requires this reduced vitamin K as well as $O_2$ and $CO_2$. During the carboxylation reaction the reduced vitamin K is converted into vitamin K 2,3-epoxide (Eq. 15-55).[508] The mechanism is uncertain but a peroxide intermediate such as that shown in Eq. 15-56 is probably involved. This could be used to generate a hydroxide ion adjacent to the *pro-S* -H of the glutamate side chain of the substrate. This hydrogen could be abstracted by the $OH^-$ to form



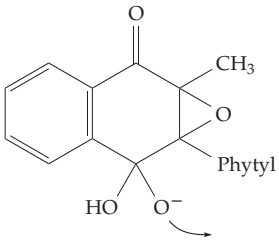

(15-55)




(15-56)

$H_2O$ and a carbanion which would be stabilized by the adjacent carboxyl group[508–511] (Eq. 15-56).

Dowd and coworkers raised doubts that a hydroxide ion released in the active site in this manner is a strong enough base to generate the anion shown in Eq. 15-52.[512,513] They hypothesized a *"base strength amplification" mechanism* that begins with a peroxide formed at C-4 followed by ring closure to form a dioxetane and rearrangement to the following hypothetical strong base.[507,512,514]



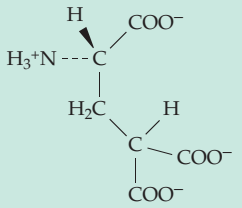

The proposal was supported by model experiments and also by observation of some incorporation of both atoms of $^{18}O_2$ into vitamin K epoxide. However, theoretical calculations support the simpler mechanism of Eq. 15-56.

The fact that L-*threo*-γ-fluoroglutamate residues, in which the fluorine atom is in the position corresponding to the *pro-S* hydrogen, are not carboxylated but that an *erythro*-γ-fluoroglutamate is carboxylated

## BOX 15-F THE VITAMIN K FAMILY

The existence of an "antihemorrhagic factor" required in the diet of chicks to ensure rapid clotting of blood was reported in 1929 by Henrik Dam at the University of Copenhagen.[a,b] The fat-soluble material, later designated vitamin K, causes a prompt (2–6 h) decrease in the clotting time when administered to deficient animals and birds. The clotting time for a vitamin K-deficient chick may be greater than 240 s, but 6 h after injection of 2 μg of vitamin $K_3$ it falls to 50–100 s.[c] Pure vitamin K (Fig. 15-24), a 1,4-naphthoquinone, was isolated from alfalfa in 1939. Within a short time two series, the phylloquinones (vitamin $K_1$) and the menaquinones (vitamin $K_2$), were recognized. The most prominant phylloquinone contains the phytyl group, which is also present in the chlorophylls. For a human being a dietary intake of about 30 μg per day is recommended.[d] Additional vitamin K is normally supplied by intestinal bacteria.

The most obvious effect of a deficiency in vitamin K in animals is delayed blood clotting, which has been traced to a decrease in the activity of **prothrombin** and of clotting factors VII, IX, and X (Chapter 12, Fig. 12-17). Prothrombin formed by the liver in the absence of vitamin K lacks the ability to chelate calcium ions essential for the binding of prothrombin to phospholipids and to its activation to thrombin. The structural differences between this abnormal protein and the normal prothrombin have been pinpointed at the N terminus of the ~560 residue glycoprotein.[e,f] Tryptic peptides from the N termini differed in electrophoretic mobility. As detailed in Chapter 12, ten residues within the first 33, which were identified as glutamate residues by the sequence analysis on normal prothrombin, are actually **γ-carboxyglutamate** (Gla). The same amino acid is present near the N termini of clotting factors VII, IX, and X.

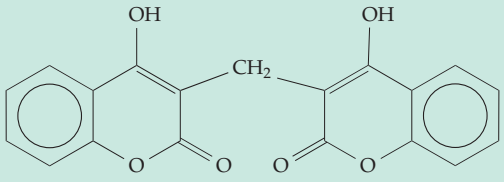


γ-Carboxyglutamate (Gla)

The fact that γ-carboxyglutamate had not been identified previously as a protein substituent is explained by its easy decarboxylation to glutamic acid during treatment with strong acid. The function of vitamin K is to assist in the incorporation of the additional carboxyl group into the glutamate residues of preformed prothrombin and other blood-clotting factors[g,h] with a resulting increase in calcium ion affinity.

Four other plasma proteins designated C, S, M, and Z contain γ-carboxyglutamate. The functions of proteins M and Z are unknown but protein C is a serine protease involved in regulation of blood coagulation and protein S is a cofactor that assists the action of protein C. Other proteins that require vitamin K for synthesis include the 49-residue **bone Gla protein** (or **osteocalcin**) and the 79-residue **matrix Gla protein** found in bone and cartilage.[i,j] These proteins contain three and five residues of Gla, respectively. Their possible functions in mineralization are considered in Chapter 8. At least two additional small human proline-rich Gla proteins of unknown function are synthesized in many tissues.[k] Gamma-carboxyglutamate also occurs in an invertebrate peptide from fish-hunting cone snails. This "sleeper peptide," which induces sleep in mice after intracerebral injection, has the sequence GEE*E*LQE*NQE*LIRE*KSN. Here E* designates the 5 residues of Gla.[l]

An interesting facet of vitamin K nutrition and metabolism was revealed by the observation that cattle fed on spoiled sweet clover develop a fatal hemorrhagic disease. The causative agent is **dicoumarol**, a compound arising from coumarin, a natural constituent of clover. Dicourmarol and the closely related synthetic **warfarin** are both potent vitamin K antagonists. Warfarin is used both as a rat poison and in the treatment of thromboembolic disease. As rodenticides hydroxycourmarin derivatives are usually safe because a single accidental ingestion by a child or pet does little harm, whereas regular ingestion by rodents is fatal.



Dicoumarol


[a] Wasserman, R. H. (1972) *Ann. Rev. Biochem.* **41**, 179–202
[b] Tim Kim, X. (1979) *Trends Biochem. Sci.* **4**, 118–119
[c] Olson, R. E. (1964) *Science* **45**, 926–928
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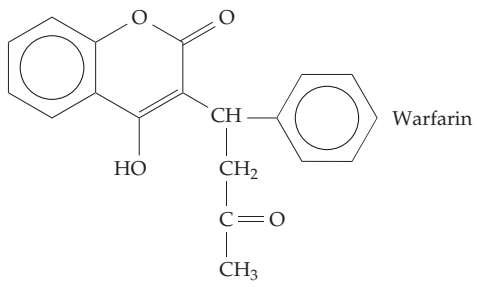
[e] Friedman, P. A. (1984) *N. Engl. J. Med.* **310**, 1458–1460
[f] Stenflo, J. (1976) *J. Biol. Chem.* **251**, 355–363
[g] Wood, G. M., and Suttie, J. W. (1988) *J. Biol. Chem.* **263**, 3234–3239
[h] Wu, S.-M., Mutucumarana, V. P., Geromanos, S., and Stafford, D. W. (1997) *J. Biol. Chem.* **272**, 11718–11722
[i] Price, P. A., and Williamson, M. K. (1985) *J. Biol. Chem.* **260**, 14971–14975
[j] Price, P. A., Rice, J. S., and Williamson, M. K. (1994) *Protein Sci.* **3**, 822–830
[k] Kulman, J. D., Harris, J. E., Haldeman, B. A., Davie, E. W. (1997) *Proc. Natl. Acad. Sci., USA* **94**, 9058–9062
[l] Prorok, M., Warder, S. E., Blandl, T., and Castellino, F. J. (1996) *Biochemistry* **35**, 16528–16534

suggested the indicated stereospecificity.[515,516] This was confirmed by observation of a kinetic isotope effect when $^2H$ is present in the *pro-S* position.[517] Addition of the carbanion to $CO_2$ would generate the Gla residue. The two glutamates in the following sequence, in which X may be various amino acids, are carboxylated if the protein also carries a suitable N-terminal signal sequence: EXXXEXC. If a suitable glutamyl peptide is not available for carboxylation the postulated peroxide intermediate (Eq. 15-55) is still converted slowly to the 2,3-epoxide of vitamin K.

A third enzyme is required to reduce the epoxide to vitamin K (Eq. 15-57). The biological reductant is uncertain but dithiols such as dithiothreitol serve in the laboratory.[518] See also Eq. 18-47. Protonation of an intermediate enolate anion would give 3-hydroxy-2, 3-dihydrovitamin K, an observed side reaction product.

$$\text{Vitamin K epoxide} + 2[H] \rightarrow H_2O + \text{Vitamin K} \qquad (15\text{-}57)$$

This reaction is of interest because of its specific inhibition by such coumarin derivatives as Warfarin:



Warfarin

This synthetic compound, as well as natural coumarin anticoagulants (Box 15-F), inhibits both the vitamin K reductase and the epoxide reductase.[518,519] The matter is of considerable practical importance because of the spread of warfarin-resistant rats in Europe and the United States. One resistance mutation has altered the vitamin K epoxide reductase so that it is much less susceptible to inhibition by warfarin.[519,520]

While glutamate residues in peptides of appropriate sequence are carboxylated by the vitamin K-dependent system, aspartate peptides scarcely react.[503] Beta-carboxyaspartate is present in protein C of the blood anticoagulant system (Fig. 12-17)[521] and in various other proteins containing EGF homology domains (Table 7-3),[522] but the mechanism of its formation is unknown.

### 6. Tocopherols (Vitamin E) as Antioxidants

The major function of the tocopherols is thought to be the protection of phospholipids of cell membranes against oxidative attack by free radicals and organic peroxides. Peroxidation of lipids, which is described in Chapter 21, can lead to rapid development of rancidity in fats and oils. However, the presence of a small amount of tocopherol inhibits this decomposition, presumably by trapping the intermediate radicals in the form of the more stable tocopherol radicals (Eq. 15-54) which may dimerize or react with other radicals to terminate the chain. Even though only one molecule of tocopherol is present for a thousand molecules of phospholipid, it is enough to protect membranes.[523] That vitamin E does function in this way is supported by the observation that much of the tocopherol requirement of some species can be replaced by *N,N'*-diphenyl-*p*-phenylenediamine, a synthetic antioxidant (see Table 18-5 for the structure of a related substance). Three generations of rats have been raised on a tocopherol-free diet containing this synthetic antioxidant. However, not all of the deficiency symptoms are prevented.

The antioxidant role of α-tocopherol in membranes is generally accepted.[524–526] It is thought to be critical to defense against oxidative injury and to help the body combat the development of tumors and to slow aging. Gamma-tocopherol may be more reactive than α-tocopherol in removing radicals created by NO and other nitrogen oxides.[526] Its actions are strongly linked to those of ascorbic acid (Box 18-D) and selenium. Ascorbate may reduce tocopherol semiquinone radicals, while selenium acts to enhance breakdown of peroxides as described in the next section.

## G. Selenium-Containing Enzymes

In 1957, Schwartz and associates showed that the toxic element selenium was also a nutritional factor essential for prevention of the death of liver cells in rats.[527] Liver necrosis would be prevented by as little as 0.1 ppm of selenium in the diet. Similar amounts of selenium were shown to prevent a muscular dystrophy called "white muscle disease" in cattle and sheep grazing on selenium-deficient soil. Sodium selenite and other inorganic selenium compounds were more effective than organic compounds in which Se had replaced sulfur. **Keshan disease**, an often fatal heart condition that is prevalent among childen in Se-deficient regions of China, can be prevented by supplementation of the diet with $NaSeO_3$.[528] Even the little crustacean "water flea" *Daphnia* needs 0.1 part per billion of Se in its water.[529]

Selenium has long been known to enhance the antioxidant activity of vitamin E. Recent work suggests that vitamin E acts as a radical scavenger, preventing

## BOX 15-G VITAMIN E: THE TOCOPHEROLS

Vitamin E was recognized in 1926 as a factor preventing sterility in rats that had been fed rancid lipids.[a–e] The curative factor, present in high concentration in wheat germ and lettuce seed oils, is a family of vitamin E compounds, the tocopherols (Fig. 15-24). The first of these was isolated by Evans and associates in 1936. Vitamin E deficiency in the rabbit or rat is accompanied by muscular degeneration (**nutritional muscular dystrophy**; see also Box 15-A) and a variety of other symptoms that vary from one species to another. Animals deficient in vitamin E display obvious physical deterioration followed by sudden death. Muscles of deficient rats show abnormally high rates of oxygen uptake, and abnormalities appear in the membranes of the endoplasmic reticulum as viewed with the electron microscope. It is thought that deterioration of lysosomal membranes may be the immediate cause of death.

The tocopherol requirement of humans is not known with certainty, but about 5 mg (7.5 IU) / day plus an additional 0.6 mg for each gram of polyunsaturated fatty acid consumed may be adequate. It is estimated that the average daily intake is about 14 mg, but the increasing use of highly refined foods may lead to dangerously low consumption. Recent interest[d–f] has been aroused by studies that show that much larger amounts of vitamin E (e.g., 100–400 mg/day) substantially reduce the risk of coronary disease and stroke in both women[g] and men[h] and also decrease oxidative modification of brain proteins.[i] The decrease in heart attacks and stroke may be in part an indirect effect of the anticlotting action of vitamin E quinone.[f] Plant oils are usually the richest sources of tocopherols, while animal products contain lower quantities.

To some extent the vitamin E requirement may be lessened by the presence in the diet of synthetic antioxidants and by selenium. Much evidence supports a relationship between the nutritional need for selenium and that for vitamin E. Lack of either causes muscular dystrophy in many animals as well as severe edema (exudative diathesis) in chicks. Since vitamin E-deficient rats have a low selenide ($Se^{2-}$) content, it has been suggested that vitamin E protects reduced selenium from oxidation.[j] Vitamin C (ascorbic acid), in turn, protects vitamin E.

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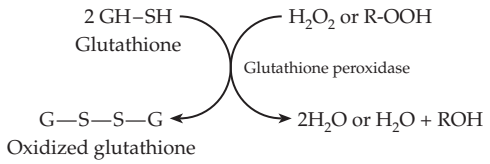
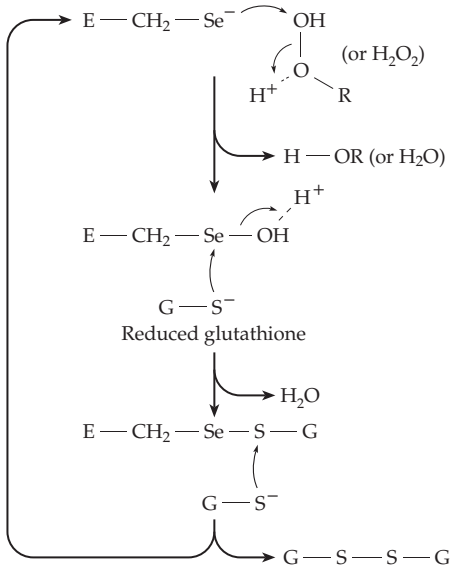
excessive peroxidation of membrane lipids, while selenium, in the enzyme **glutathione peroxidase**, acts to destroy the small amounts of peroxides that do form. This was the first established function of selenium in human beings, but there are others. If we include proteins from animals and bacteria, at least ten selenoproteins are known (Table 15-4).[530–534] Seven of these are enzymes and most catalyze redox processes. The active sites most often contain **selenocysteine**, whose selenol side chain is more acidic ($pK_a$ ~5.2) than that of cysteine and exists as $-CH_2-Se^-$ at neutral pH.[530]

Glutathione peroxidases catalyze the reductive decomposition of $H_2O_2$ or of organic peroxides by glutathione (G–SH) according to Eq. 15-58. At least three isoenzyme forms have been identified in mammals: a cellular form,[531,535–537] a plasma form, and a



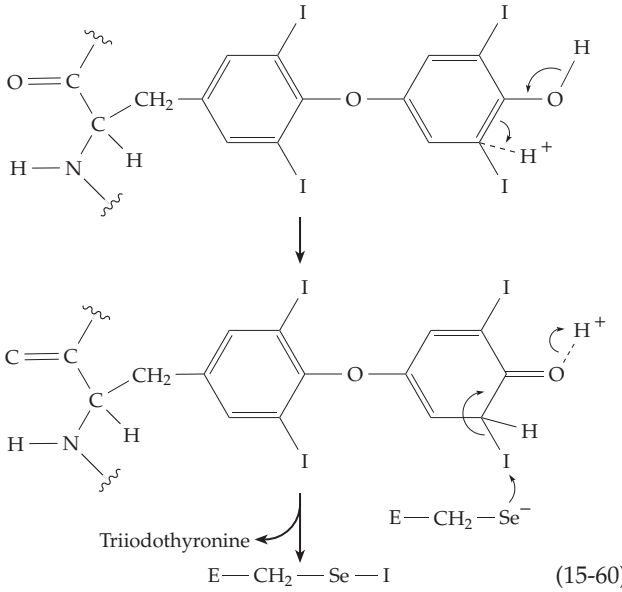

(15-58)

form with a preference for organic peroxides derived from phospholipids.[327,538–540] A related selenoprotein has been found in a human poxvirus.[540a] Selenocysteine is present at a position (residues 41–47) near the N terminus of an α helix, in the ~ 180-residue polypeptides. A possible reaction mechanism involves attack by the selenol on the peroxide to give a selenic acid intermediate which is reduced by glutathione in two nucleophilic displacement steps (Eq. 15-59).




(15-59)

Three types of **iodothyronine deiodinase** remove iodine atoms from thyroxine to form the active thyroid hormone triiodothyronine and also to inactivate the hormone by removing additional iodine[531,541–546] (see also Chapter 25). In this case the $-CH_2-Se^-$ may attach the iodine atom, removing it as $I^+$ to form $-CH_2-Se-I$. The process could be assisted by the phenolic –OH group if it were first tautomerized (Eq. 15-60).

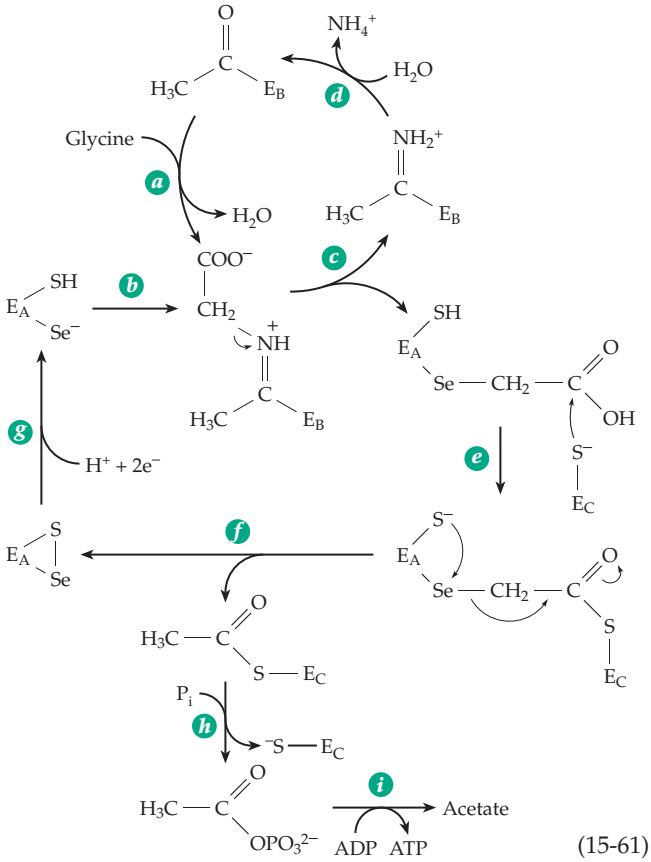



(15-60)

A recently discovered human selenoprotein is a **thioredoxin reductase** which is present in the T cells of the immune system as well as in placenta and other tissues.[189,547–549] The 55-kDa protein has one selenocysteine as the penultimate C-terminal residue. Another mammalian selenoprotein, of uncertain function, is the 57-kDa **selenoprotein P**. It contains over 60% of the selenium in rat plasma and is also present in the human body. Selenoprotein P contains ten selenocysteine residues.[550–552a] Some of these may be replaced by serine in a fraction of the molecules.[553] A smaller 9.6-kDa skeletal muscle protein, **selenoprotein W**, contains a single selenocysteine.[554–556] Another selenoprotein has been found in sperm cells, both in the tail and in a keratin-rich capsule that surrounds the mitochondria in the sperm midpiece.[557] Lack of this protein may be the cause of the abnormal immotile spermatozoa observed in Se-deficient rats and of reproductive difficulties among farm animals in Se-deficient regions.[558]

Several selenoproteins have been found in certain bacteria and archaea. A **hydrogenase** from *Methanococcus vannielii* contains selenocysteine.[559,560] This enzyme transfers electrons from $H_2$ to the C-5 *si* face of the 8-hydroxy-5-deazaflavin cofactor $F_{420}$ (Section B,4). The same bacterium synthesizes two **formate dehydrogenases** (see Fig 15-23), one of which contains Se. Two Se-containing formate dehydrogenases are made by *E. coli*. One of them, which is coupled to a hydrogenase in the formate hydrogen-lyase system (see Eq. 15-37), is a 715-residue protein containing selenocysteine at position 140.[561–563] The second has selenocysteine at position 196 and functions with a nitrate reductase in anaerobic nitrate respiration.[561]

**Glycine reductase** is a complex enzyme[530,564–566] that catalyzes the reductive cleavage of glycine to acetyl phosphate and ammonia (Eq. 15-61) with the




(15-61)

subsequent synthesis of ATP (Eq. 14-43). Electrons for reduction of the disulfide that is formed are provided by NADH. A single selenocysteine residue is present in the small 12-kDa subunits. The enzyme contains a dehydroalanine residue (Chapter 14) in subunit B and a thiol group in subunit C.[566] An acetyl-enzyme derivative of subunit C, perhaps of its –SH group, has been identified.[546] The mechanism of action is uncertain but the steps in Eq. 15-61 have been suggested.[567] The subunits are designated $E_A$, $E_B$, and $E_C$. Step *e* is particularly hard to understand because formation of a thioester in this manner is not expected to occur spontaneously and must be linked in some way to other steps.

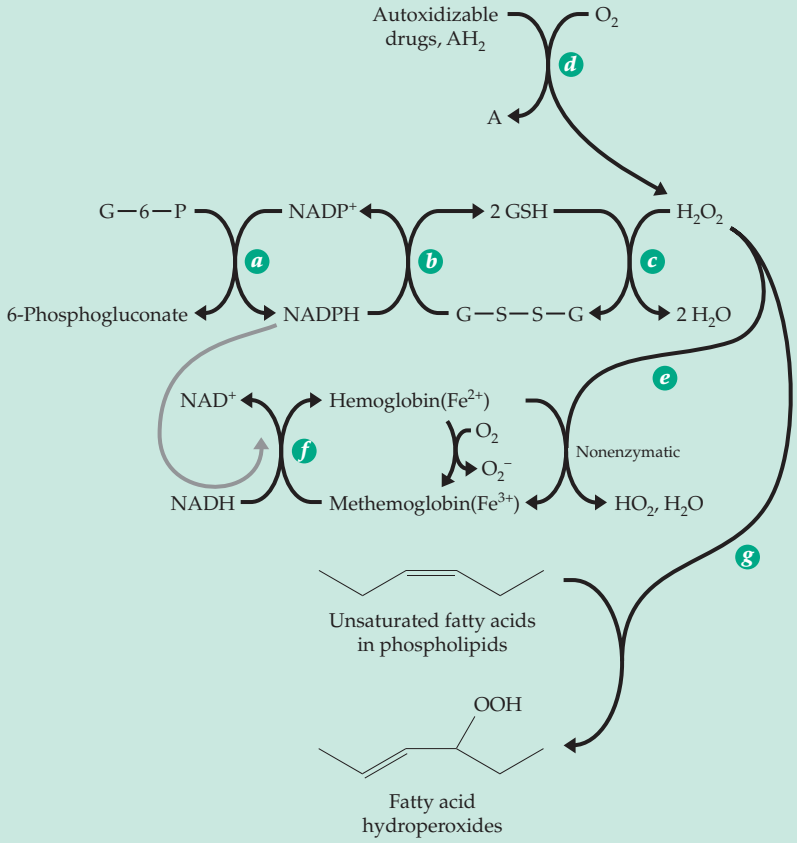
A selenium-containing **xanthine dehydrogenase** is present in purine-fermenting clostridia. Like other xanthine dehydrogenases (Chapter 16), it converts xanthine to uric acid and contains nonheme iron, molybdenum, FAD, and an Fe–S center. The selenium is probably present as $Se^{2-}$ bound to Mo as is $S^{2-}$ in xanthine oxidase (Fig. 16-32).[567a] A related reaction is catalyzed by the Fe–S protein **nicotinic acid hydroxylase** (Eq. 15-62) found in some clostridia.[568] Splitting of the Mo(V) EPR signal when $^{77}Se$ is present in the enzyme shows that the selenium is present as a ligand of molybdenum. Another member of the family is a purine hydroxylase that converts purine 2-hydroxypurine, or hypoxanthine to xanthine.[567a]

**TABLE 15-4**
**Selenium-Containing Proteins**

| Enzyme | Source | Mass (kDa) | Subunit composition | Other cofactors |
|---|---|---|---|---|
| Glutathione peroxidases | | | | None |
| Cellular | Mammals | 21 x 4 | | |
| Plasma | Humans | | | |
| Phospholipid hydroperoxide | Pig, rat | 18 | | |
| Iodothyronine deiodinases | Vertebrates | | | |
| Thioredoxin reductase | Humans | 55 × 2 | | |
| Selenoprotein *P* | Mammals | 57 | | None |
| Selenoprotein *W* | Rat | 19.6 | | |
| Formate dehydrogenase | Bacteria - *E. coli* | 600 | $\alpha_2\beta_4\gamma_{2\text{-}4}$ | Heme *b* |
| | Archaea | | | Mo; molybdopterin |
| Hydrogenase | Bacteria | | $\alpha_2\beta_4\gamma_2$ | FAD; NiFeSe |
| | Archaea | | | |
| Glycine reductase | Some clostridia | | ABC | |
| Selenoprotein A | | 12 | | |
| Selenoprotein B | | 200 | | |
| Carbonyl protein C | | 250 | | Fe? |
| Nicotinic acid hydroxylase | Clostridia | 300 | | FAD, FeS, Mo |
| Purine hydroxylase | Clostridia | | | FAD, FeS, Mo |
| Thiolase (contains selenomethionine) | *Clostridium kluyverii* | 39 × 4 | | None |
| Carbon monoxide dehydrogenase | *Oligotropha carboxidovorans* | 137 × 2 | $\alpha_2\beta_2\gamma_2$ | FAD; Mo; molybdopterin |

## BOX 15-H GLUTATHIONE PEROXIDASE AND ABNORMALITIES OF RED BLOOD CELLS

The processes by which hemoglobin is kept in the Fe(II) state and functioning normally within intact erythrocytes is vital to our health. Numerous hereditary defects leading to a tendency toward anemia have helped to unravel the biochemistry indicated in the accompanying scheme.[a]




About 90% of the glucose utilized by erythrocytes is converted by glycolysis to lactate, but about 10% is oxidized (via glucose 6-phosphate) to 6-phosphogluconate. The oxidation (reaction *a*) is catalyzed by glucose-6-phosphate dehydrogenase (Eq.15-10) using $NADP^+$. This is the principal reaction providing the red cell with NADPH for reduction of glutathione (Box 11-B) according to reaction *b*. Despite the important function of glucose-6-*P* dehydrogenase, ~400 million persons, principally in tropical and Mediterranean areas, have a hereditary deficiency of this enzyme. The genetic variations are numerous, with about 400 different ones having been identified. Although most individuals with this dificiency have no symptoms, the lack of the enzyme is truly detrimental and sometimes leads to excessive destruction of red cells and anemia during some sicknesses and in response to administration of various drugs.[b] The survival of the defective genes, like that for sickle cell hemoglobin (Box 7-B) is thought to result from increased resistance to malaria parasites.

Other erythrocyte defects that lead to drug sensitivity include a deficiency of glutathione (resulting from a decrease in its synthesis) and a deficiency of glutathione reductase (reaction *b*). The effects of drugs have been traced to the production of $H_2O_2$ (reaction *c*) in red blood cells; catalase, which converts $H_2O_2$ into $H_2O$ and $O_2$, is thought to function in a similar way. Both enzymes are probably necessary for optimal health.

An excess of $H_2O_2$ can damage erythrocytes in two ways. It can cause excessive oxidation of functioning hemoglobin to the Fe(III)-containing methemoglobin. (Methemoglobin is also formed spontaneously during the course of the oxygen-carrying function of hemoglobin. It is estimated that normally as much as 3% of the hemoglobin may be oxidized to methemoglobin daily.) The methemoglobin formed is reduced back to hemoglobin through the action of **NADH-methemoglobin reductase** (reaction *f*). A smaller fraction of the methemoglobin is reduced by a similar enzyme requiring NADPH (as indicated by the colored arrow). A hereditary lack of the NADH-methemoglobin reductase is also known.

A second destructive function of $H_2O_2$ is attack on double bonds of unsaturated fatty acids of the phospholipids in cell membranes. The resulting fatty acid hydroperoxides can react further with C–C chain cleavage and disruption of the membrane. This is thought to be the principal cause of the hemolytic anemia induced by drugs in susceptible individuals. The selenium-containing glutathione peroxidase is thought to decompose these fatty acid hydroperoxides. Vitamin E (Box 15-G), acting as an antioxidant within membranes, is also needed for good health of erythrocytes.[c,d]

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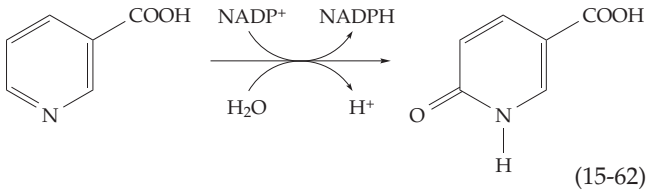
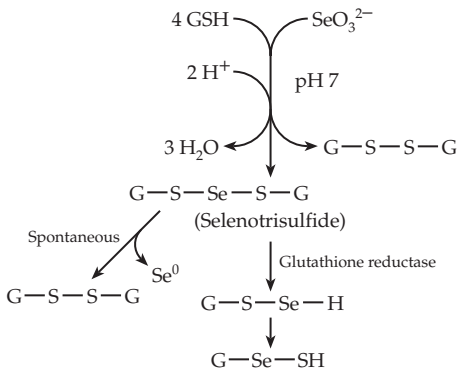



(15-62)

A **thiolase** (Eq. 13-35) from *Clostridium kluyveri* is one of only two known selenoproteins that contain selenomethionine.[569] However, the selenomethionine is incorporated randomly in place of methionine. This occurs in all proteins of all organisms to some extent and the toxicity of selenium may result in part from excessive incorporation of selenomethionine into various proteins.

Selenium is found to a minor extent wherever sulfur exists in nature. This includes the sulfur-containing modified bases of tRNA molecules. In addition to a small amount of nonspecific incorporation of Se into all S-containing bases there are, at least in bacteria, specific Se-containing tRNAs. In *E. coli* one of these is specific for lysine and one for glutamate. One of the modified bases has been identified as 5-methyl-aminomethyl-2-selenouridine.[570] It is present at the first position of the anticodon, the "wobble" position.[571]

Selenium has its own metabolism. Through the use of $^{75}Se$ as a tracer, normal rat liver has been shown to contain $Se^{2-}$, $SeO_3^{2-}$, and selenium in a higher oxidation state.[572] Glutathione may be involved in reduction of selenite to selenide.[573] The nonenzymatic reduction of selenite by glutathione yields a selenotrisulfide derivative (Eq. 15-63). The latter is spontaneously decomposed to oxidized glutathione and elemental selenium or by the action of glutathione reductase to glutathione and selenium. Selenocysteine can be converted to alanine + elemental selenium (Eq. 14-34). Some bacteria are able to oxidize elemental Se back to selenite.[574] Selenium undergoes biological methylation readily in bacteria, fungi, plants, and animals (Chapter 16).[575,576] This may in some way be related to the reported effect of selenium in protecting animals against the toxicity of mercury. Excess selenium may appear in the urine as trimethylselenonium ions.[577]




(15-63)

How is selenium incorporated into selenocysteine-containing proteins? This element does enter amino acids to a limited extent via the standard synthetic pathways for cysteine and methionine. However, the placement of selenocysteine into specific positions in selenoproteins occurs by the use of a minor serine-specific tRNA that acts as a suppressor of chain termination during protein synthesis.[532,533,578] (This topic is dealt with further in Chapter 29.) The genes for these and presumably for other selenocysteine-containing proteins have the "stop" codon TGA at the selenocysteine positions. However, when present in a suitable "context" the minor tRNA, carrying selenocysteine in place of serine, is utilized to place selenocysteine into the growing peptide chain. In bacteria, and presumably also in eukaryotes, the selenocysteinyl-tRNA is formed from the corresponding seryl-tRNA by a PLP-catalyzed β-replacement reaction. The selenium donor is not $Se^{2-}$ but selenophosphate $Se-PO_3^{2-}$ in which the Se–P bond is quite weak.[579–581] After addition to the aminoacrylate intermediate in the PLP enzyme the Se–P bond may be hydrolytically cleaved to $HPO_4^{2-}$ and selenocysteyl-tRNA.

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## Study Questions

1. *S*-adenosylmethionine is also a biological methyl group donor. The product of its methyl transferase reactions is *S*-adenosylhomocysteine. This product is further degraded by *S*-adenosylhomocysteine hydrolase, an enzyme that contains tightly bound $NAD^+$, to form homocysteine and adenosine.

   Write a step-by-step mechanism for the action of this hydrolase.

2. Compare the chemical mechanisms of enzyme-catalyzed decarboxylation of the following:
   a) a β-oxo-acid such as acetoacetate or oxaloacetate
   b) an α-oxo-acid such as pyruvate
   c) an amino acid such as L-glutamate

3. Describe the subunit structure of the enzyme pyruvate dehydrogenase. Discuss the functioning of each of the coenzymes that are associated with these subunits and write detailed mechanisms for each step in the pyruvate dehydrogenase reaction.

4. Free **formate** can be assimilated by cells via the intermediate **10-formyl-tetrahydrofolate** (10-formyl-THF).
   a) Describe the mechanism of synthesis of this compound from formate and tetrahydrofolate.
   b) Diagram a hypothetical transition state for the first step of this reaction sequence.
   c) Describe two or more uses that the human body makes of 10-formyl-THF.

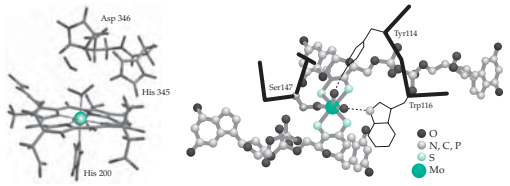
5. Using partial structural formulas, describe the reactions by which serine and methionine react to form *N*-formylmethionine needed for protein synthesis.

6. Write the equations for each of the reactions shown below. Using the $E^{0'}$ values below, calculate approximate Gibbs energies for each reaction, and show by the relative length of the arrows on which side of the reaction the equilibrium lies.
   a) The oxidation of malate by $NAD^+$
   b) The oxidation of succinate by $NAD^+$
   c) The oxidation of succinate by enzyme-bound FAD
   d) What can you say about the cofactor required for oxidation of succinate from your calculations?

   The values of $E^{0'}$ for several half reactions are given below. Everything has been rounded to one significant figure so that a calculator is unnecessary.

| Reaction | $E^{0'}$ (volts) |
|---|---|
| $NAD^+ + H^+ + 2\ e^- \rightarrow NADH$ | −0.3 |
| enzyme bound FAD + $2\ H^+ + 2\ e^- \rightarrow$ enzyme bound $FADH_2$ | 0.0 |
| fumarate + $2\ H^+ + 2\ e^- \rightarrow$ succinate | 0.0 |
| oxalacetate + $2\ H^+ + 2\ e^- \rightarrow$ malate | −0.2 |

7. Some acetogenic bacteria, which convert $CO_2$ to acetic acid, form pyruvate for synthesis of carbohydrates, etc., by formation of formaldehyde and conversion of the latter to glycine by reversal of the PLP and lipoic acid-dependent glycine decarboxylase, a 4-protein system. The glycine is then converted to serine, pyruvate, oxaloacetate, etc. Propose a detailed pathway for this sequence.




Transition metal ions function in many biological oxidation-reduction processes. (Left) A heme ring in **nitrite reductase** chelates an iron ion in its center, holding the $Fe^{2+}$ or $Fe^{3+}$ with bonds from four nitrogen atoms and a fifth bond from an imidazole ring below. Above the iron is a nitrite ion, $NO_2^-$, awaiting reduction to nitric oxide NO in a denitrifying bacterium. From Ranghino *et al.* (2000) *Biochemistry* **39**, 10958–10966. (Right) The active site of a bacterial **dimethylsulfoxide reductase** has an atom of molybdenum or tungsten at its center. The metal is held by four sulfur atoms from two molybdopterin molecules and an oxygen atom of a serine side chain. Two other oxygen atoms are bound as oxo groups and may participate in catalysis. From Stewart *et al.* (2000) *J. Mol. Biol.* **299**, 593–600.

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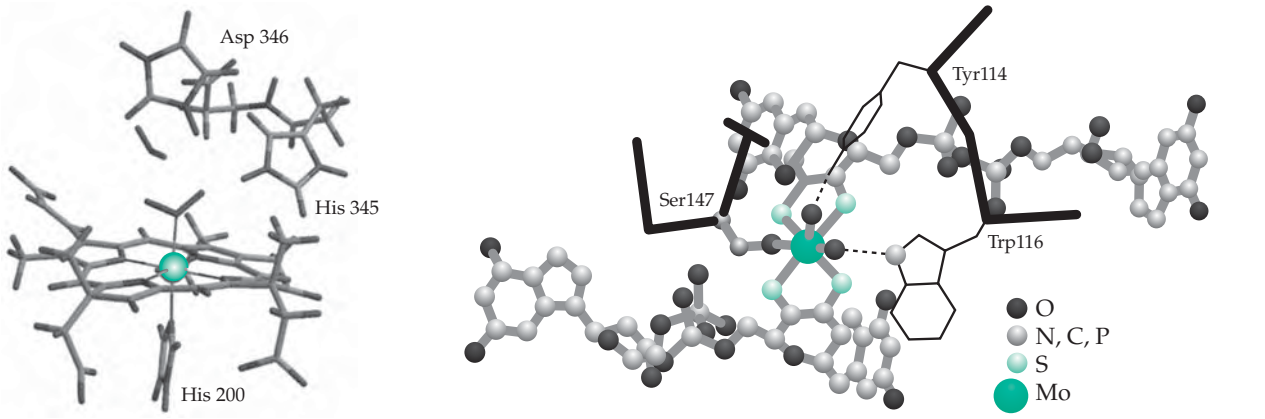
# Transition Metals in Catalysis and Electron Transport 16




Although the amounts present within living cells are very small, the ions of the transition metals Fe, Co, Ni, Cu, and Mn are extremely active centers for catalysis, especially of reactions that take advantage of the ability of these metals to exist in more than one oxidation state.[1–4] Iron, copper, and nickel are also components of the electron carrier proteins that function as oxidants or reductants in many biochemical processes. These metals are all nutritionally essential, as are chromium and vanadium. Among the heavier transition elements molybdenum is a constituent of an important group of enzymes that includes the sulfite oxidase of human liver and nitrogenase of nitrogen-fixing bacteria. Tungsten occasionally substitutes for molybdenum.

## A. Iron

Iron is one of the most abundant elements in the earth's crust, being present to the extent of ~4% in a typical soil. Its functions in living cells are numerous and diverse.[2,5–8] The average overall iron content of both bacteria and fungi is ~1 mmol / kg, but that of animal tissues is usually less. Seventy percent of the 3–5 g of iron present in the human body is located in the blood's erythrocytes, whose overall iron content is ~20 mM. In other tissues the total iron averages closer to 0.3 mM and consists principally of storage forms. The total concentration of iron in all of the iron-containing *enzymes* of tissues amounts to only about 0.01 mM. Although these concentrations are low, the iron is concentrated in oxidative enzymes of membranes and may attain much higher concentrations locally. Only a few parasitic or anaerobic bacteria, e.g. the lactic acid bacteria, possess no oxygen-requiring enzymes and are almost devoid of both iron and copper. All other organisms appear to require iron for life.

### 1. Uptake by Living Cells

A major problem for cells is posed by the relative insolubility of ferric hydroxide and other compounds from which iron must be extracted by the organism. A consequence is that iron is often taken up in a chelated form and is transferred from one organic ligand, often a protein, to another with little or no existence as free $Fe^{3+}$ or $Fe^{2+}$. As can be calculated from the estimated solubility product of $Fe(OH)_3$ (Eq. 16-1),[7] the equilibrium concentration of $Fe^{3+}$ at pH 7 is only $10^{-17}$ M.

$$K_{sp} = [Fe^{3+}][OH^-]^3 < 10^{-38}\ M^4 \qquad (16\text{-}1a)$$

$$\text{or } [Fe^{3+}] / [H^+]^3 < 10^4\ M^4 \text{ at } 25°\ C \qquad (16\text{-}1b)$$

For a 2 $\mu m^3$ bacterial cell this amounts to just one free $Fe^{3+}$ ion in almost 100 million cells at any single moment. The importance of chelated forms of iron becomes obvious. It is also evident from Eq. 16-1 that, in addition to chelation, a low external pH can also facilitate uptake of $Fe^{3+}$ by organisms.

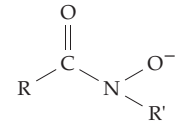
The values of the formation constants for chelates of $Fe^{2+}$ typically lie between those of $Mn^{2+}$ and $Co^{2+}$ (Fig. 6-6, Table 6-9). For example, $K_1 = 10^{14.3}\ M^{-1}$ for formation of the $Fe^{2+}$ chelate of EDTA. The smaller and more highly charged $Fe^{3+}$ is bonded more strongly ($K_1 = 10^{25}\ M^{-1}$). These binding constants are independent of pH. However, the binding of any metal ion is affected by pH, as discussed in Chapter 6. A fact of

considerable biochemical significance is the stronger binding of $Fe^{3+}$ to oxygen-containing ligands than to nitrogen atoms, while $Fe^{2+}$ tends to bind preferentially to nitrogen. It is also significant that $Fe^{3+}$ bound to oxygen ligands tends to exchange readily with other ferric ions in the medium, whereas $Fe^{3+}$ bound to nitrogen-containing ligands such as heme exchanges slowly. This fact is important for both iron-transport compounds and enzymes.

***Siderophores.*** If a suitably high content of iron (e.g., 50 μM or more for *E. coli*) is maintained in the external medium, bacteria and other microorganisms have little problem with uptake of iron. However, when the external iron concentration is low, special compounds called siderophores are utilized to render the iron more soluble.[7–11c] For example, at iron concentrations below 2 μM, *E. coli* and other enterobacteria secrete large amounts of **enterobactin** (Fig. 16-1). The stable $Fe^{3+}$–enterobactin complex is taken up by a transport system that involves receptors on the outer bacterial membrane.[9,12,13] Siderophores from many bacteria have in common with enterobactin the presence of **catechol** (*ortho*-dihydroxybenzene) groups that chelate the iron.

The three catechol groups of enterobactin are carried on a cyclic serine triester structure. A variety of both cyclic and linear structures are found among other catechol siderophores.[14–19] For example, **parabactin** and **agrobactin** (Fig. 16-1) contain a backbone of spermidine[20] (Chapter 24). After the $Fe^{3+}$–enterobactin complex enters a bacterial cell the ester linkages of a siderophore are cleaved by an esterase. Because of the extremely high formation constant of ~$10^{52}$ $M^{-1}$ for the complex[11] the only way for a cell to release the $Fe^{3+}$ is through this irreversible destruction of the iron carrier.[11d] Reduction to $Fe^{2+}$ may be involved in release of iron from some siderophores.[11e]

The first known siderophore, isolated in 1952 by Neilands,[22] is **ferrichrome** (Fig. 16-1), a cyclic hexapeptide containing **hydroxamate** groups at the iron-binding centers. Oxygen atoms form the bonds to iron



Hydroxamate group

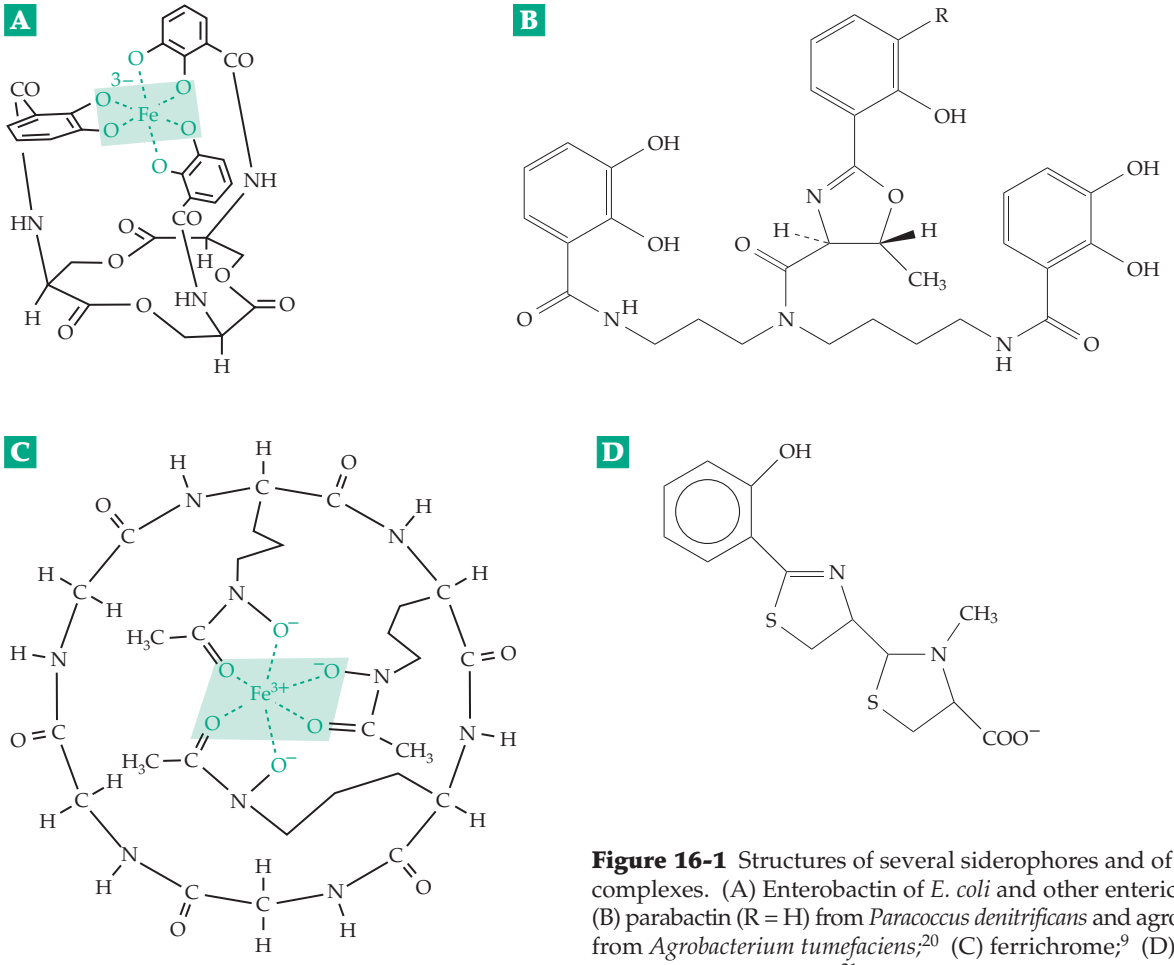



**Figure 16-1** Structures of several siderophores and of their metal complexes. (A) Enterobactin of *E. coli* and other enteric bacteria;[12] (B) parabactin (R = H) from *Paracoccus denitrificans* and agrobactin (R = OH) from *Agrobacterium tumefaciens*;[20] (C) ferrichrome;[9] (D) pyochelin from *Pseudomonas aeruginosa*.[21]

in this compound also. Ferrichrome binds $Fe^{3+}$ with a formation constant of ~$10^{29}$ $M^{-1}$. The binding is not as tight as with enterobactin and the iron can be released by enzymatic reduction to $Fe^{2+}$ which is much less tightly bound than is $Fe^{3+}$. The released ferrichrome can be secreted and used repeatedly to bring in more iron. Ferrichrome is produced by various fungi and bacilli and is only one of a series of known hydroxamate siderophores.[16] Since iron is essential to virtually all parasitic organisms the ability to obtain iron is often the limiting factor in establishing an infection.[23,24]

In *E. coli* there are seven different outer **membrane receptors** for siderophores.[25] One of these, the gated porin **FepA**, is specific for ferric enterochelin. With the assistance of another protein, **TonB**, it allows the ferric siderophore to penetrate the outer membrane.[26] A different receptor, **FhuA**, binds ferrichrome. Both FepA and FhaA are large 22-strand porins resembling the 16-strand porin shown in Fig. 8-20. However, they are nearly 7 nm long with internal diameters three times those of the 16-strand porins. In addition, loops of polypeptide chain on the outer edges can close while an N-terminal domain forms a "cork" that remains in place until the $Fe^{3+}$-siderophore complex enter the channel and binds. Like the hatches in an air lock on a spacecraft, the outer loops then close, after which the inner cork unwinds to allow the siderophore complex to enter the periplasmic space. The apparatus requires an energy supply, which apparently is provided by an additional complex consisting of proteins TonB, ExbA, and ExbD. They evidently couple the electrochemical gradient across the cell membrane (Chapter 8, B1 and C5 and Chapter 18) with the operation of the channel gates. Some bacteria use a different strategy for passage across the outer membrane.[11c] The channel of a receptor protein contains a molecule of an iron-free siderophore. When a molecule of $Fe^{3+}$-siderophore binds in the outside part of the channel the $Fe^{3+}$ jumps to the inner siderophore, which then dissociates from the receptor, carrying the $Fe^{3+}$-siderophore complex into the periplasmic space. This mechanism also seems to be available in *E. coli*. The **ferric uptake regulation** (Fur) protein binds excess free $Fe^{2+}$, the resulting complex acting as a repressor of all of the iron uptake genes in *E. coli*.[11e]

Additional proteins are required for passage through the membrane.[11b,23,25,27] These are ABC transporters, which utilize hydrolysis of ATP as an energy source (Chapter 8, Section C,4). For uptake of the $Fe^{3+}$-ferrichrome complex protein **FhuD** is the periplasmic binding protein, **FhuC** is an integral membrane component, and the cytosolic **FhuC** contains the ATPase center.[11b] Another ABC transporter, found in many bacteria, carries unchelated $Fe^{3+}$ across the inner membrane. The binding protein component for *Hemophilus influenzae*, designated Hit, resembles one lobe of mammalian transferrin (Fig. 16-2).[11b] The siderophore receptors of bacteria have been "parasitized" by various bacteriophages and toxic proteins. For example, FepA is also a receptor for the toxic colicins B and D (Box 8-D) and tonB is a receptor for bacteriophage T1.[9,13]

Some bacteria do not form siderophores but take up $Fe^{2+}$. Even *E. coli*, when grown anaerobically, synthesizes an uptake system for $Fe^{2+}$. It utilizes a 75-residue peptide encoded by gene *feoA* and a 773-residue protein encoded by *feoB*.[28,11b]

***Uptake of iron by eukaryotic cells.*** The yeast *Saccharomyces cerevisiae* utilizes two systems for uptake of iron.[29,30] A low-affinity system transports $Fe^{2+}$ with an apparent $K_m$ of ~30 μM, while a high-affinity system has a $K_m$ of ~0.15 μM. Study of these systems has been greatly assisted by the use of genetic methods developed for both bacteria and yeast (Chapter 26). The low-affinity iron uptake depends upon a protein transporter encoded by gene *FET*4 and on a reductase encoded by genes *FRE*1 and *FRE*2, proteins that are embedded in the cytoplasmic membrane.[29,31-33] It might seem reasonable that the *FET*3 copper oxidoreductase should keep $Fe^{2+}$ *reduced* while it is transported. However, it appears to *oxidize* $Fe^{2+}$ to $Fe^{3+}$. The high-affinity uptake system is more puzzling. It requires a permease encoded by *FTR I* and an additional protein encoded by *FET*3.[30,33–35] The Fet3 protein is a copper oxidoreductase related to **ceruloplasmin** (Section D). The protein **Fre1p** (encoded by *FRE*1) is a metalloreductase that reduces $Cu^{2+}$ to $Cu^{+}$, as well as $Fe^{3+}$ to $Fe^{2+}$. It is essential for copper uptake (Section D).[33] It has long been known that ceruloplasmin is required for mobilization of iron from mammalian tissues.[30] Hereditary ceruloplasmin deficiency causes accumulation of iron in tissues.[36] Yeast also contains both *mitochondrial* and *vacuolar* iron transporters.[37,37a,b]

The uptake of iron by animals is not as well understood[38–40] but it resembles that of yeast.[41] A general divalent cation transporter that is coupled to the membrane proton gradient is involved in intestinal iron uptake.[42,60] Ascorbic acid promotes the uptake of iron, presumably by reducing it to Fe(II), which is more readily absorbed than Fe(III), and also by promoting ferritin synthesis.[43] Uptake is also promoted by meat in the diet.[44] Within the body iron is probably transferred from one protein to another with only a transient existence as free $Fe^{2+}$. An average daily human diet contains ~15 mg of iron, of which ~1 mg is absorbed. This is usually enough to compensate for the small losses of the metal from the body, principally through the bile. Once it enters the body, iron is carefully conserved. The 9 billion red blood cells destroyed daily yield 20–25 mg of iron which is almost all reused or stored. The body apparently has no mechanism for excretion of large amounts of iron; a person's iron content is regulated almost entirely by the rate of

uptake. This rate is increased during pregnancy and, in young women, to compensate for iron lost in menstrual bleeding. Nevertheless, control of iron uptake is imperfect and perhaps 500 million people around the world suffer from iron deficiency.[44,45] For others, an excessive intake of iron or a genetic defect lead to accumulation of iron to toxic levels, a condition called **hemochromatosis**.[44,46a,b] This condition may also arise in any disease that leads to excessive destruction of hemoglobin or accumulation of damaged erythrocytes. Examples are β-thalassemia (Chapter 28) and cerebral malaria.[47] Treatment with chelating agents designed to remove iron is often employed.[47,48]

***Transferrins.*** Within the body iron is moved from one location to another while bound as $Fe^{3+}$ to transferrins, a family of related 680- to 700-residue 80-kDa proteins.[38,49–53] Each transferrin molecule contains two $Fe^{+3}$ binding sites, one located in each of two similar domains of the folded peptide chain. A dianion, usually $CO_3^{2-}$, is bound together with each $Fe^{3+}$. Milk transferrin (**lactoferrin**[51a,b,c] also found in leukocytes), hen egg transferrin (**ovotransferrin**),[52,52a] and rabbit and human serum transferrin[54,54a] all have similar structures. Each $Fe^{3+}$ is bonded to oxygen anions from two tyrosine side chains, an aspartate carboxylate, an imidazole group, and the bound carbonate ion (Fig. 16-2B). Transferrin of blood plasma is encoded by a separate gene but has a similar structure. Transferrin of chickens appears to be identical to conalbumin of egg whites. The iron-binding proteins of body fluids are sometimes given the group name **siderophilins**. Transferrins may function not only in transport of iron throughout the body but also as iron buffers that provide a relatively constant iron concentration within tissues.

The entrance of iron into the body through the intestinal mucosal cells may involve the transferrin present in those cells[44] and the influx of iron may also be regulated by blood plasma transferrin. There is also a nontransferrin pathway.[42,55] Transferrins bind $Fe^{2+}$ weakly and it is likely that a transferrin– $Fe^{2+}$–$HCO_3^-$ complex formed initially undergoes oxidation to the $Fe^{3+}$–$CO_3^{2-}$ complex within cells and within the bloodstream. A conformational change closes the protein around the iron ions.[56] In yeast the previously mentioned copper oxidoreductase encoded by the *FET*3 gene appears to not only oxidize $Fe^{2+}$ but also transfer the resulting $Fe^{3+}$ to transferrin. Ceruloplasmin may play a similar role in mammals.[33]

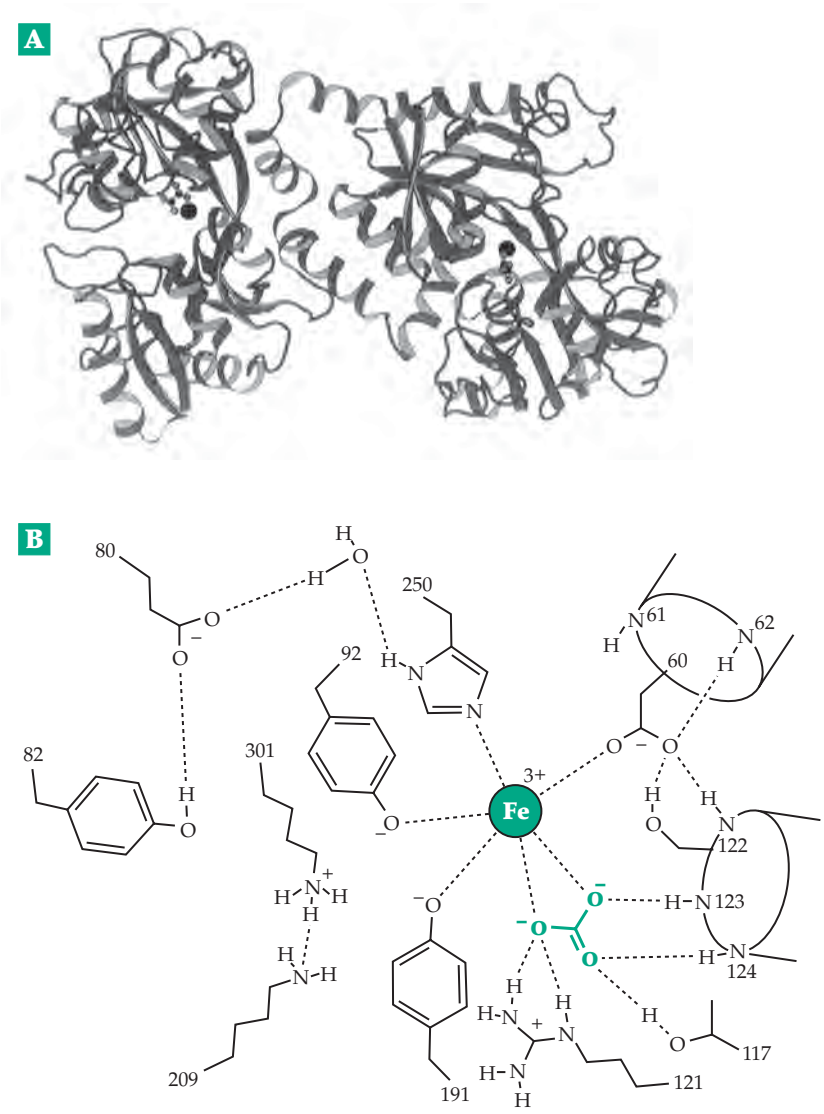



**Figure 16-2** (A) Ribbon drawing of the polypeptide chain of a transferrin, human lactoferrin. The N lobe is to the left and the C lobe to the right. Each active site contains bound $Fe^{3+}$ and a molecule of oxalate dianion which replaces the physiological $CO_3^{2-}$. From Baker *et al.*[51] Courtesy of Edward Baker. (B) Schematic diagram showing part of the hydrogen-bond network involved in binding the $Fe^{3+}$ in the N lobe of hen ovotransferrin. Some side chain groups and water molecules have been omitted. The positions of hydrogen atoms and the charge state of acid–base groups are uncertain. Most of the hydrogen-bond distances (O - - O, O - - N, N - - N) indicated by dashed lines are between 0.27 and 0.3 nm. Release of the bound $Fe^{3+}$ may be accomplished in part by protonation of the bound $CO_3^{2-}$ to form $HCO_3^-$. See Kurokawa *et al.*[52]

Iron is transferred from the plasma transferrin into cells of the body following binding of the $Fe^{3+}$–transferrin complex to specific receptors. The surface of an immature red blood cell (reticulocyte) may contain 300,000 transferrin receptors, each capable of catalyzing the entry of ~36 iron ions per hour.[38] The receptor is a 180-kDa dimeric glycoprotein. When the $Fe^{3+}$–transferrin complex is bound, the receptors aggregate in coated pits and are internalized. The mechanism of release of the $Fe^{3+}$ may occur by different mechanisms in the two lobes.[57] The pH of the endocytic vesicles containing the receptor complex is probably lowered to ~5.6 as in lysosomes. This may protonate the bound $CO_3^{2-}$ in the complex[51c,54a,58] and assists in the release of the $Fe^{3+}$, possibly after reduction to $Fe^{2+}$. Both the apotransferrin and its receptor are returned to the cell surface for reuse, the apotransferrin being released into the blood. Chelating agents such as pyrophosphate, ATP, and citrate as well as simple anions[59] may also assist in removal of iron from transferrin. The same transmembrane transporter that is involved in intestinal iron uptake[42] is needed to remove iron from the endosome after release.[60]

## 2. Storage of Iron

Within tissues of animals, plants, and fungi much of the iron is packaged into the red-brown water-soluble protein **ferritin**, which stores Fe(III) in a soluble, nontoxic, and readily available form.[61–64] Although bacteria store very little iron,[65] some of them also contain a type of ferritin.[66–67a] On the other hand, the yeast *S. cerevisieae* stores iron in polyphosphate-rich granules, even though a ferritin is also present.[65] Ferritin contains 17–23% iron as a dense core of hydrated ferric oxide ~7 nm in diameter surrounded by a protein coat made up of twenty-four subunits of mo-

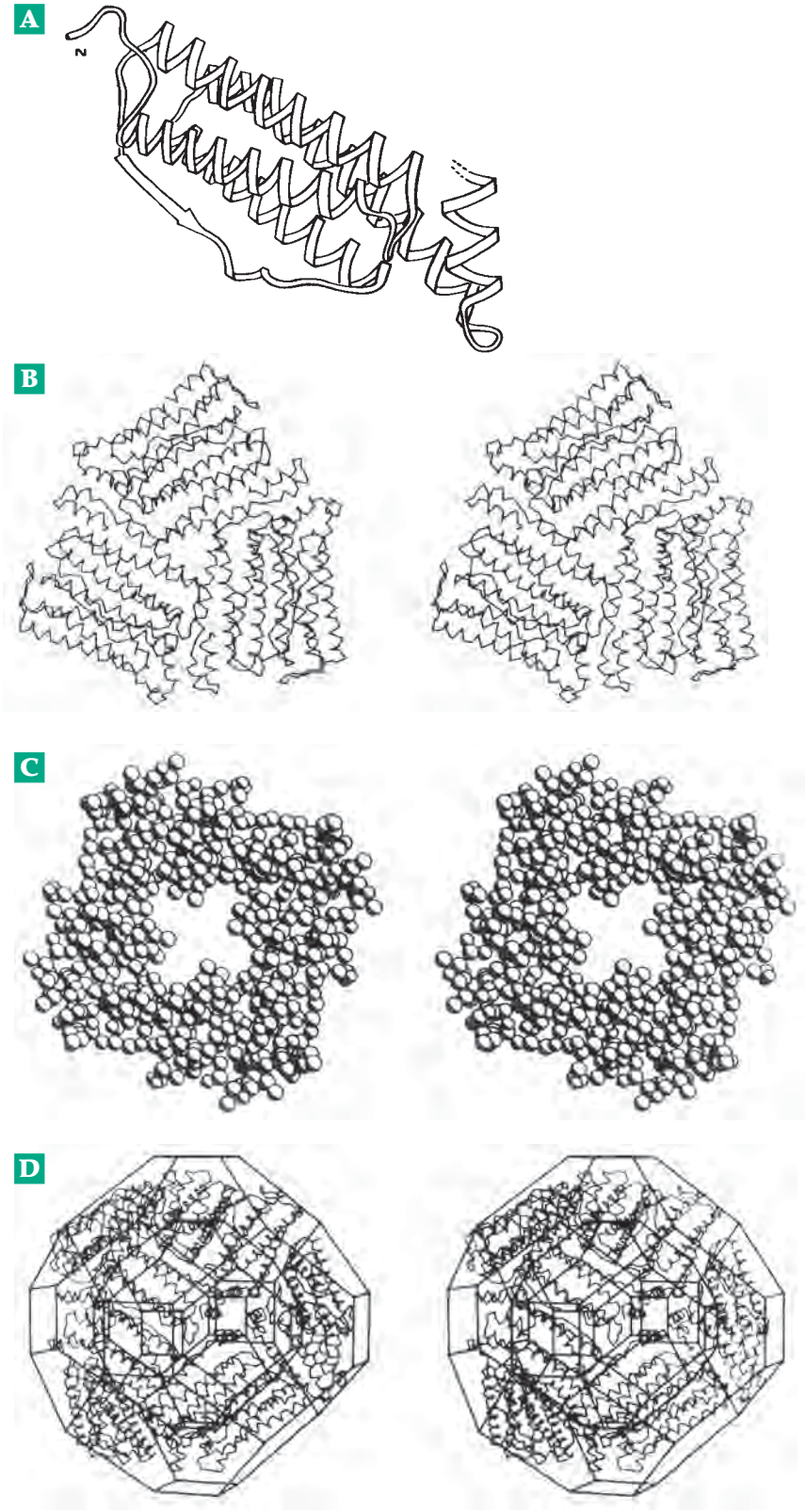



**Figure 16-3** Structure of the protein shell of ferritin (apoferritin). (A) Ribbon drawing of the 163-residue monomer. From Crichton.[62] (B) Stereo drawing of a hexamer composed of three dimers. (C) A tetrad of four subunits drawn as a space-filling diagram and viewed down the four-fold axis from the exterior of the molecule. (D) A half molecule composed of 12 subunits inscribed within a truncated rhombic dodecahedron. B–D from Bourne *et al*.[74]

lecular mass 17- to 21-kDa. Each subunit is folded as a four-helix bundle (Fig. 16-3). Mammalian ferritins consist of combinations of subunits of two or more types. For example, human ferritins contain similarly folded 19-kDa L (light) and 21-kDa H (heavy) subunits.[68] The twenty-four subunits are arranged in a cubic array (Fig. 7-13, Fig. 16-3). The outer diameter of the 444-kDa apoferritin is ~12 nm. The completely filled ferritin molecule contains 23% Fe and over 2000 atoms of iron in a crystalline lattice. Larger ferritins may contain as many as 4500 atoms of iron with the approximate composition $[Fe(O)OH]_8FeOPO_3H_2$.[69] Phosphate ions are sometimes bound into surface layers of the ferritin cores.[69a] Ferritin cores are readily visible in the electron microscope, and ferritin is often used as a labeling reagent in microscopy. Another storage form of iron, **hemosiderin**, seems to consist of ferritin partially degraded by lysosomes and containing a higher iron content than does ferritin. Depositions of hemosiderin in the liver can rise to toxic levels if excessive amounts of iron are absorbed.

Iron can be deposited in ferritin by allowing apoferritin to stand with an Fe(II) salt and a suitable oxidant, which may be $O_2$. Physiological transfer of Fe(III) from transferrin to ferritin is thought to require prior reduction to Fe(II). The reoxidation by $O_2$ to Fe(III) for deposition in the ferritin core (Eq. 16-2) is catalyzed by **ferrooxidase sites** located in the centers of the helical bundles of the H-chains.[70–73]

$$2\,Fe^{2+} + O_2 + 4\,H_2O \rightarrow 2\,Fe(O)OH_{core} + H_2O_2 + 4\,H^+ \qquad (16\text{-}2)$$

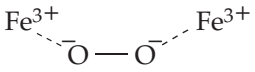
## BOX 16-A MAGNETIC IRON OXIDE IN ORGANISMS

An unusual form of stored iron is the magnetic iron oxide **magnetite** ($Fe_3O_4$). Honeybees,[a,b] monarch butterflies,[b–c] homing pigeons,[c–e] migrating birds, and even magnetotactic bacteria[f] contain deposits of $Fe_3O_4$ that are suspected of being used in nagivation.[c,g] Some bacteria have magnetic iron sulfide particles.[h-j] Human beings have magnetic bones in their sinuses[k] and in their brains[l] and may be able to sense direction magnetically. A set of possible magnetoreceptor cells, as well as associated nerve pathways, have been identified in trout.[j] In the magnetotactic bacteria found in the Northern Hemisphere the magnetic domains are oriented parallel with the axis of motility of the bacteria which tend to swim toward the geomagnetic North and downward into sediments. Similar bacteria from the Southern Hemisphere prefer to swim south and downward. The magnetic polarity of the bacterial magnetite crystals can be reversed by strong magnetic pulses, after which the bacteria swim in the direction opposite to their natural one.[m] Magnetic ferritin can be produced artificially in the laboratory.[n] The resulting particles may have practical uses, for example, in medical magnetic imaging. Magnetic materials in the human body are of interest not only in terms of a possible sensory function but also because of possible effects of electromagnetic fields on human health and behavior.[o]


Magnetotactic soil bacterium containing 36 magnetite-containing magnetosomes. Courtesy of Dennis Bazylinski.


[a] Hsu, C.-Y., and Li, C.-W. (1994) *Science* **265**, 95–97
[b] Nichol, H., and Locke, M. (1995) *Science* **269**, 1888–1889
[bc] Etheredge, J. A., Perez, S. M., Taylor, O. R., and Jander, R. (1999) *Proc. Natl. Acad. Sci. U.S.A.* **96**, 13845–13846
[c] Gould, J. L. (1982) *Nature (London)* **296**, 205–211
[d] Guilford, T. (1993) *Nature (London)* **363**, 112–113
[e] Moore, B. R. (1980) *Nature (London)* **285**, 69–70
[f] Blakemore, R. P., and Frankel, R. B. (1981) *Sci. Am.* **245** (Dec), 58–65
[g] Maugh, T. H., II,(1982) *Science* **215**, 1492 – 1493
[h] Dunin-Borkowski, R. E., McCartney, M. R., Frankel, R. B., Bazylinski, D. A., Pósfai, M., and Buseck, P. R. (1998) *Science* **282**, 1868–1870
[i] Pósfai, M., Buseck, P. R., Bazylinski, D. A., and Frankel, R. B. (1998) *Science* **280**, 880–883
[j] Walker, M. M., Diebel, C. E., Haugh, C. V., Pankhurst, P. M., Montgomery, J. C., and Green, C. R. (1997) *Nature (London)* **390**, 371–376
[k] Baker, R. R., Mather, J. G., and Kennaugh, J. H. (1983) *Nature (London)* **301**, 78–80
[l] Kirschvink, J. L., Kobayashi-Kirschvink, A., and Woodford, B. J. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 7683–7687
[m] Blakemore, R. P., Frankel, R. B., and Kalmijn, A. J. (1980) *Nature (London)* **286**, 384–385
[n] Meldrum, F. C., Heywood, B. R., and Mann, S. (1992) *Science* **257**, 522–523
[o] Barinaga, M. (1992) *Science* **256**, 967

The ferrooxidase site is a dinuclear iron center (see Section 8) in which two iron ions (probably $Fe^{2+}$) are bound as in Fig. 16-4. They are then converted to $Fe^{3+}$ ions by $O_2$, which may bind initially to the $Fe^{2+}$, forming a transient blue intermediate that is thought to have a peroxodiferric structure, perhaps of the following type.[71–73] Reaction of this intermediate with $H_2O$




may yield $H_2O_2$ plus a biomineral precursor $Fe^{2+} - O - Fe^{2+}$, which is incorporated into the core.[72a] Ferritin H subunits predominate in tissues with high oxygen levels, e.g., heart and blood cells, while the L subunits predominate in tissues with slower turnover of iron, e.g., liver.[72] The L subunits lack ferrooxidase activity but, in the centers of their helical bundles, contain polar side chains that may help to initiate growth of the mineral core.[64]

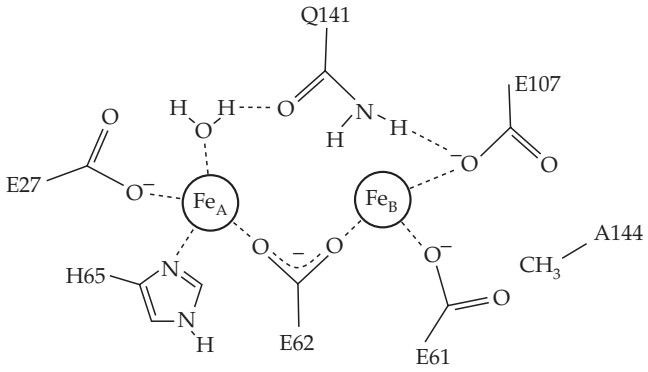


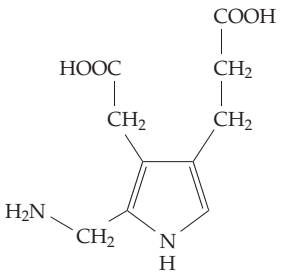

**Figure 16-4** The dinuclear iron center or ferroxidase center of human ferritin based on the structure of a terbium(III) derivative.[73] Courtesy of Pauline Harrison.

Removal of Fe(III) from storage in ferritin cores may require reduction to Fe(II) again, possibly by ascorbic acid[75] or glutathione. Some bacterial ferritins contain a bound cytochrome *b* which may assist in reduction.[67,67a] Released iron in the $Fe^{2+}$ state can be incorporated into iron-containing proteins or into heme. The enzyme **ferrochelatase**[76–76b] catalyzes the transfer of free $Fe^{2+}$ into protoporphyrin IX (Section 3) to form protoheme (Fig. 16-5). Iron in the Fe(II) state may also be oxidized to $Fe^{3+}$ through action of the copper-containing ceruloplasmin (Section D) and be incorporated into heme by direct transfer from ferritin.[53]

## 3. Heme Proteins

In 1879, German physiological chemist Hoppe-Seyler showed that two of the most striking pigments of nature are related. The red iron-containing **heme** from blood and the green magnesium complex **chlorophyll a** of leaves have similar ring structures. Later, H. Fischer proved their structures and provided them with the names and numbering systems that are used today. This information is summarized in the following section.

***Some names to remember.*** **Porphins** are planar molecules which contain large rings made by joining four pyrrole rings with methine bridges. In the **chlorins**, found in the chlorophylls, one of the rings (ring D in chlorophyll, Fig. 23-20) is reduced. The specific class of porphins known as **porphyrins** have eight substituents around the periphery of the large ring. Like the chlorins and the **corrins** of vitamin $B_{12}$ (Section B), the porphyrins are all formed biosynthetically from **porphobilinogen**. This compound is polymerized in two ways (see Fig. 24-21) to give porphyrins of types I and III (Fig. 16-5). In type I porphyrins, polymerization of porphobilinogen has taken place in a regular way so that the sequences of the carboxymethyl and carboxyethyl side chains (often referred to as acetic acid and propionic acid side chains, respectively) are the same all the way around the outside of the molecule. However, most biologically important porphyrins belong to type III, in which the first three rings A, B, and C have the same sequence of carboxymethyl and carboxyethyl side chains, but in which ring D has been incorporated in a reverse fashion. Thus, the carboxyethyl side chains of rings C and D are adjacent to each other (Fig. 16-5). Porphyrins containing all four carboxymethyl and four carboxyethyl side chains intact are known as **uroporphyrins**.




Porphobilinogen

Uroporphyrins I and III are both excreted in small amounts in the urine. Another excretion product is **coproporphyrin** III, in which all of the carboxymethyl side chains have been decarboxylated to methyl groups. The feathers of the tropical touraco are colored with copper(II) complex of coproporphyrin III and this

porphyrin as well as others are commonly found in birds' eggs. The heme proteins are all derived from protoporphyrin IX, which is formed by decarboxylation and dehydrogenation of two of the carboxyethyl side chains of uroporphyrin III to vinyl groups (Fig. 16-5).

***Hemes and heme proteins.*** Protoporphyrin IX contains a completely conjugated system of double bonds. In the center two hydrogen atoms are attached, one each to two of the nitrogens; they are free to move to other nitrogens in the center with rearrangement of the double bonds. Thus, there is tautomerism as well as resonance within the heme ring.[77–78a] The two central hydrogens can be replaced by many metal ions to form stable chelates. The complexes with $Fe^{2+}$ are known as **hemes** and the $Fe^{2+}$ complex with protoporphyrin IX as **protoheme**. Heme complexes of $Fe^{2+}$ may be designated as **ferrohemes** and the $Fe^{3+}$ compounds as **ferrihemes**. The $Fe^{3+}$ protoporphyrin IX is also called **hemin**, and may be crystallized as a chloride salt.[78b] Iron tends to have a coordination number of six, and other ligands can attach to the iron from the two axial positions on opposite sides of the planar heme. If these are nitrogen ligands, such as pyridine or imidazole, the resulting compounds, called **hemochromes**, have characteristic absorption spectra. An example is cytochrome $b_5$, which contains two axial imidazole groups.

Several modifications of protoheme are indicated in Fig. 16-5. To determine which type of heme exists in a particular protein, it is customary to split off the heme by treatment with acetone and hydrochloric acid and to convert it by addition of pyridine to the pyridine hemochrome for spectral analysis. By this means, protoheme was shown to occur in hemoglobin, myoglobin, cytochromes of the *b* and P450 types, and catalases and many peroxidases. Cytochromes *a* and $a_3$ contain **heme *a***, while one of the terminal oxidase

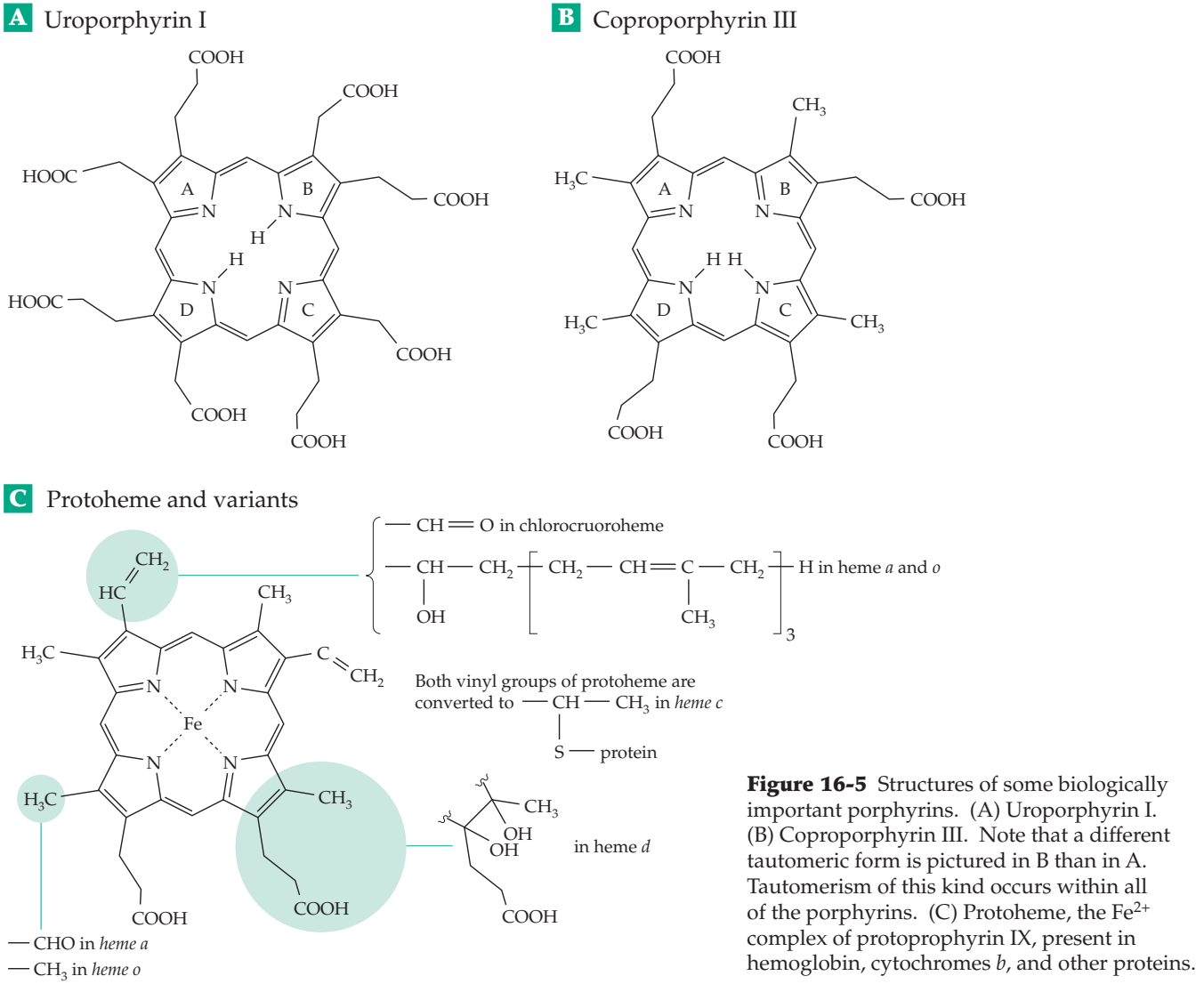



**Figure 16-5** Structures of some biologically important porphyrins. (A) Uroporphyrin I. (B) Coproporphyrin III. Note that a different tautomeric form is pictured in B than in A. Tautomerism of this kind occurs within all of the porphyrins. (C) Protoheme, the $Fe^{2+}$ complex of protoprophyrin IX, present in hemoglobin, cytochromes *b*, and other proteins.

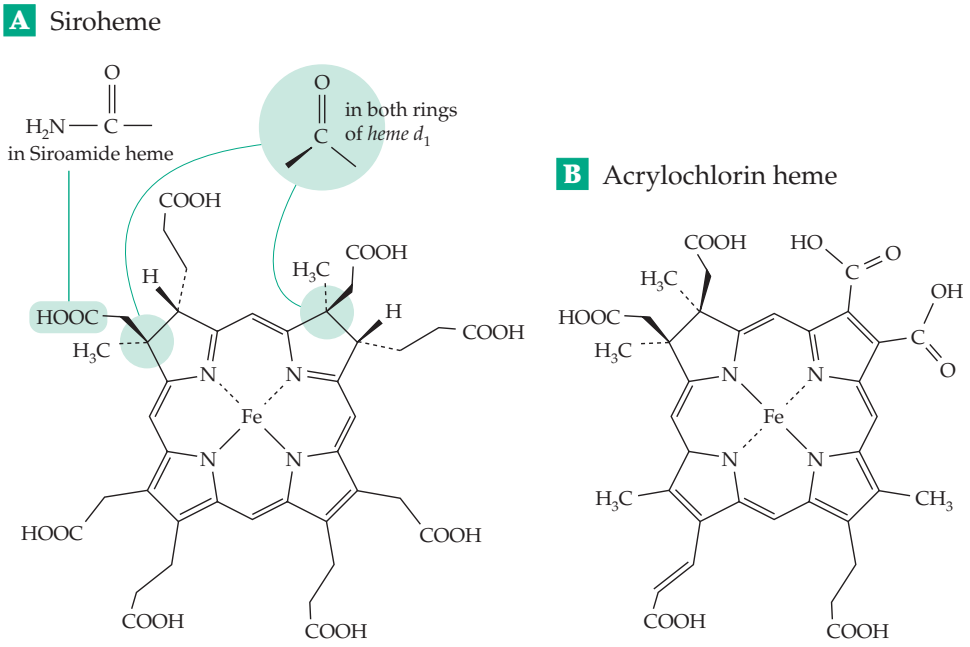



**Figure 16-6** Structures of isobacteriochlorin prosthetic groups. (A) Siroheme from nitrite and sulfite reductases; (B) acrylochlorin heme from dissimilatory nitrite reductases of *Pseudomonas* and *Paracoccus*.

systems of enteric bacteria contains the closely related heme *o* (Fig. 16-5).[79,80] A second terminal oxidase of those same bacteria contains **heme *d*** (formerly $a_2$). **Heme *c*** (present in cytochromes *c* and *f*) is a variation in which two SH groups of the protein have added to the vinyl groups of protoheme to form two thioether linkages (Fig. 16-5). A few cytochromes *c* have only one such linkage. In myeloperoxidase (Section 6) three covalent linkages, different than those in cytochrome *c*, join the heme to the protein.[81,82] There is a possibility that heme *a* may sometimes form a Schiff base with a lysyl amino group through its formyl group.[83,84]

Heme *d* is a chlorin,[85] as is **acrylochlorin heme** from certain bacterial nitrite reductases (Fig. 16-6).[86,87] **Siroheme** (Fig. 16-6), which is found in both nitrite and sulfite reductases of bacteria (Chapter 24),[88,89] is an isobacteriochlorin in which both the A and B rings are reduced. It apparently occurs as an amide **siroamide** (Fig. 16-6) in *Desulfovibrio*.[90] Heme $d_1$ of nitrite reductases of denitrifying bacteria is a dioxobacteriochlorin derivative (Fig. 16-6).[91,92]

As in myoglobin, hemoglobin (Fig. 7-23), and cytochrome *c* (see Fig 16-8), one axial coordination position on the iron of most heme proteins (customarily called the *proximal* position) is occupied by an imidazole group of a histidine side chain. However, in cytochrome P450 and chloroperoxidase a thiolate ($-S^-$) group from a cysteinyl side chain, and in catalase a phenolate anion from a tyrosyl side chain, occupies the proximal position. The sixth or *distal* coordination position is occupied by the sulfur atom of methionine in cytochrome *c* and most other cytochromes with low-spin iron but cytochromes $b_5$ and $c_3$ have histidine. The high-spin heme proteins, such as cytochromes *c*′, globins, peroxidase, and catalase, usually have no ligand other than weakly bound $H_2O$ in the distal position.[93]

Hemes are found in all organisms except the anaerobic clostridia and lactic acid bacteria. Heme proteins of blood carry oxygen reversibly, whereas those of **terminal oxidase systems**, **hydroxylases**, and **oxygenases** "activate" oxygen, catalyzing reactions with hydrogen ions and electrons or with carbon compounds. The heme-containing **peroxidases** and **catalases** catalyze reactions not with $O_2$ but with $H_2O_2$. Another group of heme proteins includes most of the cytochromes, which are purely electron-transferring compounds.

## 4. The Cytochromes

The iron in the small proteins known as cytochromes acts as an electron carrier, undergoing alternate reduction to the +2 state and oxidation to the +3 state. The cytochromes, discovered in 1884 by McMunn,[94] were first studied systematically in the 1920s by Keilin (Chapter 18) and have been isolated from many sources.[95–97] The classification into groups *a*, *b*, and *c* according to the position of the longest wavelength light absorption band (the α band; Fig. 16-7) follows a practice introduced by Keilin. However, it is now customary to designate a new cytochrome by giving the heme type (*a*, *b*, *c* or *d*) together with the wavelength of the α band, e.g., cytochrome $c_{552}$ or cyt $b_{557.5}$.

Cytochromes of the *b* type including bacterial cytochrome *o* contain protoheme. Because the sixth

position is ligated, most cytochromes *b* do not react with $O_2$. However, cytochromes *o* and *d* serve as terminal electron acceptors (cytochrome oxidases) and are oxidizable by $O_2$. Another protoheme-containing cytochrome, involved in hydroxylation (Chapter 18), is **cytochrome P450**. Here the 450 refers to the position of the intense "Soret band" (also called the γ band) of the spectrum (Fig. 16-7) in a difference spectrum run in the presence and absence of CO. Other properties are also used in arriving at designations for cytochromes. For example, cytochrome $a_3$ has a spectrum similar to that of cytochrome *a* but it reacts readily with both CO and $O_2$.

Another property that distinguishes various cytochromes is the redox potential $E°'$ (Table 6-8), which in this discussion is given for pH 7.0. Cytochromes carry electrons between other oxidoreductase proteins of widely varying values of $E^{0'}$. Because of the various heme environments cytochromes have greatly differing values of $E^{0'}$, allowing them to function in many different biochemical systems.[97a,97b] For mitochondrial cytochrome *c* the value of $E°'$ is ~ + 0.265 V but for the closely related cytochrome *f* of chloroplasts it is ~ +0.365 V and for cytochrome $c_3$ of *Desulfovibrio* about −0.330 V. There is more than an 0.6-volt difference between $E°'$ of cytochromes *f* and $c_3$. Cytochromes *b* tend to have lower $E°'$ values, close to zero, than most cytochromes *c*, while cytochrome $a_3$ has $E°' \sim +0.385$ V.

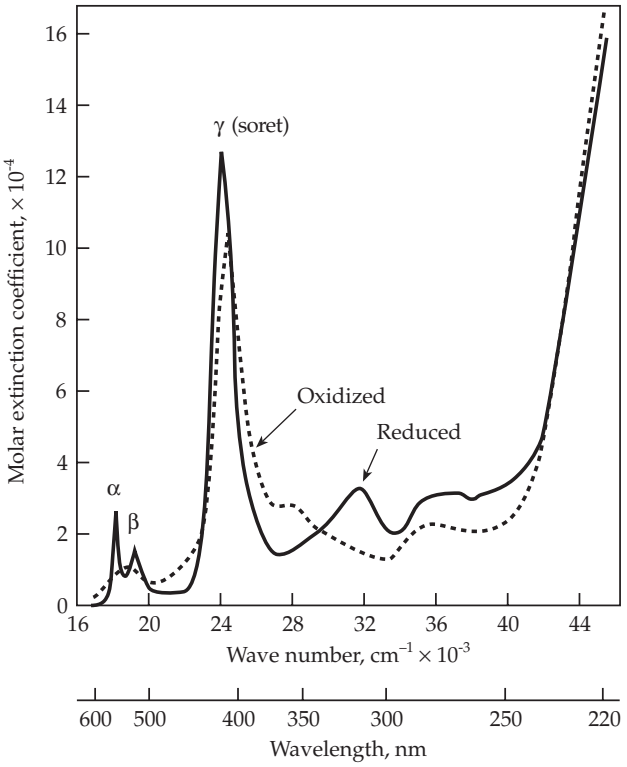



**Figure 16-7** Absorption spectra of oxidized and reduced horse heart cytochrome *c* at pH 6.8. From data of Margoliash and Frohwirt.[98]

***The c-type cytochromes.*** Mitochondrial cytochrome *c* is one of the few intracellular heme pigments that is soluble in water and that can be removed easily from membranes. A small 13-kDa protein typically containing about 104 amino acid residues, cytochrome *c* has been isolated from plants, animals, and eukaryotic microorganisms.[95–97,99,100] Complete amino acid sequences have been determined for over 100 species. Within the peptide chain 28 positions are invariant and a number of other positions contain only conservative substitutions. Cytochrome *c* was one of the first proteins to be used in attempting to trace evolutionary relationships between species by observing differences in sequence. Humans and chimpanzees have identical cytochrome *c*, but 12 differences in amino acid sequence occur between humans and the horses and 44 between human and *Neurospora*.[96] The related cytochrome $c_2$ of the photosynthetic bacterium *Rhodospillum rubrum* is thought to have diverged in evolution $2 \times 10^9$ years ago from the precursor of mammalian cytochrome *c*. Even so, 15 residues remain invariant.[101]

Structural studies[95–97,101–103] on cytochromes of the *c* and $c_2$ types show that the heme group provides a core around which the peptide chain is wound. The 104 residues of mitochondrial cytochrome *c* are enough to do little more than envelope the heme. In both the oxidized and reduced forms of the protein, methionine 80 (to the left in Fig. 16-8A) and histidine 18 (to the right) fill the axial coordination positions of the iron. The heme is nearly "buried" and inaccessible to the surrounding solvent.

The shorter chains of the 82- to 86-residue cytochromes $c_{550}$ (from *Pseudomonas*[102]), $c_{553}$,[102a] and $c_{555}$ (from *Chlorobium*[105]) as well as the longer 112-residue polypeptide of cytochrome $c_2$ from *Rhodospirillum rubrum*[93] have nearly the same folding pattern as that in mitochondrial cytochrome *c*. However, the 128-residue chain of the dimeric cytochrome *c'* from *Rhodospirillum molischianum* forms an antiparallel four-helix bundle (Fig. 16-8).[106–109] This is the same folding pattern present in the ferritin monomer (Fig. 16-3), hemerythrin (Fig. 2-22), and many other proteins including cytochrome $b_{562}$ of *E. coli*.[110] Cytochrome *f*, which functions in photosynthetic electron transport, is also a *c*-type cytochrome but with a unique protein fold.[111,112]

Most cytochromes have only one heme group per polypeptide chain,[112] but the 115-residue cytochrome $c_3$ from the sulfate-reducing bacterium *Desulfovibrio* binds four hemes (Fig. 16-8C).[104,113–115] Each one seems to have a different redox potential in the −0.20 to −0.38 V range.[114] Another *c*-type cytochrome, also from *Desulfovibrio*, contains six hemes in a much larger 66-kDa protein and functions as a nitrite reductase.[116]

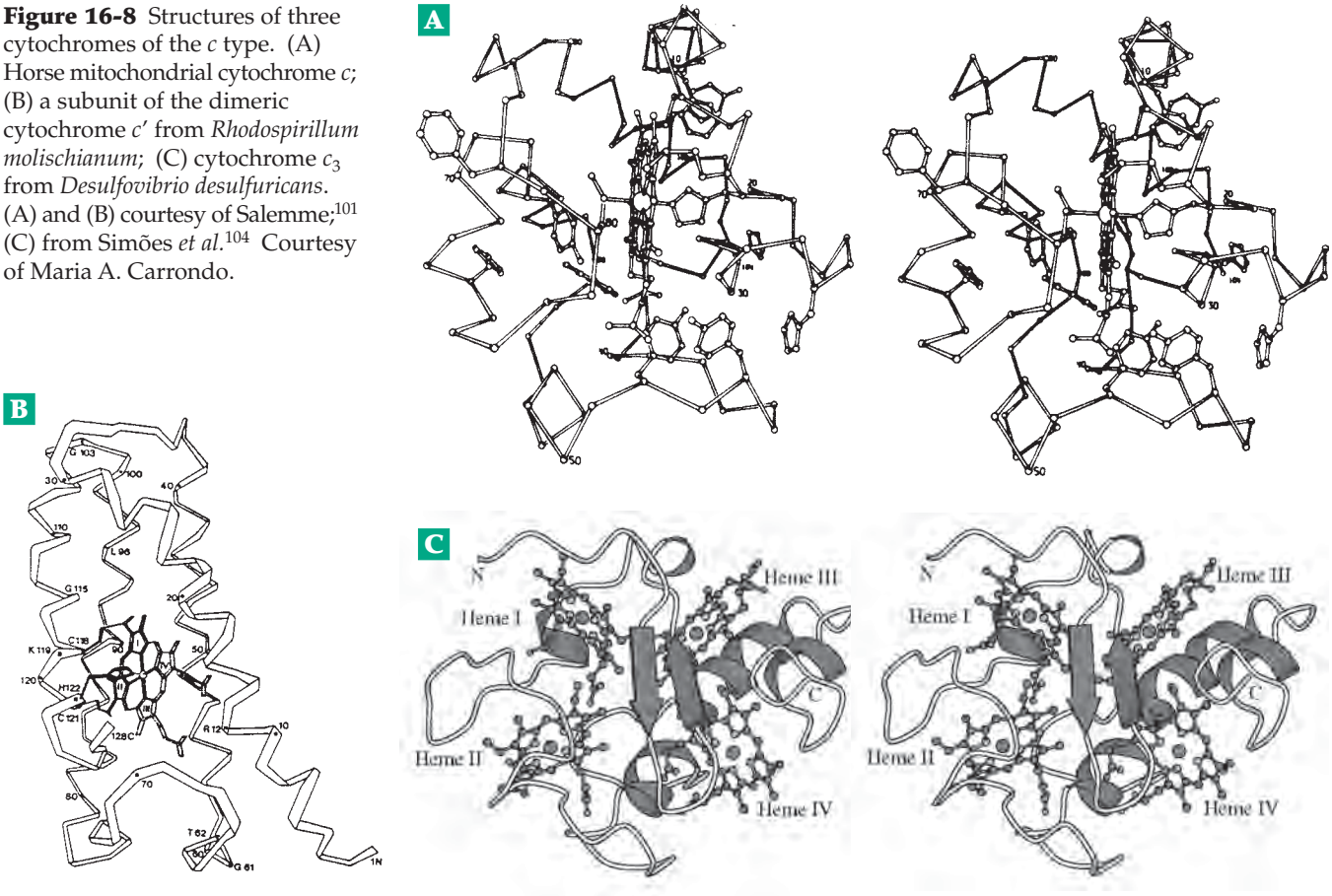



**Figure 16-8** Structures of three cytochromes of the *c* type. (A) Horse mitochondrial cytochrome *c*; (B) a subunit of the dimeric cytochrome *c'* from *Rhodospirillum molischianum*; (C) cytochrome $c_3$ from *Desulfovibrio desulfuricans*. (A) and (B) courtesy of Salemme;[101] (C) from Simões *et al.*[104] Courtesy of Maria A. Carrondo.

Triheme and octaheme proteins are also known.[117]

Many cytochromes *c* are soluble but others are bound to membranes or to other proteins. A well-studied tetraheme protein binds to the reaction centers of many purple and green bacteria and transfers electrons to those photosynthetic centers.[118–120] Cytochrome $c_2$ plays a similar role in *Rhodobacter*, forming a complex of known three-dimensional structure.[121] Additional cytochromes participate in both cyclic and noncyclic electron transport in photosynthetic bacteria and algae (see Chapter 23).[120,122–124] Some bacterial membranes as well as those of mitochondria contain a **cytochrome $bc_1$ complex** whose structure is shown in Fig. 18-8.[125,126]

***Cytochromes b, a, and o.*** Protoheme-containing cytochromes *b* are widely distributed.[127,128] There are at least five of them in *E. coli*. Whether in bacteria, mitochondria, or chloroplasts, the cytochromes *b* function within electron transport chains, often gathering electrons from dehydrogenases and passing them on to *c*-type cytochromes or to iron–sulfur proteins. Most cytochromes *b* are bound to or embedded within membranes of bacteria, mitochondria, chloroplasts, or endoplasmic reticulum (ER). For example, cytochrome $b_5$[129,129a] delivers electrons to a fatty acid desaturating system located in the ER of liver cells and to many other reductive biosynthetic enzymes.[130–132] The protein contains 132 amino acid residues plus another 85 largely hydrophobic N-terminal residues that provide a nonpolar tail which is thought to be buried in the ER membranes.[130] Solubilization of the protein causes loss of this N-terminal sequence. The heme in cytochrome $b_5$ is not covalently bonded to the protein but is held tightly between two histidine side chains. The polypeptide chain is folded differently than in either cytochrome *c* or myoglobin.

The folding pattern of cytochrome $b_5$ is also found in the complex heme protein **flavocytochrome $b_2$** from yeast (Chapter 15)[133] and probably also in liver **sulfite oxidase**[134,135] Both are 58-kDa peptides which can be cleaved by trypsin to 11-kDa fragments that have spectroscopic similarities and sequence homology with cytochrome $b_5$. Sulfite oxidase also has a molybdenum center (Section H). The 100-residue N-terminal portion of flavocytochrome $b_2$ has the cytochrome $b_5$ folding pattern but the next 386 residues form an eight-stranded $(\alpha/\beta)_8$ barrel that binds a molecule of FMN.[133,136] All of these proteins pass electrons to cytochrome *c*. In contrast, the folding of **cytochrome**

**$b_{562}$** of *E. coli* resembles that of cytochrome *c′* (Fig. 16-8).[110,137] However, it has methionine side chains as both the fifth and sixth iron ligands.

Cytochromes *b* of mitochondrial membranes are involved in passing electrons from succinate to ubiquinone in complex II[138] and also from reduced ubiquinone to cytochrome $c_1$ in the 248-kDa complex III (Fig. 18-8). A similar complex is present in photosynthetic purple bacteria.[123,139] Cytochrome $b_{560}$ functions in the transport of electrons from succinate dehydrogenase to ubiquinone,[138] and cytochrome $b_{561}$ of secretory vesicle membranes has a specific role in reducing ascorbic acid radicals.[140]

In bacteria some cytochromes *b* and $d_1$ serve as terminal electron carriers able to react with $O_2$, nitrite, or nitrate, while others act as carriers between redox systems.[141–143a] The aldehyde heme *a* is utilized by animals and by some bacteria in **cytochrome *c* oxidase**, a complex enzyme whose three-dimensional structure is known (see Fig. 18-10) and which is discussed further in Chapter 18.

## 5. Mechanisms of Biological Electron Transfer

The heme groups of the cytochromes as well as many other transition metal centers act as carriers of electrons. For example, cytochrome *c* may accept an electron from reduced cytochrome $c_1$ and pass it to cytochrome oxidase or cytochrome *c* peroxidase. The electron moves from one heme group to another over distances as great as 2 nm. Similar electron-transfer reactions between defined redox sites are met in photosynthetic reaction centers (Fig. 23-31), in metalloflavoproteins (Fig. 15-9), and in mitochondrial membranes.

What are the factors that determine the probability of an electron transfer reaction and the rate at which it may occur? They include: (1) The distance from the electron donor to the acceptor. (2) The thermodynamic driving force $\Delta G°$ for the reaction. This can be approximated using the difference in standard electrode potentials (as in Table 6-1) between donor and acceptor. $\Delta G° = -96.5\ \Delta E°$ kJ/mol at 25° C. (3) The chemical makeup of the material through which the electron transfer takes place. (4) Any changes in the geometry or charge state of the donor or acceptor that accompany the transfer. (5) The orientation of the acceptor and donor groups.[144–146] It is usually assumed that the Franck–Condon principle is obeyed, i.e., that the electron jump occurs so rapidly ($<10^{-12}$ s) that there is no change in the positions of atomic nuclei (see also Chapter 23). Subsequent rearrangement of nuclear positions may occur at rates that allow a rapid overall reaction.

The various factors that affect the rate of electron transfer were incorporated by Marcus into a quantitative theory. Electron transfer is often discussed in terms of this classic Marcus theory together with effects of quantum mechanical tunneling.[144,147–150] According to Marcus the electron transfer rate from a donor to an acceptor at a fixed separation depends upon $\Delta G°$, a nuclear reorganization parameter ($\lambda$), and the electronic coupling strength $|H_{AB}|$ between reactant and product in the transition state (Eq. 16-3):

$$k_{ET} = (4\pi^3 / h^2 \lambda k_B T)|H_{AB}|^2 \exp[-(\Delta G° + \lambda)^2 / 4\lambda k_B T] \quad (16\text{-}3)$$

Here $|H_{AB}|$ is a quantum mechanical matrix whose strength decreases exponentially with the distance of separation *R* as $e^{-\beta R}$ where $\beta$ is a coefficient of the order of 9–14 $nm^{-1}$. At the closest contact (R = 0) the rate $k_{ET}$, by extrapolation from experimental data on small synthetic compounds, is close to the molecular vibration frequency of $10^{13}$ $s^{-1}$.[151,152] At distances greater than 2 nm the rate would be negligible were it not for other factors.

Using mutant proteins as well as a variety of redox pairs and electron-transfer distances the validity of the Marcus equation with respect to the thermodynamic driving force and distance dependence has been verified.[153] This is even true for cytochrome *c* mutants functioning in living yeast cells.[146]

A huge amount of experimental work with proteins has been done to test and refine the theories of electron transport. For example, electron donor groups with various reduction potentials have been attached to various sites on the surface of a protein containing a heme or other electron accepting group. Ruthenium complexes such as Ru(III) $(NH_3)_5^{3+}$ form tight covalent linkages to imidazole nitrogens[154,155] such as that of His 33 of horse heart cytochrome *c*. This metal can be reduced rapidly to Ru(II) by an external reagent, after which the transfer of an electron from the Ru(II) across a distance of 1.2 nm to the heme Fe(III) can be followed spectroscopically. The reduction potentials *E°* for the Ru(III) / Ru(II) and Fe(III) / Fe(II) couples at pH 7 in these compounds are 0.16 and 0.27 V, respectively. Thus, an electron will jump spontaneously from the Ru(II) to the Fe(III) with $\Delta G° = -15.4$ kJ/mol. A rate constant of ~5 $s^{-1}$, which was nearly independent of temperature, was observed. Since the structures of Fe(II) and Fe(III) forms of cytochrome *c* differ only slightly,[156] the electron transfer apparently occurs with only a small amount of geometric rearrangement. The distribution of charges and dipoles within the protein may be such that the $Fe^{2+}$ and $Fe^{3+}$ complexes have almost equal thermodynamic stability.

***Electron-transfer pathways?*** In spite of the success of the Marcus theory, rates of electron-transfer from the iron of cytochrome *c* have been found to vary for different pathways.[150,153,155] For example, transfer of an electron from Fe(II) in reduced cytochrome *c* to an Ru(III) complex on His 33 was fast (~440 $s^{-1}$)[157] but

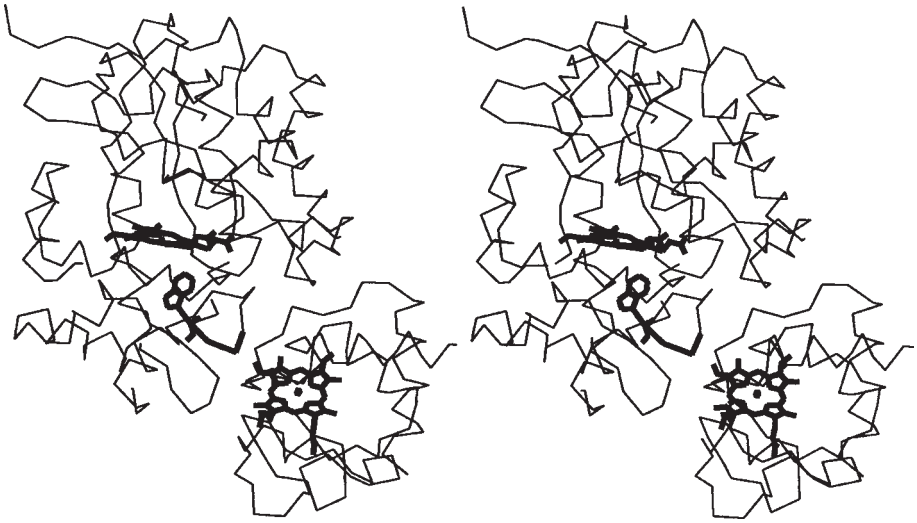


**Figure 16-9** Stereoscopic α-carbon plot of yeast cytochrome *c* peroxidase (top) and yeast cytochrome *c* (below) as determined from a cocrystal by Pelletier and Kraut.[164] The heme rings of the two proteins appear in bold lines, as does the ring of tryptophan 191 and the backbone of residues 191–193 of the cytochrome *c* peroxidase. Drawing from Miller *et al.*[165]

the rate of transfer to an equidistant Fe(III) ion on Met 65 was at most 0.6 $s^{-1}$. These results suggested that distinct electron-transfer pathways exist. One suggestion was that the sulfur atom of Met 80 donates an electron to $Fe^{3+}$ leaving an electron-deficient radical. The "hole" so created could be filled by an electron jumping in from the –OH group of the adjacent Tyr 67, which might then accept an electron from an external acceptor via Tyr 74 at the protein surface. Do electrons flow singly or as pairs from the surface through hydrogen-bonded paths? Use of both semisynthesis[155] and directed mutation[153] of cytochromes *c* is permitting a detailed study of these effects. A striking result is that substitution of the conserved residue phenylalanine 82 in a yeast cytochrome *c* with leucine or isoleucine retards electron transfer by a factor of ~$10^4$.

***"Docking."*** It is now recognized that there are distinct "docking sites" on the surface of electron-transport proteins. For rapid electron transfer to occur the two electron carriers must be properly oriented and docked by formation of correct polar and nonpolar interactions. Early indications of the importance of docking came from study of modified cytochromes *c.* Each one of the 19 lysine side chains was individually altered by acylation or alkylation to remove the positive charge or to replace it with a negative charge. The rate of electron transfer into cytochrome *c* from hexacyanoferrate was decreased by a factor of 1.3–2.0 when any one lysine at positions 8, 13, 27, 72, or 79, which are clustered around the heme edge, was modified.[158] Modification of Lys 22, 55, 99, or 100, distant from this edge, had no effect. Electron transfer *into* cytochrome *c* from its natural electron donor ubiquinol:cytochrome *c* reductase was also strongly inhibited by modification of lysines that surround the heme edge.[159] Modification of these lysines also inhibited electron transfer *out of* cytochrome *c* into its natural acceptors, cytochrome *c* oxidase and cytochrome *c* peroxidase (Fig. 16-9).[160] A major factor in these effects is probably the large dipole moment in the cytochrome *c* that arises from the unequal distribution of surface charges. This charge distribution must assist in the proper docking of the cytochrome with its natural electron donors and acceptors. The positive surface charges presumably also facilitate the reaction with hexacyanoferrate (II) or ascorbate, both of which are negatively charged reductants that react rapidly with cytochrome *c*.

Measurements of many kinds have been made between natural donor–acceptor pairs such as cytochrome *c*–cytochrome $b_5$,[161,162] cytochrome *c*–cytochrome *c* peroxidase (Fig. 16-9),[153,163–166] trimethylamine dehydrogenase–FMN to $Fe_4S_4$ center (Fig. 15-9),[167] and methylamine dehydrogenase (TTQ radical)–amicyanin ($Cu^{2+}$).[168] Designed metalloproteins are being studied as well.[169] Femtosecond laser spectroscopy is providing a new approach.[169a]

***Coupling and gating of electron transfer.*** Electrons are thought to be transferred into or out of cytochrome *c* through the exposed edge of the heme. The rate depends upon effective coupling, which in turn may depend upon orientation as well as the structure and dynamics of the protein.[170] Proteins with much β structure appear to provide stronger coupling than those that are largely composed of α helices.[144,171] The high nuclear reorganization energy λ of α helices may block electron transfer along some pathways.[153] A conformational change,[167,169] transfer of a proton, or binding of some other specific ion[172] before electron transfer occurs can be the "gating" process that determines the rate of electron transfer.[162,167,173] Electron transfer can also be "coupled" to an unfavorable, but fast, equilibrium.

***Effects of ionic equilibria on electron transfer.*** The charge on an ion of $Fe^{2+}$ in a heme is exactly balanced by two negative charges on the porphyrin ring. However, when the $Fe^{2+}$ loses an electron to become $Fe^{3+}$ an extra positive charge is suddenly present in the center of the protein. This change in charge will have a powerful electrostatic effect on charged groups in the immediate vicinity of the iron and even at the outer surface of the molecule. For example, an anion from the medium or from a neighboring protein molecule might become bound to the heme protein (Eq. 16-4).

$$X^- + [\,Fe(II)\ (heme)\,] \rightarrow [\,Fe(III)\ (heme)\,]^+\ X^- + e^- \qquad (16\text{-}4)$$

In this case the presence of a high concentration of $X^-$ in the medium would favor the oxidation of Fe(II) to Fe(III). The reduced heme would be a better reducing agent and the oxidized form a weaker oxidant than in the absence of $X^-$. If –YH were a group in the protein the loss of an electron could cause –YH to dissociate so that $Y^-$ and the Fe(III) would interact more tightly (Eq. 16-5).

$$\begin{aligned} HY &— [\ Fe\ (II)\ (heme)\ ] \rightarrow \\ Y^- &— [\ Fe\ (III)\ (heme)\ ]^+ + H^+ + e^- \end{aligned} \qquad (16\text{-}5)$$

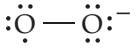
We see that electron transfer can be accompanied by loss of a proton and that $E^{\circ\prime}$ may become pH dependent. (See also Eq. 16-18.) Even with cytochrome *c*, although there is little structural change upon electron transfer, there is an increased structural mobility in the oxidized form.[156] This may be important for coupling and could also facilitate associated proton-transfer reactions. For example, it is possible that in some cytochromes the imidazole ring in the fifth coordination position may become deprotonated upon oxidation. This possibility is of special interest because cytochromes are components of proton pumps in mitochondrial membranes (Chapter 18).

## 6. Reactions of Heme Proteins with Oxygen or Hydrogen Peroxide

As Ingraham remarked,[174] "Living in a bath of 20% oxygen, we tend to forget how reactive it is." From a thermodynamic viewpoint, all living matter is extremely unstable with respect to combustion by oxygen. Ordinarily, a high temperature is required and if we are careful with fire, we can expect to escape a catastrophe. However, one mole of properly chelated copper could catalyze consumption of all of the air in an average room within one second.[174] Biochemists are interested in both the fact that $O_2$ is kinetically stable and unreactive and also that oxidative enzymes such as cytochrome *c* oxidase are able to promote rapid reactions. Two oxygen atoms, each with six valence electrons, might reasonably be expected to form dioxygen, $O_2$, as a double-bonded structure with one σ and one π bond as follows (left):

Dioxygen

However, $O_2$ is paramagnetic and contains two unpaired electrons.[175] From this evidence $O_2$ might be assigned the structure on the right.[175] The oxygen molecule is very stable, and it is relatively difficult to add an electron to form the reactive **superoxide anion radical** $O_2^-$.



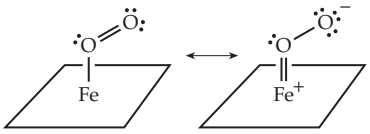
Superoxide anion radical

For this reason, oxidative attack by $O_2$ tends to be slow. However, once an electron has been acquired, it is easy for additional electrons to be added to the structure and further reduction occurs more easily. The biochemical question suggested is, "How can some heme proteins carry $O_2$ reversibly without any oxidation of the iron contained in them while others *activate* oxygen toward reaction with substrates?" Among this latter group, cytochrome *c* oxidase transfers electrons to both oxygen atoms so that only $H_2O$ is a product, whereas the hydroxylases and oxygenases, which are discussed in Chapter 18, incorporate either one or two of the atoms of $O_2$, respectively, into an organic substrate. Before examining these reactions let us reconsider the heme oxygen carriers.

***Oxygen-carrying proteins.*** In Chapter 7, we examined the behavior of hemoglobin in the cooperative binding of four molecules of $O_2$ and studied its structural relationship to the monomeric muscle protein myoglobin.[176] The iron in functional hemoglobin and myoglobin is always Fe(II) and is only very slowly converted by $O_2$ into the Fe(III) forms methemoglobin or metmyoglobin.[177,178] Erythrocytes contain an enzyme system for immediately reducing methemoglobin back to the Fe(II) state (see Box 15-H).

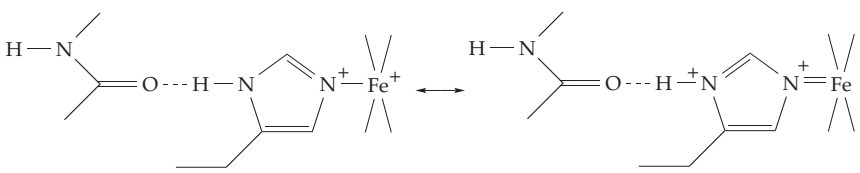
Binding of $O_2$ to the iron in the heme is usually considered not to cause a change in the oxidation state of the metal. However, oxygenated heme has some of the electronic characteristics of an $Fe^{3+}$–$OO^-$ peroxide anion. Bonding of the heme iron to oxygen is thought to occur by donation of a pair of electrons by the oxygen to the metal. In deoxyhemoglobin the Fe(II) ion is in the "high-spin" state; four of the five 3*d* orbitals in the valence shell of the iron contain one unpaired electron and the fifth orbital contains two paired electrons. The binding of oxygen causes the iron to revert

to the "low-spin" state in which all of the electrons are paired and the paramagnetism of hemoglobin is lost. The stability of heme–oxygen complexes is thought to be enhanced by "back-bonding," i.e., the donation of an electron pair from one of the filled *d* orbitals of the iron atom to form a bond with the adjacent oxygen.[179] This can be indicated symbolically as follows:




These structures, which have been formulated by assuming that one of the unshared electron pairs on $O_2$ forms the initial bond to the metal, are expected to lead to an angular geometry which has been observed in X-ray structures of model compounds,[180] in oxymyoglobin (Fig. 16-10),[181,182] and in oxyhemoglobin.[183] Neutron diffraction studies have shown that the outermost oxygen atom of the bound $O_2$ is hydrogen bonded to the H atom on the $N^{\varepsilon}$ atom of the distal imidazole ring of His E7 (Fig. 16-10). Carbon monoxide binds with the C≡O axis perpendicular to the heme plane and unable to form a corresponding hydrogen bond.[184] This decreases the affinity for CO and helps to protect us from carbon monoxide poisoning.

All oxygen-carrying heme proteins have another imidazole group that binds to iron on the side opposite the oxygen site. Without this proximal imidazole group, heme does not combine with oxygen. Coordination with heterocyclic nitrogen compounds favors formation of low-spin iron complexes and simple synthetic compounds that closely mimic the behavior of myoglobin have been prepared by attaching an imidazole group by a chain of appropriate length to the edge of a heme ring.[179,185] Similar compounds bearing a pyridine ring in the fifth coordination position have a low affinity for oxygen. Thus, the polarizable imidazole ring itself seems to play a role in promoting oxygen binding. The π electrons of the imidazole ring may also participate in bonding to the iron as is indicated in the following structures.[179] The π bonding to the iron would allow the iron to back-bond more strongly to an $O_2$ atom entering the sixth coordination position. These diagrams illustrate another feature found frequently in heme proteins: The N–H group of the imidazole is hydrogen bonded to a peptide backbone carbonyl group.



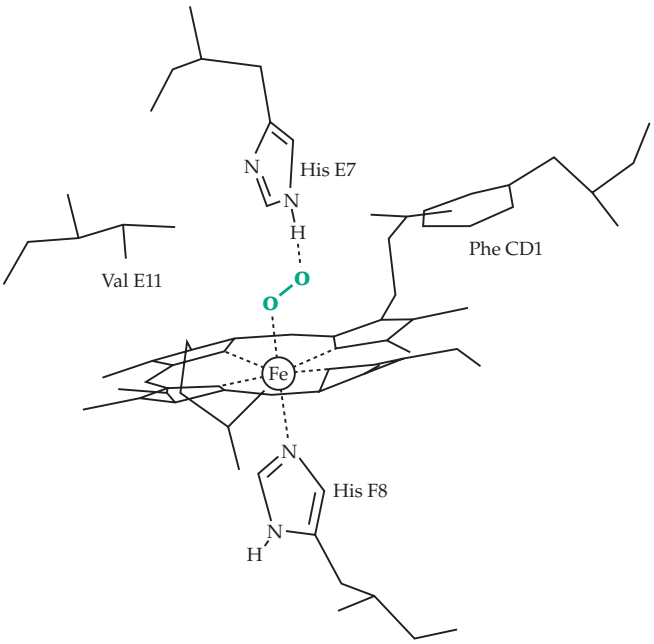




**Figure 16-10** Geometry of bonding of $O_2$ to myoglobin and position of hydrogen bond to $N^{\varepsilon}$ of the distal histidine E7 side chain. After Perutz.[182]

The coordination of the heme iron to histidine also appears to provide the basis for the cooperativity in binding of oxygen by hemoglobin.[186] The radius of high-spin iron, whether Fe(II) or Fe(III), is so large that the iron cannot fit into the center of the porphyrin ring but is displaced toward the coordinated imidazole group by a distance of ~0.04 nm for Fe(II).[187] Thus, in deoxyhemoglobin both iron and the imidazole group lie further from the center of the ring than they do in oxyhemoglobin. In the latter, the iron lies in the center of the porphyrin ring because the change to the low-spin state is accompanied by a decrease in ionic radius.[186,188] The change in protein conformation induced by this small shift in the position of the iron ion was described in Chapter 7. However, the exact nature of the linkage between the Fe position and the conformational changes is not clear.

The mechanical response to the movement of the iron and proximal histidine, described in Chapter 7, may explain this linkage. However, oxygenation may also induce a change in the charge distribution within the hydrogen-bond network of the protein. The carbonyl group shown in the foregoing structure is attached to the F helix (see Fig. 7-23) and is also hydrogen bonded to other amide groups. Electron withdrawal into the heme–oxygen complex would tend to strengthen the hydrogen bond as indicated by the resonance forms shown and also to weaken

competing hydrogen bonds.[189,190] This could affect the charge distribution in the upper end of the F helix and could conceivably induce a momentary conformational change that could facilitate the rearrangement of structure that was discussed in Chapter 7 (Fig. 7-25). The $\alpha_1\beta_2$ contact in which a change of hydrogen bonding takes place is located nearby behind the F and G helices. In any event, it is remarkable that nature has so effectively made use of the subtle differences in the properties of iron induced by changes in the electron distribution within the *d* orbitals of this transition metal.

A few groups of invertebrates, e.g., the sipunculid worms, use a nonheme iron-containing protein, **hemerythrin**, as an oxygen carrier.[191,192] Its 113-residue subunits are often associated as octamers of $C_4$ symmetry, each peptide chain having a four-helix bundle structure (Fig. 2-22). Instead of a heme group, each monomer contains two atoms of high-spin Fe(II) held by a cluster of histidine and carboxylate side chains (see Fig. 16-20).[193,194] Hemerythrin is a member of a group of such diiron oxoproteins which are considered further in Section 8. The copper oxygen carrier **hemocyanin** is discussed in Section D.

***Catalases and peroxidases.*** Many iron and copper proteins do not bind $O_2$ reversibly but "activate" it for further reaction. We will look at such metalloprotein oxidases in Chapter 18. Here we will consider heme enzymes that react not with $O_2$ but with peroxides. The peroxidases,[194a] which occur in plants, animals, and fungi, catalyze the following reactions (Eq. 16-6, 16-7):

$$H_2O_2 + AH_2 \rightarrow 2\,H_2O + A \qquad (16\text{-}6)$$

$$ROOH + AH_2 \rightarrow R{-}OH + H_2O + A \qquad (16\text{-}7)$$

Here $AH_2$ is an oxidizable organic compound such as an alcohol or a pair of one-electron donor molecules. Catalases, which are found in almost all aerobic cells,[194b] may sometimes account for as much as 1% of the dry weight of bacteria. The enzyme catalyzes the breakdown of $H_2O_2$ to water and oxygen by a mechanism similar to that employed by peroxidases. If Eq. 16-7 is rewritten with $H_2O_2$ for $AH_2$ and $O_2$ for A, we have the following equation:

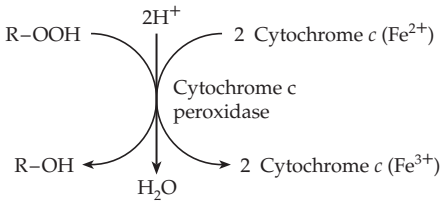
$$2\,H_2O_2 \xrightarrow{\text{Catalase}} 2\,H_2O + O_2 \qquad (16\text{-}8)$$

The action of catalase is very fast, almost $10^4$ times faster than that of peroxidases. The molecular activity per catalytic center is about $2 \times 10^5\ s^{-1}$.

Catalase exerts a protective function by preventing the accumulation of $H_2O_2$ which might be harmful to cell constituents. The complete intolerance of obligate anaerobes to oxygen may result from their lack of this enzyme. Support for this protective function comes from the existence of the human hereditary condition **acatalasemia**.[195,196] Persons with extremely low catalase activity are found worldwide but are especially numerous in Korea. In Japan it is estimated that there are 1800 persons lacking catalase. Because about half of them have no symptoms, catalase might be judged unessential. However, many of the individuals affected develop ulcers around their teeth. Apparently, hydrogen peroxide produced by bacteria accumulates and oxidizes hemoglobin to methemoglobin (Box 15-H) depriving the tissues of oxygen.

Catalase from most eukaryotic species is tetrameric.[197] The protein from beef liver consists of 506-residue subunits.[198] Human catalase is similar.[198a] The proximal ligand to the heme $Fe^{3+}$ is a tyrosinate anion (Tyr 358), while side chains of His 75 and Asn 148 lie close to the heme on the distal $H_2O_2$-binding side (Fig. 16-11). Larger ~650-residue fungal and bacterial catalases have a similar folding pattern but an extra C-terminal domain with a flavodoxin-like structure.[197,199] Catalase is gradually inactivated by its very reactive substrate. As isolated, beef liver catalase usually contains about two subunits in which the heme ring has been oxidatively cleaved to **biliverdin**[200] (Fig. 24-24) and various other alterations have been found.[197] Each subunit of mammalian catalases normally contains a bound molecule of NADPH which helps to protect against inactivation by $H_2O_2$.[201,202] Catalases from *Neurospora* and from *E. coli* contain heme *d* rather than protoporphyrin.[197,203] Some lactobacilli, lacking heme altogether, form a manganese-containing pseudocatalase.[204,205]

Of the plant peroxidases, which are found in abundance in the peroxisomes, the 40-kDa monomeric **horseradish peroxidase** has been studied the most.[206–208a] It occurs in over 30 isoforms and has an extracellular role in generating free radical intermediates for polymerization and crosslinking of plant cell wall components.[209] Secreted fungal peroxidases, e.g., such as those from *Coprinus*[210] and *Arthromyces*,[211] form a second class of peroxidases with related structues.[212] A third class is represented by **ascorbate peroxidase** from the cytosol of the pea[212–214] and by the small 34-kDa **cytochrome c peroxidase** from yeast mitochondria[215] (Fig. 16-11). The latter has a strong preference for reduced cytochrome *c* as a substrate (Eq. 16-9).[216,217]




(16-9)

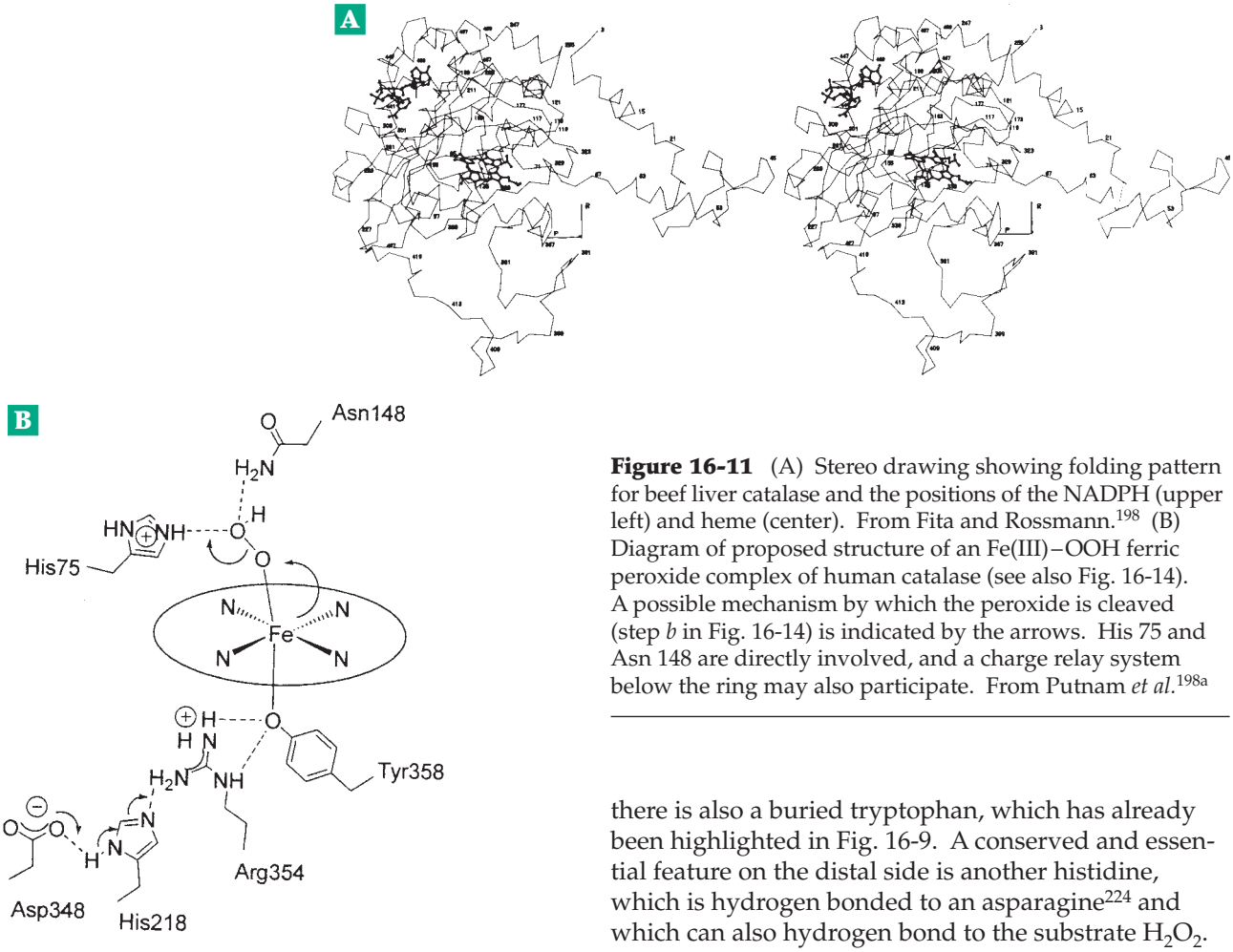



**Figure 16-11** (A) Stereo drawing showing folding pattern for beef liver catalase and the positions of the NADPH (upper left) and heme (center). From Fita and Rossmann.[198] (B) Diagram of proposed structure of an Fe(III)–OOH ferric peroxide complex of human catalase (see also Fig. 16-14). A possible mechanism by which the peroxide is cleaved (step *b* in Fig. 16-14) is indicated by the arrows. His 75 and Asn 148 are directly involved, and a charge relay system below the ring may also participate. From Putnam *et al.*[198a]

Because the three-dimensional structures of the peroxidase, its reductant cytochrome *c*, and the complex of the two (Fig. 16-9) are known, cytochrome *c* peroxidase is the subject of much experimental study. Other fungal peroxidases, some of which contain manganese rather than iron, act to degrade lignin (Chapter 25).[218] A lignin peroxidase from the white wood-rot fungus *Phanerochaete chrysosporium* has a surface tryptophan with a specifically hydroxylated Cβ carbon atom which may have a functional role in catalysis.[218a,b]

The human body contains **lactoperoxidase**, a product of exocrine secretion into milk, saliva, tears, etc., and peroxidases with specialized functions in **saliva**, the **thyroid, eosinophils**,[219] and **neutrophils**.[220] The functions are largely protective but the enzymes also participate in biosynthesis. Mammalian peroxidases have heme covalently linked to the proteins, as indicated in Fig. 16-12.[220–222a]

The active site structure of peroxidases (Fig. 16-13) is quite highly conserved. As in myoglobin, an imidazole group is the proximal heme ligand, but it is usually hydrogen bonded to an aspartate carboxylate as a catalytic diad (Fig. 16-13).[223] In cytochrome *c* peroxidase there is also a buried tryptophan, which has already been highlighted in Fig. 16-9. A conserved and essential feature on the distal side is another histidine, which is hydrogen bonded to an asparagine[224] and which can also hydrogen bond to the substrate $H_2O_2$. Fungal peroxidases also have a conserved arginine on the distal side. However, even an octapeptide with a bound heme cut from cytochrome *c* acts as a "microperoxidase" with properties similar to those of natural peroxidases.[225]

Peroxidases and catalases contain high-spin Fe(III) and resemble metmyoglobin in properties. The enzymes are reducible to the Fe(II) state in which form they are able to combine (irreversibly) with $O_2$. We see that the same active center found in myoglobin and hemoglobin is present but its chemistry has been modified by the proteins. The affinity for $O_2$ has been altered drastically and a new group of catalytic activities for ferriheme-containing proteins has emerged.

***Mechanisms of catalase and peroxidase catalysis.*** Attention has been focused on a series of strikingly colored intermediates formed in the presence of substrates. When a slight excess of $H_2O_2$ is added to a solution of horseradish peroxidase, the dark brown enzyme first turns olive green as **compound I** is formed, and then pale red as it turns into **compound II**. The latter reacts slowly with substrate $AH_2$ or with another $H_2O_2$ molecule to regenerate the original enzyme. This sequence of reactions is indicated by the colored arrows in Fig. 16-14, steps *a*–*d*.

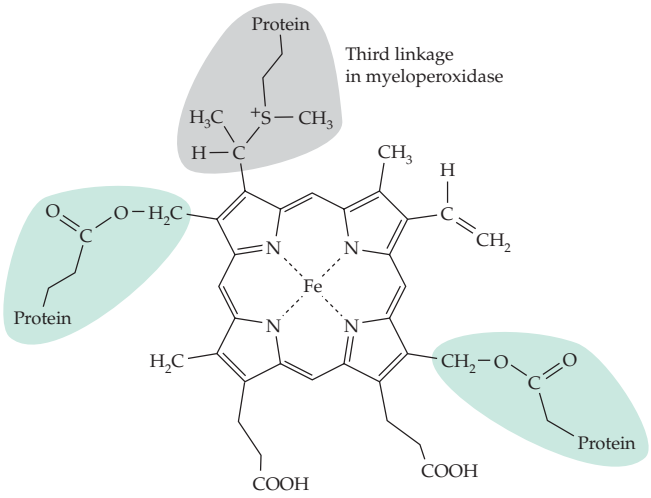



**Figure 16-12** Linkage of heme to mammalian peroxidases. There are two ester linkages to carboxylate side chains from the protein.[220,221] Myeloperoxidase contains a third linkage.[222,222a]

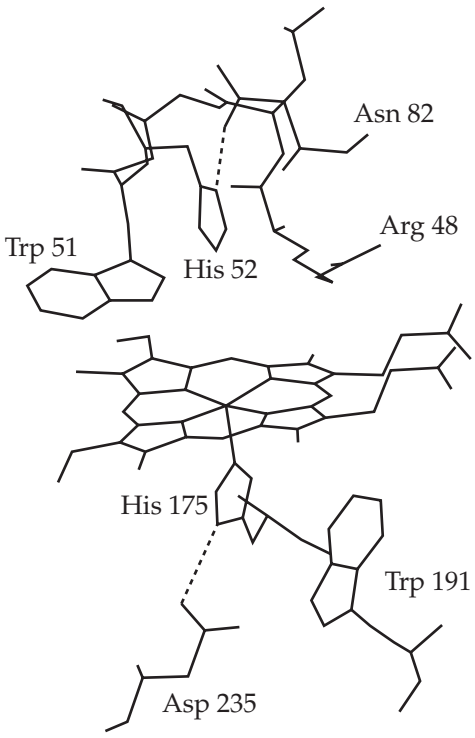



**Figure 16-13** The active site of yeast cytochrome *c* peroxidase. Access for substrates is through a channel above the front edge of the heme ring as viewed by the reader. A pathway for entrance of electrons may be via Trp 191 and His 175. From Holzbaur *et al.*[206] Based on coordinates of Finzel *et al.*[215]

Titrations with such reducing agents as ferrocyanide or $K_2IrBr_6$ have established that compound I is converted into compound II by a one-electron reduction and compound II to free peroxidase by another one-electron reduction. Thus, the iron in compound I may formally be designated Fe(V) and that in II as Fe(IV). However, this does not tell us whether or not the oxygen atoms of $H_2O_2$ are present in compounds I and II. The enzyme in the $Fe^{3+}$ form can be reduced to $Fe^{2+}$ (Fig. 16-14, step *e*), as previously mentioned, and when the $Fe^{2+}$ enzyme reacts with $H_2O_2$ it is apparently converted into compound II (Fig. 16-14, step *f*). This suggests that the latter is an $Fe^{2+}$ complex of the peroxide anion. Here, $P^{2-}$ represents the porphyrin ring:

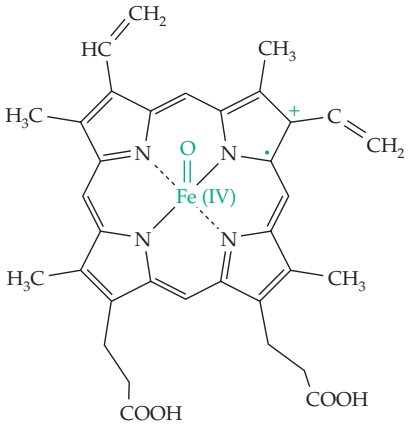
$$P^{2-}—Fe^{+}(II)—OOH$$

However, spectroscopic evidence suggests that compound II is a **ferryl iron** complex which could be derived from the preceding structure by addition of a proton and loss of water.[226,227]

$$P^{2-}—Fe^{2+}(IV)=O$$
Compound II

High concentrations of $H_2O_2$ convert II into compound III, which is thought to be the same as the **oxyperoxidase** that is formed upon addition of $O_2$ to the Fe(II) form of the free enzyme (Fig. 16-14, step *g*) and corresponds in structure to oxyhemoglobin.[228]

Compound I was at one time thought to be a complex of $H_2O_2$ or its anion with Fe(III), but its magnetic and spectral properties are inconsistent with this structure. Rather, it too appears to contain ferryl iron bound to an electron-deficient porphyrin π-cation radical.[229] The reaction with peroxide probably involves initial formation of a peroxide anion complex (Fig. 16-14, step *a*) which is cleaved with release of water (step *b*).[215,230] The resulting $Fe(V)^{+}=O$ compound is converted to compound I by transfer of a single elec-




Compound I. The unpaired electron and the positive charge are delocalized over the porphyrin ring and perhaps into the proximal histidine ring

tron from the porphyrin to the iron. In cytochrome *c* peroxidase compound I contains a free radical on the nearby Trp 191 ring instead of on the porphyrin radical.[216] Consistent with this is the fact that horseradish peroxidase contains phenylalanine in place of Trp 191.

If we consider the fate of substrate $AH_2$ during the action of a peroxidase, we see that donation of an electron to compound I to convert it into II (Fig. 16-14, step *c*) will generate a free radical •AH as well as a proton. The radical may then donate a second electron to II to form the free enzyme. Alternatively, a second molecule of $AH_2$ may react (Fig. 16-14, step *c*) to form a second radical •AH. The two •AH radicals may then disproportionate to form A and $AH_2$ or they may leave the enzyme and react with other molecules in their environment. Compound II of horse radish peroxidase is able to exchange the oxygen atom of its Fe(IV)=O center with water rapidly at pH 7, presumably by donation of a proton from the nearby histidine side chain (corresponding to His 52 of Fig. 16-13).[227,230a,b] This histidine presumably also functions in proton transfer during reactions with substrates (see Fig. 16-11B).[224]

The catalase compound I appears to be converted in a two-electron reduction by $H_2O_2$ directly to free ferricatalase without intervention of compound II (Fig. 16-14, step *c'*). The catalytic histidine probably donates a proton to help form water from one of the oxygen

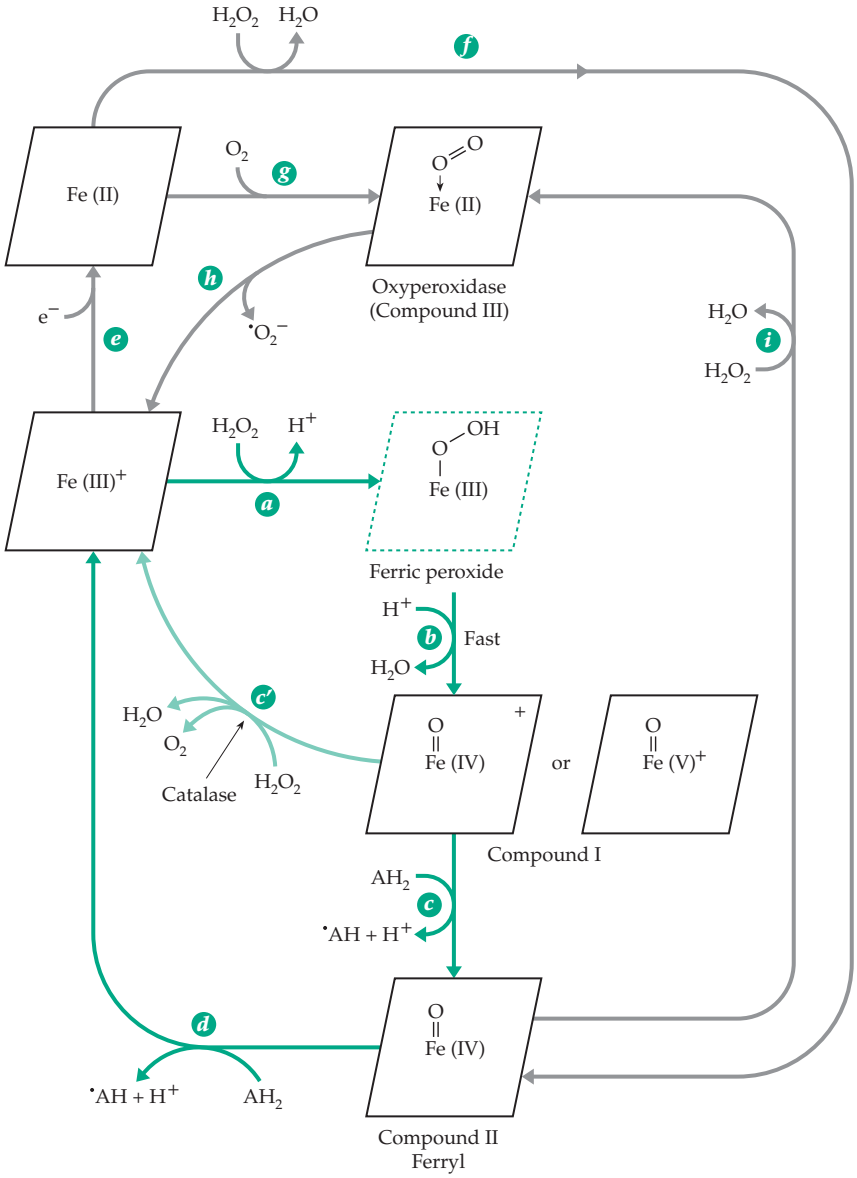


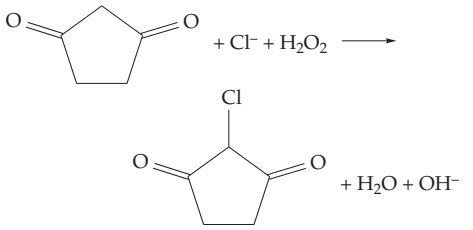

**Figure 16-14** The catalytic cycles and other reactions of peroxidases and catalases. The principal cycle for peroxidases is given by the colored arrows. That of catalases is smaller, making use of step *a*, *b*, and *c'*, which is marked by a light green line.

$$\underset{\text{Compound I}}{{}^{\bullet}P^{+}\text{—Fe(IV)=O}} + H_2O_2 \rightarrow H_2O + O_2 + \underset{}{P\text{—Fe(III)}^{+}} \qquad (16\text{-}10)$$

atoms of the $H_2O_2$. Nevertheless, compound II does form slowly, especially if a slow substrate such as ethanol is present. The previously mentioned bound NADPH apparently reduces compound II formed in this way, converting the inactivated enzyme back to active catalase.[201,231] This may involve unusual one-electron oxidation steps for the NADPH.[231] Under some circumstances compound II of peroxidases reacts with substrates in a two-electron process[232–235] with transfer of an oxygen atom to the substrate, a characteristic also of reactions catalyzed by cytochromes P450 (see Eq. 18-57).

***Haloperoxidases.*** Many specialized peroxidases are active in halogenation reactions. **Chloroperoxidases** from fungi[236,237] catalyze chlorination reactions like that of Eq. 16-11 using $H_2O_2$ and $Cl^-$ as well as the usual peroxidase reaction.

Chloroperoxidase is isolated in a low-spin Fe(III) state. The reduced Fe(II) enzyme is a high-spin form

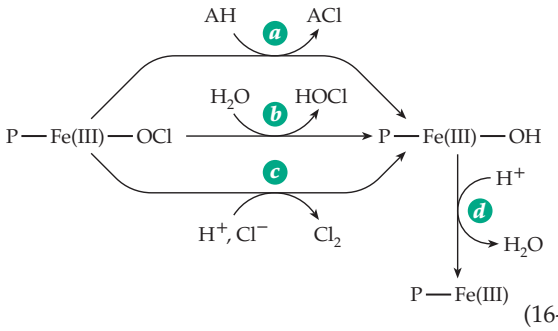



(16-11)

with spectroscopic properties similar to those of the oxygenases of the cytochrome P450 family, which are discussed in Chapter 18.[238,239] Like the cytochromes P450 chloroperoxidase contains a thiolate group of a cysteine side chain as the fifth iron ligand.[235,239,240] Chloroperoxidase forms compounds I and II, as do other peroxidases. A chloride ion may combine with compound I to form a complex of hypochlorite with the Fe(III) heme.

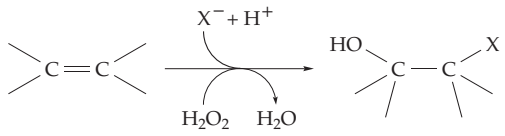
$$\bullet P^{+}\text{—Fe(IV)=O} + Cl^{-} \rightarrow P\text{—Fe(III)}^{+}\text{—OCl} \qquad (16\text{-}12)$$

This intermediate could then halogenate substrate AH (Eq. 16-13, step *a*), lose HOCl (Eq. 16-13, step *b*), or generate $Cl_2$ by reaction with $Cl^-$ (Eq. 16-13, step *c*). These are all well-established reactions for the enzyme. In each case the chlorine in the peroxidase complex can be viewed as an electrophile which is transferred to an attacking nucleophile. A fourth reaction that can go




(16-13)

through the same intermediate is conversion of alkenes to α,β-halohydrins (Eq. 16-14).[241] **Lactoperoxidase** of milk, reacting with $I^- + H_2O_2$, promotes an analogous iodination of tyrosine and histidine residues of proteins. With radioactive $^{125}I^-$ or $^{131}I^-$ it provides a convenient and much used method for labeling of proteins in

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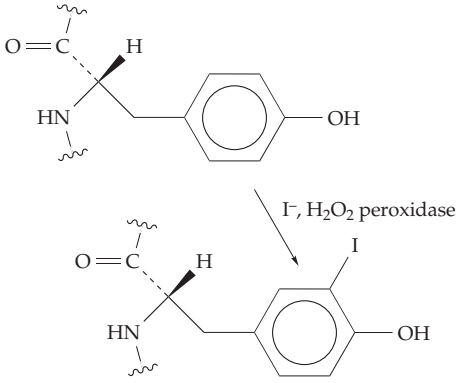



(16-15)

exposed surfaces of membranes (Eq. 16-15).[242] Iodinated tyrosine derivatives are formed in the thyroid gland by a similar reaction catalyzed by **thyroid peroxidase**.[232] Even horseradish peroxidase can oxidize iodide ions but neither it nor lactoperoxidase will carry out chlorination or bromination reactions.

**Myeloperoxidase**, present in specialized lysosomes of polymorphonuclear leukocytes (neutrophils),[243] utilizes $H_2O_2$ and a halide ion to kill ingested bacteria.[244,245] Phagocytosis induces increased respiration by the leukocyte and generation of $H_2O_2$, partly by the membrane-bound NADPH oxidase described in Chapter 18. Some of the $H_2O_2$ is used by myeloperoxidase to attack the bacteria, apparently through generation of HOCl by peroxidation of $Cl^-$. Human myeloperoxidase is a tetramer of two 466-residue chains and two 108-residue chains, which carry the covalently linked heme.[222a,246] Another oxygen-dependent killing mechanism that may also be used by neutrophils is the generation of the reactive **singlet oxygen**.[247] This can occur by reaction of hypochlorite with $H_2O_2$ (Eq. 16-16) or from an enzyme-bound hypochlorite intermediate such as that shown in Eq. 16-13. Hereditary deficiency of myeloperoxidase is relatively common.[245]

$$OCl^- + H_2O_2 \rightarrow \underset{\text{singlet } O_2}{O_2(^1\Delta g)} + H_2O + Cl^- \qquad (16\text{-}16)$$

Lactoperoxidase[248] and chloroperoxidase[249] also generate singlet oxygen. The possible biological significance is discussed in Chapter 18. Eosinophil peroxidase appears to promote formation of **hydroxyl radicals**.[250] **Bromoperoxidases** are found in many red and green marine algae.[251] Many of them contain **vanadium** and function by a mechanism different than that used by heme peroxidases (see Section G).[252,253]

Another related nonheme enzyme is the selenoprotein glutathione peroxidase. It reacts by a mechanism very different (Eq. 15-59) from those discussed

here, as does **NADPH peroxidase**, a flavoprotein with a cysteine sulfinate side chain in the active site.[254–255a] A lignin-degrading peroxidase from the white wood rot fungus *Phanerochaete chrysosporium* is a simple heme protein,[256] while other peroxidases secreted by this organism contain Mn.[257,258]

## 7. The Iron–Sulfur Proteins

Not all of the iron within cells is chelated by porphyrin groups. Hemerythrin (see Fig. 16-20) has been known for many years, but the general significance of nonheme iron proteins was not appreciated until large-scale preparation of mitochondria was developed by Crane in about 1945. The iron content of mitochondria was found to far exceed that of the heme proteins present. In 1960, Beinert, who was studying the mitochondrial dehydrogenase systems for succinate and for NADH, observed that when the electron transport chain was partially reduced by these substrates and the solutions were frozen at low temperature and examined, a strong EPR signal was observed at $g = 1.94$. The signal was obtained only upon reduction by substrate, and fractionation pointed to the nonheme iron proteins. Six or more proteins of this type are involved in the mitochondrial electron transport chain (Eq. 18-1), and numerous others have become recognized as members of the same large family of **iron–sulfur proteins** (Fe–S proteins).[259,260]

***Ferredoxins, high-potential iron proteins, and rubredoxins.*** The presence of nonheme iron proteins is most evident in the anaerobic clostridia, which contain no heme. It was from these bacteria that the first Fe–S protein was isolated and named **ferredoxin**. This protein has a very low reduction potential of $E°'$(pH 7) = –0.41 V. It participates in the pyruvate–ferredoxin oxidoreductase reaction (Eq. 15-35), in nitrogen fixation in some species, and in formation of $H_2$. A small green-brown protein, the ferredoxin of *Peptococcus aerogenes* contains only 54 amino acids but complexes eight atoms of iron. If the pH is lowered to ~1, eight molecules of $H_2S$ are released. Thus, the protein contains eight "labile sulfur" atoms in an iron sulfide linkage. There are also eight iron atoms.

Another group of related electron carriers, the **high-potential iron proteins** (HIPIP) contain four labile sulfur and four iron atoms per peptide chain.[261–266] X-ray studies showed that the 86-residue polypeptide chain of the HIPIP of *Chromatium* is wrapped around a single **iron–sulfur cluster** which contains the side chains of four cysteine residues plus the four iron and four sulfur atoms (Fig. 16-15D).[261] This kind of cluster is referred to as [4Fe–4S], or as $Fe_4S_4$. Each cysteine sulfur is attached to one atom of Fe, with the four iron atoms forming an irregular tetrahedron with an Fe–Fe

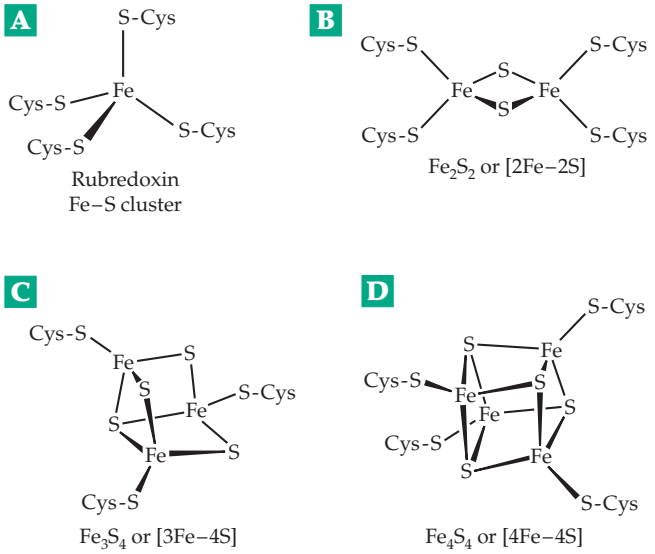



**Figure 16-15** Four different iron–sulfur clusters of a type found in many proteins. From Beinert[259] with permission.

distance of ~0.28 nm. The four labile sulfur atoms ($S^{2-}$) form an interpenetrating tetrahedron 0.35 nm on a side with each of the sulfur atoms bonded to three iron atoms. The cluster is ordinarily able to accept only a single electron. The iron–sulfur cluster structure was a surprise, but after its discovery it was found that ions such as $[Fe_4S_4(S–CH_2CH_2COO^-)_4]^{6-}$ assemble spontaneously from their components and have a similar cluster structure.[259,266a] Thus, living things have simply improved upon a natural bonding arrangement.

The bacterial ferredoxins from *Peptococcus, Clostridium* (Fig. 16-16B),[267,268] *Desulfovibrio,* and other anaerobes each contain two $Fe_4S_4$ clusters with essentially the same structure as that of the *Chromatium* HIPIP.[267,269] Each cluster can accept one electron. Much of the amino acid sequence in the first half of the ferredoxin chain is repeated in the second half, suggesting that the chain may have originated as a result of gene duplication. Many invariant positions are present in the sequence, including those of the cysteine residues forming the Fe–S cluster. Ferredoxins with single $Fe_4S_4$ clusters are also known.[270]

The ferredoxins have reduction potentials $E°'$ (pH 7) from about –0.4 V to as low as –0.6 V. However, the corresponding values for HIPIP proteins range from +0.05 to +0.50 V at pH 7.[271] This wide range of potentials initially seemed strange because the structures of the active centers of both the clostridial ferredoxins and the *Chromatium* HIPIP appear virtually identical.[272] Part of the explanation lies in the fact that $Fe_4S_4$ clusters can exist in three oxidation states (Eq. 16-17) that differ, one from another, by a single electron.[273]

$$[Fe_4S_4]^{+} \xrightarrow[e^-]{} [Fe_4S_4]^{2+} \xrightarrow[e^-]{} [Fe_4S_4]^{3+}$$

Reduced Fd | Oxidized Fd; reduced high potential iron protein | Oxidized high potential iron protein; Super-oxidized Fd (16-17)

Here the charges shown are those on the cluster. The cysteine ligands from the protein each add an additional negative charge. The *Chromatium* HIPIP and the clostridial ferredoxins have the middle oxidation state in common. The cluster is a little smaller in the more oxidized states; in the *Chromatium* HIPIP the Fe–Fe distance changes from 0.281 to 0.272 nm upon oxidation.

***Rubredoxins.*** The simplest of the Fe–S proteins are the rubredoxins. These proteins contain iron but no labile sulfur. The rubredoxin of *Clostridium pasteurianum* is a 54-residue peptide containing four cysteines whose side chains form a distorted tetrahedron about a single iron atom (Fig. 16-16A).[274] Not shown for any

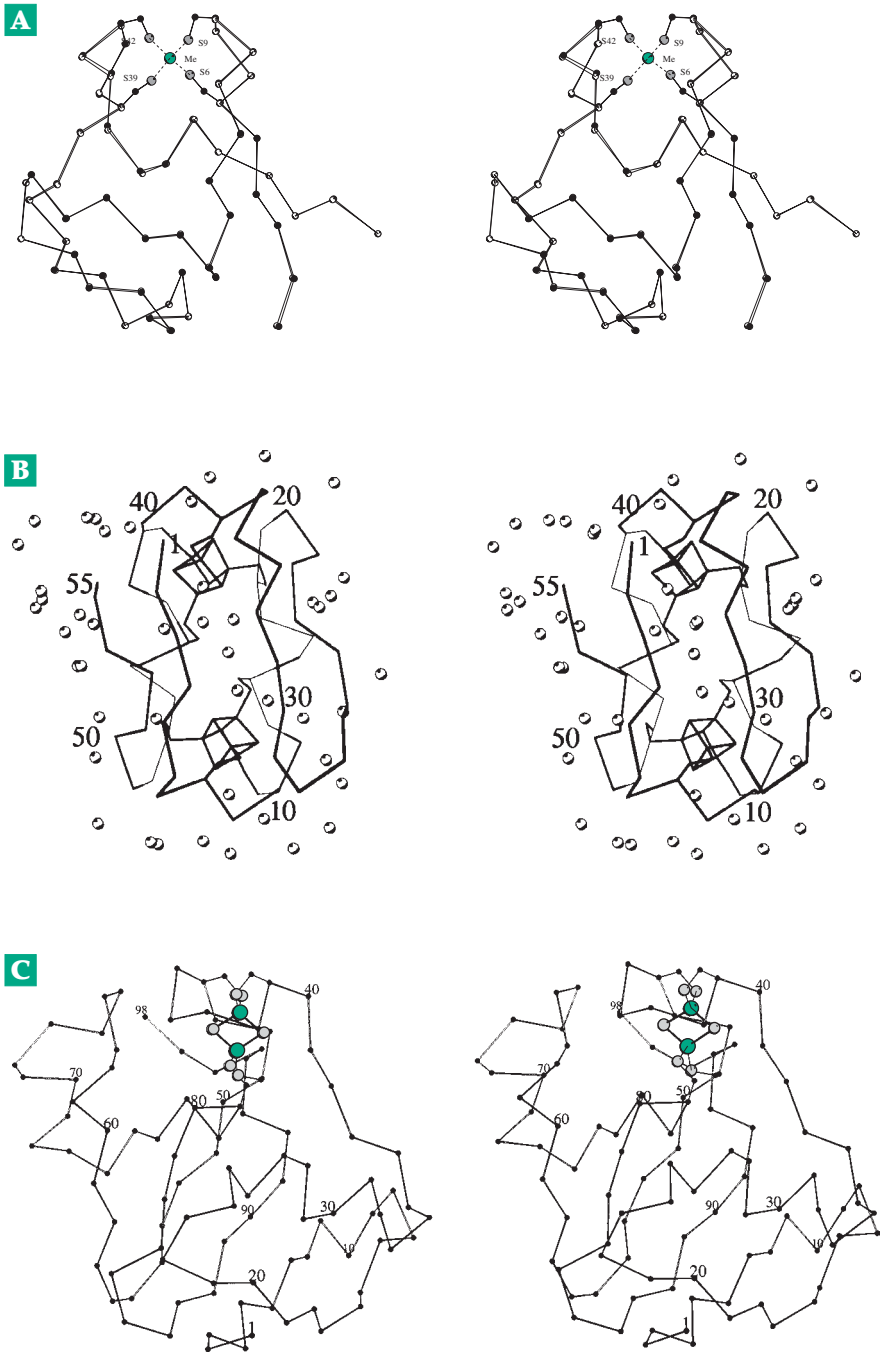
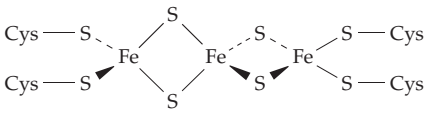



**Figure 16-16** (A) Superimposed stereoscopic α-carbon traces of the peptide chain of rubredoxin from *Clostridium pasteurianum* with either $Fe^{3+}$ (solid circles) or $Zn^{2+}$ (open circles) bound by four cysteine side chains. From Dauter *et al.*[274] (B) Alpha-carbon trace for ferredoxin from *Clostridium acidurici*. The two $Fe_4S_4$ clusters attached to eight cysteine side chains are also shown. The open circles are water molecules. Based on a high-resolution X-ray structure by Duée *et al.*[267] Courtesy of E. D. Duée. (C) Polypeptide chain of a chloroplast-type ferredoxin from the cyanobacterium *Spirulina platensis*. The $Fe_2S_2$ cluster is visible at the top of the molecule. From Fukuyama *et al.*[276] Courtesy of K. Fukuyama.

of the structures in Fig. 16-16 are NH—S hydrogen bonds that connect backbone NH groups of the peptide chain to the sulfur atoms of the cysteine groups, forming the clusters.[275] These bonds may have important effects on properties of the cluster. Rubredoxins also participate in electron transport and can substitute for ferredoxins in some reactions. Larger 14-kDa and 18-kDa rubredoxins able to bind two iron ions participate in electron transport in a hydroxylase system of *Pseudomonas* (Chapter 18).[277] A smaller 7.9-kDa 2-Fe **desulforedoxin** functions in sulfate-reducing bacteria.[278]

***Chloroplast-type ferredoxins.*** Members of a large class of [2Fe–2S] or $Fe_2S_2$ ferredoxins each contain two iron atoms and two labile sulfur atoms with the linear structure of Fig. 16-15B.[279,279a] Best known are the chloroplast ferredoxins, which transfer electrons from photosynthetic centers of chloroplasts to the flavoprotein reductase that reduces $NADP^+$ to NADPH.[280,280a] The structure of a cyanobacterial protein of this type is shown in Fig. 16-16C.[276] A second group of $Fe_2S_2$ ferredoxins are found in bacteria including *E. coli*[281] and in human mitochondria.[282] For example, in steroid hormone-forming tissues the ferredoxin **adrenodoxin**[282a] carries electrons to cytochromes P450. Its $Fe_2S_2$ center receives electrons from adrenodoxin reductase (Chapter 15 banner).[282b] The nitrogen-fixing *Clostridium pasteurianum* also contains a ferredoxin of this class.[283] In *Pseudomonas putida* (Chapter 18) the related 106-residue **putidaredoxin** transfers electrons to cytochromes P450.[284]

***The 3Fe–4S clusters.*** A 106-residue ferredoxin from *Azotobacter vinelandii* contains seven iron atoms in two Fe–S clusters that operate at very different redox potentials of –0.42 and +0.32 V.[285] Other similar seven-iron proteins are known.[286] From EPR measurements it appeared that both clusters function between the $2^+$ and $3^+$ (oxidized and superoxidized) states of Eq. 16-17, despite the widely differing potentials. A super-reduced all-Fe(II) form with $E^{\circ\prime}$ (pH 7) = –0.70 V can also be formed.[286] X-ray crystallographic studies have revealed that the protein contains one $Fe_4S_4$ cluster and one $Fe_3S_4$ cluster (Fig. 16-15C).[286–288] The structures and environments of the $Fe_3S_4$ clusters are similar to those of $Fe_4S_4$ clusters but they lack one iron and one cysteine side chain. An $Fe_4S_4$ cluster may sometimes lose $S^{2-}$ to form an $Fe_3S_4$ cluster such as the one in the *A. vinelandii* ferredoxin. A less likely possibility is isomerization to a linear $Fe_3S_4$ structure.[289]




Aconitase (Eq. 13-17) isolated under aerobic conditions contains an $Fe_3S_4$ cluster and is catalytically inactive. Incubation with $Fe^{2+}$ activates the enzyme and reconverts the $Fe_3S_4$ to an $Fe_4S_4$ cluster (Fig. 13-4).[260,289,290]

***Properties of iron–sulfur clusters.*** These clusters were viewed for many years as unstable and unable to exist outside of a protein. However, if protected from oxygen and manipulated in the presence of soluble organic thiols they are stable and "cofactor-like."[260] Intact clusters can be "extruded" from proteins by treatment with thiols in nonaqueous media. Both $Fe_4S_4$ and $Fe_2S_2$ clusters as well as more complex forms have been synthesized[291] and nonenzymatic cluster interconversions have been demonstrated. Binding to proteins stabilizes the clusters further, but some (Fe–S) proteins are labile and difficult to study. This is evidently because of partial exposure of the cluster to the surrounding solvent. Not only can $O_2$ cause oxidation of exposed clusters[291a] but also superoxide,[292] nitric oxide, and peroxynitrite can react with the iron. Aconitase has only three cysteine side chains available for coordination with Fe and the protein is unstable. Apparently, a superoxidized $[Fe_4S_4]^{3+}$ cluster is formed in the presence of $O_2$ but loses $Fe^{2+}$ to give an $[Fe_3S_4]^+$ cluster.[293]

Another interesting cluster conversion is the joining of two $Fe_2S_2$ clusters in a protein to form a single $Fe_4S_4$ cluster at the interface between a dimeric protein. Such a cluster is present in the nitrogenase iron protein (Fig. 24-2) and probably also in biotin synthase.[294] The clusters in such proteins can also be split to release the monomers.

Synthetic iron–sulfur clusters have weakly basic properties[273] and accept protons with a p$K_a$ of from 3.9 to 7.4. Similarly, one clostridial ferredoxin, in the oxidized form, has a p$K_a$ of 7.4; it is shifted to 8.9 in the reduced form.[295] If we designate the low-pH oxidized form of such a protein as $HOx^+$ and the reduced form as HRed, we can depict the reduction of each $Fe_4S_4$ cluster as follows.

$$HOx^+ + e^- \rightarrow HRed \qquad (16\text{-}18)$$

Comparing with Eq. 6-64 and using the Michaelis pH functions (first two terms of Eq. 7-13) for $HOx^+$ and HRed, it is easy to show that the value of $E^{\circ\prime}$ ($E_{1/2}$) at which equal amounts of oxidized protein ($HOx^+$ + Ox) and reduced protein (HRed + $Red^-$) are present is given by Eq. 16-19, in which $K_{ox}$ and $K_{red}$ are the $K_a$ values for dissociation of the protonated oxidized and reduced forms, respectively.

$$E_{1/2} = E^0\ (\text{low pH}) + 0.0592 \log\left[\frac{(1 + K_{red}/[H^+])}{(1 + K_{ox}/[H^+])}\right] \text{V} \qquad (16\text{-}19)$$

At the high pH limit this becomes $E_{1/2} = E°$ (low pH) + 0.0592 ($pK_{ox} - pK_{red}$) and V = −0.371 + 0.0592 (7.4−8.9) V = −0.431 V. Thus, the value of $E_{1/2}$ changes from −0.371 to −0.460 V as the pH is increased. In the pH range between the $pK_a$ values of 7.4 and 8.9 reduction of the protein will lead to binding of a proton from the medium and oxidation to loss of a proton. Human and other vertebrate ferredoxins also show pH-dependent redox potentials.[282] This suggests, as with the cytochromes, a possible role of Fe−S centers in the operation of proton pumps in membranes. Nevertheless, many ferredoxins, such as that of *C. pasteurianum*, show a constant value of $E°'$ from pH 6.3 to 10[296] and appear to be purely electron carriers.

Both the iron and labile sulfur can be removed from Fe−S proteins and the active proteins can often be reconstituted by adding sulfide and $Fe^{2+}$ ions. Using this approach, the natural isotope $^{56}Fe$ (nuclear spin zero) has been exchanged with $^{57}Fe$, which has a magnetic nucleus[297] and $^{32}S$ has been replaced by $^{77}Se$. The resulting proteins appear to function naturally and give EPR spectra containing hyperfine lines that result from interaction of these nuclei with unpaired electrons in the $Fe_4S_4$ clusters (Fig. 16-17). These observations suggest that electrons accepted by $Fe_4S_4$ clusters are not localized on a single type of atom but interact with nuclei of both Fe and S. The native proteins as well as many mutant forms are being studied by NMR[299,300] and other spectroscopic techniques (Fig. 16-18),[260] by theoretical computations,[301–304] and by protein engineering and "rational design."[305,306]

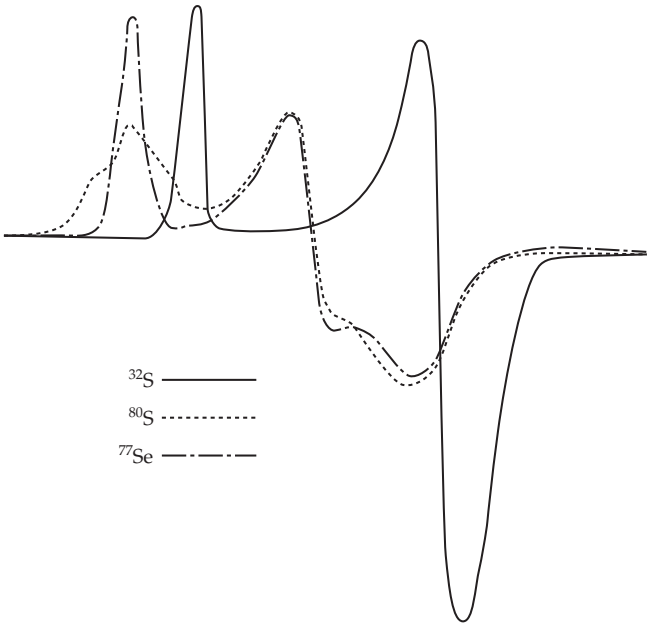



**Figure 16-17** Electron paramagnetic resonance spectrum of the Fe−S protein putidaredoxin in the natural form ($^{32}S$) and with labile sulfur replaced by selenium isotopes. Well-developed shoulders are seen in the low-field end of the spectrum of the $^{77}Se$ (spin = 1/2)-containing protein. From Orme-Johnson *et al.*[298] Courtesy of W. H. Orme-Johnson.

***Functions of iron–sulfur enzymes.*** Numerous iron−sulfur clusters are present within the membrane-bound electron transport chains discussed in Chapter 18. Of special interest is the $Fe_2S_2$ cluster present in a protein isolated from the cytochrome *bc* complex (complex III) of mitochondria. First purified by Rieske *et al.*,[307] this protein is often called the **Rieske iron–sulfur protein**.[308] Similar proteins are found in cytochrome *bc* complexes of chloroplasts.[125,300,309,310] In

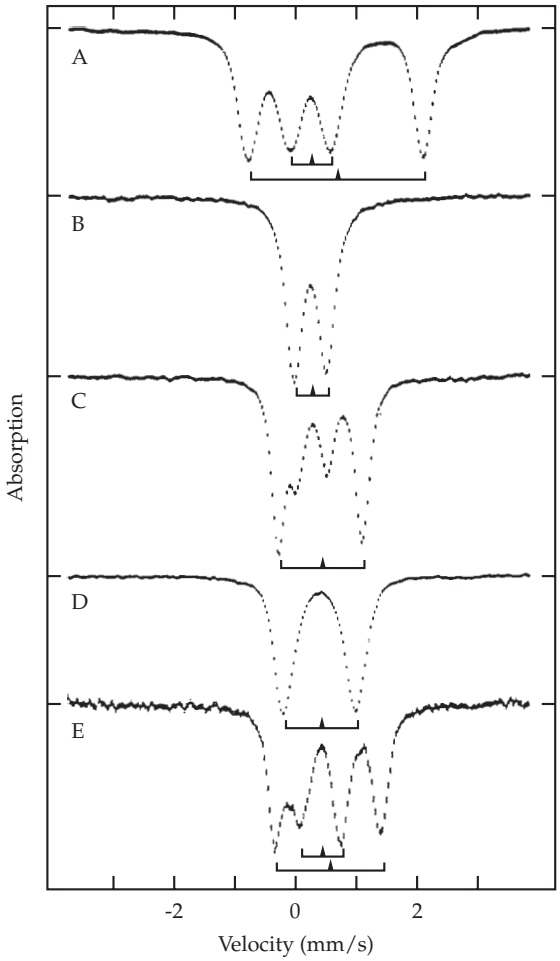
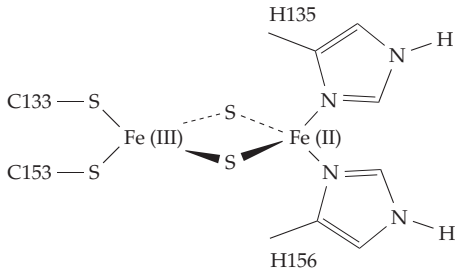



**Figure 16-18** Mössbauer X-ray absorption spectra of iron−sulfur clusters. (See Chapter 23 for a brief description of the method.) Quadrupole doublets are indicated by brackets and isomer shifts are marked by triangles. (A) $[Fe_2S_2]^{1+}$ cluster of the Rieske protein from *Pseudomonas mendocina*, at temperature $T$ = 200 K. (B) $[Fe_3S_4]^{1+}$ state of *D. gigas* ferredoxin II, $T$ = 90 K. (C) $[Fe_3S_4]^{0}$ state of *D. gigas* ferredoxin II, $T$ = 15 K. (D) $[Fe_4S_4]^{2+}$ cluster of *E. coli* FNR protein, $T$ = 4.2 K. (E) $[Fe_4S_4]^{1+}$ cluster of *E. coli* sulfite reductase, $T$ = 110 K. From Beinert *et al.*[260]

some bacteria Rieske-type proteins deliver electrons to oxygenases.[300] The 196-residue mitochondrial protein has an unusually high midpoint potential, $E_m$ of ~ 0.30 V. The $Fe_2S_2$ cluster, which is visible in the atomic structure of complex III shown in Fig. 18-8, is coordinated by two cysteine thiolates and two histidine side chains. In a *Rhodobacter* protein they occur in the following conserved sequences:[311] C133-T-H-L-G-C138 and C153-P-C-H-G-S158. One iron is bound by C133 and C153 and the other by H135 and H156 as follows:

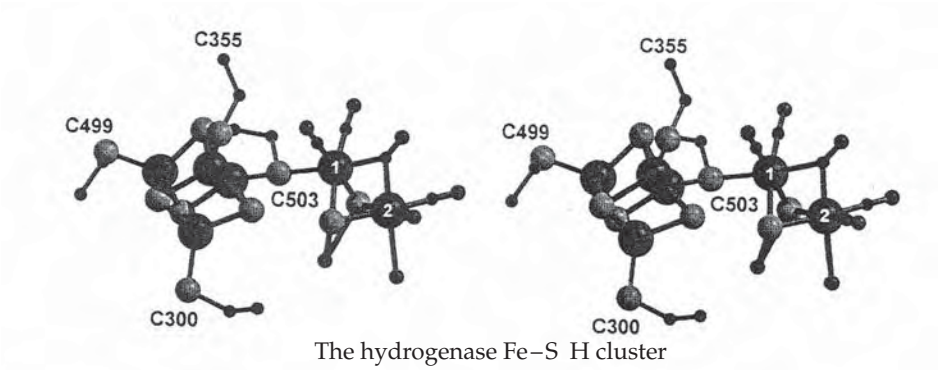



These proteins may also have an ionizable group with a p$K_a$ of ~8.0, perhaps from one of the histidines that is linked to the oxidation–reduction reaction.

Iron–sulfur clusters are found in flavoproteins such as NADH dehydrogenase (Chapter 18) and trimethylamine dehydrogenase (Fig. 15-9) and in the siroheme-containing **sulfite reductases** and **nitrite reductases**.[312] These two reductases are found both in bacteria and in green plants. Spinach nitrite reductase,[313] which is considered further in Chapter 24, utilizes reduced ferredoxin to carry out a six-electron reduction of $NO_2^-$ to $NH_3$ or of $SO_3^{2-}$ to $S^{2-}$. The 61-kDa monomeric enzyme contains one siroheme and one $Fe_4S_4$ cluster. A sulfite reductase from *E. coli* utilizes NADPH as the reductant. It is a large $\beta_8\alpha_4$ oligomer.[312] The 66-kDa α chains contain bound flavin (4 FAD + 4 FMN),[312,314,315] while the 64-kDa β subunits contain both siroheme and a neighboring $Fe_4S_4$ cluster (Fig. 16-19).[89,304,312,316] The iron of the siroheme and the closest iron atom of the cluster are bridged by a single sulfur atom of a cysteine side chain.

A somewhat similar double cluster is present in **all-Fe hydrogenases** from *Clostridium pasteurianum*.[316a,b,c] These enzymes also contain two or three $Fe_4S_4$ clusters and, in one case, an $Fe_2S_2$ cluster.[316a] At the presumed active site a special **H cluster** consists of an $Fe_3S_4$ cluster with one cysteine sulfur atom shared by an adjoining $Fe_2S_2$ cluster: Because many hydrogenases contain nickel, their chemistry and functions are discussed in Section C,2.

The role of the iron–sulfur clusters in many of the proteins that we have just considered is primarily one of single-electron transfer. The Fe–S cluster is a place for an electron to rest while waiting for a chance to react. There may sometimes be an associated proton pumping action. In a second group of enzymes, exemplified by aconitase (Fig. 13-4), an iron atom of a cluster functions as a **Lewis acid** in facilitating removal of an –OH group in an α,β dehydration of a carboxylic acid (Chapter 13). A substantial number of other bacterial dehydratases as well as an important plant dihydroxyacid dehydratase also apparently use Fe–S clusters in a catalytic fashion.[317] Fumarases A and B from *E. coli*,[317] L-serine dehydratase of a *Peptostreptococcus* species,[317–319] and the dihydroxyacid




The hydrogenase Fe–S H cluster

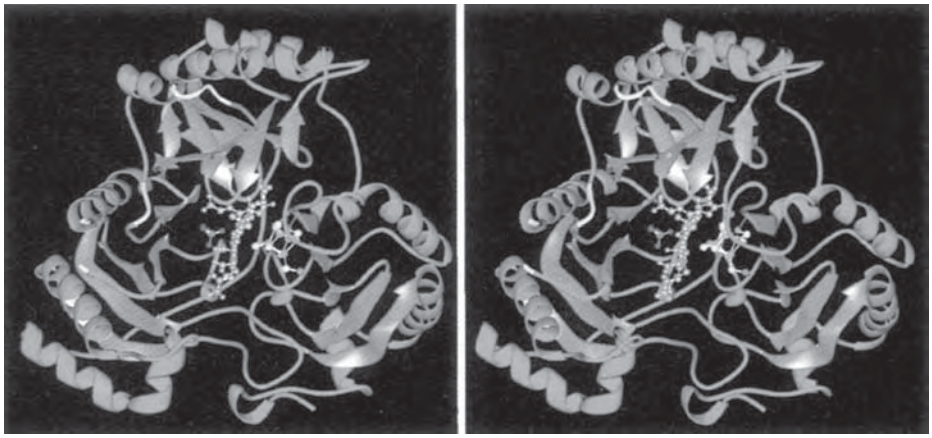


**Figure 16-19** Stereoscopic view of *E. coli* assimilatory sulfite reductase. The siroheme (Fig. 16-6) is in the center with one edge toward the viewer and the $Fe_4S_4$ cluster is visible on its right side. A single S atom from a cysteine side chain bridges between the Fe of the siroheme and the $Fe_4S_4$ cluster. A phosphate ion is visible in the sulfite-binding pocket at the left center of the siroheme. From Crane *et al.*[312] Courtesy of E. D. Getzoff.

dehydratase[320,321] may all use their $Fe_4S_4$ clusters in a manner similar to that of aconitase (Eq. 13-17). However, the Fe–S enzymes that dehydrate *R*-lactyl-CoA to crotonyl-CoA,[322] 4-hydroxybutyryl-CoA to crotonyl-CoA,[323,324] and *R*-2-hydroxyglutaryl-CoA to *E*-glutaconyl-CoA[325] must act by quite different mechanisms, perhaps similar to those utilized in vitamin $B_{12}$-dependent reactions. In these enzymes, as in pyruvate formate lyase (Eq. 15-38), the Fe–S center may act as a radical generator.

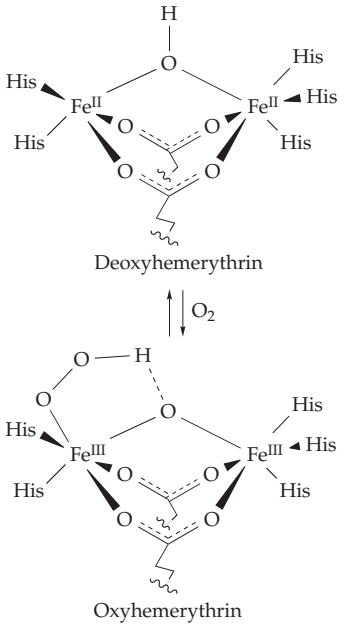
The molybdenum-containing enzymes considered in Section F also contain Fe–S clusters. Nitrogenases (Chapter 24) contain a more complex Fe–S–Mo cluster. Carbon monoxide dehydrogenase (Section C) contains 2 Ni, ~11 Fe, and 14 $S^{2-}$ as well as Zn in a dimeric structure. In these enzymes the Fe–S clusters appear to participate in catalysis by undergoing alternate reduction and oxidation.

For a few enzymes such as aconitase and amido-transferases,[326] an Fe–S cluster plays a **regulatory role** in addition to or instead of a catalytic function.[327] Cytosolic aconitase is identical to **iron regulatory proteins 1** (IRP1), which binds to iron responsive elements in RNA to inhibit translation of genes asso-ciated with iron uptake. A high iron concentration promotes assembly of the $Fe_4S_4$ cluster (see Chapter 28, Section C,6). Another example is provided by the *E. coli* transcription factor SOXR. This protein, which controls a cellular defense system against oxygen-derived superoxide radicals, contains two $Fe_2S_2$ centers. Oxidation of these centers by superoxide radicals appears to induce the transcription of genes encoding superoxide dismutase and other proteins involved in protecting cells against oxidative injury (Chapter 18).[328–330]

## 8. The (μ-oxo) Diiron Proteins

Both the ferroxidase center of ferritin (Fig. 16-4) and the oxygen-carrying hemerythrin are members of a family of diiron proteins with similar active site structures.[331,332] The pair of iron atoms, with the bridging (μ) ligands such as $O_2$, $HO^-$, $HOO^-$, $O_2^{2-}$, is often held between four α helices, as can be seen in Fig. 2-22 and in Fig. 16-20C. In most cases each iron is ligated by at least one histidine, one glutamate side chain, and frequently a tyrosinate side chain. As many as three side chains may bind to each iron. In addition, one or two carboxylate groups from glutamate or aspartate side chains bridge to both irons, as does the μ-oxo group, which is typically $H_2O$ or $^-OH$. Examples of this structure are illustrated in Figs. 16-4 and 16-20. Although the active sites of all of the diiron proteins appear similar, the chemistry of the catalyzed reactions is varied.

***Hemerythrin.*** When both iron atoms are in the Fe(II) state, hemerythrin, like hemoglobin, functions as a carrier of $O_2$. In the oxidized Fe(III) **methemerythrin** form the iron atoms are only 0.32 nm apart. Three bridging (μ) groups lie between them: two carboxylate groups and a single oxygen atom which may be either $O^{2-}$ or $OH^-$. One coordination position on one of the hexacoordinate iron atoms is open and appears to be the site of binding of oxygen. The $O_2$ is thought to accept two electrons, oxidizing the two iron atoms to Fe(III) and itself becoming a peroxide dianion $O_2^{2-}$. The process is completely reversible. The conversion of the oxygen to a bound peroxide ion is supported by studies of resonance Raman spectra (see Chapter 23) which also suggest that the peroxide group is protonated. In the diferrous protein the μ-oxo bridge is thought to be an $^-OH$ group. Upon oxygenation the proton could be shared with or donated to the peroxo group (Eq. 16-20).[334] A similar binding of $O_2$ as a peroxide dianion appears to occur in the copper-containing hemocyanins (Section C,3).

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***Purple acid phosphatases.*** Diiron-tyrosinate proteins with acid phosphatase activity occur in mammals, plants, and bacteria. Most are basic glycoproteins with an intense 510- to 550-nm light absorption band. Well-studied members come from beef spleen, from the uterine fluid of pregnant sows (**uteroferrin**),[335] and from human macrophages and osteoclasts.[336–336b] One of the two iron atoms is usually in the Fe(III) oxidation state, but the second can be reduced to Fe(II) by mild reductants such as ascorbate. This half-reduced form is enzymatically active and has a pink color and a characteristic EPR signal. Treatment with oxidants such as $H_2O_2$ or hexacyannoferrate (III)

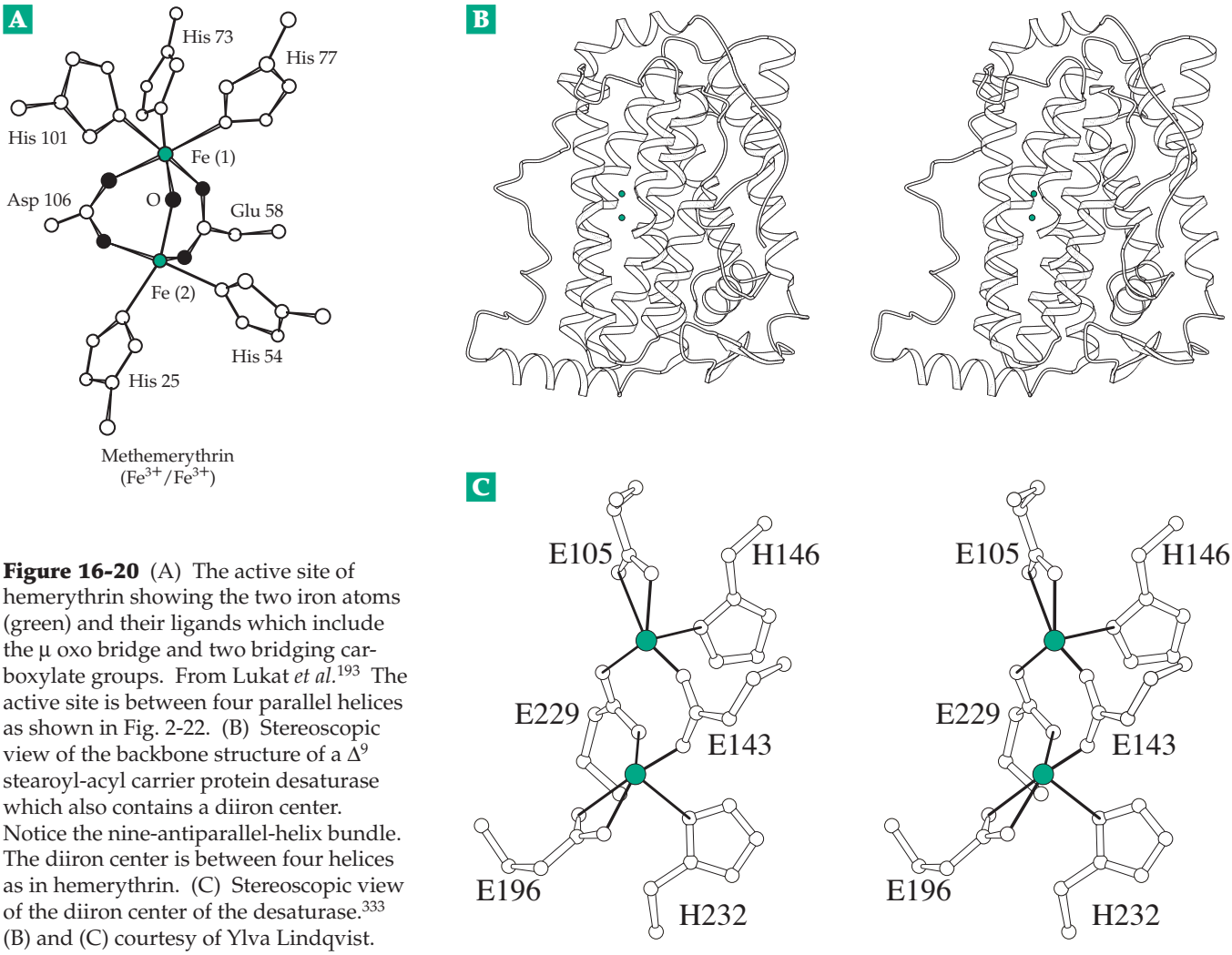



**Figure 16-20** (A) The active site of hemerythrin showing the two iron atoms (green) and their ligands which include the μ oxo bridge and two bridging carboxylate groups. From Lukat *et al.*[193] The active site is between four parallel helices as shown in Fig. 2-22. (B) Stereoscopic view of the backbone structure of a $\Delta^9$ stearoyl-acyl carrier protein desaturase which also contains a diiron center. Notice the nine-antiparallel-helix bundle. The diiron center is between four helices as in hemerythrin. (C) Stereoscopic view of the diiron center of the desaturase.[333] (B) and (C) courtesy of Ylva Lindqvist.

generates purple inactive forms which lack a detectable EPR signal.[337,338] The $Fe^{3+}$ of the active enzyme can be replaced[339] with $Ga^{3+}$ and the $Fe^{2+}$ with $Zn^{2+}$ with retention of activity; also, some plants contain phosphatases with Fe–Zn centers.[340,341] The catalytic mechanism resembles those of other metallophosphatases (Chapter 12) and the change of oxidation state of the Fe may play a regulatory role. On the other hand, a principal function of uteroferrin may be in transplacental transport of iron to the fetus.[342]

***Diiron oxygenases and desaturases.*** In the (μ-oxo) diiron oxygenases $O_2$ is initially bound in a manner similar to that in hemerythrin but one atom of the bound $O_2$ is reduced to $H_2O$ using electrons supplied by a cosubstrate such as NADPH. The other oxygen atom enters the substrate. This is illustrated by Eq. 16-21 for methane monooxygenase.[332,343–345] A toluene monooxygenase has similar properties.[346]

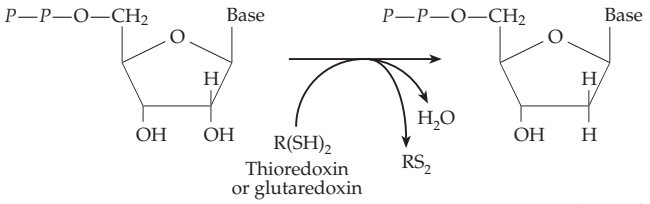
$$CH_4 + NADH + H^+ + O_2 \rightarrow CH_3OH + NAD^+ + H_2O \quad (16\text{-}21)$$

In green plants a soluble $\Delta^9$ stearoyl-acyl carrier protein desaturase uses $O_2$ and NADH or NADPH to introduce a double bond into fatty acids. The structure of this protein (Fig. 16-20B,C) is related to those of methane oxygenase and ribonucleotide reductase.[333,347] The desaturase mechanism is discussed in Chapter 21.

## 9. Ribonucleotide Reductases

Ribonucleotides are reduced to the 2'-deoxyribonucleotides (Eq. 16-21) that are needed for DNA synthesis by enzymes that act on either the di- or triphosphates of the purine and pyrimidine nucleosides[348–351] (Chapter 25). These ribonucleotide reductases utilize either thioredoxin or glutaredoxin (Box 15-C) as the immediate hydrogen donors (Eq. 16-22). The pair of closely spaced –SH groups in the reduced thioredoxin or glutaredoxin are converted into a disulfide bridge at the same time that the 2'-OH of the ribonucleotide di- (or tri-) phosphate is converted to $H_2O$. While some organisms employ a vitamin $B_{12}$-

dependent enzyme for this purpose, most utilize iron–tyrosinate enzymes (Class I ribonucleotide reductases). These are two-protein complexes of composition $\alpha_2\beta_2$. The enzyme from *E. coli* contains 761-residue α chains and 375-residue β chains. That from *Salmonella typhimurium*[351a] is similar, as are corresponding mammalian enzymes and a virus-encoded ribonucleotide reductase formed in *E. coli* following infection by T4 bacteriophage.[352] In every case the larger $\alpha_2$ dimer, which is usually called the **R1 protein**, contains the substrate binding sites, allosteric effector sites, and redox-active SH groups. Each α chain is folded into an unusual $(\alpha/\beta)_{10}$ barrel.[353,354] As in the more familiar $(\alpha/\beta)_8$ barrels, the active site is at the N termini of the β strands.

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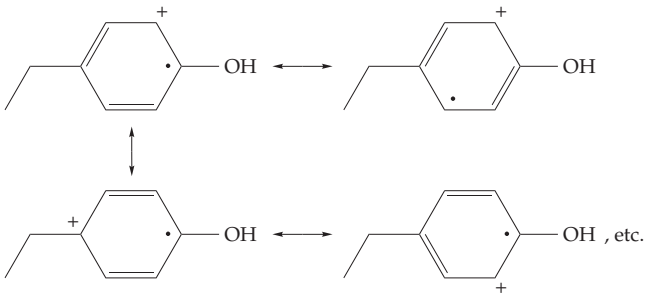
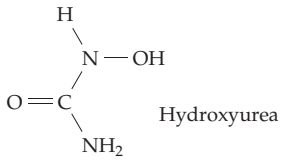
Each polypeptide chain of the $\beta_2$ dimer or **R2 protein** contains a diiron center which serves as a free radical generator.[354a,b,c] A few bacteria utilize a dimanganese center.[355] Oxygenation of this center is linked to the uptake of both a proton and an electron and to the removal of a hydrogen atom from the ring of tyrosine 166 to form $H_2O$ and an organic radical (Eq. 16-23):[356–360]

$$2\ Fe(II)^{2+} + O_2 + H^+ + e^- + Y122\text{-}OH \rightarrow Fe(III)^{3+} - O^{2-} - Fe(III)^{3+} - Y122 - O\bullet + H_2O \quad (16\text{-}23)$$

The tyrosyl radical is used to initiate the ribonucleotide reduction at the active site in the R1 protein ~3.5 nm away. The tyrosyl radical is very stable and was discovered by a characteristic EPR spectrum of isolated enzyme. Alteration of this spectrum when bacteria were grown in deuterated tyrosine indicated that the radical is located on a tyrosyl side chain and that the spin density is delocalized over the tyrosyl ring.[361,362] Using protein engineering techniques the ring was located as Tyr 122 of the *E. coli* enzyme. A few of the resonance structures that can be used to depict the radical are the following:




A chain of hydrogen-bonded side chains apparently provides a pathway for transfer of an unpaired electron from the active site to the Tyr 122 radical and from there to the radical generating center.[363] The tyrosyl radical can be destroyed by removal of the iron by exposure to $O_2$ or by treatment of ribonucleotide reductases with hydroxyurea, which reduces the radical and also destroys catalytic activity:




Hydroxyurea

A second group of ribonucleotide reductases (Class II), found in many bacteria, depend upon the cobalt-containing **vitamin $B_{12}$ coenzyme** which is discussed in Section B. These enzymes are monomeric or homodimeric proteins of about the size of the larger α subunits of the Class I enzymes. The radical generating center is the 5'-deoxyadenosyl coenzyme.[350,364,365]

Class III or **anaerobic nucleotide reductases** are used by various anaerobic bacteria including *E. coli* when grown anaerobically[350,366–369a] and also by some bacteriophages.[370] Like the Class I reductases, they have an $\alpha_2\beta_2$ structure but each β subunit contains an $Fe_4S_4$ cluster which serves as the free radical generator,[369] that forms a stable glycyl radical at G580.[369a] In this respect the enzyme resembles pyruvate formate-lyase (Eq. 15-40). As with other enzymes using $Fe_4S_4$ clusters as radical generators, *S*-adenosylmethionine is also required. All ribonucleotide reductases may operate by similar radical mechanisms.[350,351]

When a 2'-Cl or -F analog of UDP was used in place of the substrate an irreversible side reaction occurred by which $Cl^-$ or $F^-$, inorganic pyrophosphate, and uracil were released.[349] When one of these enzyme-activated inhibitors containing $^3H$ in the 3' position was tested, the tritium was shifted to the 2' position with loss of $Cl^-$ and formation of a reactive 3'-carbonyl compound (Eq. 16-24) that can undergo β elimination at each end to give an unsaturated ketone which inactivates the enzyme. This suggested that the Fe-tyrosyl radical abstracts an electron (through a

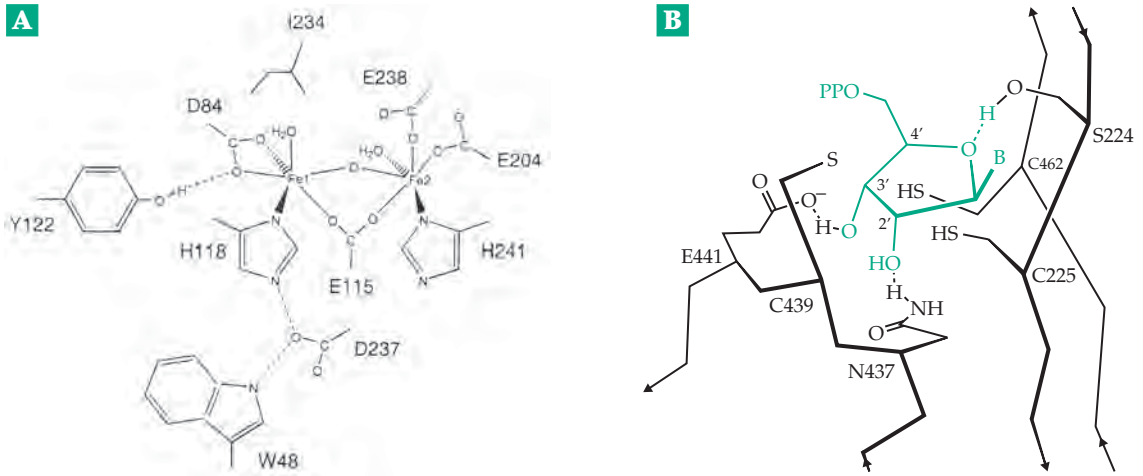
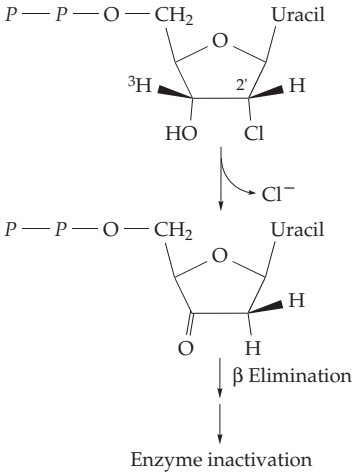



**Figure 16-21** (A) Scheme showing the diiron center of the R2 subunit of *E. coli* ribonucleotide reductase. Included are the side chains of tyrosine 122, which loses an electron to form a radical, and of histidine 118, aspartate 237, and tryptophan 48. These side chains provide a pathway for radical transfer to the R1 subunit where the chain continues to tyrosines 738 and 737 and cysteine 429.[354a–c] From Andersson *et al.*[354c] (B) Schematic drawing of the active site region of the *E. coli* class III ribonucleotide reductase with a plausible position for a model-built substrate molecule. Redrawn from Lenz and Giese[373] with permission.

chain of intermediate groups) from an –SH group, now identified as C439 in the *E. coli* enzyme[371–372] (Fig. 16-21). The resulting thiyl radical is thought to abstract a hydrogen atom from C′-3 of a true substrate to form a substrate radical (Eq. 16-25, step *a*) which, with the help of the C462 –SH group, facilitates the loss of $^{-}$OH from C-2 in step *b*. The resulting C2 radical would be reduced by the nearby redox-active thiol pair C462 and C225 (Fig. 16-21 and Eq. 16-25, step *c*). In Eq. 16-25 the reaction is shown as a hydride transfer with an associated one-electron shift but the mechanism is uncertain. In step *d* of Eq. 16-25 the thiyl radical is regenerated and continues to function in subsequent rounds of catalysis. In the final step (step *e*) the redox active pair is reduced by reduced thioredoxin or glutaredoxin. The active site must open to release the product and to permit this reduction, which may involve participation of still other –SH groups in the protein.

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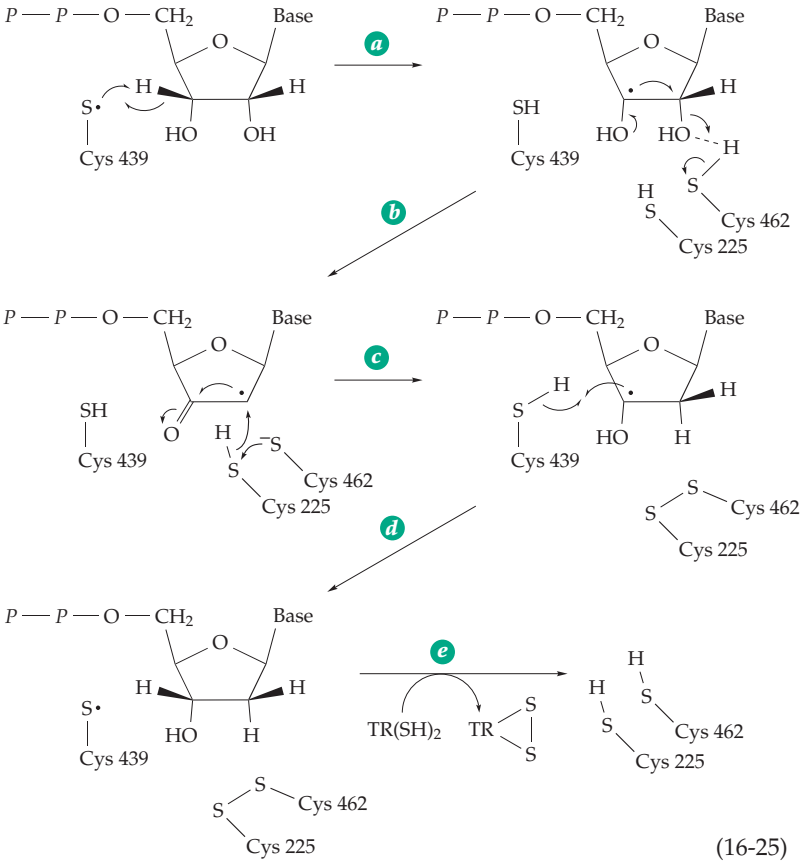


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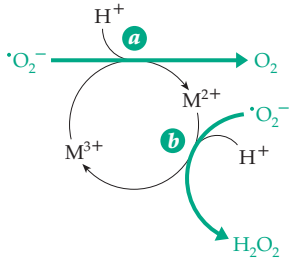
## 10. Superoxide Dismutases

Metalloenzymes of at least three different types catalyze the destruction of superoxide radicals that arise from reactions of oxygen with heme proteins, reduced flavoproteins, and other metalloenzymes. These superoxide dismutases (SODs) convert superoxide anion radicals $^{\bullet}O_2^-$ into $H_2O_2$ and $O_2$ (Eq. 16-26). The $H_2O_2$ can then be destroyed by catalase (Eq. 16-8).

$$2\ O_2^- + 2\ H^+ \longrightarrow H_2O_2 + O_2 \qquad (16\text{-}26)$$

The much studied Cu / Zn superoxide dismutase of eukaryotic cytoplasm is described in Section D. However, eukaryotic mitochondria contain manganese SOD and some eukaryotes also synthesize an iron-containing SOD. For example, the protozoan *Leishmania tropica*, which takes up residence in the phagolysosomes of a victim's macrophages, synthesizes an iron-containing SOD[374] to protect itself against superoxide generated by the macrophages. *Mycobacterium tuberculosis* secretes an iron SOD which assists its survival in living tissues and is also a target for the immune response of human hosts.[375]

Iron and manganese SODs have ~20-kDa subunits in each of which a single ion of Fe or Mn is bound by three imidazole groups and a carboxylate group.[376,377] The metal ion undergoes a cyclic change in oxidation state as illustrated by Eq. 16-27. Notice that two protons must be taken up for formation of $H_2O_2$. In Cu / Zn SOD the copper cycles between $Cu^{2+}$ and $Cu^+$. The structure of the active site of an Fe SOD is shown in Fig. 16-22. That of Mn SOD is almost identical.[376] In addition to the histidine and carboxylate ligands, the metal binds a hydroxyl ion $^-OH$ or $H_2O$ and has a site




(16-27)

open for binding of $^{\bullet}O_2^-$. As indicated in Eq. 16-27, uptake of one proton is associated with each reaction step. As illustrated in Fig 16-22 for step *a* of Eq. 16-27, the first proton may be taken up to convert the bound $^-OH$ to $H_2O$. The enzyme in the $Fe^{2+}$ form has a $pK_a$ of 8.5 that has been associated with tyrosine 34. Perhaps this residue is involved in the proton uptake process.[378] A similarly located Tyr 41 from *Sulfolobus* is covalently modified, perhaps by phosphorylation.[378a]

## B. Cobalt and Vitamin $B_{12}$

The human body contains only about 1.5 mg of cobalt, almost all of it is in the form of **cobalamin**, vitamin $B_{12}$. Ruminant animals, such as cattle and sheep, have a relatively high nutritional need for cobalt and in regions with a low soil cobalt content, such as Australia, cobalt deficiency in these animals is a serious problem. This need for cobalt largely reflects the high requirement of the microorganisms of the rumen (paunch) for vitamin $B_{12}$. All bacteria require vitamin $B_{12}$ but not all are able to synthesize it. For example, *E. coli* lacks one enzyme in the biosynthetic

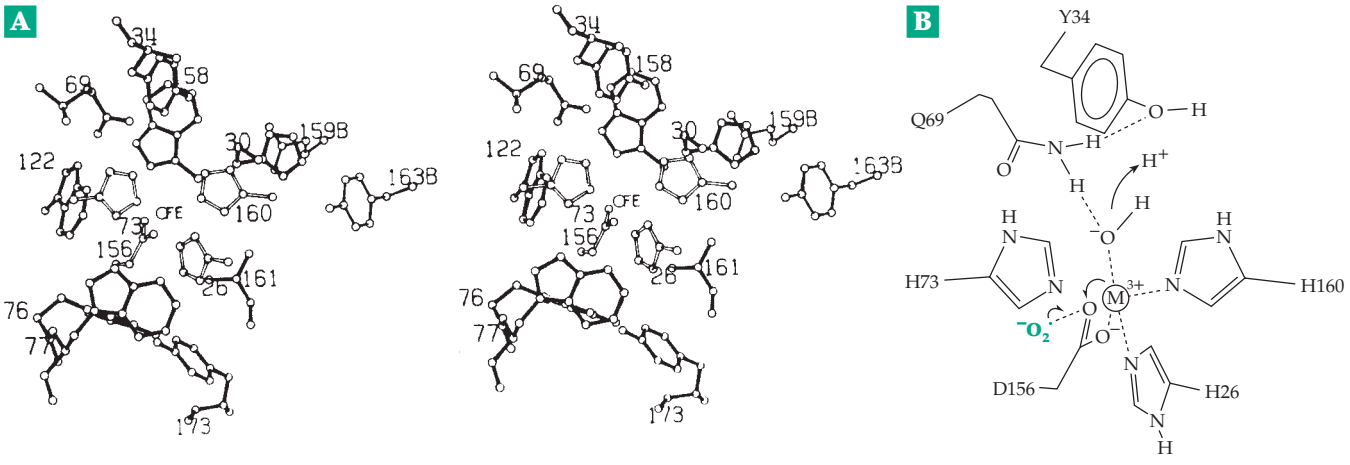
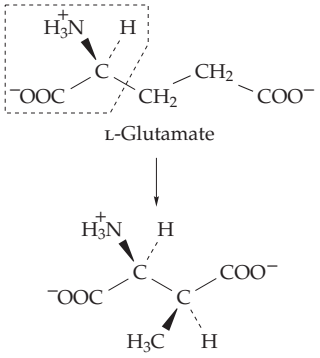



**Figure 16-22** (A) Structure of the active site of iron superoxide dismutase from *E. coli*. From Carlioz *et al.*[379] Courtesy of M. Ludwig. (B) Interpretive drawing illustrating the single-electron transfer from a superoxide molecule to the $Fe^{3+}$ of superoxide dismutase and associated proton uptake. Based on Lah *et al.*[376]

pathway and must depend upon other bacteria to complete the synthesis.

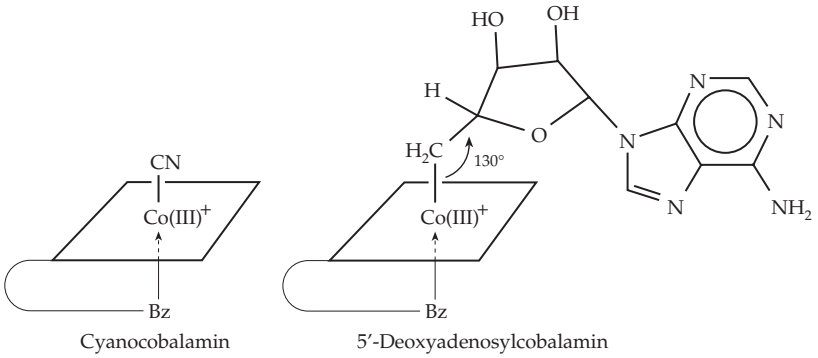
## 1. Coenzyme Forms

For several years after the discovery of cobalamin its biochemical function remained a mystery, a major reason being the extreme sensitivity of the coenzymes to decomposition by light. Progress came after Barker and associates discovered that the initial step in the anaerobic fermentation of glutamate by *Clostridium tetanomorphum* is rearrangement to β-methylaspartate[380,381] (Eq. 16-28).




(16-28)

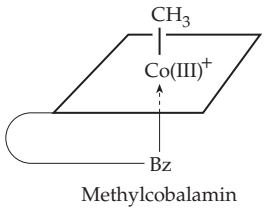
The latter compound can be catabolized by reactions that cannot be used on glutamate itself. Thus, the initial rearrangement is an indispensable step in the energy metabolism of the bacterium. A new coenzyme required for this reaction was isolated in 1958 after it was found that protection from light during the preparation was necessary. The coenzyme was characterized in 1961 by X-ray diffraction[382] as **5'-deoxyadenosylcobalamin**. It is related to cyanocobalamin (Box 16-B) by replacement of the CN group by a 5'-deoxyadenosyl group as indicated in the following abbreviated formulas.[383–385] Here the planes represent the corrin ring system and Bz the dimethylbenzimidazole that is coordinated with the cobalt from below the ring.




The most surprising structural feature is the Co–C single bond of length 0.205 nm. Thus, the coenzyme is an alkyl cobalt, the first such compound found in nature. In fact, alkyl cobalts were previously thought to be unstable. Vitamin $B_{12}$ contains Co(III), and cyanocobalamin can be imagined as arising by replacement of the single hydrogen on the inside of the corrin ring by $Co^{3+}$ plus $CN^-$. However, bear in mind that three other nitrogens of the corrin ring and a nitrogen of dimethylbenzimidazole also bind to the cobalt. Each nitrogen atom donates an electron pair to form coordinate covalent linkages. Because of resonance in the conjugated double-bond system of the corrin, all four of the Co–N bonds in the ring are nearly equivalent and the positive charge is distributed over the nitrogen atoms surrounding the cobalt.

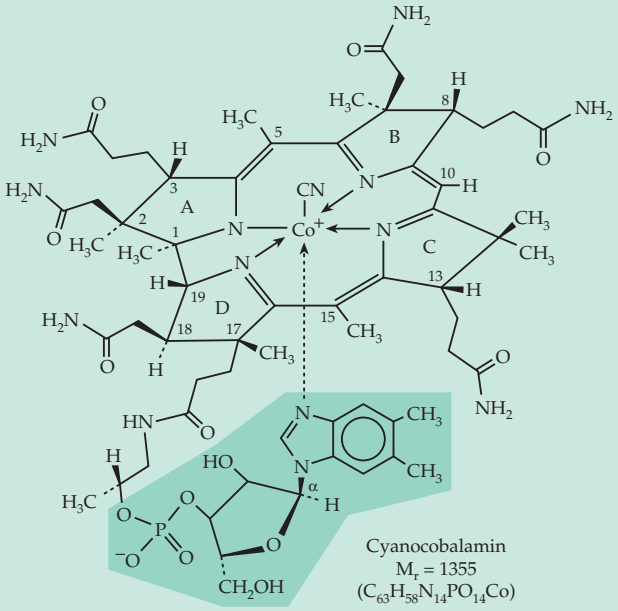
The strength of the axial Co–C bond is directly influenced by the strength of bonding of the dimethylimidazole whose conjugate base has a microscopic $pK_a$ of 5.5.[386] Protonation of this base breaks its bond to cobalt and may thereby strengthen the Co–C bond.[387] Steric factors are also important in determining the strength of this bond. NMR techniques are now playing an important role in investigation of these factors.[386–388]

In both bacteria and liver, the 5'-deoxyadenosyl coenzyme is the most abundant form of vitamin $B_{12}$, while lesser amounts of **methylcobalamin** are present. Other naturally occurring analogs of the coenzymes include **pseudo vitamin $B_{12}$** which contains adenine in place of the dimethylbenzimidazole.



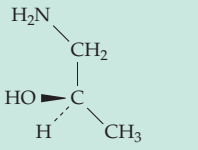

Like dimethylbenzimidazole, it is combined with ribose in the unusual α linkage. A compound called factor A is the vitamin $B_{12}$ analog with 2-methyladenine. Related compounds have been isolated from such sources as sewage sludge which abounds in anaerobic bacteria. It has been suggested that plants may contain vitamin $B_{12}$-like materials which do not support growth of bacteria. Thus, we may not have discovered all of the alkyl cobalt coenzymes.

## BOX 16-B COBALAMIN (VITAMIN $B_{12}$)

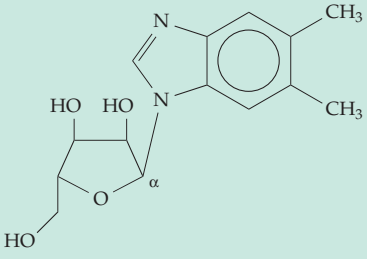


Cyanocobalamin
$M_r$ = 1355
($C_{63}H_{58}N_{14}PO_{14}Co$)

The story of vitamin $B_{12}$ began with pernicious anemia, a disease that usually affects only persons of age 60 or more but which occasionally strikes children.[a] Before 1926 the disease was incurable and usually fatal. Abnormally large, immature, and fragile red blood cells are produced but the total number of erythrocytes is much reduced from 4–6 x $10^6$ $mm^{-3}$ to 1–3 x $10^6$ $mm^{-3}$. Within the bone marrow mitosis appears to be blocked and DNA synthesis is suppressed. The disease also affects other rapidly growing tissues such as the gastric mucous membranes (which stop secreting HCl) and nervous tissues. Demyelination of the central nervous system with loss of muscular coordination (ataxia) and psychotic symptoms is often observed.



*R*-1-Amino-2-propanol



α-Ribofuranoside of dimethylimidazole

In 1926, Minot and Murphy discovered that pernicious anemia could be controlled by eating one-half pound of raw or lightly cooked liver per day, a treatment which not all patients accepted with enthusiasm. Twenty-two years later vitamin $B_{12}$ was isolated (as the crystalline derivative cyanocobalamin) and was shown to be the curative agent. It is present in liver to the extent of 1 mg $kg^{-1}$ or ~$10^{-6}$ M. Although much effort was expended in preparation of concentrated liver extracts for the treatment of pernicious anemia, the lack of an assay other than treatment of human patients made progress slow.

In the early 1940s nutritional studies of young animals raised on diets lacking animal proteins and maintained out of contact with their own excreta (which contained vitamin $B_{12}$) demonstrated the need for "animal protein factor" which was soon shown to be the same as vitamin $B_{12}$. The animal feeding experiments also demonstrated that waste liquors from streptomyces fermentations used in production of antibiotics were extremely rich in vitamin $B_{12}$. Later this vitamin was recognized as a growth factor for a strain of *Lactobacillus lactis* which responded with half-maximum growth to as little as 0.013 μg / l ($10^{-11}$ M).

In 1948, red cobalt-containing crystals of vitamin $B_{12}$ were obtained almost simultaneously by two pharmaceutical firms. Charcoal adsorption from liver extracts was followed by elution with alcohol and numerous other separation steps. Later fermentation broths provided a richer source. Chemical studies revealed that the new vitamin had an enormous molecular weight, that it contained one atom of phosphorus which could be released as $P_i$, a molecule of aminopropanol, and a ribofuranoside of dimethyl benzimidazole with the unusual α configuration.

Note the relationship of the dimethylbenzimidazole to the ring system of riboflavin (Box 15-B). Several molecules of ammonia could be released from amide linkages by hydrolysis, but all attempts to remove the cobalt reversibly from the ring system were unsuccessful. The structure was determined in 1956 by Dorothy C. Hodgkin and coworkers using X-ray diffraction.[b] At that time, it was the largest organic structure determined by X-ray diffraction. The complete laboratory synthesis was accomplished in 1972.[c]

The ring system of vitamin $B_{12}$, like that of porphyrins (Fig. 16-5), is made up of four pyrrole rings whose biosynthetic relationship to the corresponding rings in porphyrins is obvious from the structures. In addition, a number of "extra" methyl groups are present. A less extensive conjugated system of double bonds is present in the **corrin** ring of vitamin $B_{12}$ than in porphyrins, and as a result, many chiral centers are found around the periphery

**BOX 16-B (continued)**

of the somewhat nonplanar rings.

Cyanocobalamin, the form of vitamin $B_{12}$ isolated initially, contains cyanide attached to cobalt. It occurs only in minor amounts, if at all, in nature but is generated through the addition of cyanide during the isolation. **Hydroxocobalamin** (vitamin $B_{12a}$) containing $OH^-$ in place of $CN^-$ does occur in nature. However, the predominant forms of the vitamin are the coenzymes in which an alkyl group replaces the $CN^-$ of cyanocobalamin.

Intramuscular injection of as little as 3–6 µg of crystalline vitamin $B_{12}$ is sufficient to bring about a remission of pernicious anemia and 1 µg daily provides a suitable maintenance dose (often administered as hydroxocobalamin injected once every 2 weeks). For a normal person a dietary intake of 2–5 µg / day is adequate. There is rarely any difficulty in meeting this requirement from ordinary diets. Vitamin $B_{12}$ has the distinction of being synthesized only by bacteria, and plants apparently contain none. Consequently, strict vegetarians sometimes have symptoms of vitamin $B_{12}$ deficiency.

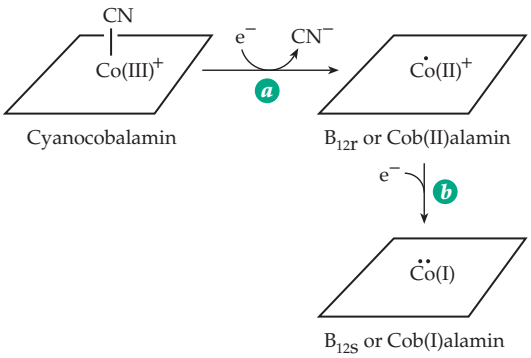
Pernicious anemia is usually caused by poor absorption of the vitamin. Absorption depends upon the **intrinsic factor**, a mucoprotein (or mucoproteins) synthesized by the stomach lining.[a,d–f] Pernicious anemia patients often have a genetic predilection toward decreased synthesis of the intrinsic factor. Gastrectomy, which decreases synthesis of the intrinsic factor, or infection with fish tapeworms, which compete for available vitamin $B_{12}$ and interfere with absorption, can also induce the disease. Also essential are a plasma membrane receptor[g,h] and two blood transport proteins **transcobalamin**[d,i] and **cobalophilin**. The latter is a glycoprotein found in virtually every human biological fluid and which may protect the vitamin from photodegradation by light that penetrates tissues.[j] A variety of genetic defects involving uptake, transport, and conversion to vitamin $B_{12}$ coenzyme forms are known.[f,k]

Normal blood levels of vitamin $B_{12}$ are ~2 x $10^{-10}$ M or a little more, but in vegetarians the level may drop to less than one-half this value. A deficiency of folic acid can also cause megaloblastic anemia, and a large excess of folic acid can, to some extent, reverse the anemia of pernicious anemia and mask the disease.

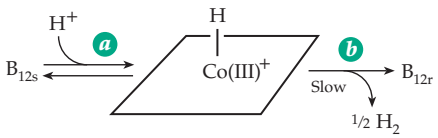

[a] Karlson, P. (1979) *Trends Biochem. Sci.* **4**, 286
[b] Hodgkin, D. C. (1965) *Science* **150**, 979–988
[c] Maugh, T. H., II (1973) *Science* **179**, 266–267
[d] Gräsbeck, R., and Kouvonen, I. (1983) *Trends Biochem. Sci.* **8**, 203–205
[e] Allen, R. H., Stabler, S. P., Savage, D. G., and Lindenbaum, J. (1993) *FASEB J.* **7**, 1344–1353
[f] Fenton, W. A., and Rosenberg, L. E. (1995) in *The Metabolic and Molecular Bases of Inherited Disease*, 7th ed., Vol. 2 (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., eds), pp. 3129–3149, McGraw-Hill, New York
[g] Seetharam, S., Ramanujam, K. S., and Seetharam, B. (1992) *J. Biol. Chem.* **267**, 7421–7427
[h] Birn, H., Verroust, P. J., Nexo, E., Hager, H., Jacobsen, C., Christensen, E. I., and Moestrup, S. K. (1997) *J. Biol. Chem.* **272**, 26497–26504
[i] Fedosov, S. N., Berglund, L., Nexo, E., and Petersen, T. E. (1999) *J. Biol. Chem.* **274**, 26015–26020
[j] Frisbie, S. M., and Chance, M. R. (1993) *Biochemistry* **32**, 13886–13892
[k] Rosenblatt, D. S., Hosack, A., Matiaszuk, N. V., Cooper, B. A., and Laframboise, R. (1985) *Science* **228**, 1319–1321


## 2. Reduction of Cyanocobalamin and Synthesis of Alkyl Cobalamins

Cyanocobalamin can be reduced in two one-electron steps (Eq. 16-29).[385,388] The cyanide ion is lost in the first step (Eq. 16-29, step *a*), which may be accomplished with chromous acetate at pH 5 or by catalytic hydrogenation. The product is the brown paramagnetic compound $B_{12r}$, a tetragonal low-spin cobalt(II) complex. In the second step (Eq. 16-29, step *b*), an additional electron is added, e.g., from sodium borohydride or from chromous acetate at pH 9.5, to give the gray-green exceedingly reactive $B_{12s}$. The latter is thought to be in equilibrium with cobalt(III) hydride, as shown in Eq. 16-30, step *a*. The hydride is unstable and breaks down slowly to $H_2$ and $B_{12r}$ (Eq. 16-30, step *b*).[389]

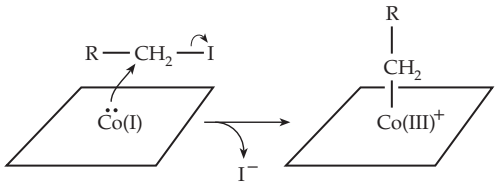



(16-29)




(16-30)

Vitamin $B_{12s}$ reacts rapidly with alkyl iodides (e.g., methyl iodide or a 5'-chloro derivative of adenosine) via nucleophilic displacement to form the alkyl cobalt forms of vitamin $B_{12}$ (Eq. 16-31). These reactions provide a convenient way of preparing isotopically labeled alkyl cobalamins, including those selectively

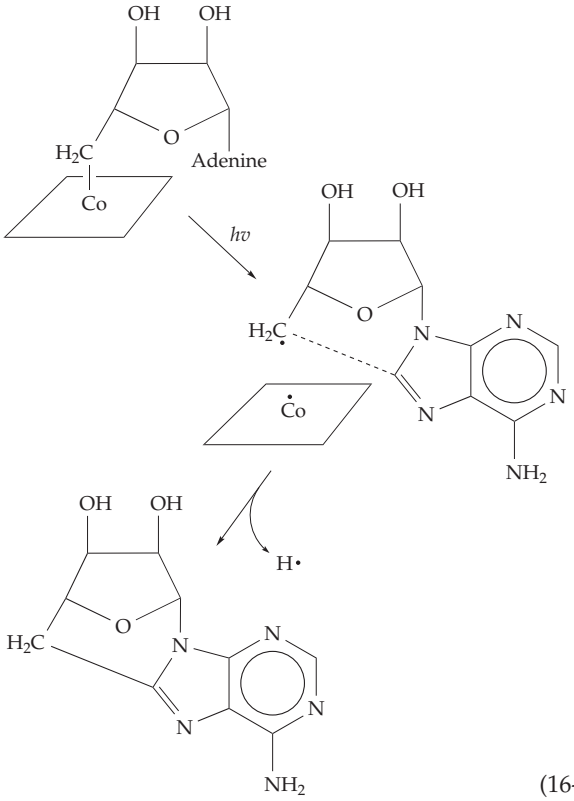



(16-31)

enriched in $^{13}C$ for use in NMR studies.[390] The biosynthesis of 5'-deoxyadenosylcobalamin utilizes the same type of reaction with ATP as a substrate.[391] A **$B_{12s}$ adenosyltransferase** catalyzes nucleophilic displacement on the 5' carbon of ATP with formation of the coenzyme and displacement of inorganic tripolyphosphate $PPP_i$.

## 3. Three Nonenzymatic Cleavage Reactions of Vitamin $B_{12}$ Coenzymes

The 5'-deoxyadenosyl coenzyme is easily decomposed by a variety of agents. Anaerobic irradiation with visible light yields principally vitamin $B_{12r}$ and a cyclic 5',8-deoxyadenosine which is probably formed through an intermediate radical[391a] (Eq. 16-32):

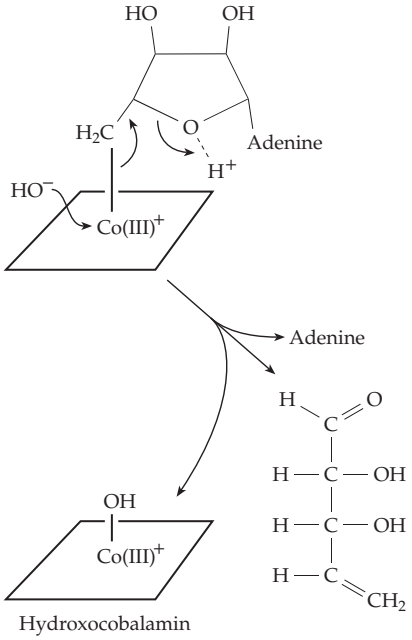



(16-32)

Irradiation in the presence of air gives a variety of products.

Hydrolysis of deoxyadenosylcobalamin by acid (1 M HCl, 100°, 90 min) yields hydroxycobalamin, adenine, and an unsaturated sugar (Eq. 16-33). The initial reaction step is thought to be protonation of the oxygen of the ribose ring.

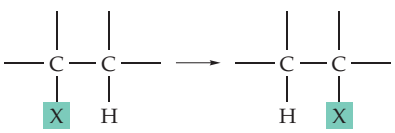
A related cleavage by alkaline cyanide can be viewed as a nucleophilic displacement of the deoxyadenosyl anion by cyanide. The end product is **dicyanocobalamin**, in which the loosely bound nucleotide containing dimethyl benzimidazole is replaced by a second cyanide ion. Methyl and other simple alkyl cobalamins are stable to alkaline cyanide. A number of other cleavage reactions of alkyl cobalamins are known.[392,393]




(16-33)

## 4. Enzymatic Functions of $B_{12}$ Coenzymes

Three types of enzymatic reactions depend upon alkyl corrin coenzymes. The first is the reduction of ribonucleotide triphosphates by cobalamin-dependent ribonucleotide reductase, a process involving *intermolecular* hydrogen transfer (Eq. 16-21). The second type of reaction encompasses the series of isomerizations shown in Table 16-1. These can all be depicted as in Eq. 16-34. Some group X, which may be attached by a C–C, C–O, or C–N bond, is transferred to an adjacent carbon atom bearing a hydrogen. At the same time,



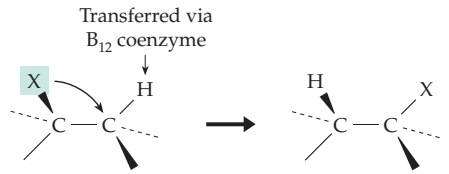
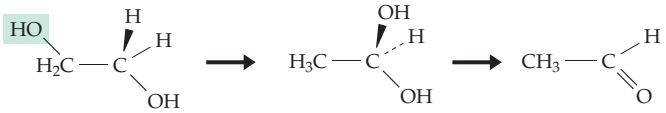
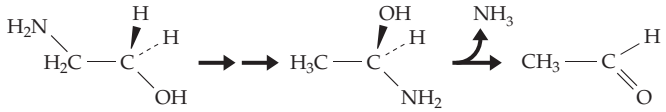
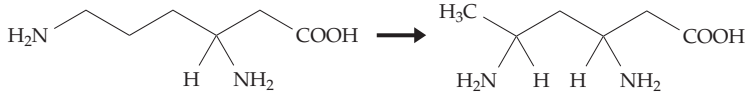
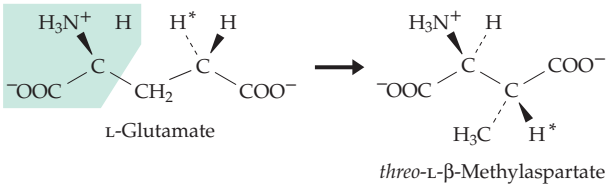
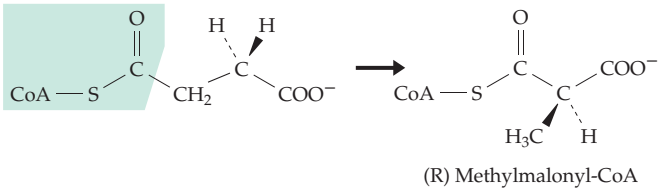
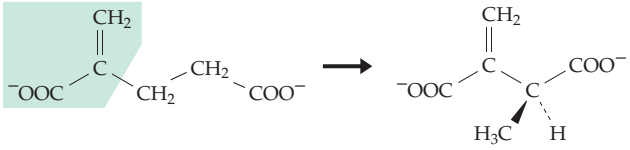

(16-34)

the hydrogen is transferred to the carbon to which X was originally attached. The third type of reaction is the transfer of methyl groups via methylcobalamin and some related bacterial metabolic reactions.

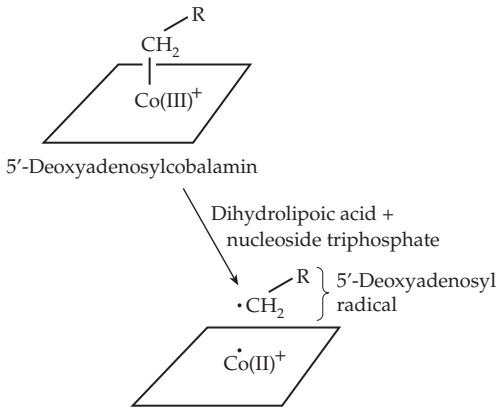
***Cobalamin-dependent ribonucleotide reductase.*** Lactic acid bacteria such as *Lactobacillus leichmanni* and many other bacteria utilize a 5'-deoxyadenosylcobalamin-containing enzyme to reduce nucleoside triphosphates according to Eq. 16-21. Thioredoxin or dihydrolipoic acid can serve as the hydrogen donor. Early experiments showed that protons from water are reversibly incorporated at C-2' of the reduced nucleotide with retention of configuration. A more important finding was a large kinetic isotope effect of 1.8 when 3'-$^3$H-containing UTP was reduced by the enzyme.[394]

Reaction of the reductase with dihydrolipoic acid in the presence of deoxy-GTP, which apparently serves as an allosteric activator, leads to formation, within a few milliseconds, of a radical with a characteristic EPR spectrum that can be studied when the reaction mixture is rapidly cooled to 130°K. When GTP (a true substrate) is used instead of dGTP, the radical signal reaches a maximum in about 20 ms and then decays. Of the various oxidation states of cobalt (3+, 2+, and 1+)

**TABLE 16-1**
**Isomerization Reactions Involving Hydrogen Transfer and Dependent upon a Vitamin $B_{12}$ Coenzyme**

| | |
|---|---|
|  | General reaction<br>Migrating group is enclosed in a box |
|  | Dioldehydratase<br>Glycerol dehydratase catalyzes the same type of reaction |
|  | Ethanolamine ammonia-lyase |
|  | L-β-Lysine mutase<br>D-α-Lysine mutase and ornithine mutase catalyze the same type of reaction |
|  | Glutamate mutase |
|  | Methylmalonyl-CoA mutase<br>Isobutyryl-CoA mutase catalyzes the same type of reaction |
|  | α-Methyleneglutarate mutase |

only the 2+ state of vitamin $B_{12r}$ is paramagnetic and gives rise to an EPR signal. The electronic absorption spectrum of the coenzyme of ribonucleotide reductase is also changed rapidly by substrate in a way that suggests formation of $B_{12r}$. Thus, it was proposed that a *homolytic* cleavage occurs to form $B_{12r}$ and a stabilized 5'-deoxyadenosyl radical (Eq. 16-35).[395–396a] However, $H^3$ is not transferred from the 3' position of the substrate into the deoxyadenosyl part of the coenzyme.[394] The enzyme has many properties in common with the previously discussed iron–tyrosinate ribonucleotide reductases (Fig. 16-21) including limited peptide sequence homology.[350] Stubbe and coworkers suggested that the deoxyadenosyl radical is formed as a radical chain initiator[394,396b] and that the mechanism of ribonucleotide reduction is as shown in Eq. 16-25. Studies of enzyme-activated inhibitors support this mechanism.[364]



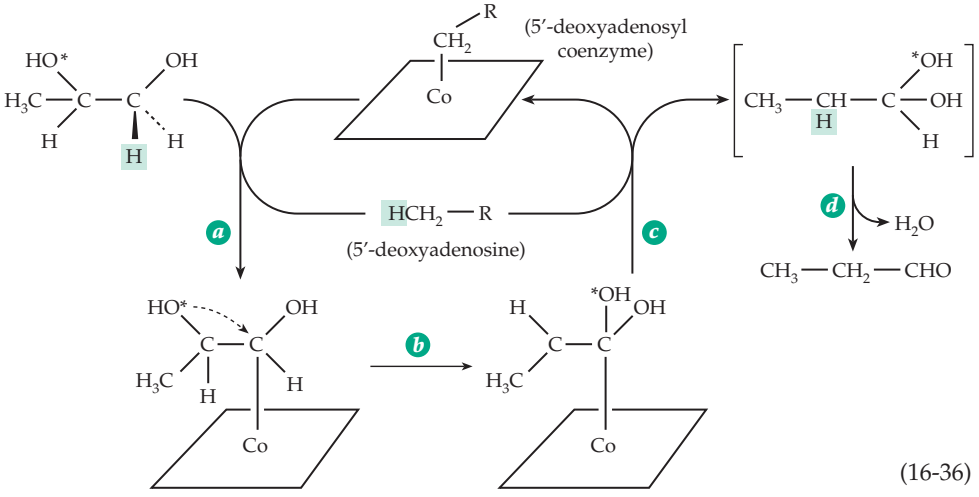

(16-35)

***The isomerization reactions.*** At least 10 reactions of the type described by Eq. 16-34 are known[397] (Table 16-1). They can be subdivided into three groups. First, X = OH or $NH_2$ in Eq. 16-34; isomerization gives a *geminal*-diol or aminoalcohol that can eliminate $H_2O$ or $NH_3$ to give an aldehyde. All of these enzymes, which are called **hydro-lyases** or **ammonia-lyases**, specifically require $K^+$ as well as the vitamin $B_{12}$ coenzyme. Second, X = $NH_2$ in Eq. 16-34; For this group of **aminomutases** PLP is required as a second coenzyme. Third, X is attached via a carbon atom; the enzymes are called **mutases**. **Methylmalonyl-Co mutase** is required for catabolism of propionate in the human body, and is one of only two known vitamin $B_{12}$-dependent enzymes. The related isobutyryl-CoA mutase participates in the microbial synthesis of such polyether antibiotics as monensin A.[397a,b] Other mutases are involved in anaerobic bacterial fermentations.

In these reactions the hydrogen is always transferred via the $B_{12}$ coenzyme. No exchange with the medium takes place. Since X may be an electronegative group such as OH, the reactions could all be treated formally as hydride ion transfers but it is more likely that they occur via homolytic cleavages. Such cleavage is indicated by the observation of EPR signals for several of the enzymes in the presence of their substrates.[398]

Abeles and associates showed that when **dioldehydratase** (Table 16-1) catalyzes the conversion of 1,2-[1-$^3$H]propanediol to propionaldehyde, tritium appears in the coenzyme as well as in the final product. When $^3$H-containing coenzyme is incubated with unlabeled propanediol, the product also contains $^3$H, which was shown by chemical degradation to be exclusively on C-5'. Synthetic 5'-deoxyadenosyl coenzyme containing $^3$H in the 5' position transferred $^3$H to product. Most important, using a mixture of propanediol and ethylene glycol, a small amount of *intermolecular* transfer was demonstrated; that is, $^3$H was transferred into acetaldehyde, the product of dehydration of ethylene glycol. Similar results were also obtained with ethanolamine ammonia-lyase.[399]

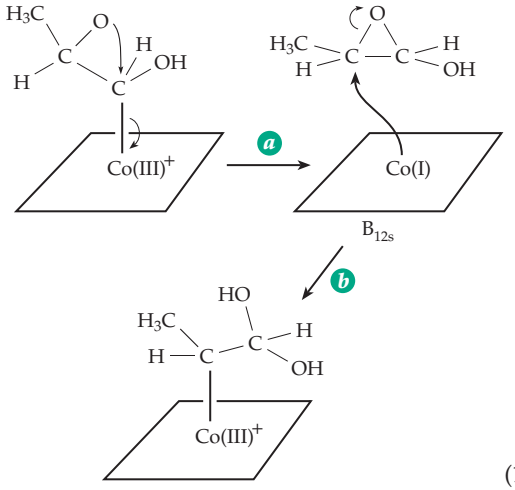
Another important experiment[398] showed that $^{18}$O from [2-$^{18}$O]propanediol was transferred into the 1 position without exchange with solvent. Furthermore, $^{18}$O from (*S*)-[1-$^{18}$O]propanediol was retained in the product while that from the (*R*) isomer was not. Thus, it appears that the enzyme stereospecifically dehydrates the final intermediate. From these and other experiments, it was concluded that initially a 5'-deoxyadenosyl radical is formed via Eq. 16-35. This radical then abstracts the hydrogen atom marked by a shaded box in Eq. 16-36 to form a substrate radical and 5'-deoxyadenosine. One proposal, illustrated in Eq. 16-36, is that the substrate radical immediately recombines with




(16-36)

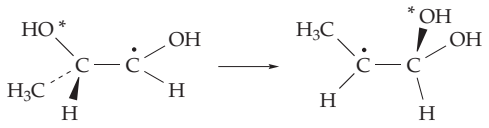
the Co(II) of the coenzyme to form an organo-cobalt substrate compound (step *a* of Eq. 16-36). The 5'-deoxyadenosine now contains hydrogen from the substrate; because of rotation of the methyl group this hydrogen becomes equivalent to the two already present in the coenzyme. The substrate–cobalamin compound formed in this step then undergoes isomerization, which, in the case of dioldehydratase, leads to intramolecular transfer of the OH group (step *b*). In step *c* the hydrogen atom is transferred back from the 5'-deoxyadenosine to its new location in the product and in step *d* the resulting *gem*-diol is dehydrated to form the aldehyde product.[401a]

Carbocation, carboanion, and free radical intermediates have all been proposed for the isomerization step in the reaction. A carbocation would presumably be cyclized to an epoxide which could react with the $B_{12s}$ (Eq. 16-37, step *b*) to complete the isomerization.




(16-37)

At present most evidence favors, for the isomerization reactions, an enzyme-catalyzed rearrangement of the substrate radical produced initially during formation of the 5'-deoxyadenosine (Eq. 16-38).[400–401c]

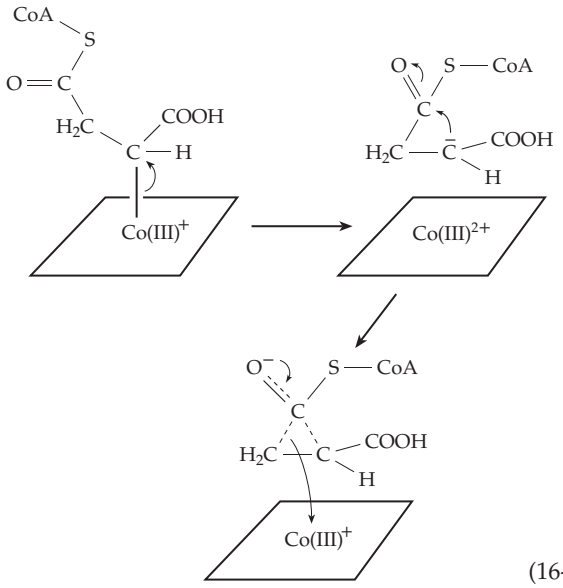



(16-38)

According to this mechanism the Co(II) of the $B_{12r}$ formed in Eq. 16-35 has no active role in the isomerization and does not form an organocobalt intermediate as in Eq. 16-36. Its only role is to be available to recombine with the 5'-deoxyadenosyl radical at the end of the reaction sequence. Support for this interpretation has been obtained from study of model reactions and of organic radicals generated in other ways.[400]

Recently, EPR spectroscopy with $^{2}H$- and $^{13}C$-labeled glutamates as substrates for glutamate mutase permitted identification of a 4-glutamyl radical as a probable intermediate for that enzyme.[402,402a,b]

On the other hand, for methylmalonyl-CoA mutase Ingraham suggested cleavage of the Co–C bond of an organocobalt intermediate to form a carbanion, the substrate–cobalamin compound serving as a sort of "biological Grignard reagent." The carbanion would be stabilized by the carbonyl group of the thioester forming a "homoenolate anion" (Eq. 16-39). The latter could break up in either of two ways reforming a C–Co bond and causing the isomerization.[403] Some experimental results also favor an ionic or organocobalt pathway.[404]




(16-39)

The three-dimensional structure of methylmalonyl-CoA mutase from *Propionibacterium shermanii* shows that the vitamin $B_{12}$ is bound in a base-off conformation with the dimethylbenzimidazole group bound to the protein far from the corrin ring (Fig. 16-23; see also Fig. 16-24).[405,405a] A histidine of the protein coordinates the cobalt, as also in methionine synthase. The entrance to the deeply buried active site is blocked by the coenzyme part of the substrate. The buried active site may be favorable for free radical rearrangement reactions. The structure of substrate complexes shows that the coenzyme is in the cob(II)alamin ($B_{12r}$) form with the 5'-deoxyadenosyl group detached from the cobalt, rotated, shifted, and weakly bound to the protein.[405a] Side chains from neighboring Y89, R207, and H244 all hydrogen bond to the substrate. The R207 guanidinium group makes an ion pair with the substrate carboxylate, and the phenolic and imidazole groups may have catalytic functions.[405b,c,d,406] Studies are also in progress on crystalline glutamate mutase.[406,406a]

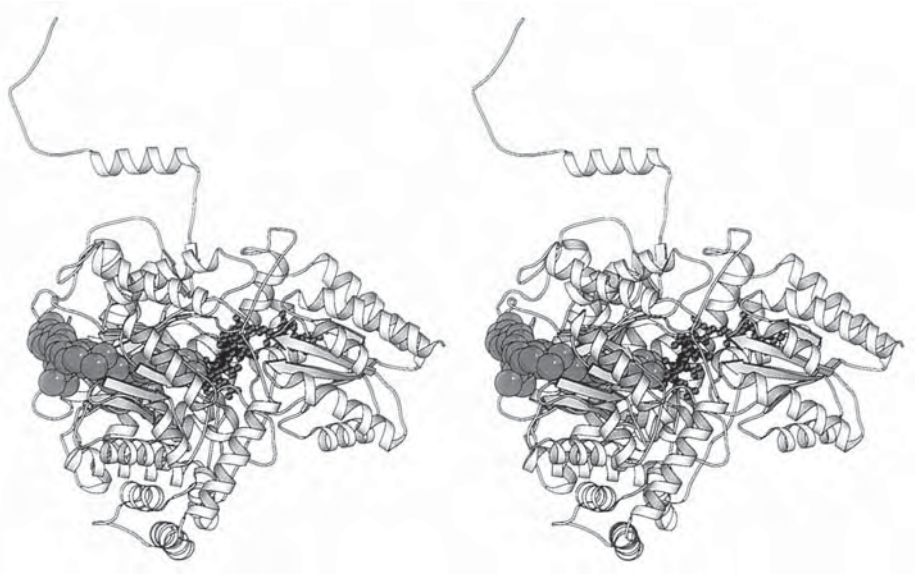
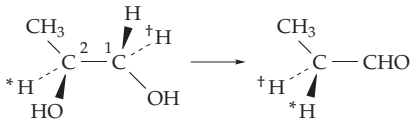


**Figure 16-23** Three-dimensional structure of methylmalonyl-CoA mutase from *Propionobacterium shermanii*. The $B_{12}$ coenzyme is deeply buried, as is the active site. A molecule of bound desulfocoenzyme A, a substrate analog, blocks the active site entrance on the left side. From Mancia *et al.*[405] Courtesy of Philip R. Evans.

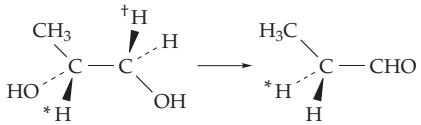
According to all of these mechanisms, 5'-deoxyadenosine is freed from its bond to cobalt during the action of the enzymes. Why then does the deoxyadenosine not escape from the coenzyme entirely, leading to its inactivation? Substrate-induced inactivation is not ordinarily observed with coenzyme $B_{12}$-dependent reactions, but some quasi-substrates do inactivate their enzymes. Thus, glycolaldehyde converts the coenzyme of dioldehydratase to 5'-deoxyadenosine and ethylene glycol does the same with ethanolamine deaminase. When 5'-deoxyinosine replaces the 5'-deoxyadenosine of the normal coenzyme in dioldehydratase, 5'-deoxyinosine is released quantitatively by the substrate. This suggests that the dehydratase may normally hold the adenine group of 5'-deoxyadenosine through hydrogen bonding to the amino group. Because the OH group of inosine tautomerizes to C=O, inosine may not be held as tightly. The deeply buried active site (Fig. 16-23) may also prevent escape of the deoxyadenosine.

Despite the evidence in its favor, there was initially some reluctance to accept 5'-deoxyadenosine as an intermediate in vitamin $B_{12}$-dependent isomerization reactions. It was hard to believe that a methyl group could exchange hydrogen atoms so rapidly. It was suggested that protonation of the oxygen of the ribose ring as in Eq. 16-33 might facilitate release of a hydrogen atom. However, substitution of the ring oxygen by $CH_2$ in a synthetic analog did not destroy the coenzymatic activity.[407] Another possibility is that the methyl group has an unusual reactivity if the cobalt is reduced to Co(II).

***Stereochemistry of the isomerization reactions.*** Dioldehydratase acts on either the (*R*) or (*S*) isomers of 1,2-propanediol (Eqs. 16-40 and 16-41; asterisks and daggers mark positions of labeled atoms in specific experiments).



(*S*)-1,2-Propanediol (16-40)



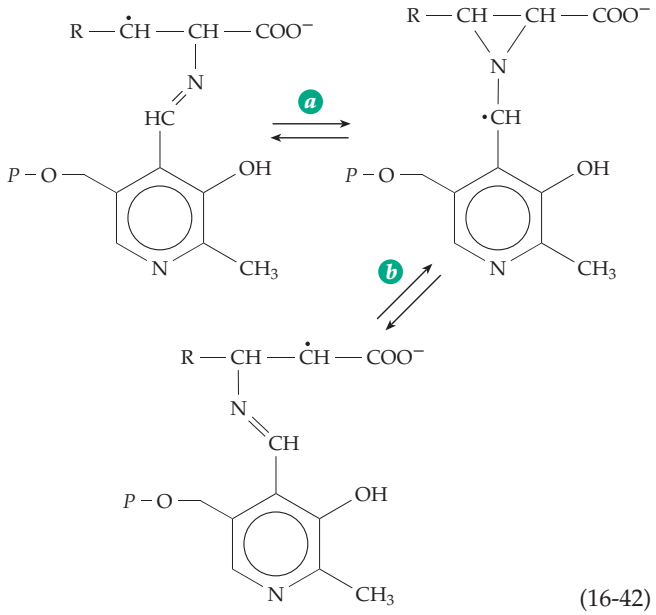
(*R*)-1,2-Propanediol (16-41)

In both cases the reaction proceeds with retention of configuration at C-2 and with stereochemical specificity[408] for one of the two hydrogens at C-1. The reaction catalyzed by methylmalonyl-CoA mutase likewise proceeds with retention of configuration at C-2 (Table 16-1)[409] but the glutamate mutase reaction is accompanied by inversion (Eq. 16-28).

***Aminomutases.*** The enzymes **L-β-lysine mutase** (which is also **D-α-lysine mutase**) and **D-ornithine mutase** catalyze the transfer of an ω-amino group to an adjacent carbon atom[410] (Table 16-1). Two proteins are needed for the reaction; pyridoxal phosphate is required and is apparently directly involved in the amino group migration. In the β-lysine mutase the 6-amino group of L-β-lysine replaces the pro-*S* hydrogen at C-5 but with inversion at C-5 to yield (3*S*, 5*S*)-3,5-diaminohexanoic acid.[411] A bacterial D-lysine 5,6-aminomutase interconverts D-lysine with 2,5-diaminohexanoic acid.[411a] Another related enzyme

is **L-leucine 2,3-aminomutase**, which catalyzes the reversible interconversion of L-leucine and β-leucine.[410,412] It was reported to be present in plants and also in the human body, but the latter could not be confirmed.[413]

The interconversion of L-α-lysine and L-β-lysine is catalyzed by a lysine 2,3-aminomutase found in certain clostridia.[414-415a] This enzyme also requires pyridoxal phosphate and catalyzes a reaction with the same stereochemistry as that of β-lysine mutase. However, it does not contain vitamin $B_{12}$ but depends upon *S*-adenosylmethionine (AdoMet) and an iron–sulfur cluster. The adenosyl group of AdoMet may function in the same manner as does the deoxyadenosyl group of the adenosylcobalamin coenzyme. In these mutases a 5'-deoxyadenosyl radical may abstract a hydrogen atom from the β position of a Schiff base of PLP with the amino acid substrate. The radical isomerizes (Eq. 16-42) and then accepts a hydrogen atom back from the 5'-deoxyadenosine to complete the reaction. Its properties suggest that lysine 2,3-aminomutase is related to the pyruvate formate-lyase of *E. coli* (Eq. 15-37),[410] class I ribonucleotide reductases, and other enzymes that act by homolytic mechanisms.




(16-42)

***Transfer reactions of methyl groups.*** The generation and utilization of methyl groups is a quantitatively important aspect of the metabolism of all cells. As we have seen (Fig. 15-18), methyl groups can be created by the reduction of one-carbon compounds attached to tetrahydrofolic acid. Methyl groups of methyltetrahydrofolic acid ($N^5$-$CH_3$THF) can then be transferred to the sulfur atom of homocysteine to form methionine (Eq. 16-43). The latter is converted to *S*-adenosylmethionine, the nearly universal methyl group donor for transmethylation reactions (Eq. 12-3). In some bacteria, fungi, and higher plants, the methyl-THF-homocysteine transmethylase does not depend upon vitamin $B_{12}$ but is a metalloenzyme with zinc at the active center.[416] However, human beings share with certain strains of *E. coli* and other bacteria the need for methylcobalamin.

The structure of the *E. coli* enzyme (Fig. 16-24) shows methylcobalamin bound in a base-off conformation, with histidine 759 of the protein replacing dimethylbenzimidazole in the distal coordination position on the cobalt. This histidine is part of a sequence Asp-X-His-X-X-Gly that is found not only in methionine synthase but also in methylmalonyl-CoA mutase, glutamate mutase, and 2-methyleneglutarate mutase. However, diol dehydratase lacks this sequence and binds adenosylcobalamin with the dimethylbenzimidazole–cobalt bond intact.[417]

The coenzyme evidently functions in a cyclic process. The cobalt alternates between the +1 and +3 oxidation states as shown in Eq. 16-43. The first indication of such a cyclic process was the report by Weissbach that $^{14}C$-labeled methylcobalamin could be isolated following treatment of the enzyme with such methyl donors as AdoMet and methyl iodide

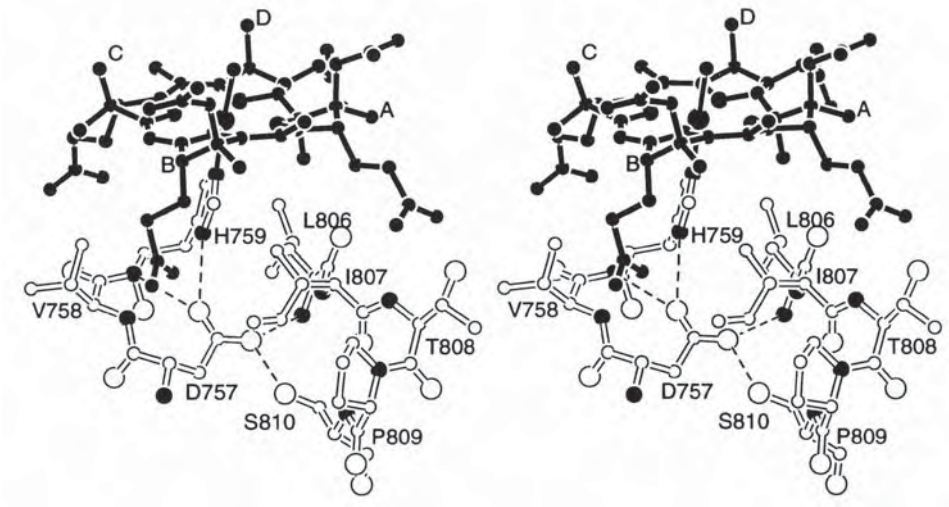
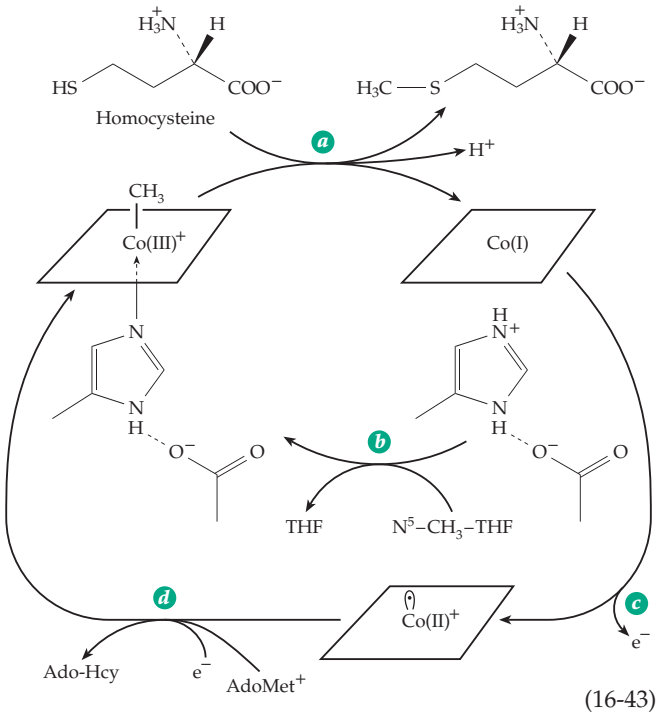



**Figure 16-24** Stereoscopic views of the active site of methionine synthase from *E. coli*. Methylcobalamin (black) is in the active site with His 759 of the protein in the distal position of the coenzyme in a base-off conformation. The dimethylbenzimidole nucleotide has been omitted for clarity. Notice the hydrogen-bonded His 759 – D757 – S810 triad. From Jarret *et al.*[418a] Courtesy of R. G. Matthews.

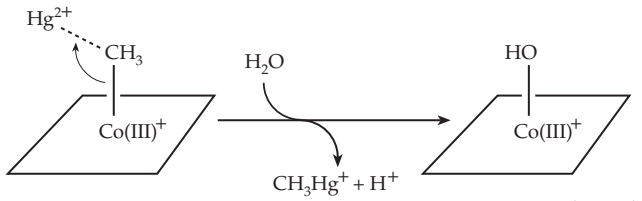



(16-43)

after reduction (e.g., with reduced riboflavin phosphate). The sequence parallels that of Eq. 16-31 for the laboratory synthesis of methylcobalamin. Nevertheless, the transmethylase demonstrates some complexities. Initially, it must be "activated" by AdoMet or methyl iodide, after which it cycles according to Eq. 16-43, steps *a* and *b*, but is gradually inactivated. This apparently happens by oxidation to a Co(II) form of the enzyme (Eq. 16-43, step *c*) that must be reductively methylated with AdoMet and reduced flavodoxin (step *d*) to regenerate the active form.[418] It is also possible that the methyl group is not transferred as a formal $CH_3^+$ as pictured in Eq. 16-43 but as a $^{\bullet}CH_3$ radical generated by homolytic cleavage of methylcobalamin to cob(II)alamin. Whatever the mechanism, a chiral methyl group is transferred from 5-methyl-THF to homocysteine with overall retention of configuration.[419]

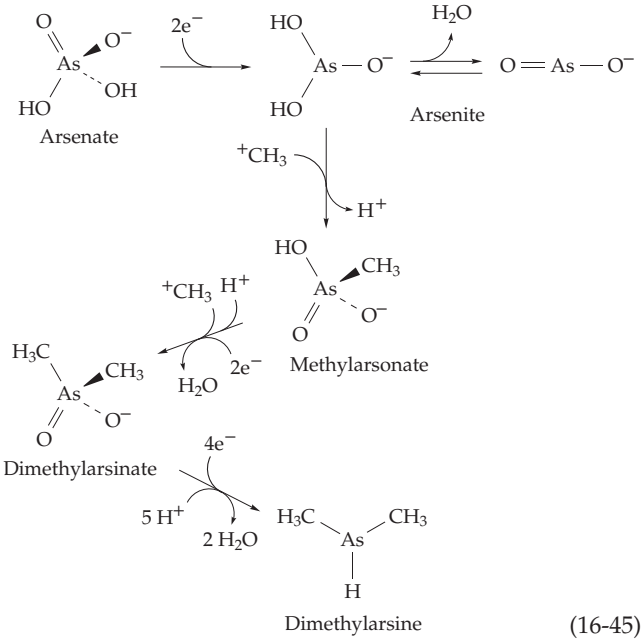
Another important group of methyl transfer reactions are those from methyl corrinoids to mercury, tin, arsenic, selenium, and tellurium. For example, Eq. 16-44 describes the methylation of $Hg^{2+}$. These reactions are of special interest because of the generation of toxic methyl and dimethyl mercury and dimethylarsine.

Notice that whereas in Eq. 16-43 the methyl group is transferred as $CH_3^+$ by nucleophilic displacement on a carbon atom, the transfer to $Hg^{2+}$ in Eq. 16-44 is that of a carbanion, $CH_3^-$, with no valence change occurring in the cobalt. However, it is also possible that transfer occurs as a methyl radical.[420] Methyl corrinoids are able to undergo this type of reaction nonenzymatically, and the ability to transfer a methyl anion is a property of methyl corrinoids not shared by other transmethylating agents such as AdoMet. At the conclusion of the reaction in Eq. 16-44 the cobalt is in the +3 state. To be remethylated, it must presumably be reduced to Co(II). A second methyl group can be transferred by the same type of reaction to form $(CH_3)_2Hg$.




(16-44)

Methylation of arsenic is an important pollution problem because of the widespread use of arsenic compounds in insecticides and because of the presence of arsenate in the phosphate used in household detergents.[421,422] After reduction to arsenite, methylation occurs in two steps (Eq. 16-45). Additional reduction steps result in the formation of **dimethylarsine**, one of the principal products of action of methanogenic bacteria on arsenate. The methyl transfer is shown as occurring through $CH_3^+$, with an accompanying loss of a proton from the substrate. However, a $CH_3$ radical may be transferred with formation of a cobalt(II) corrinoid.[423]




(16-45)

### *Corrinoid-dependent synthesis of acetyl-CoA.*

The anaerobic bacterium *Clostridium thermoaceticum* obtains its energy for growth by reduction of $CO_2$ with hydrogen (Eq. 16-46). One of the $CO_2$ molecules is reduced to formate which is converted via 5-methyl-THF to the methyl corrinoid 5-methoxybenzimidazolyl-

cobamide. The methyl group of the latter

$$4\ H_2 + 2\ CO_2 \longrightarrow CH_3COO^- + H^+ + 2\ H_2O$$
$$\Delta G'\ (\text{pH } 7) = -94.9\ \text{kJ mol}^{-1} \qquad (16\text{-}46)$$

combines with another $CO_2$ to form acetyl-CoA. The process, which requires the nickel-containing carbon monoxide dehydrogenase, is discussed in Section C.

## C. Nickel

It was not until the 1970s that nickel was first recognized as a dietary essential for animals.[424–426] Nickel-deficient chicks grew poorly, had thickened legs, and developed dermatitis. Tissues of deficient animals contained swollen mitochondria and swollen perinuclear space suggesting a function for Ni in membranes. However, doubts have been raised about the conclusions based on these experiments.[427] Within tissues the nickel content ranges from 1 to 5 μg / l. Some of the metal in serum is present as complexes of low molecular mass and some is bound to serum albumin[428] and to a specific nickel-containing protein of the macroglobulin class, known as **nickeloplasmin**.[429] Nickel is also present in plants; in some, e.g., *Allysum*, it accumulates to high concentrations.[430] It is essential for legumes and possibly for all plants.[431] Nickel uptake proteins have been identified in bacteria and fungi.[432,432a] Because of its ubiquitous occurrence it is difficult to prepare a totally Ni-free diet. The acute toxicity of orally ingested Ni(II) is low, and homeostatic mechanisms exist in the animal body for regulating its concentration. However, the volatile nickel carbonyl $Ni(CO)_4$ is very toxic[426] and Ni from jewelry is a common cause of dermatitis.[428,433]

In its compounds nickel usually has the +2 oxidation state but the +3 and +4 states occur rarely in complexes. The $Ni^{2+}$ ion contains eight 3d electrons, a configuration that favors square-planar coordination of four ligands. However, the ion is also able to form a complex with six ligands and an octahedral geometry. It has been suggested that this "ambivalence" may be of biochemical significance.

Nickel is found in at least four enzymes: **urease**, certain **hydrogenases, methyl-CoM reductase** (in its **cofactor $F_{430}$**) of methanogenic bacteria, and **carbon monoxide dehydrogenase** of acetogenic and methanogenic bacteria.[434]

### 1. Urease

Urease, which was first isolated from the jack bean has a special place in biochemical history as the first enzyme to be crystallized. This was accomplished by J. B. Sumner in 1926, and although Sumner eventually obtained the Nobel Prize, his first reports were greeted with skepticism and outright disbelief. The presence of two atoms of nickel in each molecule of urease[435] was not discovered until 1975. The metal ions had been overlooked previously, despite the fact that the absorption spectrum of the purified enzyme contains an absorption "tail" extending into the visible region with a shoulder at 425 nm and weak maxima at 725 and 1060 nm. Urease catalyzes the hydrolytic cleavage of urea to two molecules of ammonia and one of bicarbonate and is useful in the analytical determination of urea.

Jack bean urease is a trimer or hexamer of identical 91-kDa subunits while that of the bacterium *Klebsiella* has an $(\alpha\beta_2\gamma_2)_2$ stoichiometry. Nevertheless, the enzymes are homologous and both contain the same binickel catalytic center (Fig. 16-25).[435–437a] The three-dimensional structure of the *Klebsiella* enzyme revealed that the two nickel ions are bridged by a carbamyl group of a carbamylated lysine. Like ribulose bisphosphate carboxylase (Fig. 13-10), urease also requires $CO_2$ for formation of the active enzyme.[438] Formation of the metallocenter also requires four additional proteins, including a chaperonin and a nickel-binding protein.[438,439]

The mechanism of urease action is probably related to those of metalloproteases such as carboxypeptidase A (Fig. 12-16) and of the zinc-dependent carbonic

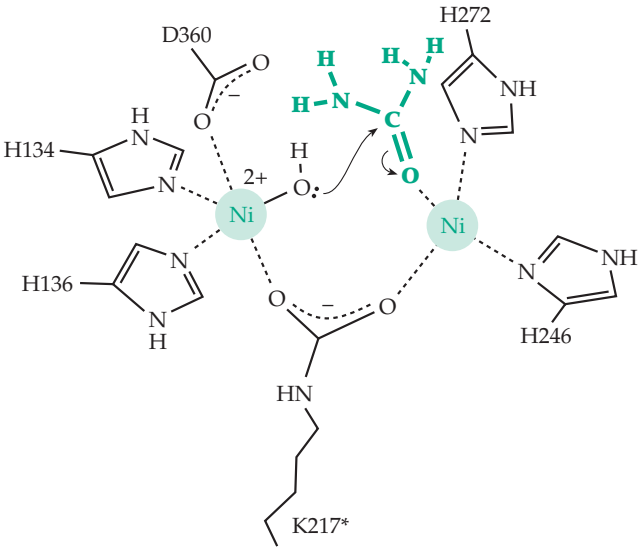
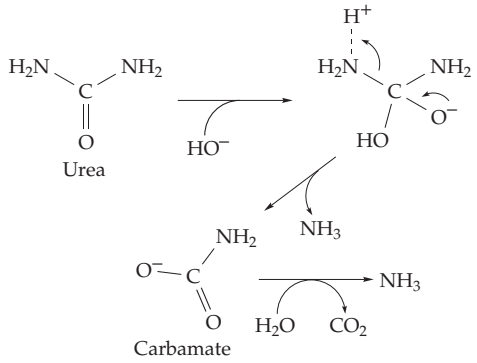



**Figure 16-25** The active site of urease showing the two $Ni^+$ ions held by histidine side chains and bridged by a carbamylated lysine (K217*). A bound urea molecule is shown in green. It has been placed in an open coordination position on one nickel and is shown being attacked for hydrolytic cleavage by a hydroxyl group bound to the other nickel. Based on a structure by Jabri *et al*.[436] and drawing by Lippard.[437]




(16-47)

anhydrase (Fig. 13-1). In Fig. 16-25 one nickel ion is shown polarizing the carbonyl group, while the second provides a bound hydroxyl ion that serves as the attacking nucleophile. A probable intermediate product is a carbamate ion (Eq. 16-47).

Urease is an essential enzyme for bacteria and other organisms that use urea as a primary source of nitrogen. The peptic ulcer bacterium *Helicobacter pylori* uses urease to hydrolyze urea in order to defend against the high acidity of the stomach.[439a] The enzyme is also present in plant leaves and may play a necessary role in nitrogen metabolism.[431] In nitrogen-fixing legumes urea derivatives, the **ureides**, have an important function (Chapter 25) but urease may not be involved in the catabolism of these compounds.[440]

## 2. Hydrogenases

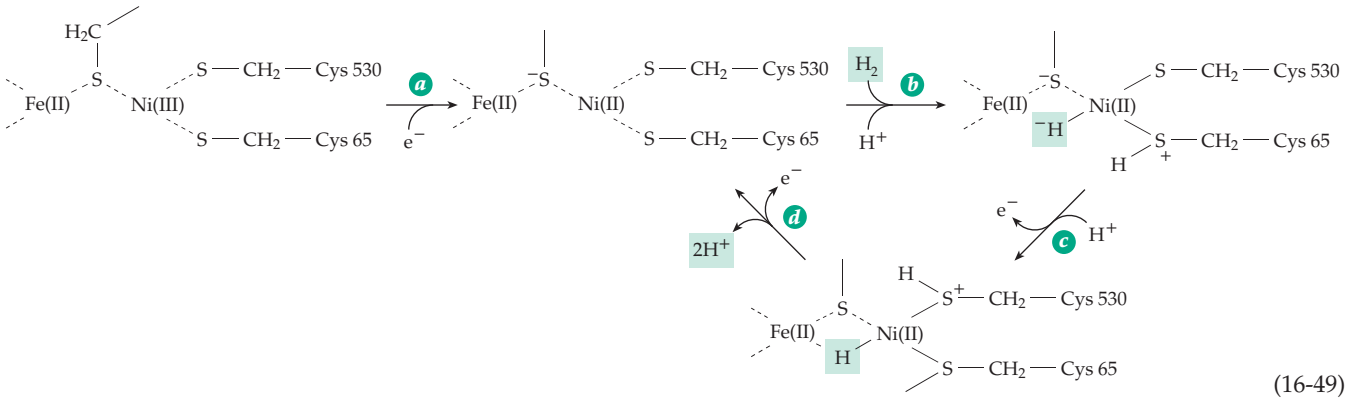
Many plants, animals, and microorganisms are able to evolve $H_2$ by reduction of hydrogen ions (Eq. 16-48) or to oxidize $H_2$ by the reverse of this reaction.[441,442]

$$2H^+ + 2e^- \rightarrow H_2 \qquad (16\text{-}48)$$

Hydrogenases have been classified into two main types: **Fe-hydrogenases**, which contain iron as the only metal,[443] and **Ni-hydrogenases**, which contain both iron and nickel.[444] In a few Ni-hydrogenases a selenocysteine residue replaces a conserved cysteine side chain.[445,446] Fe-hydrogenases are often extremely active and are utilized to rid organisms of an excess of electrons by evolution of $H_2$. Since they may also be used to acquire electrons by oxidation of $H_2$, they are often described as *bidirectional*. Ni-hydrogenases, as well as some Fe-hydrogenases, are involved primarily in *uptake* of $H_2$.[447] All hydrogenases contain one or more Fe–S centers in addition to the $H_2$-forming catalytic center.[441] Some hydrogenases are membrane bound and are often coupled through unidentified carriers to formate dehydrogenase (Chapter 17). In the strict anaerobes such as clostridia, hydrogenases are linked to ferredoxins. Hydrogenases are inactivated readily by $O_2$, which oxidizes the catalytic centers but can sometimes be reactivated by treatment with reducing agents.[448]

The 60-kDa all-iron monomeric hydrogenase I of *Clostridium pasteurianum* (mentioned on p. 861) contains ferredoxin-like $Fe_4S_4$ clusters plus additional Fe and sulfur atoms organized as a special H cluster. The EPR spectrum of the catalytic center, recognized because the spectrum is altered by the binding of carbon monoxide, is unusual. Its $g$ values of 2.00, 2.04, and 2.10 are similar to those of oxidized high-potential iron proteins.[449] The *C. pasteurianum* hydrogenase II is a 53-kDa monomer containing eight Fe and eight $S^{2-}$ ions. These are organized into one ferredoxin-like $Fe_4S_4$ cluster plus a three-Fe cluster and one iron ion in a unique environment.[449]

In contrast to these iron-only hydrogenases, the large periplasmic hydrogenase of *Desulfovibrio gigas* consists of one 28-kDa subunit and one 60-kDa subunit and contains two $Fe_4S_4$ clusters, one $Fe_3S_4$ cluster, and another dimetal center containing a single atom of Ni.[450,451] These are seen clearly in the three-dimensional structure depicted in Fig. 16-26. The three Fe–S clusters, at 5- to 6-nm intervals, form a chain from the external surface to the deeply buried nickel–iron center.[450–453a] A plausible pathway for transport of protons to the active center can also be seen.[450,451] The nickel center also contains an atom of Fe. Four cysteine side chains participate in forming the Ni–Fe cluster, two of them provide sulfur atoms that bridge between the metals while two others are ligands to Ni (Fig. 16-26).




(16-49)

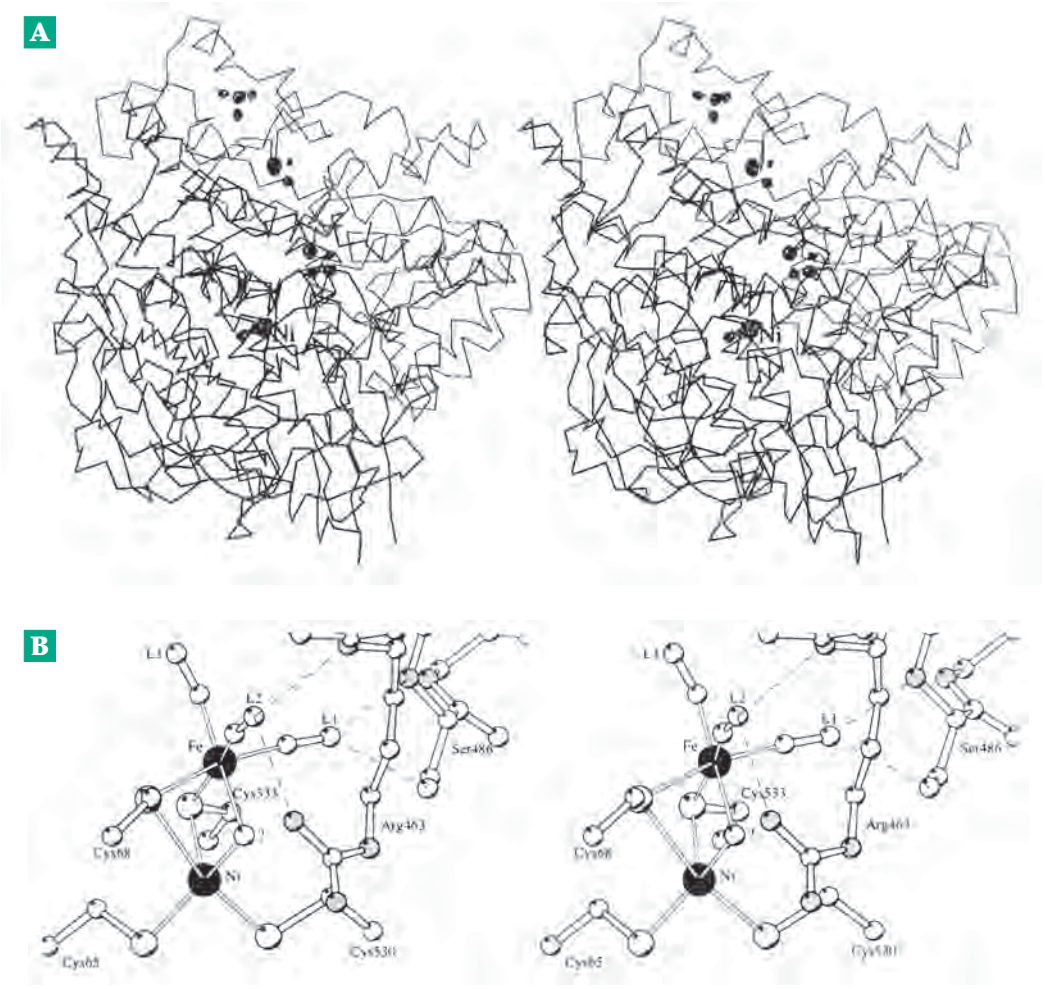



**Figure 16-26** (A) Stereoscopic view of the structure of the *Desulfovibrio gigas* hydrogenase as an α-carbon plot. The electron density map at the high level of 8σ is superimposed and consists of dark spheres representing the Fe and Ni atoms. The iron atoms of the two $Fe_4S_4$ and one $Fe_3S_4$ clusters are seen clearly forming a chain from the surface of the protein to the Ni–Fe center. (B) The structure of the active site Ni–Fe pair. The two metals are bridged by two cysteine sulfur atoms and an unidentified atom, perhaps O, and the nickel is also coordinated by two additional cysteine sulfurs. Unidentified small molecules L1, L2, and L3 are also present. From Volbeda *et al.*[453] Courtesy of M. Frey.

A hydrogenase from *chromatium vinosum* has a similar structure.[453b] There are still uncertainties about other nonprotein ligands such as $H_2O$.[452–453a]

All of the Ni-hydrogenases display an EPR signal that can be assigned to Ni(III).[452] However, the active enzyme from *D. gigas* contains Ni(II). A proposed mechanism[452] is indicated in Eq. 16-49. Step *a* of this equation is a reductive activation. In step *b* a molecule of $H_2$ is bound as a hydride ion on Ni and a proton on a nearby sulfur. Protonation of a second sulfur ligand to Ni is needed to promote the cleavage of $H_2$ prior to the two-step oxidation of the bound $H^-$. One of two Ni-containing hydrogenases of *Methanobacterium thermoautotrophicum* contains FAD as well as Fe–S clusters.[454] It specifically reduces the 5-deazaflavin cofactor $F_{420}$ (Chapter 15). A major function of this deazaflavin is reduction of the nickel-containing cofactor $F_{430}$.

### 3. Cofactor $F_{430}$ and Methyl-Coenzyme M Reductase

Cofactor $F_{430}$ is a nickel tetrapyrrole with a structure (Fig. 16-27)[455,456] similar to those of vitamin $B_{12}$ and of siroheme. The tetrapyrrole ring is the most highly reduced in cofactor $F_{430}$, which functions in reduction of methyl-CoM to methane in methanogens (Fig. 16-28). The methyl CoM reductase of *Methanobacterium thermoautotrophicum* is a large 300-kDa protein with subunit composition $\alpha_2\beta_2\gamma_2$ and containing two molecules of bound $F_{430}$. The nickel in $F_{430}$ is first thought to be reduced, in an activation step, to Ni(I),[456a] which may attack the methyl group of methyl-CoM homolytically to yield a methyl nickel complex and a sulfur radical.[457,458] Alkyl nickel compounds react with protons, and in this case they would yield methane and would regenerate the Ni(II) form of the cofactor. The CoM radical could be reduced back to free CoM.

High-resolution crystal structures of the enzyme in two inactive Ni(II) forms[458] show the two $F_{430}$ molecules. Each is bound in an identical channel about 3 nm in length and extending from the surface deep into the interior of the protein. The $F_{430}$ lies at the bottom of this channel with its nickel atom coordinated with the oxygen atom of a glutamine side chain. In one form CoM lies directly above the nickel, with its

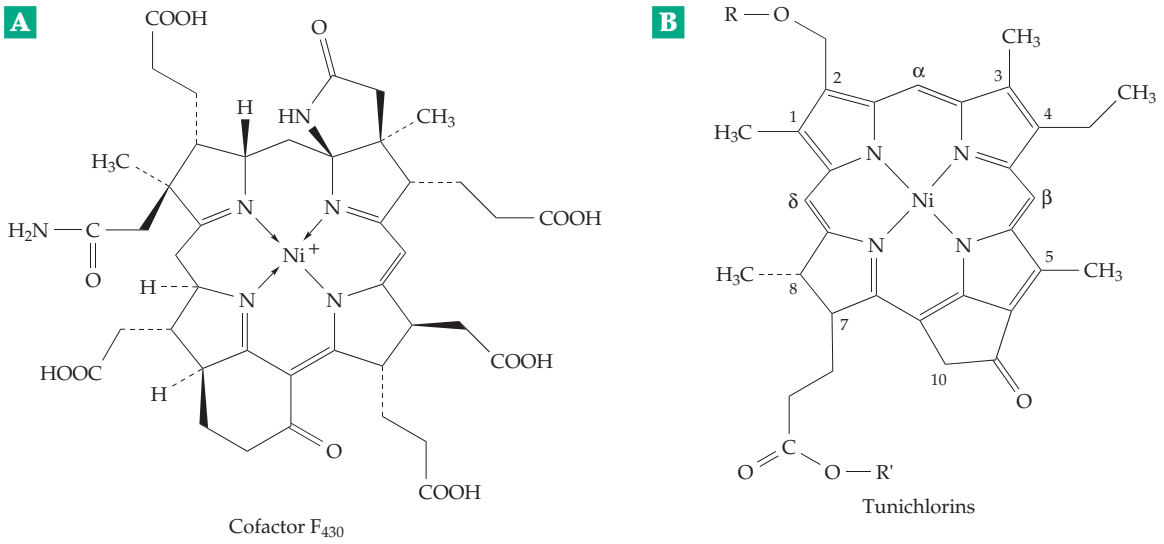



**Figure 16-27** (A) Structure of the nickel-containing prosthetic group $F_{430}$ as isolated in the esterified (methylated) form. From Pfaltz *et al.*[459] The "front" face, which reacts with methyl-coenzyme M, is toward the reader.[458] (B) Structure of a representative member of a family of tunichlorins isolated from marine tunicates.[460] For tunichlorin R = R′ = H. Related compounds have R′ = $CH_3$ and/or R = an alkyl group with 13–21 carbon atoms and up to six double bonds.

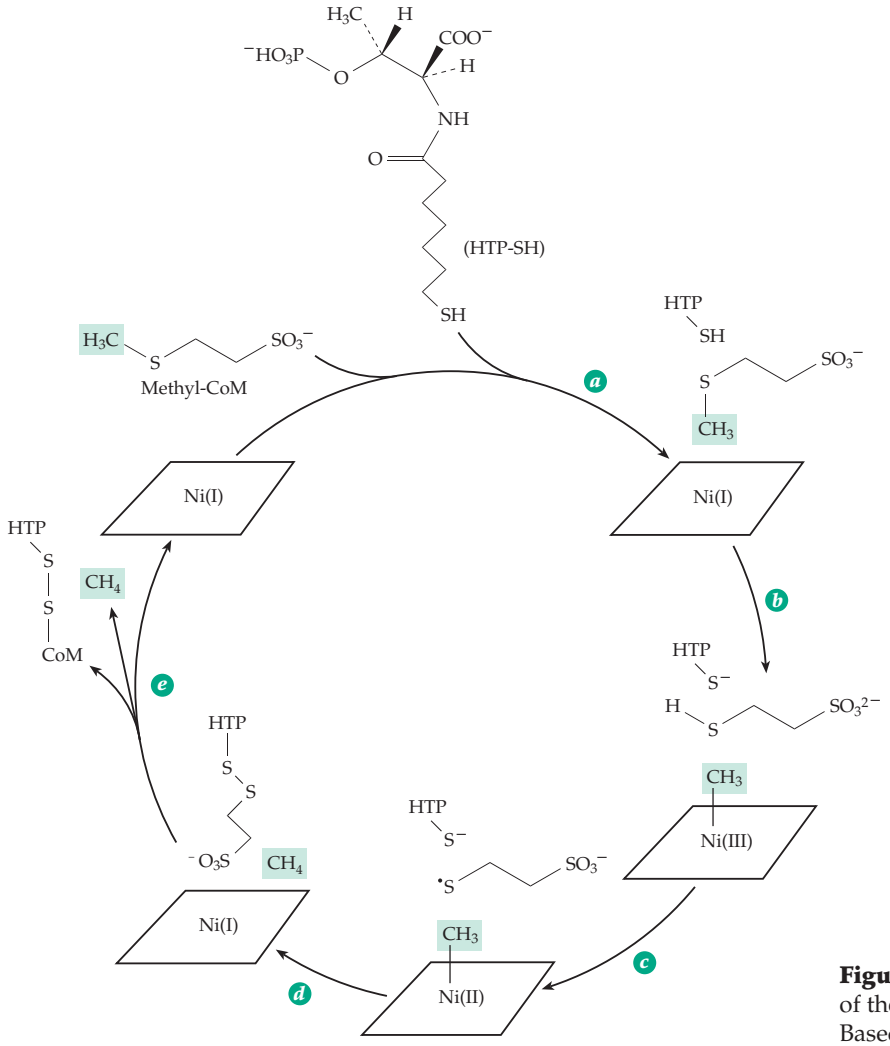



**Figure 16-28** Proposed mechanism of action of the methane-forming coenzyme M reductase. Based on the crystal structure.

thiolate sulfur providing the sixth ligand for the nickel. The long –SH-containing side chain of **heptanoyl threonine phosphate** (HTP; also called coenzyme B; Chapter 15, Section E) also lies within the channel with its amino acid head group blocking the entrance. In a second crystal form the mixed disulfide HTP–S–S–CoM, an expected product of the reaction (Fig. 16-28), is present in the channel.[458] Because of the distance from the –SH group of HTP and the nickel atom it is clear that there must be some motion of the methyl-CoM and that the methane formed may stay trapped in the active site until the HTP–S–S–CoA product leaves.

A proposed mechanism for the catalytic cycle based on the X-ray results as well as previous chemical studies and EPR spectroscopy is shown in Fig. 16-28. The substrates enter in step *a*. The position of the HTP, with its extended side chain, is probably the same as that seen in the X-ray structures of the Ni(II) complexes but the conformation of the methyl-CoM is different. The methyl transfer in step *b* is reminiscent of that of methionine synthase (Eq. 16-43). Although the distance from the CoM sulfur and the HTP–SH is too great for direct proton transfer between the two, as indicated for step *b*, there are two tyrosine hydroxyls that could provide a pathway for proton transfer. The region around the surface of the nickel coenzyme is largely hydrophobic and could facilitate formation of the thiyl radical in step *c*. In the structure with the bound HTP–S–S–CoM heterodisulfide an oxygen atom of the sulfonate group of CoM is bonded tightly to the Ni(II). However, in the active Ni(I) form the nickel is nucleophilic and would probably repel the sulfonate, perhaps assisting the product release.[458]

The enzyme contains five posttranslationally modified amino acids near the active site: *N*-methylhistidine, 5-methylarginine, 2-methylglutamine, 2-methylcysteine, and thioglycine in a thiopeptide bond. The latter may be the site of radical formation.[458a,b]

## 4. Tunichlorins

Nickel is found in various marine invertebrates. In the tunicates (sea squirts and their relatives) it occurs in a fixed ratio with cobalt, suggesting a metabolic role.[460] A new class of nickel chelates called tunichlorins have been isolated. An example is shown in Fig. 16-27B. The function of tunichlorins is unknown but their existence suggests the possibility of unidentified biochemical roles for nickel.

## 5. Carbon Monoxide Dehydrogenases and Carbon Monoxide Dehydrogenase/Acetyl-CoA Synthase

There are several bacterial carbon monoxide dehydrogenases that catalyze the reversible oxidation of CO to $CO_2$:

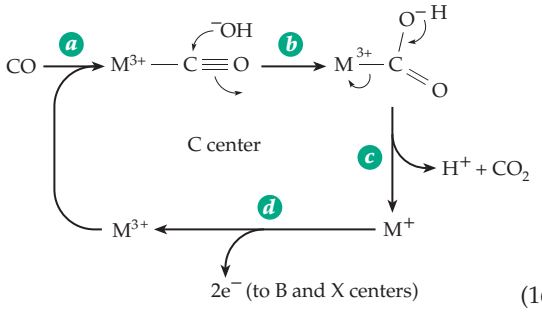
$$CO + H_2O \rightarrow CO_2 + H^+ + 2\ e^- \qquad (16\text{-}50a)$$

$$CO + H_2O \rightarrow CO_2 + H_2 \qquad \Delta G^\circ = -38.1\ \text{kJ / mol} \qquad (16\text{-}50b)$$

Some bacteria use CO as both a source of energy and for synthesis of carbon compounds. The purple photosynthetic bacterium *Rhodospirillum rubrum* employs a relatively simple monomeric Ni-containing CO dehydrogenase containing one atom of Ni and seven or eight iron atoms, apparently arranged in $Fe_4S_4$ clusters. These bacteria can grow anaerobically with CO as the sole source of both energy and carbon.[461] Some aerobic bacteria oxidize CO using a molybdenum-containing enzyme (Section H). However, the most studied CO dehydrogenase is a complex enzyme that also synthesizes, reversibly, acetyl-CoA from CO and a methyl corrin. Employed by methanogens, acetogens, and sulfate-reducing bacteria, it is at the heart of the **Wood–Ljundahl pathway** of autotrophic metabolism, which is discussed further in Chapter 17.

Both oxidation of CO to $CO_2$ and reduction of $CO_2$ to CO are important activities of CO dehydrogenase / acetyl-CoA synthase. During growth on CO, some CO must be oxidized to $CO_2$ and then reduced by the pathways of Fig. 15-22 to form a methyl-tetrahydropterin which can be used to form the methyl group of acetyl-CoA. During growth on any other carbon compound $CO_2$ must be reduced to CO to form the carbonyl group of acetyl-CoA which can serve as a precursor to all other carbon compounds. Native CO dehydrogenase/acetyl CoA synthase was isolated from cells of *Clostridium thermoaceticum* grown in the presence of radioactive $^{63}Ni$. The protein is a 310-kDa $\alpha_2\beta_2$ oligomer. Each αβ dimer contains 2 atoms of Ni, 1 of Zn, ~12 of Fe and ~12 sulfide ions,[462–464] which are organized into three metal clusters referred to as A, B, and C. Each cluster contains 4 Fe atoms and clusters A and C also contain 1 Ni each. Oxidation of CO occurs in the β subunits, each of which contains both cluster B, an $Fe_4S_4$ ferredoxin-type cluster, and cluster C, where the oxidation of CO is thought to occur. Cluster C contains 1 nickel ion as well as an $Fe_4S_4$ cluster that resembles that of aconitase (Fig. 13-4). Cluster A, which is in the α subunit, also contains 1 atom of Ni and 4 Fe ions and is probably the site of synthesis of acetyl-CoA.

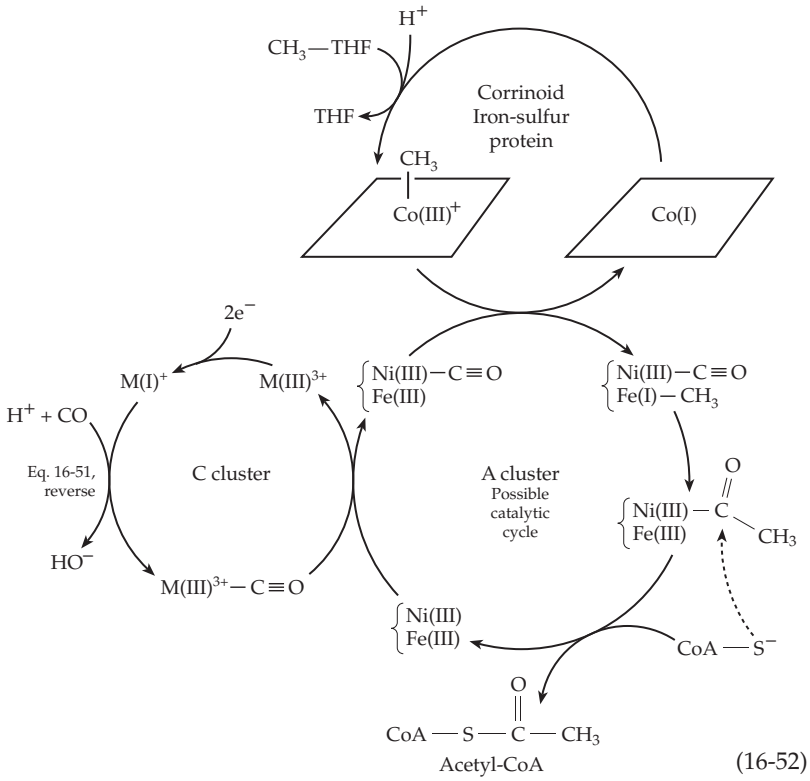
Oxidation of CO may require cooperation of the nickel ion and the $Fe_4S_4$ group within the C cluster.




(16-51)

CO probably binds to one of these metals and is attacked by a hydroxyl ion (Eq. 16-51, step *b*) which may be donated by the other metal of the pair.[465,465a] $CO_2$ and a proton are released rapidly (step *c*), after which the reduced metal center is reoxidized (step *d*). One electron is thought to be transferred directly to the $Fe_4S_4$ cluster B and the second by an alternative route.[465] A multienzyme complex isolated from the methanogen *Methanosarcina* has an $(\alpha\beta\gamma\delta\varepsilon)_6$ structure, with the subunits having masses of 89, 60, 50, 48, and 20 kDa, respectively.[466] In this complex the CO dehydrogenase/acetyl-CoA synthase activity appears to reside in the $\alpha_2\varepsilon_2$ complex, the $\gamma\delta$ complex has a tetrahydropteridine: cob(I)amide–protein transferase, and the β subunit has an acetyltransferase that binds acetyl-CoA and transfers the acetyl group to a group on the β subunit.[466]

Although the *Clostridium* and *Methanosarcina* systems are not identical, similar mechanisms are presumably involved.[467,467a] To generate acetyl-CoA a methyl group is first transferred from a tetrahydropterin such as tetrahydrofolate or, in methanogens, tetrahydromethanopterin or tetrahydrosarcinapterin (Fig. 15-17) to form a methylcorrinoid. At the A center of the CO dehydrogenase a molecule of CO, which may be bound to the Ni, equilibrates with the methyl group, and with acetyl-CoA. As depicted in Eq. 16-52, acetyl-Ni may be an intermediate. Other details shown here are hypothetical. It is possible that the methyl group is transferred to the Ni atom in the M cluster before reaction with the CO, which might be bound to either Ni of cluster C or to Fe. This reaction of two transition-metal-bound ligands parallels a proposed industrial process for synthesis of acetic acid from methanol and CO and involving catalysis by rhodium metal and methyl iodide. It is thought that rhodium-bound CO is inserted into bound Rh–$CH_3$ to form an intermediate Rh–$\overset{\overset{O}{\|}}{C}$–$CH_3$. The acetyl group is released as acetyl iodide which is hydrolyzed to acetic acid. An acetyl-nickel intermediate may be involved in the corresponding biological reaction of Eq. 16-52. The stereochemistry of the sequence has been investigated using methyl-THF containing a chiral methyl group. Overall retention of the configuration of the methyl group in acetyl-CoA was observed.[468,469]




(16-52)

## D. Copper

Copper was recognized as nutritionally essential by 1924 and has since been found to function in many cellular proteins.[470–474] Copper is so broadly distributed in foods that a deficiency has only rarely been observed in humans.[474a] However, animals may sometimes receive inadequate amounts because absorption of $Cu^{2+}$ is antagonized by $Zn^{2+}$ and because copper may be tied up by molybdate as an inert complex. There are copper-deficient desert areas of Australia where neither plants nor animals survive. Copper-deficient animals have bone defects, hair color is lacking, and hemoglobin synthesis is impaired. Cytochrome oxidase activity is low. The protein elastin of arterial walls is poorly crosslinked and the arteries are weak. Genetic defects in copper metabolism can have similar effects.

An adult human ingests ~2–5 mg of copper per day, about 30% of which is absorbed. The total body content of copper is ~100 mg (~2 x $10^{-4}$ mol / kg), and both uptake and excretion (via the bile) are regulated. Since an excess of copper is toxic, regulation is important.

Because $Cu^{2+}$ is the most tightly bound metal ion in most chelating centers (Table 6-9), almost all of the copper present in living cells is complexed with proteins. Copper is transported in the blood by a 132-kDa, 1046-residue sky-blue glycoprotein called **ceruloplasmin**.[471,475–477] This one protein contains 3% of the total body copper.

Regulation of copper uptake has been studied in most detail in the yeast *Saccharomyces cerevisiae*. Uptake of $Cu^{2+}$ is similar to that of $Fe^{3+}$. The same plasma membrane reductase system, consisting of proteins Fre1p and Fre2p (encoded by genes *FRE*1 and *FRE*2), acts to reduce both $Fe^{3+}$ and $Cu^{2+}$.[478–481] These two genes are controlled in part by a transcriptional activator that responds to the internal copper concentration.[410,482,483] Similar regulation is thought to occur in both plants[484] and animals.

The human hereditary disorders **Wilson's disease** and **Menkes' disease** have provided further insight into copper metabolism.[485,486] In Wilson's disease the ceruloplasmin content is low and copper gradually accumulates to high levels in the liver and brain. In Menkes syndrome, there is also a low ceruloplasmin level and an accumulation of copper in the form of copper metallothionein.[487,488] Persons with this disease have abnormalities of hair, arteries, and bones and die in childhood of cerebral degeneration.[489,490] Similar symptoms are seen in some patients with Ehlers–Danlos syndrome (Box 8-E).[491] Genes for the proteins that are defective in both Wilson's and Menkes' diseases have been cloned and both proteins have been identified as P-type ATPase cation transporters (Chapter 8).[492–495c] The two proteins must be similar in structure as indicated by a 55% sequence identity.[496] Homologous genes involved in copper homeostasis have been located in both yeast[497] and the cyanobacterium *Synechococcus*.[498] The transporter encoded by this yeast gene, designated *Ccc*2, apparently functions to export copper from the cytosol into an extracytosolic compartment. In a similar way the Wilson and Menkes disease proteins, which reside in the *trans*-Golgi network, are thought to export copper or to provide copper for incorporation into essential proteins.[493,499] The Wilson disease protein is also found in a shortened form in mitochondrial membranes.[492] Other proteins associated with intracellular copper metabolism seem to be chaperones for Cu(I).[500–501a]

The ability of copper ions to undergo reversible changes in oxidation state permits them to function in a variety of oxidation–reduction processes. Like iron, copper also provides sites for reaction with $O_2$, with superoxide radicals, and with nitrite ions.

## 1. Electron-Transferring Copper Proteins

A large group of small, intensely blue copper proteins function as single-electron carriers within bacteria and plants. Best known is **plastocyanin**, which is ubiquitous in green plants and functions in the electron transport chain between the light-absorbing photosynthetic centers I and II of chloroplasts (Chapter 23). The bacterial **azurins**[502] are thought to carry electrons between cytochrome $c_{441}$ and cytochrome oxidase. **Amicyanin** accepts electrons from the coenzyme TTQ of methylamine dehydrogenase of methylotrophic bacteria and passes them to a cytochrome *c* (Chapter 15).[168,503,504] A basic blue copper protein **phytocyanin** of uncertain function occurs in cucumber seeds.[505]

The 10.5-kDa peptide chain of plastocyanin is folded into an eight-stranded β barrel (Fig. 2-16), which contains a single copper atom. In poplar plastocyanin, the Cu is coordinated by the side chains of His 37, His 87, Met 92, and Cys 84 in a tetrahedral but distorted toward a trigonal bipyramidal geometry. Since copper-free apoplastocyanin has essentially the same structure, this geometry may be imposed by the protein onto the $Cu^{2+}$, which usually prefers square-planar or tetrahedral coordination (Chapter 7).[506] Calculations suggest that there is little or no strain and that the "Franck–Condon barrier" to electron transfer is low.[507,508] The three-dimensional structure and copper environment of azurin are similar to those of plastocyanin.[509–511] Messerschmidt *et al*.[512] suggested that the copper site in these proteins is perfectly adapted to its function because its geometry is a compromise between the optimal geometries of the Cu(I) and Cu(II) states between which it alternates. **Stellacyanin**, present in the Japanese lac tree and some other plants, is a mucoprotein; the 108-residue protein is over 40% carbohydrate.[513] While its spectrum resembles that of plastocyanins and azurins, stellacyanin contains no methionine and this amino acid cannot be a ligand to copper.[514] **Rusticyanin** functions in the periplasmic space of some chemolithotropic sulfur bacteria to transfer electrons from $Fe^{2+}$ to cytochrome *c* as part of the energy-providing reaction for these organisms (Eq. 18-23).[515–517] **Halocyanin** functions in membranes of the archaeobacterium *Natronobacterium*,[518] and **aurocyanin** functions in green photosynthetic bacteria.[519]

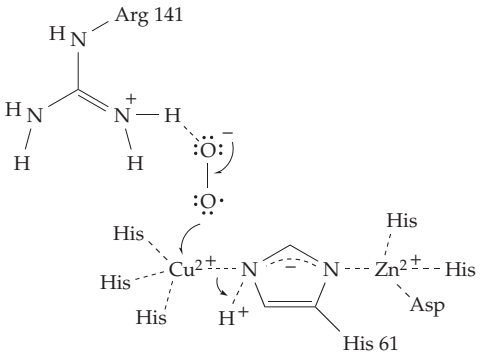
The blue color of these "type 1" copper proteins is much more intense than are the well known colors of the hydrated ion $Cu(H_2O)_4^{2+}$ or of the more strongly absorbing $Cu(NH_3)_4^{2+}$. The blue color of these simple complexes arises from a transition of an electron from one *d* orbital to another within the copper atom. The absorption is somewhat more intense in copper peptide chelates of the type shown in Eq. 6-85. However, the ~600 nm absorption bands of the blue proteins are an order of magnitude more intense, as is illustrated by the absorption spectrum of azurin (Fig. 23-8). The intense blue is thought to arise as a result of transfer of electronic charge from the cysteine thiolate to the $Cu^{2+}$ ion.[520,521]

A third type of copper center, first recognized in cytochrome *c* oxidase (see Fig. 18-10) is called $Cu_A$ or **purple CuA**. Each copper ion is bonded to an imidazole and two cysteines serve as bridging ligands. The two copper ions are about 0.24 nm apart, and the two $Cu^{2+}$ ions together can accept a single electron from an external donor such as cytochrome *c* or azurin to give a half-reduced form.[521a,b]

## 2. Copper, Zinc-Superoxide Dismutase

Although of similar topology to the blue electron-transferring proteins, Cu, Zn-superoxide dismutase, has a different function. This dimeric 153-residue protein has been demonstrated in the cytoplasm of virtually all eukaryotic cells[522] and in the periplasmic space of some bacteria[523] where it converts superoxide ions $^{\bullet}O_2^-$ to $O_2$ and $H_2O_2$. The enzyme, which has a major protective role against oxidative damage to cells, presumably functions in a manner similar to that indicated in Eq. 16-27 for iron or manganese. However, copper cycles between $Cu^{2+}$ and $Cu^+$, alternately accepting and donating electrons.

The active site of cytosolic superoxide dismutase (SOD) contains both $Cu^{2+}$ and $Zn^{2+}$. The copper ion is of "type 2": nonblue and paramagnetic. It is surrounded by four imidazole groups with an irregular square planar geometry.[524–527] One of these imidazole groups (that of His 61) is shared with the $Zn^{2+}$, which is also bonded to two additional imidazole groups and a side chain carboxylate. The metal ions have evidently replaced the hydrogen atom that would otherwise be present on the imidazole of His 61 (see the following diagram). It has been suggested, as is also indicated in the diagram, that when the bound superoxide




donates an electron to the Cu(II) to become $O_2$ (first step of Eq. 16-27), a proton becomes attached to the bridging imidazole with breakage of its linkage to the Cu(I). The structure of a new crystalline form of reduced yeast SOD shows that the Cu(I) has moved 0.1 nm away from the bridging imidazole in agreement with this possibility.[528] In the second half reaction the imidazole proton, together with a second proton from the medium and an electron from the Cu(I), would react to convert the second $O_2^-$ into $H_2O_2$. The role of the $Zn^{2+}$ may be in part structural but it may also serve to ensure that His 61 is protonated on the correct nitrogen atom. Arg 141 may assist in binding the $O_2^-$ as is shown in the diagram. However, the fact that a mutant containing leucine in place of the active site arginine has over 10% of the activity of the native enzyme shows that the arginine is not absolutely essential.[529] Additional nearby positively charged arginine and lysine side chains may provide "electrostatic guidance" that increases the velocity of reaction of superoxide ions.[530,531] Cu, Zn-SOD is one of the fastest enzymes known.

In addition to the cytosolic SOD there is a longer ~222-residue extracellular form that binds to the proteoglycans found on cell surfaces.[522,527] Manganese SODs are found in mitochondria and in bacteria and iron SODs in plants and bacteria. They all appear to be important in protecting cells from superoxide radicals.[522,532,533] This importance was dramatically emphasized when it was found that a defective SOD is present in persons (about 1 in 100,000) with a hereditary form of **amylotrophic lateral sclerosis** (ALS), which is also called Lou Gehrig's disease after the baseball hero who was stricken with this terrible disease of motor neurons in 1939 at the age of 36.[534–536]

## 3. Nitrite and Nitrous Oxide Reductases

Copper enzymes participate in two important reactions catalyzed by denitrifying bacteria. Nitrite reductases from species of *Achromobacter*[537,538] and *Alcaligenes*[539–542] are trimeric proteins[543] made up of 37-kDa subunits, each of which contains one type 1 (blue) copper and one type 2 (nonblue) copper. The first copper serves as an electron acceptor from a small blue **pseudoazurin**.[544,544a] The second copper, which is in the active site, is thought to bind to nitrite throught its nitrogen atom and to reduce it to NO.

$$NO_2^- + 2H^+ + e^- \rightarrow NO + H_2O \qquad (16\text{-}53)$$

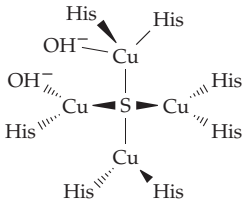
Crystallographic studies on the 343-residue *Alcaligenes* enzyme reveal two β barrel domains with the type 1 copper embedded in one of them and the type 2 copper in an interface between the domains. Studies of EPR[541] and ENDOR[545] spectra and of various mutant forms have shown that, as for other copper enzymes, the type 1 copper is an electron-transferring center, accepting electrons from the pseudoazurin and passing them to the type 2 copper which binds and reduces nitrite.[540,545a]

The immediate product of nitrite reductase is NO, which is reduced in two one-electron steps to $N_2O$,

and then to $N_2$. The second of these steps is catalyzed by another copper enzyme, nitrous oxide reductase.[546]

$$N_2O + 2H^+ + 2e^- \rightarrow N_2 + H_2O \qquad (16\text{-}54)$$

The biological significance of these reactions is considered further in Chapters 18 and 24. The 132-kDa dimeric $N_2O$ reductase from *Pseudomonas stutzeri* contains four copper atoms per subunit.[546] One of its copper centers resembles the $Cu_A$ centers of cytochrome *c* oxidase. A second copper center consists of four copper ions, held by seven histidine side chains in a roughly tetrahedral array around one sulfide ($S^{2-}$) ion. Rasmussen *et al.* speculate that this copper-sulfide cluster may be an acceptor of the oxygen atoms of $N_2O$ in the formation of $N_2$.[546a] There is also a cytochrome $cd_1$ type of nitrite reductase.[143a]



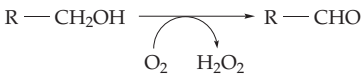

## 4. Hemocyanins

While many copper proteins are catalysts for oxidative reactions of $O_2$, hemocyanin reacts with $O_2$ reversibly. This water-soluble $O_2$ carrier is found in the blue blood of many molluscs and arthropods, including snails, crabs, spiders, and scorpions. Hemocyanins are large oligomers ranging in molecular mass from 450 to 13,000 kDa. Molluscan hemocyanins are cylindrical oligomers which have a striking appearance under the electron microscope. Simpler hemocyanins, found in arthropods, are hexamers of 660-residue 75-kDa subunits. Each subunit of the hemocyanin from the spiny lobster is folded into three distinct domains, one of which contains a pair of Cu(I) atoms which bind the $O_2$. Each copper ion is held by three imidazole groups without any bridging groups between them, the Cu–Cu distance being 0.36–0.46 nm.[547] *Octopus* hemocyanin has a different fold and forms oligomers of ten subunits. However, the active sites are very similar.[548] The $O_2$ is thought to bind between the two copper atoms. An allosteric mechanism may involve changes in the distance between the copper atoms.[549] The oxygenated compound is distinctly blue with a molar extinction coefficient 5–10 times greater than that of cupric complexes. This fact suggests that the Cu(I) has been oxidized to Cu(II) and that the $O_2$ has been reduced to the peroxide dianion $O_2^{2-}$ in the complex.[550–552] Further support for this idea comes from the observation that treatment of oxygenated hemocyanin with glacial acetic acid leads to the formation of equal amounts of $Cu^{2+}$ and $Cu^+$ and protonated superoxide.

$$(CuO_2Cu)^{2+} + H^+ \longrightarrow Cu^{2+} + Cu^+ + HO_2 \qquad (16\text{-}55)$$

## 5. Copper Oxidases

A large group of copper-containing proteins activate oxygen toward chemical reactions of dehydrogenation, hydroxylation, or oxygenation. **Galactose oxidase** (Fig. 16-29), from the mushroom *Polyporus*, is a dehydrogenase which converts the 6-hydroxymethyl group of galactose to an aldehyde while $O_2$ is reduced to $H_2O_2$.

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Galactose oxidase has been used frequently to label glycoproteins of external cell membrane surfaces. Exposed terminal galactosyl or *N*-acetylgalactosaminyl residues are oxidized to the corresponding C-6 aldehydes and the latter are reduced under mild conditions with tritiated sodium borohydride.[553]

The single 639-residue polypeptide chain contains one type 2 copper ion.[554] Neither oxygen nor galactose affects the absorption spectrum of the light murky green enzyme, but the combination of the two does, suggesting that both substrates bind to the enzyme before a reaction takes place. A side reaction releases

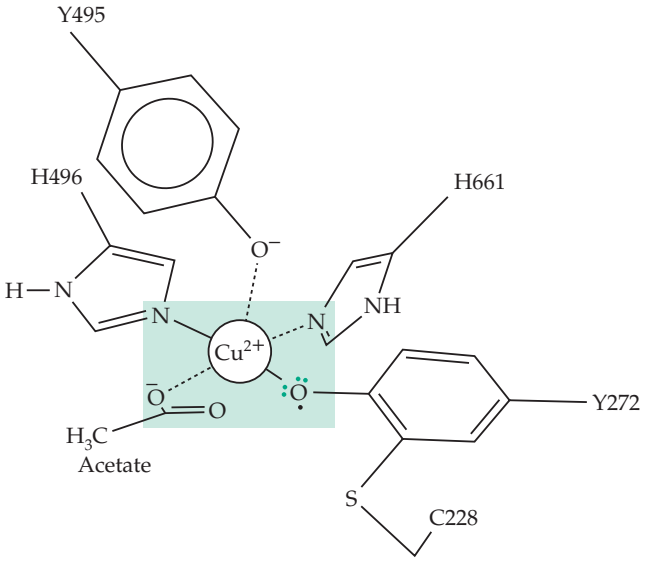



**Figure 16-29** Drawing of the active site of galactose oxidase showing both the Cu(II) atom and the neighboring free radical on tyrosine 272, which has been modified by addition of the thiol of cysteine 228 and oxidation. See Halfen *et al.*[557] Based on a crystal structure of Ito *et al.*[558]

superoxide ion and leaves the enzyme in the inactive Cu(II) state. EPR spectroscopic observations on the enzyme were puzzling. The active enzyme shows no EPR signal but a one-electron reduction gives an inactive form with an EPR signal that arises from Cu(II). Experimental studies eventually pointed to the presence of a second reducible center which contains an organic free radical. In the active form this radical is **antiferromagnetically coupled** (spin-coupled) giving an "EPR-silent" enzyme able to accept two electrons.[555–557]

Another surprise was the discovery, from the X-ray structure,[558–559] that a tyrosine side chain at the active site has been modified by addition of a thiolate group from a cysteine residue. This structure, which is also the site of the organic free radical, is shown in Fig. 16-29. A possible mechanism of action is portrayed in Fig. 16-30. The substrate binds in step *a* and its –OH group is deprotonated, perhaps by transfer of $H^+$ to the phenolate oxygen of tyrosine 495 (Fig. 16-29) in step *b* of Fig. 16-30. In step *c* a free radical hydrogen transfer occurs to form a ketyl radical which is immediately oxidized by Cu(II) in step *d*. In steps *c* and *f* the oxidant $O_2$ is converted to $H_2O$ and the aldehyde product is also released. A fungal glyoxal oxidase has similar characteristics.[560] The galactose oxidase proenzyme is self-processing. The Tyr-Cys cofactor arises as a result of copper-catalyzed oxidative modification via a tyrosine free radical.[560a]

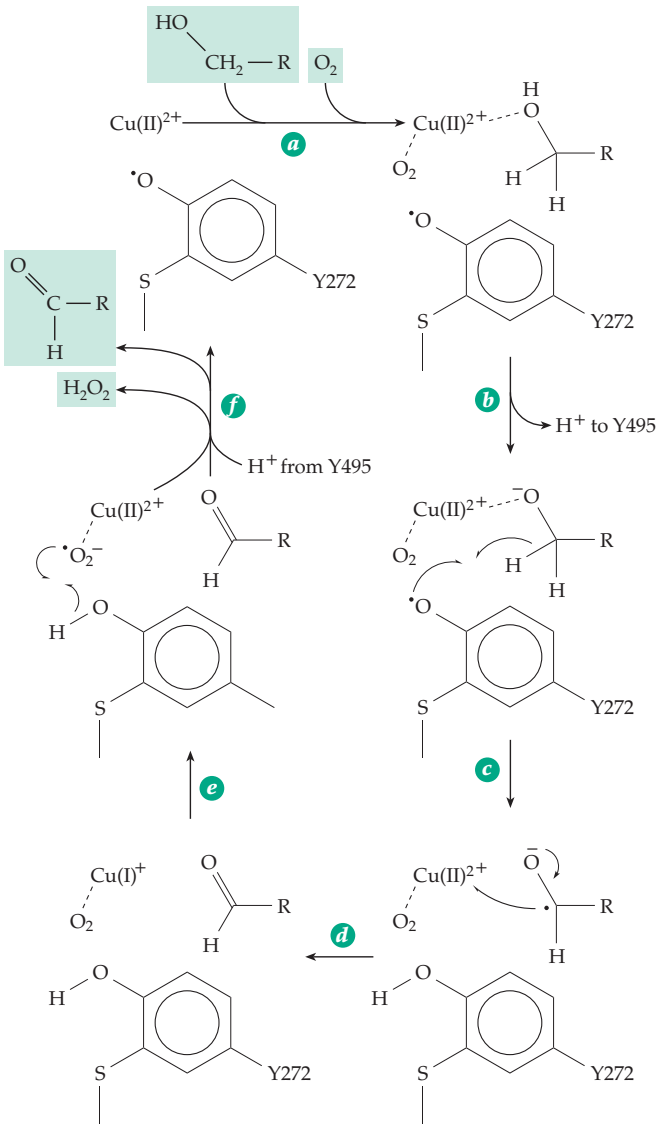
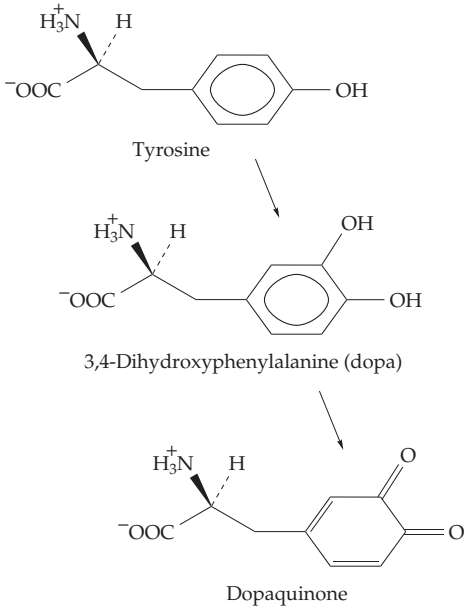



**Figure 16-30** Possible reaction cycle for catalysis of the 6–OH of D-galactose or other suitable alcohol substrate by galactose oxidase. See Wachter *et al.*[566] and Whittaker *et al.*[567]

Several copper-containing amine oxidases[561,561a] convert amines to aldehydes and $H_2O_2$. They also contain one of the organic quinone cofactors discussed in Chapter 15. The dimeric **plasma amine oxidase** contains a molecule of the coenzyme TPQ and one $Cu^{2+}$ per 90-kDa subunit.[562–563a] Whether the $O_2$ binds only to the single atom Cu(I) or also interacts directly with a cofactor radical in step *d* of Eq. 15-53 is uncertain.[562] **Lysyl oxidase**, which is responsible for conversion of ε-amino groups of side chains of lysine into aldehyde groups in collagen and elastin (Chapter 8) contains coenzyme LTQ as well as Cu.[564] The enzyme is specifically inhibited by β-aminopropionitrile (Box 8-G) and its activity is decreased in genetic diseases of copper metabolism. The **glycerol oxidase** of *Aspergillus* is a large 400 kDa protein containing one heme and two atoms of Cu.[565] It converts glycerol + $O_2$ into glyceraldehyde and $H_2O_2$. Also containing copper is **urate oxidase**, whose action is indicated in Fig. 25-18.

**Tyrosinase** catalyzes hydroxylation followed by dehydrogenation (Eq. 16-57). First identified in mushrooms, the enzyme has a widespread distribution in




(16-57)

nature. It is present in large amounts in plant tissues and is responsible for the darkening of cut fruits. In animals tyrosinase participates in the synthesis of **dihydroxyphenylalanine** (dopa) and in the formation of the black **melanin** pigment of skin and hair. Either a lack of or inhibition of this enzyme in the melanin-producing melanocytes causes **albinism** (Chapter 25).

The 46-kDa monomeric tyrosinase of *Neurospora* contains a pair of spin-coupled Cu(II) ions.[568,569] The structure of this copper pair (**type 3 copper**) has many properties in common with the copper pair in hemocyanin.[569a] For example, in the absence of other substrates, tyrosinase binds $O_2$ to form "oxytyrosinase," a compound with properties resembling those of oxyhemocyanin and containing a bound peroxide dianion.[569]

Tyrosinase is both an oxidase and a hydroxylase. Some other copper enzymes have only a hydroxylase function. One of the best understood of these is the **peptidylglycine α-hydroxylating monoxygenase**, which catalyzes the first step of the reaction of Eq. 10-11. The enzyme is a colorless two-copper protein but the copper atoms are 1.1 nm apart and do not form a binuclear center.[570] Ascorbate is an essential cosubstrate, with two molecules being oxidized to the semidehydroascorbate radical as both coppers are reduced to Cu(I). A ternary complex of reduced enzyme, peptide, and $O_2$ is formed and reacts to give the hydroxylated product.[570] A related two-copper enzyme is **dopamine β-monooxygenase**, which utilizes $O_2$ and ascorbate to hydroxylate dopamine to noradrenaline (Chapter 25).[571,572] These and other types of hydroxylases are compared in Chapter 18.

The **blue multicopper oxidases** couple the oxidation of substrates to the four-electron reduction of molecular oxygen to $H_2O$.[573] In this respect they resemble cytochrome *c* oxidase, which also contains copper. However, they do not contain iron. The best known member of this group is the plant enzyme **ascorbate oxidase**, which dehydrogenates ascorbic acid to dehydroascorbic acid (See Box 18-D). It is a dimeric blue copper with identical 70-kDa subunits. The three-dimensional structure revealed one type 1 copper ion held in a typical blue copper environment as in plastocyanin or azurin and also a **three-copper center**. In this center a pair of copper ions, each held by three imidazole groups, and bridged by a μ-oxo group as in hemerythrin (Fig. 16-20), lie 0.51 nm apart and 0.41–0.44 nm away from the third copper, which is held by two other imidazoles.[574] The type 1 copper shows typical intense 600-nm absorption and characteristic EPR signal, while the pair with the oxo bridge are antiferromagnetically coupled and EPR silent but with strong near ultraviolet light absorption (type 3 copper). The additional metal ion in the trinuclear center is a type 2 copper which lacks characteristic spectroscopic features.[575,576] Reduction of $O_2$ to 2 $H_2O$ is thought to procede via superoxide radical intermediates. When substrate is added to the enzyme, the blue color fades and it can be shown that the copper is reduced to the +1 state. The reduced enzyme then reacts with $O_2$, converting it into two molecules of $H_2O$. Similar to ascorbate oxidase in structure and properties are **laccase**, found in the latex of the Japanese lac tree and in the mushroom *Polyporus*, and the previously discussed **ceruloplasmin.**[477] Laccase is a catalyst for oxidation of phenolic compounds by a free radical mechanism involving the trinuclear copper center.[577] Studied by Gabriel Bertrand in the 1890s, it was one of the first oxidative enzymes investigated.[578]

In addition to its previously mentioned role in copper transport, ceruloplasmin is an amine oxidase, a superoxide dismutase, and a ferrooxidase able to catalyze the oxidation of $Fe^{2+}$ to $Fe^{3+}$. Ceruloplasmin contains three consecutive homologous 350-residue sequences which may have originated from an ancestral copper oxidase gene. Like ascorbate oxidase, this blue protein contains copper of the three different types. Blood clotting factors V and VIII (Fig. 12-17), and the iron uptake protein Fet3 (Section A,1) are also closely related.

### 6. Cytochrome *c* Oxidase

The most studied of all copper-containing oxidases is cytochrome *c* oxidase of mitochondria. This multisubunit membrane-embedded enzyme accepts four electrons from cytochrome *c* and uses them to reduce $O_2$ to 2 $H_2O$. It is also a proton pump. Its structure and functions are considered in Chapter 18. However, it is appropriate to mention here that the essential catalytic centers consist of two molecules of heme *a* (*a* and $a_3$) and three $Cu^+$ ions. In the fully oxidized enzyme two metal centers, one $Cu^{2+}$ (of the two-copper center $Cu_A$) and one $Fe^{3+}$ (heme *a*), can be detected by EPR spectroscopy. The other $Cu^{2+}$ ($Cu_B$) and heme $a_3$ exist as an EPR-silent exchange-coupled pair just as do the two copper ions of hemocyanin and of other type 3 binuclear copper centers.

## E. Manganese

Tissues usually contain less than one part per million of manganese on a dry weight basis, less than 0.01 mM in fresh tissues. This compares with a total content in animal tissues of the more abundant $Mg^{2+}$ of 10 mM. A somewhat higher Mn content (3.5 ppm) is found in bone. Nevertheless, manganese is nutritionally essential[579,580] and its deficiency leads to well-defined symptoms. These include ovarian and testicular degeneration, shortening and bowing of legs, and other skeletal abnormalities such as the

"slipped tendon disease" of chicks. In Mn deficiency the organic matrix of bones and cartilage develops poorly. The galactosamine, hexuronic acids, and chondroitin sulfates content of cartilage is decreased. Manganese is also essential for plant growth and plays a unique and essential role in the photosynthetic reaction centers of chloroplasts. Two magnetically coupled pairs of manganese ions bound in a protein act as the $O_2$ evolving center in photosynthetic system II. This function is considered in Chapter 23. An ABC transporter for managanese uptake has been identified in the cyanobacterium *Synechocystis*.[581]

Manganese lies in the center of the first transition series of elements. The stable $Mn^{2+}$ (manganous ion) contains five 3*d* electrons in a high-spin configuration. The less stable $Mn^{3+}$ (manganic ion) appears to be of importance in some enzymes and is essential to the photosynthetic evolution of oxygen. Many enzymes specifically require or prefer $Mn^{2+}$. These include galactosyl and *N*-acetylgalactosaminyltransferases[582] needed for synthesis of mucopolysaccharides (Chapter 20), lactose synthetase (Eq. 20-15), and a muconate-lactonizing enzyme (Eq. 13-23).[583]

**Arginase**, essential to the production of urea in the human body (Fig. 24-11), specifically requires $Mn^{2+}$ which exists as a spin-coupled dimetal center with a bridging water or $^{-}OH$ ion. The $Mn^{2+}$ may act much as does the $Ni^{2+}$ ions of urease (Fig. 16-25).[584,585] Pyruvate carboxylase (Eq. 14-3) contains four atoms of tightly bound $Mn^{2+}$, one for each biotin molecule present. This manganese is essential for the transcarboxylation step in the action of this enzyme. Either $Mn^{2+}$ or $Mg^{2+}$ is also needed in the initial step of carboxylation of biotin (Eq. 14-5). Another $Mn^{2+}$-containing protein is the lectin concanavalin A (Chapter 4). The joining of *O*-linked oligosaccharides to secreted glycoproteins also seems to require manganese.[586]

Manganese is a component of a "pseudocatalase" of *Lactobacillus*,[204] of lignin-degrading peroxidases,[257,258] and of the wine-red superoxide dismutases found in bacteria and in the mitochondria of eukaryotes.[376,587] The dimeric dismutase from *E. coli* has a structure nearly identical to that of bacterial iron SOD (Fig. 16-22). The manganese ions are presumed to alternate between the +3 and +2 states during catalysis (Eq. 16-27). "Knockout" mice with inactivated Mn SOD genes live no more than three weeks, indicating that this enzyme is essential to life. However, mice lacking CuZn SOD appear normal in most circumstances.[588] Some dioxygenases contain manganese.[589] Many enzymes that require $Mg^{2+}$ can utilize $Mn^{2+}$ in its place, a fact that has been exploited in study of the active sites of enzymes.[590] The highly paramagnetic $Mn^{2+}$ is the most useful ion for EPR studies (Box 8-C) and for investigations of paramagnetic relaxation of NMR signals. Manganese can also replace $Zn^{2+}$ in some enzymes and may alter catalytic properties.

Manganese may function in the regulation of some enzymes. For example, glutamine synthetase (Fig. 24-7) in one form requires $Mg^{2+}$ for activity but upon adenylylation binds $Mn^{2+}$ tightly.[591] Nucleases and DNA polymerases often show altered specificity when $Mn^{2+}$ substitutes for $Mg^{2+}$. However, the significance of these differences *in vivo* is uncertain. Manganese is mutagenic in living organisms, apparently because it diminishes the fidelity of DNA replication.[592]

A striking accumulation of $Mn^{2+}$ often occurs within bacterial spores (Chapter 32). *Bacillus subtilus* absolutely requires $Mn^{2+}$ for initiation of sporulation. During logarithmic growth the bacteria can concentrate $Mn^{2+}$ from 1 μM in the external medium to 0.2 mM internally; during sporulation the concentrations become much higher.[593]

## F. Chromium

Animals deficient in chromium grow poorly and have a reduced life span.[594–596] They also have decreased "glucose tolerance," i.e., glucose injected into the blood stream is removed only half as fast as it is normally.[597,598] This is similar to the effect of a deficiency of insulin. Fractionation of yeast led to the isolation of a chromium-containing **glucose tolerance factor** which appeared to be a complex of $Cr^{3+}$, nicotinic acid, and amino acids.[597] The chromium in this material is apparently well absorbed by the body but is probably not an essential cofactor.[596] Nevertheless, dietary supplementation with chromium appears to improve glucose utilization, apparently by enhancing the action of insulin.[596] Ingestion of glucose not only increases insulin levels in blood but also causes increased urinary loss of chromium,[599] perhaps as a result of insulin-induced mobilization of stored chromium.[596] It has been suggested that a specific **chromium-binding oligopeptide** isolated from mammalian liver[596,600] may be released in response to insulin and may activate a membrane phosphotyrosine phosphatase.[596]

Chromium concentrations in animal tissues are usually less than 2 μM but tend to be much higher in the caudate nucleus of the brain. High concentrations of $Cr^{3+}$ have also been found in RNA–protein complexes.[601] While several oxidation states, including +2, +3, and +6, are known for chromium, only Cr(III) is found to a significant extent in tissues. The Cr(VI) complex ions, chromate and dichromate, are toxic and chronic exposure to chromate-containing dust can lead to lung cancer. Ascorbate is a principal biological reductant of chromate and can create mutagenic Cr(V) compounds that include a Cr(V)–ascorbate–peroxo complex.[602] However, Cr(III) compounds administered orally are not significantly toxic. Evidently, the Cr(VI) compounds can cross cell membranes and be reduced

to Cr(III), which forms stable complexes with many constituents of cells including DNA.[603] The use of such "exchange-inert" Cr(III) complexes of ATP in enzymology was considered in Chapter 12.

Most forms of Cr(III) are not absorbed and utilized by the body. For this reason, and because of the increased use of sucrose and other refined foods, a marginal human chromium deficiency may be widespread.[604,605] This may result not only in poor utilization of glucose but also in other effects on lipid and protein metabolism.[597] However, questions have been raised about the use of chromium picolinate as a dietary supplement. High concentrations have been reported to cause chromosome damage[606] and there may be danger of excessive accumulation of chromium in the body.[607]

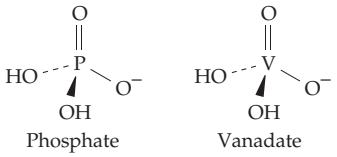
## G. Vanadium

Vanadium is a dietary essential for goats and presumably also for human beings,[427] who typically consume ~2 mg / day. However, because vanadium compounds have powerful pharmacological effects it has been difficult to establish the nutritional requirement for animals.[427,604,607a] The adult body contains only about 0.1 mg of vanadium. Typical tissue concentrations are 0.1–0.7 μM[608,609] and serum concentration may be 10 nM or less. Vanadium can assume oxidation states ranging from +2 to +5, the vanadate ion $VO_4^{3-}$ being the predominant form of V(V) in basic solution and in dilute solutions at pH 7. However, at millimolar concentrations, $V_3O_9^{3-}$, $V_4O_{12}^{4-}$, and other polynuclear forms predominate.[610–612] In plasma most exists as metavanadate, $VO_2^-$, but within cells it is reducted to the vanadyl cation $VO^{2+}$ which is an especially stable double-bonded unit in compounds of V(IV).[609] Only at very low pH is V(III) stable.

The first suggestion of a possible biochemical function for vanadium came from the discovery that **vanadocytes**, the green blood cells of tunicates (sea squirts), contain ~1.0 M V(III) and 1.5–2 M $H_2SO_4$ .[613] It was proposed that a V-containing protein is an oxygen carrier. However, the $V^{3+}$ appears not to be associated with proteins[612] and it does not carry $O_2$. It may be there to poison predators.[614] The vanadium-accumulating species also synthesize several complex, yellow catechol-type chelating agents (somewhat similar to enterobactin; Fig. 16-1) which presumably complex V(V) and perhaps also reduce it to V(III).[615] Vanadium is also accumulated by other marine organisms and by the mushroom *Amanita muscaria*.

**Vanadoproteins** are found in most marine algae and seaweed and in some lichens.[616] Among these are **haloperoxidases**,[252,253,617–618b] enzymes that are quite different from the corresponding heme peroxidases discussed in Section A,6. The vanadium is bound as hydrogen vanadate, $HVO_4^{2-}$, in trigonal bipyrimidal coordination with the three oxygens in equatorial positions and a histidine in one axial position. In the crystal structure an azide ($N_3^-$) ion occupies the other axial position, but it is presumably the site of interaction with peroxide.[619] The structure is similar to that of acid phosphatases inhibited by vanadate.[620,621] Many nitrogen-fixing bacteria contain genes for a vanadium-dependent nitrogenase that is formed only if molybdenum is not available.[622] The nitrogenases are discussed in Chapter 24.

Much of current interest in vanadium stems from the discovery that vanadate ($HVO_4^{2-}$ at pH 7) is a powerful inhibitor of ATPases such as the sodium pump protein $(Na^+ + K^+)$ATPase (Chapter 8), of phosphatases,[623] and of kinases.[624] This can be readily understood from comparison of the structure of phosphate and vanadate ions.



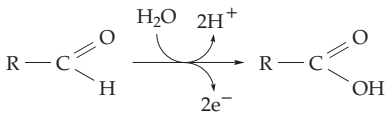
Phosphate Vanadate

Other enzymes such as the cyclic AMP-dependent protein kinase are *stimulated* by vanadium.[624] Vanadate seems to inhibit most strongly those enzymes that form a phosphoenzyme intermediate. This inhibition may be diminished within cells because vanadate is readily reduced by glutathione and other intracellular reductants. The resulting vanadyl ion is a much weaker inhibitor and also stimulates several metabolic processes.[608]

Also of great interest is an insulin-like action of vanadium[607a,625] and evidence that vanadium may be essential to proper cardiac function.[626] A role in lipid metabolism was suggested by the observation that in high doses vanadium inhibits cholesterol synthesis and lowers the phospholipid and cholesterol content of blood. Vanadium is reported to inhibit development of caries by stimulating mineralization of teeth. Unlike tungsten, vanadium does not compete with molybdenum in the animal body.[627] The sometimes dramatic effects of vanadate as an inhibitor, activator, and metabolic regulator are shared also by molybdate and tungstate.[628,629] Even greater effects are observed with vanadate, molybdate, or tungstate plus $H_2O_2$.[630] The resulting **pervanadate**, **permolybdate**, and **pertungstate** are often assumed to be monoperoxo compounds, e.g., vanodyl hydroperoxide. However, there is some uncertainty.[631]

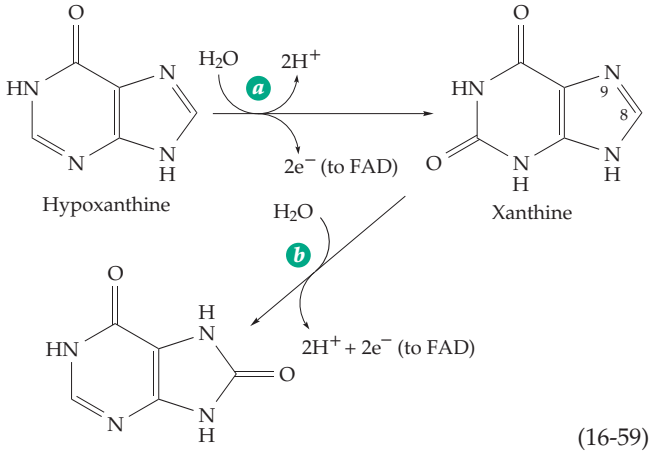
## H. Molybdenum

Long recognized as an essential element for the growth of plants, molybdenum has never been directly demonstrated as a necessary animal nutrient. Nevertheless, it is found in several enzymes of the human body, as well as in 30 or more additional enzymes of bacteria and plants.[632] **Aldehyde oxidases**,[633] **xanthine oxidase** of liver and the related **xanthine dehydrogenase**, catalyze the reactions of Eqs. 16-58 and 16-59 and contain molybdenum that is essential for catalytic activity. Xanthine oxidase also contains two $Fe_2S_2$ clusters and bound FAD. The enzymes can also




(16-58)

oxidize xanthine further (Eq. 16-59, step *b*) by a repetition of the same type of oxidation process at positions 8 and 9 to form **uric acid**. The much studied xanthine dehydrogenase has been isolated from milk,[634,635] liver, fungi,[636] and some bacteria.[637] In the dehydrogenase $NAD^+$ is the electron acceptor that oxidizes the bound $FADH_2$ formed in Eq. 16-59. Xanthine dehydrogenase, in the absence of thiol compounds, is converted spontaneously into xanthine oxidase, probably as a result of a conformational change and formation of a disulfide bridge within the protein. Treatment with thiol compounds such as dithiothreitol reconverts the enzyme to the dehydrogenase. Evidently in the oxidase form the $NAD^+$ binding site has moved away from the FAD, permitting oxidation of $FADH_2$ by $O_2$ with formation of hydrogen peroxide.[635,638]




(16-59)

A purine hydroxylase from fungi,[639] bacterial quinoline and isoquinoline oxidoreductases,[640,641] and a selenium-containing nicotinic acid hydroxylase from *Clostridium barberei*[642] are members of the **xanthine oxidase family** (or molybdenum hydroxylase family).[632,641,643] Also included in the family are aldehyde oxidoreductases from the sulfate-reducing *Desulfovibrio gigas*[633] and from the tomato.[644]

Two other families of molybdoenzymes are the **sulfite oxidase family**[646a,b] and the **dimethylsulfoxide reductase family**.[632,641] **Nitrogenase** (Chapter 24) constitutes a fourth family. Sulfite oxidase (Eq. 16-60) is an essential human liver enzyme (see also Chapter 24).[645,646]

$$SO_3^{2-} + H_2O \rightarrow SO_4^{2-} + 2\ e^- + 2\ H^+ \qquad (16\text{-}60)$$

The **assimilatory nitrate reductase** (Eq. 16-61) of fungi and green plants (Chapter 24) also belongs to the sulfite oxidase family.

$$NO_3^{2}\ + 2\ e^- + 2\ H^+ \rightarrow NO_2^- + H_2O \qquad (16\text{-}61)$$

**DMSO reductase** reduces dimethylsulfoxide to dimethylsulfide (Eq. 16-62) as part of the biological sulfur cycle.[647–648d]

$$(H_3C)_2S{=}O + 2\ H^+ + 2\ e^- \rightarrow (CH_3)_2S \qquad (16\text{-}62)$$

A number of other reductases and dehydrogenases, including **dissimilatory nitrate reductases** of *E. coli* and of denitrifying bacteria (Chapter 18), belong to the DMSO reductase family. Other members are reductases for biotin *S*-oxide,[649] trimethylamine *N*-oxide, and polysulfides as well as **formate dehydrogenases** (Eq. 16-63), formylmethanofuran dehydrogenase (Fig. 15-22, step *b*), and arsenite oxidase.[632] Several other molybdoenzymes, such as pyridoxal oxidase, had not been classified by 1996.[632]

$$HCOO^- + 2\ e^- + 3\ H^+ \rightarrow CO_2 + 2\ H_2O \qquad (16\text{-}63)$$

### 1. Molybdenum Ions and Coenzyme Forms

Molybdenum is a metal of the second transition series, one of the few heavy elements known to be essential to life. Its most stable oxidation state, Mo(VI), has 4*d* orbitals available for coordination with anionic ligands. Coordination numbers of 4 and 6 are preferred, but molybdenum can accommodate up to eight ligands. Most of the complexes are formed from the oxycation $Mo(VI)O_2^{2+}$. If two molecules of water are coordinated with this ion, the protons are so acidic that they dissociate completely to give $Mo(VI)O_4^{2-}$, the molybdate ion. Other oxidation states vary from Mo(III) to Mo(V). In these lower oxidation states, the tendency for protons to dissociate from coordinated ligands is less, e.g., $Mo(III)(H_2O)_6^{3+}$ does not lose protons even in a very basic medium. Molybdenum tends to form dimeric

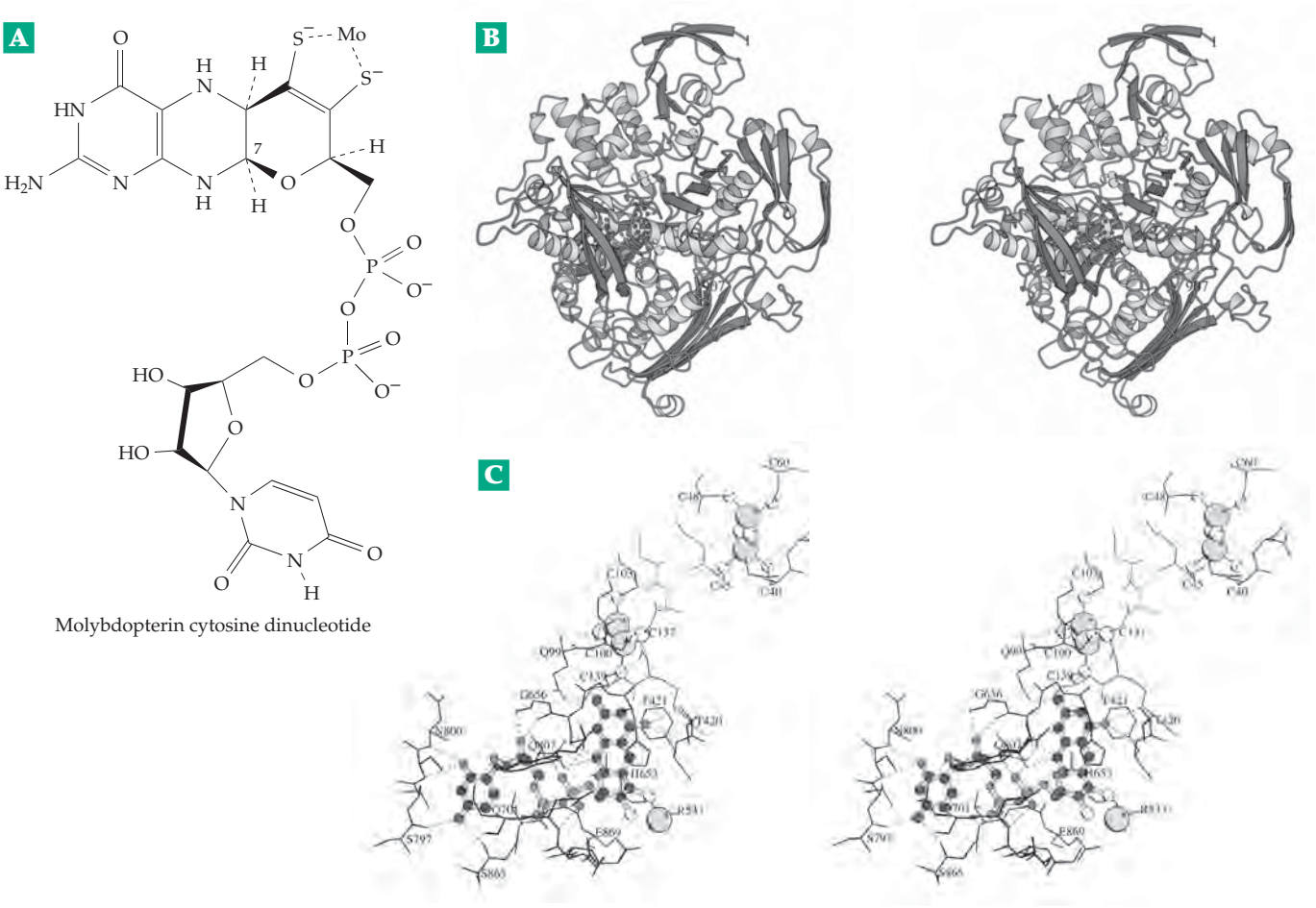


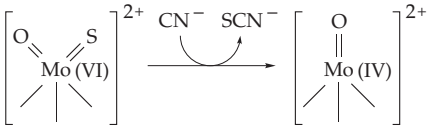

**Figure 16-31** (A) Structure of molybdopterin cytosine dinucleotide complexed with an atom of molybdenum. (B) Stereoscopic ribbon drawing of the structure of one subunit of the xanthine oxidase-related aldehyde oxidoreductase from *Desulfovibrio gigas*. Each 907-residue subunit of the homodimeric protein contains two $Fe_2S_2$ clusters visible at the top and the molybdenum–molybdopterin coenzyme buried in the center. (C) Alpha-carbon plot of portions of the protein surrounding the molybdenum–molybdopterin cytosine dinucleotide and (at the top) the two plant-ferredoxin-like $Fe_2S_2$ clusters. Each of these is held by a separate structural domain of the protein. Two additional domains bind the molybdopterin coenzyme and there is also an intermediate connecting domain. In xanthine oxidase the latter presumably has the FAD binding site which is lacking in the *D. gigas* enzyme. From Romão *et al.*[633] Courtesy of R. Huber.

or polymeric oxygen-bridged ions. However, within the enzymes it exists as the unique **molybdenum coenzymes.** The Mo-containing enzymes usually also contain additional bound cofactors, including Fe – S clusters and flavin coenzymes or heme.

The recognition that the Mo in the molybdoproteins exists in organic cofactor forms came from studies of mutants of *Aspergillus* and *Neurospora*.[650] In 1964, Pateman and associates discovered mutants that lacked both nitrate reductase and xanthine dehydrogenase. Later, it was shown that acid-treated molybdoenzymes released a material that would restore activity to the inactived nitrate reductase from the mutant organisms. This new coenzyme, a phosphate ester of molybdopterin (Fig. 15-17), was characterized by Rajagopalan and coworkers.[650,651] A more complex form of the coenzyme, **molybdopterin cytosine dinucleotide** (Fig. 16-31), is found in the *D. gigas* aldehyde oxidoreductase. Related coenzyme forms include nucleotides of adenine, guanine (see chapter banner, p. 837), and hypoxanthine.[651a,651b] The structure of molybdopterin is related to that of **urothione** (Fig. 15-17), a normal urinary constituent. The relationship to urothione was strengthened by the fact that several children with severe neurological and other symptoms were found to lack both sulfite oxidase and xanthine dehydrogenase as well as the molybdenum cofactor and urinary urothione.[646,646a,646b]

Study by X-ray absorption spectroscopy of the extended **X-ray absorption fine structure** (EXAFS) has provided estimates of both the nature and the number of the nearest neighboring atoms around the Mo. The EXAFS spectra of xanthine dehydrogenase and of nitrate reductase from **Chlorella** confirmed the

presence of both the Mo(VI)$O_2$ unit with Mo–O distances of 0.17 nm and two or three sulfur atoms at distances of 0.24 nm.[652,653] The two sulfur atoms were presumed to come from the molybdopterin. A peculiarity of the xanthine oxidase family is the presence on the molybdenum of a "cyanolyzable" sulfur.[654] This is a sulfide attached to the molybdenum, which is present as Mo(VI)OS rather than Mo(VI)$O_2$. Reaction with cyanide produces thiocyanate (Eq. 16-64).

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The active site structures of the three classes of molybdenum-containing enzymes are compared in Fig. 16-32. In the DMSO reductase family there are two identical molybdopterin dinucleotide coenzymes complexed with one molybdenum. However, only one of these appears to be functionally linked to the $Fe_2S_2$ center.

Nitrogenase, which catalyzes the reduction of $N_2$ to two molecules of $NH_3$, has a different **molybdenum–iron cofactor (FeMo-co)**. It can be obtained by acid denaturation of the very oxygen-labile iron–molybdenum protein of nitrogenase followed by extraction with dimethylformamide.[655,656] The coenzyme is a complex Fe–S–Mo cluster also containing **homocitrate** with a composition $MoFe_7S_9$–homocitrate (see Fig. 24-3). Nitrogenase and this coenzyme are considered further in Chapter 24.

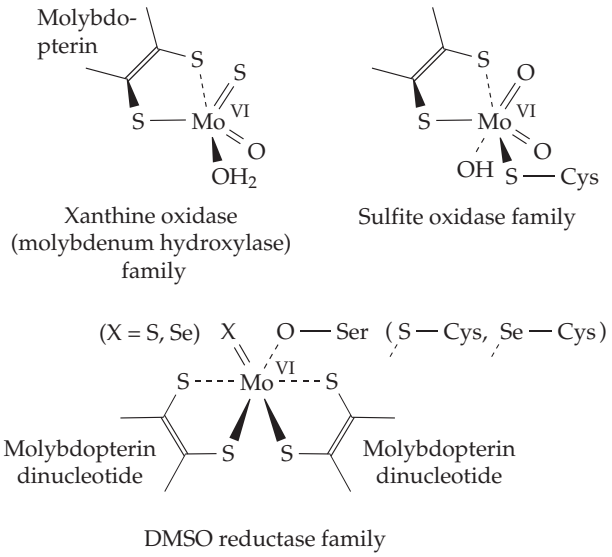



**Figure 16-32** Structures surrounding molybdenum in three families of molybdoenzymes. See Hille.[632]

## 2. Enzymatic Mechanisms

Although several of the reactions catalyzed by molybdoenzymes are classified as dehydrogenases, all of them except nitrogenase involve $H_2O$ as either a reactant or a product. The EXAFS spectra suggest that the Mo(VI)$O_2$ unit is converted to Mo(IV)O during reaction with a substrate Sub (Eq. 16-65, step *a*). Reaction of the Mo(IV)O with water (step *b*) completes the catalysis.

$$\textit{a}\qquad [Mo(VI)O_2]^{2+} + Sub \rightarrow [Mo(IV)O]^{2+} + Sub\text{–}O$$

$$\textit{b}\qquad [Mo(IV)O]^{2+} + H_2O \rightarrow 2H^+ + 2e^- + [Mo(VI)]^{2+}O_2$$

$$SumSub + H_2O \rightarrow Sub\text{–}O + 2H^+ + 2e^- \qquad (16\text{-}65)$$

Step *a* of all of these reactions can be regarded as an **oxo-transfer**.[653]

To complete the reaction, two electrons must be passed from Mo(IV) to a suitable acceptor, usually an Fe–S cluster or a bound heme group. FAD is also often present. Xanthine oxidase[634,635,643,657,657a] contains two $Fe_2S_2$ clusters and a FAD for each of the two atoms of Mo in the dimer. Since this enzyme acts like a typical flavin oxidase that generates $H_2O_2$ from $O_2$, it may be that electrons pass from Mo to the Fe–S center and then to the flavin. Since the EPR signal of the paramagnetic Mo(V), with its characteristic six-line hyperfine structure, is seen during the action of xanthine oxidase and other molybdenum-containing enzymes, single-electron transfers are probably involved.

In bacteria such as *E. coli* a dissimilatory nitrate reductase allows nitrate to serve as an oxidant in place of $O_2$. An oxygen atom is removed from the nitrate to form nitrite as two electrons are accepted from a membrane-bound cytochrome *b*. The nitrate reductase consists of a 139-kDa Mo-containing catalytic subunit, a 58-kDa electron-transferring subunit that contains both $Fe_3S_4$ and $Fe_4S_4$ centers, and a 26-kDa heme-containing membrane anchor subunit.[658–660] The assimilatory nitrate reductase of fungi, green algae, and higher plants contains both a *b*-type cytochrome and FAD and a molybdenum coenzyme in a large oligomeric complex.[661–663a]

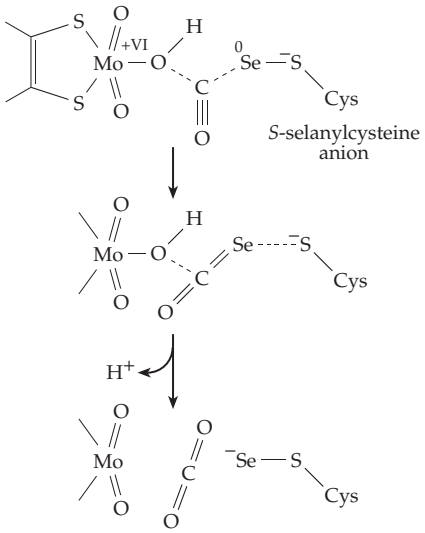
Formate dehydrogenases from many bacteria contain molybdopterin and also often selenium (Table 15-4).[664,665] A membrane-bound Mo-containing formate dehydrogenase is produced by *E. coli* grown anaerobically in the presence of nitrate. Under these circumstances it is coupled to nitrate reductase via an electron-transport chain in the membranes which permits oxidation of formate by nitrate (Eq. 18-26). This enzyme is also a multisubunit protein.[665,666] Two other Mo- and Se- containing formate dehydrogenases are produced

by *E. coli*.[667,668] The three-dimensional structure is known for one of them, **formate dehydrogenase H**, a component of the anaerobic formate hydrogen lyase complex (Eq. 17-25).[669,670] The structure shows Mo held by the sulfur atoms of two molybdopterin molecules, as in DMSO reductase. The Se atom of SeCys 140 is also coordinated with the Mo atom, and the imidazole of His 141 is in close proximity. When $^{13}C$-labeled formate was oxidized in $^{18}O$-enriched water no $^{18}O$ was found in the released product, $CO_2$.[671] This suggested that formate may be bound to Mo and dehydrogenated, with Mo(VI) being reduced to Mo(IV). The formate hydrogen might be transferred as $H^+$ to the His 140 side chain. Mo(IV) could then be reoxidized by electron transfer in two one-electron steps.[670] However, recent X-ray absorption spectra suggest the presence in the enzyme of a selenosulfide ligand to Mo.[672] Mechanistic uncertainties remain!

A flavin-dependent formate dehydrogenase system found in *Methanobacterium* passes electrons from dehydrogenation of formate to FAD and then to the deazaflavin coenzyme $F_{420}$.[673] In contrast to these Mo-containing enzymes, the formate dehydrogenase from *Pseudomonas oxalaticus*, which oxidizes formate with $NAD^+$ (Eq. 16-66), contains neither Mo or Se.[674]

$$HCOO^- + NAD^+ \longrightarrow CO_2 + NADH \qquad (16\text{-}66)$$

It is a large 315-kDa oligomer containing 2 FMN and ~20 Fe / S. Formate dehydrogenases of green plants and yeasts are smaller 70- to 80-kDa proteins lacking bound prosthetic groups.[674] A key enzyme in the metabolism of carbon monoxide-oxidizing bacteria is CO oxidase, another membrane-bound molybdoenzyme.[675–676c] It also contains selenium, which is attached to a cysteine side chain as ***S*-selanylcysteine.** A proposed reaction requence[676a] is shown in Eq. 16-67.




(16-67)

## 3. Nutritional Need for Mo

The first hint of an essential role of molybdenum in metabolism came from the discovery that animals raised on a diet deficient in molybdenum had decreased liver xanthine oxidase activity. There is no evidence that xanthine oxidase is essential for all life, but a human genetic deficiency of sulfite oxidase or of its molybdopterin coenzyme can be lethal.[646,646a,b] The conversion of molybdate into the molybdopterin cofactor in *E. coli* depends upon at least five genes.[677] In *Drosophila* the addition of the cyanolyzable sulfur (Eq. 16-64) is the final step in formation of xanthine dehydrogenase.[678] It is of interest that sulfur ($S^0$) can be transferred from rhodanese (see Eq. 24-45), or from a related mercaptopyruvate sulfurtransferase[679] into the desulfo form of xanthine oxidase to generate an active enzyme.[680]

Uptake of molybdate by cells of *E. coli* is accomplished by an ABC-type transport system.[681] In some bacteria, e.g., the nitrogen-fixing *Azotobacter*, molybdenum can be stored in protein-bound forms.[682]

# I. Tungsten

For many years tungsten was considered only as a potential antagonist for molybdenum. However, in 1970 growth stimulation by tungsten compounds was observed for some acetogens, some methanogens, and a few hyperthermophilic bacteria. Since then over a dozen tungstoenzymes have been isolated.[683,683a] These can be classified into three categories: aldehyde oxidoreductases, formaldehyde oxidoreductases, and the single enzyme acetylene hydratase. In most cases the tungstoenzymes resemble the corresponding molybdoenzymes and in most instances organisms containing a tungsten-requiring enzyme also contain the corresponding molybdenum enzyme. However, a few hyperthermophilic archaea appear to require W and are unable to use Mo.

The aldehyde ferredoxin oxidoreductase from the hyperthermophile *Pyrococcus furiosus* was the first molybdopterin-dependent enzyme for which a three-dimensional structure became available.[683,684] The tungstoenzyme resembles that of the related molybdoenzyme (Fig. 16-31). A similar ferredoxin-dependent enzyme reduces glyceraldehyde-3-phosphate.[685] Another member of the tungstoenzyme aldehyde oxidoreductase family is **carboxylic acid reductase**, an enzyme found in certain acetogenic clostridia. It is able to use reduced ferredoxin to convert unactivated carboxylic acids into aldehydes, even though $E^{o'}$ for the acetaldehyde / acetate couple is –0.58 V.[686]

Tungsten- and sometimes Se-containing formate dehydrogenases together with *N*-formylmethanofuran dehydrogenases (Fig. 15-22, step *b*) form a second family.

Again, these appear to resemble the corresponding Mo-dependent enzymes. The unique **acetylene hydratase** from the acetylene-utilizing *Pelobacter acetylenicus* catalyzes the hydration of acetylene to acetaldehyde.[687]

$$H{-}C{\equiv}C{-}H + H_2O \rightarrow H_3C{-}CHO \qquad (16\text{-}68)$$

In *Thermotoga maritima*, the most thermophilic organism known, tungsten promotes synthesis of an Fe-containing hydrogenase as well as some other enzymes but seems to have a regulatory rather than a structural role.[688]

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## Study Questions

1. Describe one or more metabolic functions of ions or chelate complexes of ions derived from each of the following metallic elements: Ca, Mg, Fe, Cu, Ni, Co.

2. If the concentration of Cu, Zn-superoxide dismutase (SOD) in a yeast cell is 10 μM, the total copper (bound and free) is 70 μM, and the dissociation constant for loss of $Cu^+$ from SOD is 6 fM, what will be the concentration of free $Cu^+$ within the cell? If the cell volume is $10^{-14}$ liter, how many copper ions will be present in a single cell? See Roe *et al.* (1999) *Science* **284**, 805–808.

3. Outline the metabolic pathways that are utilized by acetic acid-producing bacteria (acetogens) in the stoichiometric conversion of one molecule of glucose into three molecules of acetic acid. Indicate briefly the nature of any unusual coenzymes or metalloproteins that are required.

4. Suppose that you could add a solution containing micromolar concentrations of $Cu^{2+}$, $Mn^{2+}$, $Fe^{3+}$, $Co^{2+}$, $Zn^{2+}$, and $MoO_4^{2-}$ and millimolar amounts of $Mg^{2+}$, $Ca^{2+}$, and $K^+$ to a solution that contains a large excess of a mixture of many cellular proteins. What would be the characteristics of the sites that would become occupied by each of these metal ions? How tightly do you think they would be bound?

5. What factors affect the rate of electron transfer from an electron **donor** (atom or molecule) to an **acceptor**?

6. List some mechanism that cells can use to combat the toxicity of metal ions?

7. NADH peroxidase (p. 857, Eq. 15-59) does not contain a transition metal ion. Propose a reasonable detailed mechanism for its action and compare it with mechanisms of action of heme peroxidases.

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Page numbers set in **boldface** refer to major discussions.
The symbol *s* after a page number refers to a chemical structure.

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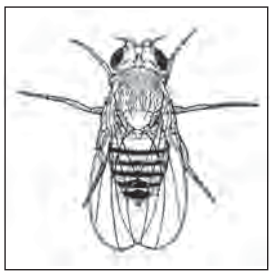

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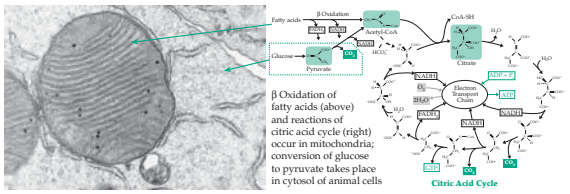


β Oxidation of fatty acids (above) and reactions of citric acid cycle (right) occur in mitochondria; conversion of glucose to pyruvate takes place in cytosol of animal cells

Metabolism, a complex network of chemical reactions, occurs in several different compartments in eukaryotic cells. Fatty acids, a major source of energy for many human cells, are oxidized in the mitochondria via β oxidation and the citric acid cycle. Glucose, a primary source of energy, is converted to pyruvate in the cytosol. Biosynthetic reactions occurring in both compartments form proteins, nucleic acids, storage polymers such as glycogen, and sparingly soluble lipid materials which aggregate to form membranes. Hydrophobic groups in proteins and other polymers also promote self-assembly of the cell. At the same time, oxidative processes, initiated by $O_2$, increase the water solubility of molecules, leading to metabolic turnover. Micrograph courtesy of Kenneth Moore.

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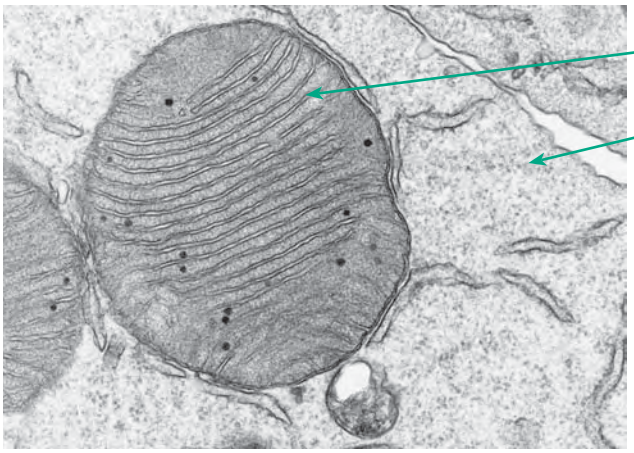
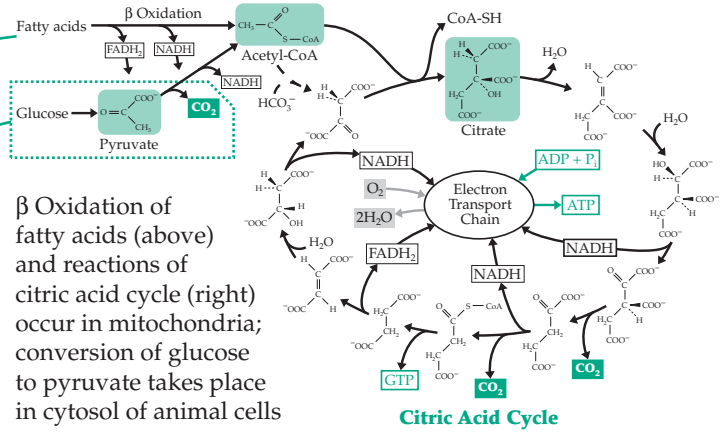
# The Organization of Metabolism

# 17






β Oxidation of fatty acids (above) and reactions of citric acid cycle (right) occur in mitochondria; conversion of glucose to pyruvate takes place in cytosol of animal cells

Metabolism involves a bewildering array of chemical reactions, many of them organized as complex cycles which may appear difficult to understand. Yet, there is logic and orderliness. With few exceptions, metabolic pathways can be regarded as sequences of the reactions considered in Chapters 12–16 (and summarized in the table inside the back cover) which are organized to accomplish specific chemical goals. In this chapter we will examine the chemical logic of the major pathways of catabolism of foods and of cell constituents as well as some reactions of biosynthesis (anabolism). A few of the sequences have already been discussed briefly in Chapter 10.

## A. The Oxidation of Fatty Acids

Hydrocarbons yield more energy upon combustion than do most other organic compounds, and it is, therefore, not surprising that one important type of food reserve, the fats, is essentially hydrocarbon in nature. In terms of energy content the component fatty acids are the most important. Most aerobic cells can oxidize fatty acids completely to $CO_2$ and water, a process that takes place within many bacteria, in the matrix space of animal mitochondria, in the peroxisomes of most eukaryotic cells, and to a lesser extent in the endoplasmic reticulum.

The carboxyl group of a fatty acid provides a point for chemical attack. The first step is a priming reaction in which the fatty acid is converted to a water-soluble acyl-CoA derivative in which the α hydrogens of the fatty acyl radicals are "activated" (step *a*, Fig. 17-1). This synthetic reaction is catalyzed by **acyl-CoA synthetases** (fatty acid:CoA ligases). It is driven by the hydrolysis of ATP to AMP and two inorganic phosphate ions using the sequence shown in Eq. 10-1 (p. 508). There are isoenzymes that act on short-, medium-, and long-chain fatty acids. Yeast contains at least five of these.[1] In every case the acyl group is activated through formation of an intermediate acyl adenylate; hydrolysis of the released pyrophosphate helps to carry the reaction to completion (see discussion in Section H).

### 1. Beta Oxidation

The reaction steps in the oxidation of long-chain acyl-CoA molecules to acetyl-CoA were outlined in Fig. 10-4. Because of the great importance of this β oxidation sequence in metabolism the steps are shown again in Fig. 17-1 (steps *b*–*e*). The chemical logic becomes clear if we examine the structure of the acyl-CoA molecule and consider the types of biochemical reactions available. If the direct use of $O_2$ is to be avoided, the only reasonable mode of attack on an acyl-CoA molecule is dehydrogenation. Removal of the α hydrogen as a proton is made possible by the activating effect of the carbonyl group of the thioester. The β hydrogen can be transferred from the intermediate enolate, as a hydride ion, to the bound FAD present in the **acyl-CoA dehydrogenases** that catalyze this reaction[2–5] (step *b*, Fig. 17-1; see also Eq. 15-23). These enzymes contain FAD, and the reduced coenzyme $FADH_2$ that is formed is reoxidized by an **electron transferring flavoprotein** (Chapter 15), which also contains FAD. This protein carries the electrons abstracted in the oxidation process to the inner membrane of the mitochondrion where they enter the mitochondrial electron transport system,[5a] as depicted in Fig. 10-5 and as discussed in detail in

Chapter 18.

The product of step *b* is always a **trans-$\Delta^2$-enoyl-CoA**. One of the few possible reactions of this unsaturated compound is nucleophilic addition at the β position. The reacting nucleophile is an $HO^-$ ion from water. This reaction step (step *c*, Fig. 17-1) is completed by addition of $H^+$ at the α position. The resulting **β-hydroxyacyl-CoA** (3-hydroxyacyl-CoA) is dehydrogenated to a ketone by $NAD^+$ (step *d*).[5b] This series of three reactions is the β oxidation sequence.

At the end of this sequence, the β-oxoacyl-CoA derivative is cleaved (Fig. 17-1, step *e*) by a **thiolase** (see also Eq. 13-35). One of the products is acetyl-CoA, which can be catabolized to $CO_2$ through the citric acid cycle. The other product of the thiolytic cleavage is an acyl-CoA derivative that is *two carbon atoms shorter than the original acyl-CoA*. This molecule is recycled through the β oxidation process, a two-carbon acetyl unit being removed as acetyl-CoA during each turn of the cycle (Fig. 17-1). The process continues until the fatty acid chain is completely degraded. If the original fatty acid contained an *even* number of carbon atoms in a straight chain, acetyl-CoA is the only product. However, if the original fatty acid contained an *odd* number of carbon atoms, **propionyl-CoA** is formed at the end.

For every step of the β oxidation sequence there is a small family of enzymes with differing chain length preferences.[6,7] For example, in liver mitochondria one acyl-CoA dehydrogenase acts most rapidly on *n*-butyryl and other short-chain acyl-CoA; a second prefers a substrate of medium chain length such as *n*-octanoyl-CoA; a third prefers long-chain substrates such as palmitoyl-CoA; and a fourth, substrates with 2-methyl branches. A fifth enzyme acts specifically on isovaleryl-CoA. Similar preferences exist for the other enzymes of the β oxidation pathway. In *Escherichia coli*

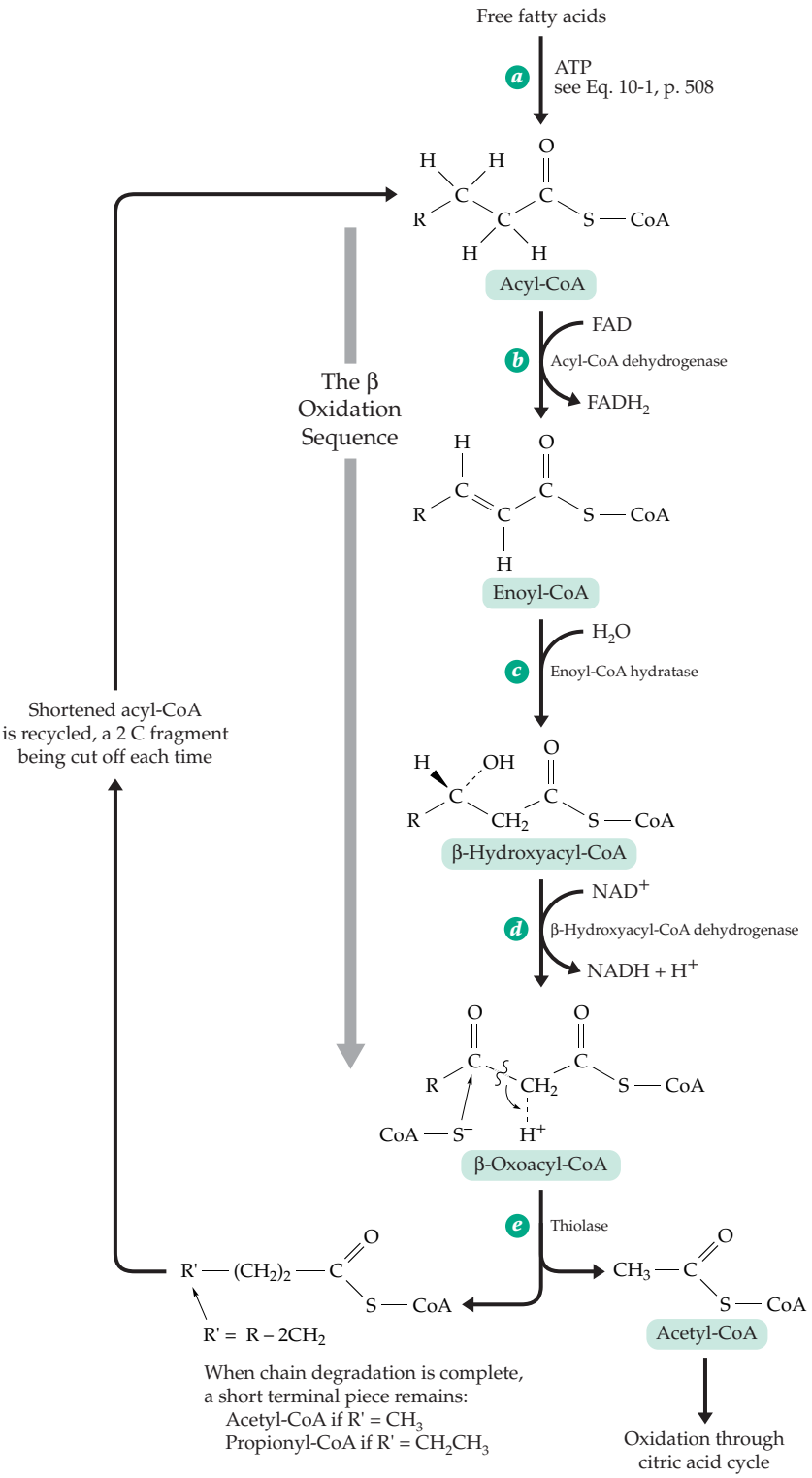



**Figure 17-1** The β oxidation cycle for fatty acids. Fatty acids are converted to acyl-CoA derivatives from which 2-carbon atoms are cut off as acetyl-CoA to give a shortened chain which is repeatedly sent back through the cycle until only a 2- or 3-carbon acyl-CoA remains. The sequence of steps *b*, *c*, and *d* also occurs in many other places in metabolism.

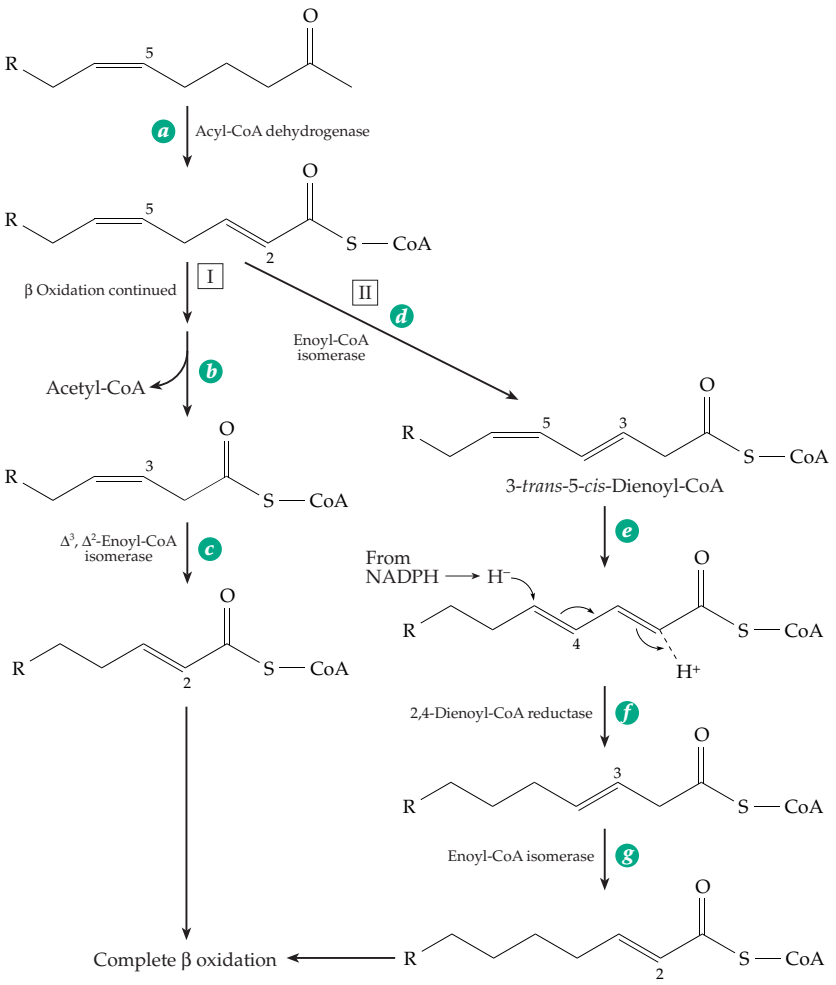
most of these enzymes are present as a complex of multifunctional proteins[8] while the mitochondrial enzymes may be organized as a multiprotein complex.[9,10]

***Peroxisomal beta oxidation.*** In animal cells β oxidation is primarily a mitochondrial process,[5] but it also takes place to a limited extent within peroxisomes and within the endoplasmic reticulum.[11–14] This "division of labor" is still not understood well. Straight-chain fatty acids up to 18 carbons in length appear to be metabolized primarily in mitochondria, but in the liver fatty acids with very long chains are processed largely in peroxisomes.[13] There, a very long-chain acyl-CoA synthetase acts on fatty acids that contain 22 or more carbon atoms.[15] In yeast all β oxidation takes place in peroxisomes,[15,16] and in most organisms, including green plants,[17–18a] the peroxisomes are the most active sites of fatty acid oxidation. However, animal peroxisomes cannot oxidize short-chain acyl-CoA molecules; they must be returned to the mitochondria.[16] The activity of peroxisomes in β oxidation is greatly increased by the presence of a variety of compounds known as **peroxisome proliferators**. Among them are drugs such as aspirin and clofibrate and environmental xenobiotics such as the plasticizer bis-(2-ethyl-hexyl)-phthalate. They may induce as much as a tenfold increase in peroxisomal β oxidation.[11,12,19,19a]

Several other features also distinguish β oxidation in peroxisomes. The peroxisomal flavoproteins that catalyze the dehydrogenation of acyl-CoA molecules to unsaturated enoyl-CoAs (step *b* of Fig. 17-1) are **oxidases** in which the $FADH_2$ that is formed is reoxidized by $O_2$ to form $H_2O_2$.[13,20] In peroxisomes the enoyl-hydratase and the $NAD^+$-dependent dehydrogenase catalyzing steps *c* and *d* of Fig. 17-1 are present together with an enoyl-CoA isomerase (next section) as a trifunctional enzyme consisting of a single polypeptide chain.[21] As in mitochondrial β oxidation the 3-hydroxyacyl-CoA intermediates formed in both animal peroxisomes and plant peroxisomes (glyoxysomes) have the L configuration. However, in fungal peroxisomes as well as in *E. coli* they have the D configuration.[22,23] Further metabolism in these organisms requires an epimerase that converts the D-hydroxyacyl-CoA molecules to L.[24] In the past it has often been assumed that peroxisomal membranes are freely permeable to $NAD^+$, NADH, and acyl-CoA molecules. However, genetic experiments with yeast and other recent evidence indicate that they are impermeable *in vivo* and that carrier and shuttle mechanisms similar to those in mitochondria may be required.[14,25]

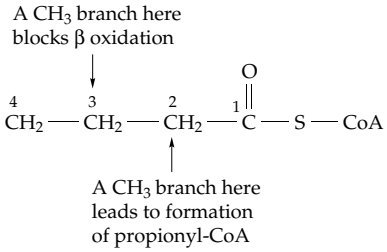
***Unsaturated fatty acids.*** Mitochondrial β oxidation of such unsaturated acids as the $\Delta^9$-oleic acid begins with removal of two molecules of acetyl-CoA to form a $\Delta^5$-acyl-CoA. However, further metabolism is slow. Two pathways have been identified (Eq. 17-1).[26–29b] The first step for both is a normal dehydrogenation to a 2-*trans*-5-*cis*-dienoyl-CoA. In pathway I this intermediate reacts slowly by the normal β oxidation sequence to form a 3-*cis*-enoyl-CoA intermediate which must then be acted upon by an auxiliary enzyme, a *cis*-$\Delta^3$-*trans*-$\Delta^2$-enoyl-CoA isomerase (Eq. 17-1, step *c*), before β oxidation can continue.

The alternative reductase pathway (II in Eq. 17-1) is often faster. It makes use of an additional isomerase which converts 3-*trans*, 5-*cis*-dienoyl-CoA into the 2-*trans*, 4-*trans* isomer in which the double bonds are conjugated with the carbonyl group.[29] This permits removal of one double bond by reduction with NADPH as shown (Eq. 17-1, step *f*).[29a,29b] The peroxisomal



pathway is similar.[21] However, the intermediate formed in step *e* of Eq. 17-1 may sometimes have the 2-*trans*, 4-*cis* configuration.[17] The NADH for the reductive step *f* may be supplied by an NADP-dependent isocitrate dehydrogenase.[29c] Repetition of steps *a*, *d*, *e*, and *f* of Eq. 17-1 will lead to β oxidation of the entire chain of polyunsaturated fatty acids such as linoleoyl-CoA or arachidonoyl-CoA. Important additional metabolic routes for polyunsaturated fatty acid derivatives are described in Chapter 21.

***Branched-chain fatty acids.*** Most of the fatty acids in animal and plant fats have straight unbranched chains. However, branches, usually consisting of methyl groups, are present in lipids of some microorganisms, in waxes of plant surfaces, and also in polyprenyl chains. As long as there are not too many branches and if they occur only in the even-numbered positions (i.e., on carbons 2, 4, etc.) β oxidation proceeds normally. Propionyl-CoA is formed in addition to acetyl-CoA as a product of the chain degradation. On the other hand, if methyl groups occur in positions 3, 5, etc., β oxidation is blocked at step *d* of Fig. 17-1. A striking example of the effect of such blockage was

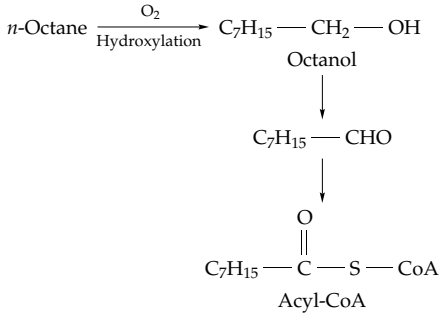



provided by the synthetic detergents in common use until about 1966. These detergents contained a hydrocarbon chain with methyl groups distributed more or less randomly along the chain. Beta oxidation was blocked at many points and the result was a foamy pollution crisis in sewage plants in the United States and in some other countries. Since 1966, only biodegradable detergents having straight hydrocarbon chains have been sold.

In fact, cells *are* able to deal with small amounts of these hard-to-oxidize substrates. The $O_2$-dependent reactions called α oxidation and ω oxidation are used. These are related also to the oxidation of hydrocarbons which we will consider next.

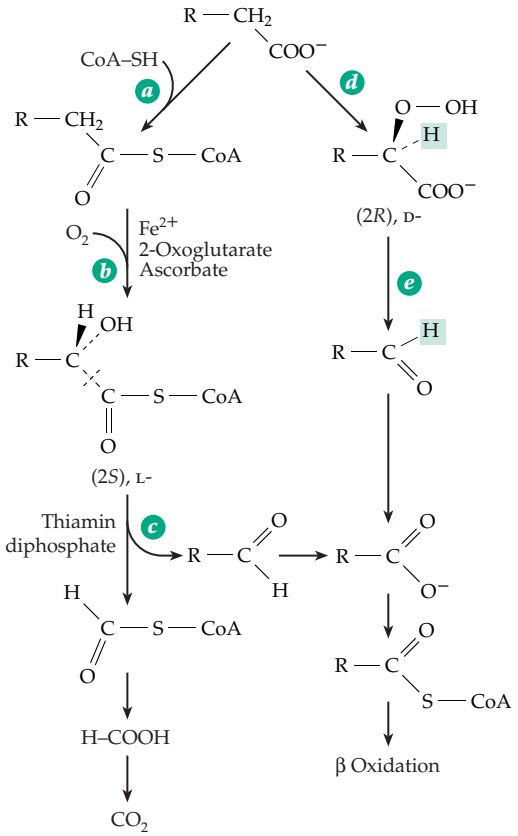
***Oxidation of saturated hydrocarbons.*** Although the initial oxidation step is chemically difficult, the tissues of our bodies are able to metabolize saturated hydrocarbons such as *n*-heptane slowly, and some microorganisms oxidize straight-chain hydrocarbons rapidly.[30,31] Strains of *Pseudomonas* and of the yeast *Candida* have been used to convert petroleum into edible proteins.[9]

The first step in oxidation of alkanes is usually an $O_2$-requiring **hydroxylation** (Chapter 18) to a primary alcohol. Further oxidation of the alcohol to an acyl-CoA derivative, presumably via the aldehyde (Eq. 17-2), is a frequently encountered biochemical oxidation sequence.




(17-2)

***Alpha oxidation and omega oxidation.*** Animal tissues degrade such straight-chain fatty acids as palmitic acid, stearic acid, and oleic acid almost entirely by β oxidation, but plant cells often oxidize fatty acids one carbon at a time. The initial attack may involve hydroxylation on the α-carbon atom (Eq. 17-3) to form either the D- or the L-2-hydroxy acid.[17,18,32,32a] The L-hydroxy acids are oxidized rapidly, perhaps by dehydrogenation to the oxo acids (Eq. 17-3, step *b*) and oxidative decarboxylation, possibly utilizing $H_2O_2$ (see Eq. 15-36). The D-hydroxy acids tend to accumulate



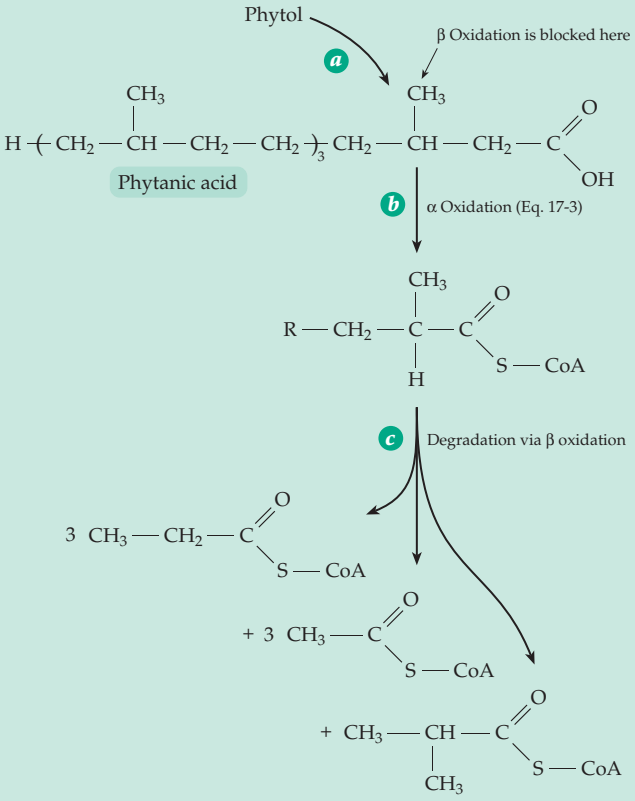

(17-3)

and are normally present in green leaves. However, they too are oxidized further, with retention of the α hydrogen as indicated by the shaded squares in Eq. 17-3, step *e*. This suggests a new type of dehydrogenation with concurrent decarboxylation. Alpha oxidation also occurs to some extent in animal tissues. For example, when β oxidation is blocked by the presence of a methyl side chain, the body may use α oxidation to get past the block (see **Refsum disease**, Box 17-A). As in plants, this occurs principally in the peroxisomes[33–35] and is important for degradation not only of polyprenyl chains but also bile acids. In the brain some of the fatty acyl groups of sphingolipids are hydroxylated to α-hydroxyacyl groups.[36] Alpha oxidation in animal cells occurs after conversion of free fatty acids to their acyl-CoA derivatives (Eq. 7-3, step *a*). This is followed by a 2-oxoglutarate-dependent hydroxylation (step *b*, see also Eq. 18-51) to form the 2-hydroxyacyl-CoA, which is cleaved in a standard thiamin diphosphate-requiring α cleavage (step *c*). The products are formyl-CoA, which is hydrolyzed and oxidized to $CO_2$, and a fatty aldehyde which is metabolized further by β oxidation.[34a]

In plants α-dioxygenases (Chapter 18) convert free fatty acids into 2(*R*)-hydroperoxy derivatives (Eq. 7-3, step *d*).[32a] These may be decarboxylated to fatty aldehydes (step *e*, see also Eq. 15-36) but may also give rise to a variety of other products. Compounds arising from linoleic and linolenic acids are numerous and include epoxides, epoxy alcohols, dihydroxy acids, short-chain aldehydes, divinyl ethers, and jasmonic acid (Eq. 21-18).[32a]

On other occasions, **omega (ω) oxidation** occurs at the opposite end of the chain to yield a dicarboxylic acid. Within the human body 3,6-dimethyloctanoic acid and other branched-chain acids are degraded largely via ω oxidation. The initial oxidative attack is by a hydroxylase of the cytochrome P450 group (Chapter 18). These enzymes act not only on fatty acids but also on prostaglandins, sterols, and many other lipids. In the animal body fatty acids are sometimes hydroxylated both at the terminal (ω) position and at the next (ω-2 or ω2) carbon. In plants hydroxylation may occur at the ω2, ω3, and ω4 positions as well.[17,37] Dicarboxylates resulting from ω oxidation of straight-chain fatty acids

## BOX 17-A REFSUM DISEASE




In this autosomally inherited disorder of lipid metabolism the 20-carbon branched-chain fatty acid **phytanic acid** accumulates in tissues. Phytanic acid is normally formed in the body (step *a* in the accompanying scheme) from the polyprenyl plant alcohol **phytol**, which is found as an ester in the chlorophyll present in the diet (Fig. 23-20). Although only a small fraction of the ingested phytol is oxidized to phytanic acid, this acid accumulates to a certain extent in animal fats and is present in dairy products. Because β oxidation is blocked, the first step (step *b*) in degradation of phytanic acid is α oxidation in peroxisomes.[a] The remainder of the molecule undergoes β oxidation (step *c*) to three molecules of propionyl-CoA, three of acetyl-CoA, and one of isobutyryl-CoA. The disease, which was described by Refsum in 1946, causes severe damage to nerves and brain as well as lipid accumulation and early death.[b–d] This rare disorder apparently results from a defect in the initial hydroxylation. The causes of the neurological symptoms of Refsum disease are not clear, but it is possible that the isoprenoid phytanic acid interferes with prenylation of membrane proteins.[b]


[a] Singh, I., Pahan, K., Dhaunsi, G. S., Lazo, O., and Ozand, P. (1993) *J. Biol. Chem.* **268**, 9972–9979

[b] Steinberg, D. (1995) in *The Metabolic and Molecular Bases of Inherited Disease*, 7th ed., Vol. 2 (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., eds), pp. 2351–2369, McGraw-Hill, New York

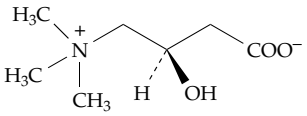
[c] Steinberg, D., Herndon, J. H., Jr., Uhlendorf, B. W., Mize, C. E., Avigan, J., and Milne, G. W. A. (1967) *Science* **156**, 1740–1742

[d] Muralidharan, V. B., and Kishimoto, Y. (1984) *J. Biol. Chem.* **259**, 13021–13026

can undergo β oxidation from both ends. The resulting short-chain dicarboxylates, which appear to be formed primarily in the peroxisomes,[38] may be converted by further β oxidation into succinyl-CoA and free succinate.[39] Incomplete β oxidation in mitochondria (Fig. 17-1) releases small amounts of 3(β)-hydroxy fatty acids, which also undergo ω oxidation and give rise to free 3-hydroxydicarboxylic acids which may be excreted in the urine.[40]

## 2. Carnitine and Mitochondrial Permeability

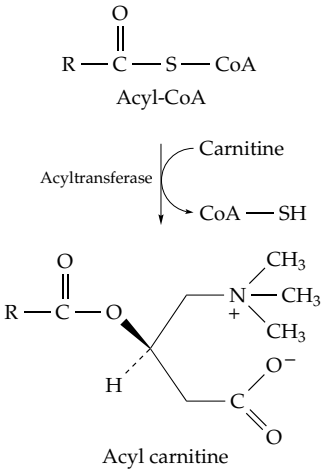
A major factor controlling the oxidation of fatty acids is the rate of entry into the mitochondria. While some long-chain fatty acids (perhaps 30% of the total) enter mitochondria as such and are converted to CoA derivatives in the matrix, the majority are "activated" to acyl-CoA derivatives on the inner surface of the outer membranes of the mitochondria. Penetration of these acyl-CoA derivatives through the mitochondrial inner membrane is facilitated by **L-carnitine**.[41–44]



L-Carnitine

Carnitine is present in nearly all organisms and in all animal tissues. The highest concentration is found in muscle where it accounts for almost 0.1% of the dry matter. Carnitine was first isolated from meat extracts in 1905 but the first clue to its biological action was obtained in 1948 when Fraenkel and associates described a new dietary factor required by the mealworm, *Tenebrio molitor*. At first designated **vitamin $B_t$**, it was identified in 1952 as carnitine. Most organisms synthesize their own carnitine from lysine side chains (Eq. 24-30). The inner membrane of mitochondria contains a long-chain acyltransferase (carnitine palmitoyltransferase I) that catalyzes transfer of the fatty acyl group from CoA to the hydroxyl group of carnitine (Eq. 17-4).[45–47a] Perhaps acyl carnitine derivatives pass through the membrane more easily than do acyl-CoA derivatives because the positive and negative charges can swing together and neutralize each other as shown in Eq. 17-4. Inside the mitochondrion the acyl group is transferred back from carnitine onto CoA (Eq. 17-4, reverse) by carnitine palmitoyltransferase II prior to initiation of β oxidation.

Tissues contain not only long-chain acylcarnitines but also **acetylcarnitine** and other short-chain acylcarnitines, some with branched chains.[41] By accepting acetyl groups from acetyl-CoA, carnitine causes the release of free coenzyme A which can then be reused.



(17-4)

Thus, carnitine may have a regulatory function. In flight muscles of insects acetylcarnitine serves as a reservoir for acetyl groups. Carnitine acyltransferases that act on short-chain acyl-CoA molecules are also present in peroxisomes and microsomes, suggesting that carnitine may assist in transferring acetyl groups and other short acyl groups between cell compartments. For example, acetyl groups from peroxisomal β oxidation can be transferred into mitochondria where they can be oxidized in the citric acid cycle.[41]

## 3. Human Disorders of Fatty Acid Oxidation

Mitochondrial β oxidation of fatty acids is the principal source of energy for the heart. Consequently, inherited defects of fatty acid oxidation or of carnitine-assisted transport often appear as serious heart disease (inherited cardiomyopathy). These may involve heart failure, pulmonary edema, or sudden infant death. As many as 1 in 10,000 persons may inherit such problems.[48–50a] The proteins that may be defective include a plasma membrane carnitine transporter; carnitine palmitoyltransferases; carnitine/acylcarnitine translocase; long-chain, medium-chain, and short-chain acyl-CoA dehydrogenases; 2,4-dienoyl-CoA reductase (Eq. 17-1); and long-chain 3-hydroxyacyl-CoA dehydrogenase. Some of these are indicated in Fig. 17-2.

Several cases of genetically transmitted carnitine deficiency in children have been recorded. These children have weak muscles and their mitochondria oxidize long-chain fatty acids slowly. If the inner mitochondrial membrane carnitine palmitoyltransferase II is lacking, long-chain acylcarnitines accumulate in the mitochondria and appear to have damaging effects on membranes. In the unrelated condition of **acute myocardial ischemia** (lack of oxygen, e.g., during a heart attack) there is also a large accumulation of long-chain acylcarnitines.[51,52] These compounds may induce cardiac arrhythmia and may also account for

sudden death from deficiency of carnitine palmitoyltransferase II. Treatment of disorders of carnitine metabolism with daily oral ingestion of several grams of carnitine is helpful, especially for deficiency of the plasma membrane transporter.[50a,53] Metabolic abnormalities may be corrected completely.[50a]

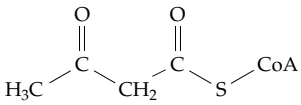
One of the most frequent defects of fatty acid oxidation is deficiency of a mitochondrial acyl-CoA dehydrogenase.[50] If the long-chain-specific enzyme is lacking, the rate of β oxidation of such substrates as octanoate is much less than normal and afflicted individuals excrete in their urine hexanedioic (adipic), octanedioic, and decanedioic acids, all products of ω oxidation.[54] Much more common is the lack of the mitochondrial *medium-chain* acyl-CoA dehydrogenase. Again, dicarboxylic acids, which are presumably generated by ω oxidation in the peroxisomes, are present in blood and urine. Patients must avoid fasting and may benefit from extra carnitine.

A deficiency of very long-chain fatty acid oxidation in peroxisomes is apparently caused by a defective transporter of the ABC type (Chapter 8).[55] The disease, **X-linked adrenoleukodystrophy (ALD)**, has received considerable publicity because of attempts to treat it with "Lorenzo's oil," a mixture of triglycerides of oleic and the $C_{22}$ monoenoic **erucic acid**. The hope has been that these acids would flush out the very long-chain fatty acids that accumulate in the myelin sheath of neurons in the central nervous system and may be responsible for the worst consequences of the disease. However, there has been only limited success.[56,57]

Several genetic diseases involve the development of peroxisomes.[14,35,58,59] Most serious is the **Zellweger syndrome** in which there are no functional peroxisomes. Only "ghosts" of peroxisomes are present and they fail to take up proteins containing the C-terminal peroxisome-targeting sequence SKL.[60,60a] There are many symptoms and death occurs within the first year. Less serious disorders include the presence of catalaseless peroxisomes.[60a]

## 4. Ketone Bodies

When a fatty acid with an even number of carbon atoms is broken down through β oxidation the last intermediate before complete conversion to acetyl-CoA is the four-carbon **acetoacetyl-CoA**:



Acetoacetyl-CoA

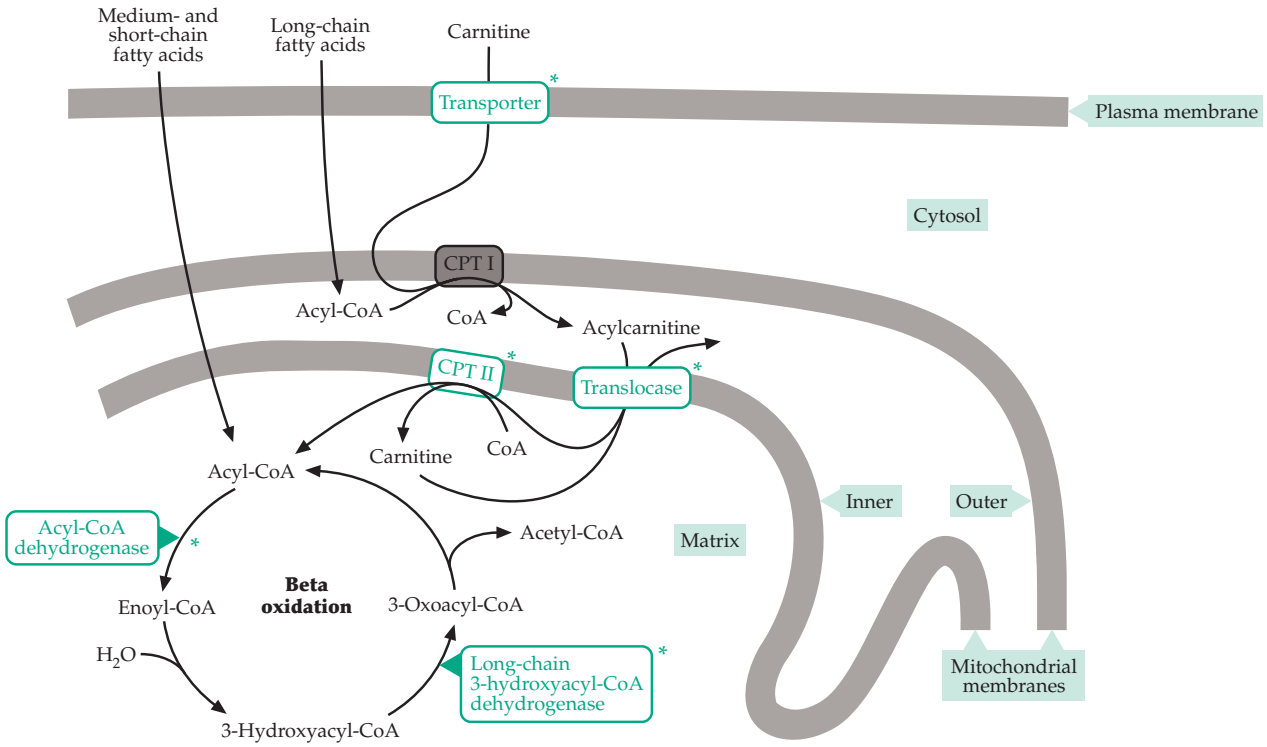
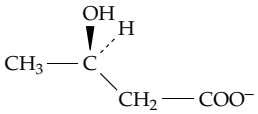



**Figure 17-2** Some specific defects in proteins of β oxidation and acyl-carnitine transport causing cardiomyopathy are indicated by the green asterisks. CPT I and CPT II are carnitine palmitoyltransferases I and II. After Kelly and Strauss.[48]

Acetoacetyl-CoA appears to be in equilibrium with acetyl-CoA within the body and is an important metabolic intermediate. It can be cleaved to two molecules of acetyl-CoA which can enter the citric acid cycle. It is also a precursor for synthesis of polyprenyl (isoprenoid) compounds, and it can give rise to free **acetoacetate**, an important constituent of blood. Acetoacetate is a β-oxoacid that can undergo decarboxylation to acetone or can be reduced by an NADH-dependent dehydrogenase to D-3-hydroxybutyrate. Notice that the configuration of this compound is opposite to that of L-3-hydroxybutyryl-CoA which is



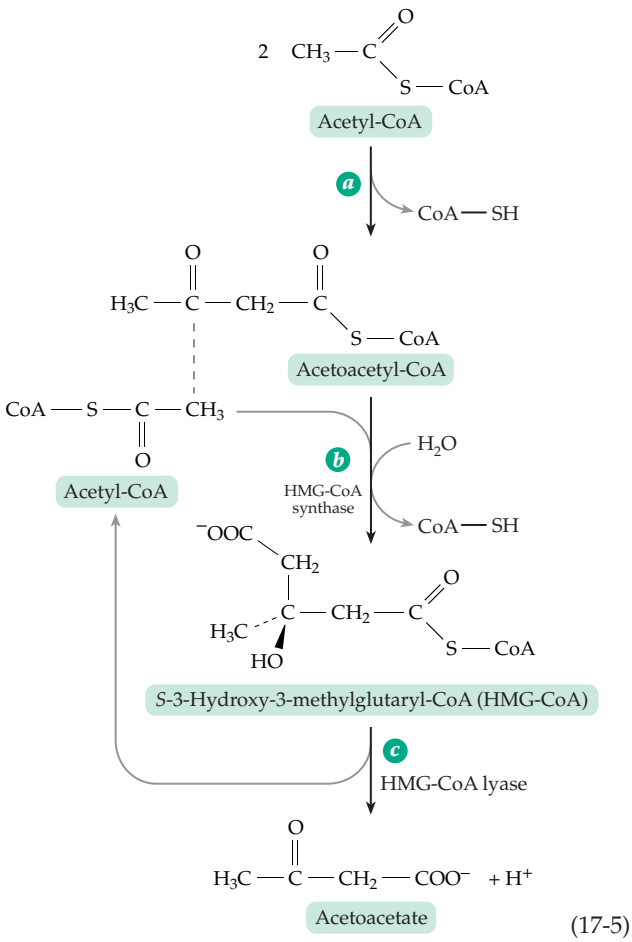
D-3-Hydroxybutyrate

formed during β oxidation of fatty acids (Fig. 17-1). D-3-Hydroxybutyrate is sometimes stored as a polymer in bacteria (Box 21-D).

The three compounds, acetoacetate, acetone, and 3-hydroxybutyrate, are known as **ketone bodies**.[60b] The inability of the animal body to form the glucose precursors, pyruvate or oxaloacetate, from acetyl units sometimes causes severe metabolic problems. The condition known as **ketosis**, in which excessive amounts of ketone bodies are present in the blood, develops when too much acetyl-CoA is produced and its combustion in the critic acid cycle is slow. Ketosis often develops in patients with Type I **diabetes mellitus** (Box 17-G), in anyone with high fevers, and during starvation. Ketosis is dangerous, if severe, because formation of ketone bodies produces hydrogen ions (Eq. 17-5) and acidifies the blood. Thousands of young persons with insulin-dependent diabetes die annually from ketoacidosis.

Rat blood normally contains about 0.07 mM acetoacetate, 0.18 mM hydroxybutyrate, and a variable amount of acetone. These amounts increase to 0.5 mM acetoacetate and 1.6 mM hydroxybutyrate after 48 h of starvation. On the other hand, the blood glucose concentration falls from 6 to 4 mM after 48 h starvation.[61] Under these conditions acetoacetate and hydroxybutyrate are an important alternative energy source for muscle and other tissues.[62,63] Acetoacetate can be thought of as a transport form of acetyl units, which can be reconverted to acetyl-CoA and oxidized in the citric acid cycle.

Some free acetoacetate is formed by direct hydrolysis of acetoacetyl-CoA. In rats, ~11% of the hydroxybutyrate that is excreted in the urine comes from acetoacetate generated in this way.[64] However, most acetoacetate arises in the liver indirectly in a two-step process (Eq. 17-5) that is closely related to the synthesis

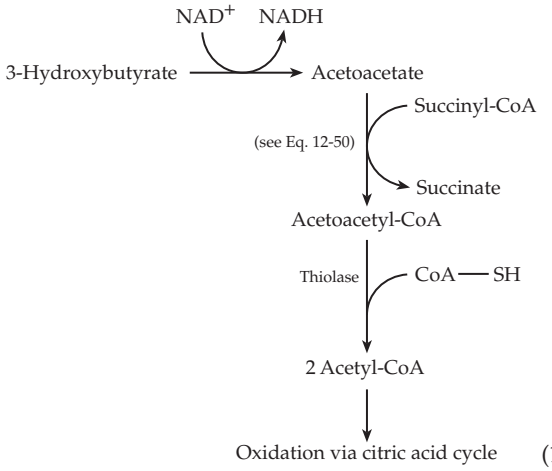



(17-5)

of cholesterol and other polyprenyl compounds. Step *a* of this sequence is a Claisen condensation, catalyzed by 3-hydroxy-3-methyl-glutaryl CoA synthase (HMG-CoA synthase)[64a–c] and followed by hydrolysis of one thioester linkage. It is therefore similar to the citrate synthase reaction (Eq. 13-38). Step *c* is a simple aldol cleavage. The overall reaction has the stoichiometry of a direct hydrolysis of acetoacetyl-CoA. Liver mitochondria contain most of the body's HMG-CoA synthase and are the major site of ketone body formation (**ketogenesis**). Cholesterol is synthesized from HMG-CoA that is formed in the cytoplasm (Chapter 22).

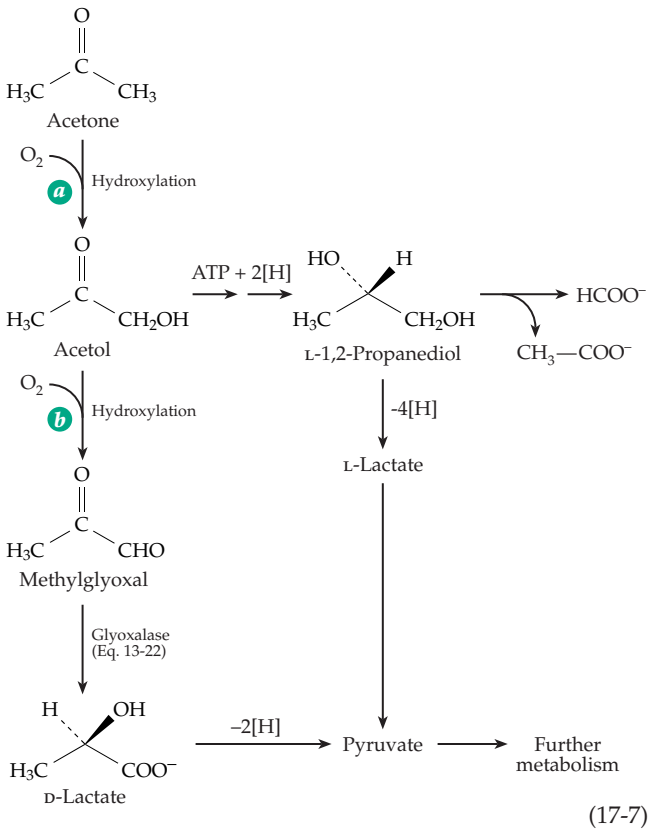
Utilization of 3-hydroxybutyrate or acetoacetate for energy requires their reconversion to acetyl-CoA as indicated in Eq. 17-6. All of the reactions of this sequence may be nearly at equilibrium in tissues that use ketone bodies for energy.[61]

Acetone, in the small amounts normally present in the body, is metabolized by hydroxylation to acetol (Eq. 17-7, step *a*), hydroxylation and dehydration to methylglyoxal (step *b*), and conversion to D-lactate and pyruvate. A second pathway via 1,2-propanediol and L-lactate is also shown in Eq. 17-7. During fasting the acetone content of human blood may rise to as much as 1.6 mM. As much as two-thirds of this may be converted to glucose.[65–69] Accumulation of acetone




(17-6)

appears to induce the synthesis of the hydroxylases needed for methylglyoxal formation,[68] and the pyruvate formed by Eq. 17-7 may give rise to glucose by the gluconeogenic pathway. However, at high acetone concentrations most metabolism may take place through a poorly understood conversion of 1,2-propanediol to acetate and formate or $CO_2$.[69] No net conversion of acetate into glucose can occur in animals, but isotopic labels from acetate can enter glucose via acetyl-CoA and the citric acid cycle.




(17-7)

## B. Catabolism of Propionyl Coenzyme A and Propionate

Beta oxidation of fatty acids with an odd number of carbon atoms leads to the formation of propionyl-CoA as well as acetyl-CoA. The three-carbon propionyl unit is also produced by degradation of cholesterol and other isoprenoid compounds and of isoleucine, valine, threonine, and methionine. Human beings ingest small amounts of free propionic acid, e.g., from Swiss cheese (which is cultivated with propionic acid-producing bacteria) and from propionate added to bread as a fungicide. In **ruminant** animals, such as cattle and sheep, the ingested food undergoes extensive fermentation in the **rumen**, a large digestive organ containing cellulose-digesting bacteria and protozoa. Major products of the rumen fermentations include acetate, propionate, and butyrate. Propionate is an important source of energy for these animals.

### 1. The Malonic Semialdehyde Pathways

The most obvious route of metabolism of propionyl-CoA is further β oxidation which leads to 3-hydroxypropionyl-CoA (Fig. 17-3, step *a*). This appears to be the major pathway in green plants.[17] Continuation of the β oxidation via steps *a*–*c* of Fig. 17-3 produces the CoA derivative of malonic semialdehyde. The latter can, in turn, be oxidized to malonyl-CoA, a β-oxoacid which can be decarboxylated to acetyl-CoA. The necessary enzymes have been found in *Clostridium kluyveri*,[70] but the pathway appears to be little used.

Nevertheless, malonyl-CoA is a major metabolite. It is an intermediate in fatty acid synthesis (see Fig. 17-12) and is formed in the peroxisomal β oxidation of odd chain-length dicarboxylic acids.[70a] Excess malonyl-CoA is decarboxylated in peroxisomes, and lack of the decarboxylase enzyme in mammals causes the lethal **malonic aciduria**.[70a] Some propionyl-CoA may also be metabolized by this pathway. The modified β oxidation sequence indicated on the left side of Fig. 17-3 is used in green plants and in many microorganisms. 3-Hydroxypropionyl-CoA is hydrolyzed to *free* β-hydroxypropionate, which is then oxidized to malonic semialdehyde and converted to acetyl-CoA by reactions that have not been completely described. Another possible pathway of propionate metabolism is the direct conversion to pyruvate via α oxidation into lactate, a mechanism that may be employed by some bacteria. Another route to lactate is through addition of water to acrylyl-CoA, the product of step *a* of Fig. 17-3. The water molecule adds in the "wrong way," the $OH^-$ ion going to the α carbon instead of the β (Eq. 17-8). An enzyme with an active site similar to that of histidine ammonia-lyase (Eq. 14-48) could

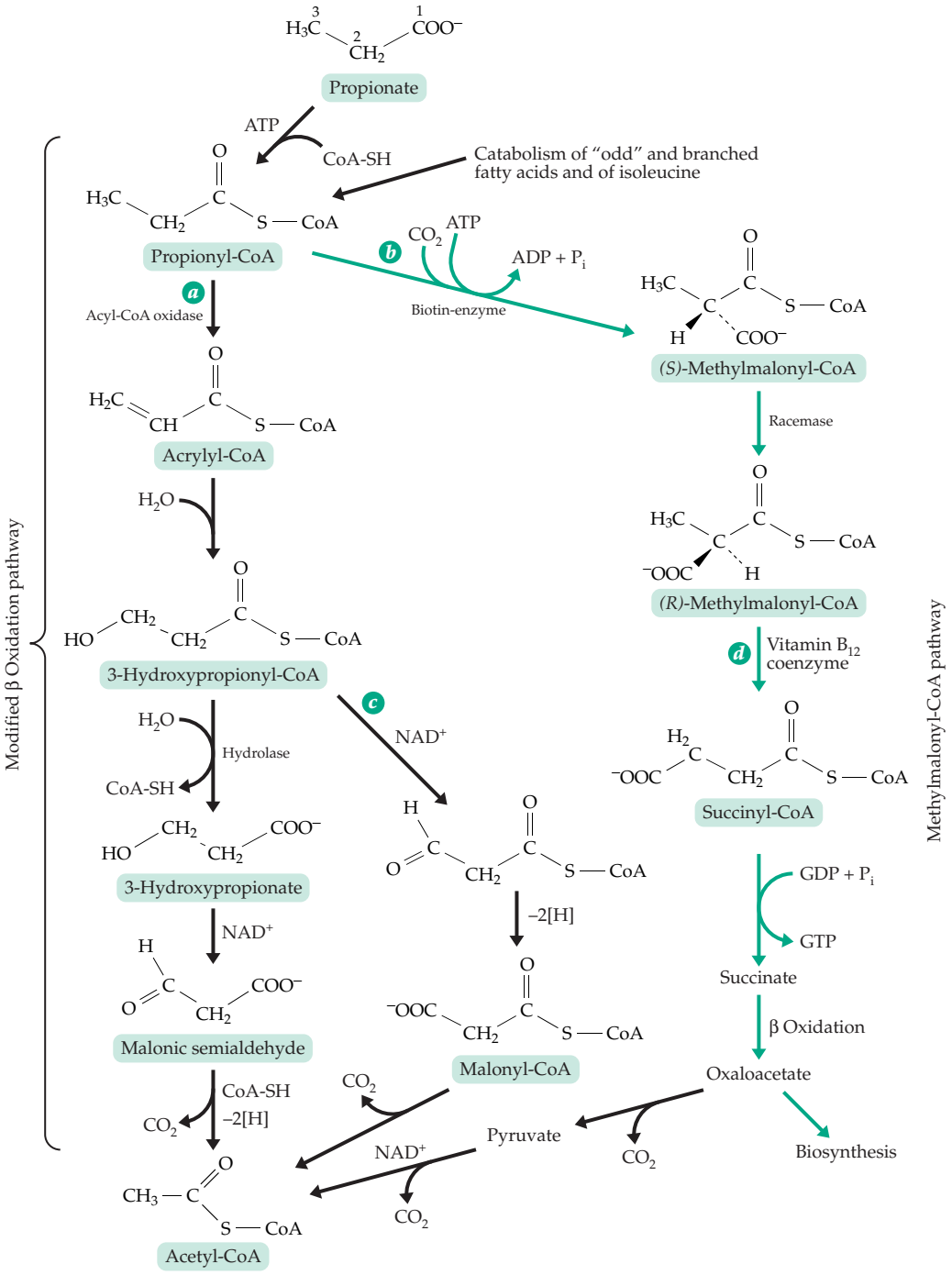
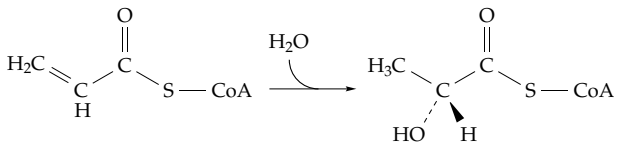



**Figure 17-3** Catabolism of propionate and propionyl-CoA. In the names for methylmalonyl-CoA the *R* and *S* refer to the methylmalonyl part of the structure. Coenzyme A is also chiral.

presumably catalyze such a reaction. Lactyl-CoA could be converted to pyruvate readily. *Clostridium propionicum* does interconvert propionate, lactate, and pyruvate via acrylyl-CoA and lactyl-CoA as part of a fermentation of alanine (Fig. 24-19).[71–74] The enzyme that catalyzes hydration of acrylyl-CoA in this case is a complex flavoprotein that may function via a free radical mechanism.[71,72,74]




(17-8)

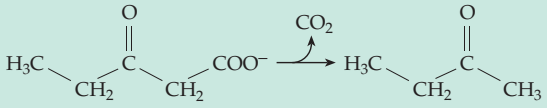
## BOX 17-B METHYLMALONIC ACIDURIA

In this hereditary disease up to 1–2 g of methylmalonic acid per day (compared to a normal of <5 mg/day) is excreted in the urine, and a high level of the compound is present in blood. Two causes of the rare disease are known.[a–d] One is the lack of functional vitamin $B_{12}$-containing coenzyme. This can be a result of a mutation in any one of several different genes involved in the synthesis and transport of the cobalamin coenzyme.[e] Cultured fibroblasts from patients with this form of the disease contain a very low level of the vitamin $B_{12}$ coenzyme (Chapter 16), and addition of excess vitamin $B_{12}$ to the diet may restore coenzyme synthesis to normal. Among elderly patients a smaller increase in methylmalonic acid excretion is a good indicator of vitamin $B_{12}$ deficiency. A second form of the disease, which does not respond to vitamin $B_{12}$, arises from a defect in the methylmalonyl mutase protein. Methylmalonic aciduria is often a very severe disease, frequently resulting in death in infancy. Surprisingly, some children with the condition are healthy and develop normally.[a,f]

A closely related disease is caused by a deficiency of propionyl-CoA carboxylase.[a] This may be a result of a defective structural gene for one of the two subunits of the enzyme, of a defect in the enzyme that attaches biotin to carboxylases, or of biotinitase, the enzyme that hydrolytically releases biotin from linkage with lysine (Chapter 14). The latter two defects lead to a multiple carboxylase deficiency and to methylmalonyl aciduria as well as ketoacidosis and propionic acidemia.[g]

Both methylmalonic aciduria and propionyl-CoA decarboxylase deficiency are usually accompanied by severe ketosis, hypoglycemia, and hyperglycinemia. The cause of these conditions is not entirely clear. However, methylmalonyl-CoA, which accumulates in methylmalonic aciduria, is a known inhibitor of pyruvate carboxylase. Therefore, ketosis may develop because of impaired conversion of pyruvate to oxaloacetate.

Patients with propionic or methylmalonic acidemia also secrete 2,3-butanediols (D-,L- or meso) and usually also 1,2-propandiol in their urine. Secretion of 1,2-propanediol is also observed during starvation and in diabetic ketoacidosis. Propanediol may be formed from acetone (Eq. 17-7), and butanediols may originate from acetoin, which is a side reaction product of pyruvate dehydrogenase. However, in the metabolic defects under consideration here, acetoin may be formed by hydroxylation of methylethyl ketone which can arise, as does acetone, by decarboxylation of an oxoacid precursor formed by β oxidation.[h]




Methylmalonic aciduria is rare and can be diagnosed incorrectly. In 1989 a woman in St. Louis, Missouri, was convicted and sentenced to life in prison for murdering her 5-month-old son by poisoning with ethylene glycol. While in prison she gave birth to another son who soon fell ill of methylmalonyl aciduria and was successfully treated. Reexamination of the evidence revealed that the first boy had died of the same disease and the mother was released.[i]

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[a] Fenton, W. A., and Rosenberg, L. E. (1995) in *The Metabolic and Molecular Bases of Inherited Disease*, 7th ed., Vol. 1 (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., eds), pp. 1423–1449, McGraw-Hill, New York

[b] Matsui, S. M., Mahoney, M. J., and Rosenberg, L. E. (1983) *N. Engl. J. Med.* **308**, 857–861

[c] Hubbard, S. R., Wei, L., Ellis, L., and Hendrickson, W. A. (1994) *Nature (London)* **372**, 746–754

[d] Luschinsky Drennan, C., Matthews, R. G., Rosenblatt, D. S., Ledley, F. D., Fenton, W. A., and Ludwig, M. L. (1996) *Proc. Natl. Acad. Sci. U.S.A.* **93**, 5550–5555

[e] Fenton, W. A., and Rosenberg, L. E. (1995) in *The Metabolic and Molecular Bases of Inherited Disease*, 7th ed., Vol. 2 (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., eds), pp. 3129–3149, McGraw-Hill, New York

[f] Ledley, F. D., Levy, H. L., Shih, V. E., Benjamin, R., and Mahoney, M. J. (1984) *N. Engl. J. Med.* **311**, 1015–1018

[g] Wolf, B. (1995) in *The Metabolic and Molecular Bases of Inherited Disease*, 7th ed., Vol. 2 (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., eds), pp. 3151–3177, McGraw-Hill, New York

[h] Casazza, J. P., Song, B. J., and Veech, R. L. (1990) *Trends Biochem. Sci.* **15**, 26–30

[i] Zurer, P. (1991) *Chem. Eng. News* **69** Sep 30, 7–8

### 2. The Methylmalonyl-CoA Pathway of Propionate Utilization

Despite the simplicity and logic of the β oxidation pathway of propionate metabolism, higher animals use primarily the more complex methylmalonyl-CoA pathway (Fig. 17-3, step *b*). This is one of the two processes in higher animals presently known to depend upon vitamin $B_{12}$. This vitamin has never been found in higher plants, nor does the methylmalonyl pathway occur in plants. The pathway (Fig. 17-3) begins with the biotin- and ATP-dependent carboxylation of propionyl-CoA. The *S*-methylmalonyl-CoA so formed is isomerized to *R*-methylmalonyl-CoA, after which the methylmalonyl-CoA is converted to succinyl-CoA in a vitamin $B_{12}$ coenzyme-requiring reaction step *d* (Table 16-1). The succinyl-CoA is converted to free succinate (with the formation of GTP compensating for the ATP used initially). The succinate, by β oxidation, is converted to oxaloacetate which is decarboxylated to pyruvate. This, in effect, removes the carboxyl group that was put on at the beginning of the sequence in the ATP-dependent step. Pyruvate is converted by oxidative decarboxylation to acetyl-CoA.

A natural question is "Why has this complex pathway evolved to do something that could have been done much more directly?" One possibility is that the presence of too much malonyl-CoA, the product of the β oxidation pathway of propionate metabolism (Fig. 17-3, pathways *a* and *c*), would interfere with lipid metabolism. Malonyl-CoA is formed in the cytosol during fatty acid biosynthesis and retards mitochondrial β oxidation by inhibiting carnitine palmitoyltransferase I.[46,70a,75] However, a relationship to mitochondrial propionate catabolism is not clear. On the other hand, the tacking on of an extra $CO_2$ and the use of ATP at the beginning suggests that the *methylmalonyl-CoA pathway* (Fig. 17-3) *is a biosynthetic rather than a catabolic route* (see Section H,4). The methylmalonyl pathway provides a means for converting propionate to oxaloacetate, a transformation that is chemically difficult.

In this context it is of interest that cows, whose metabolism is based much more on acetate than is ours, often develop a severe ketosis spontaneously. A standard treatment is the administration of a large dose of propionate which is presumably effective because of the ease of its conversion to oxaloacetate via the methylmalonyl-CoA pathway. It is possible that this pathway was developed by animals as a means of capturing propionyl units, scanty though they may be, for conversion to oxaloacetate and use in biosynthesis. In ruminant animals, the pathway is especially important. Whereas we have 5.5 mM glucose in our blood, the cow has only half as much, and a substantial fraction of this glucose is derived, in the liver, from the propionate provided by rumen microorganisms.[76] The need for vitamin $B_{12}$ in the formation of propionate by these organisms also accounts for the high requirement for cobalt in the ruminant diet (Chapter 16).

## C. The Citric Acid Cycle

To complete the oxidation of fatty acids the acetyl units of acetyl-CoA generated in the β oxidation sequence must be oxidized to carbon dioxide and water.[77] The citric acid (or tricarboxylic acid) cycle by which this oxidation is accomplished is a vital part of the metabolism of almost all aerobic creatures. It occupies a central position in metabolism because of the fact that acetyl-CoA is also an intermediate in the catabolism of carbohydrates and of many amino acids and other compounds. The cycle is depicted in detail in Fig. 10-6 and in an abbreviated form, but with more context, in Fig. 17-4.

### 1. A Clever Way to Cleave a Reluctant Bond

Oxidation of the chemically resistant two-carbon acetyl group to $CO_2$ presents a chemical problem. As we have seen (Chapter 13), cleavage of a C–C bond occurs most frequently between atoms that are α and β to a carbonyl group. Such β cleavage is clearly impossible within the acetyl group. The only other common type of cleavage is that of a C–C bond adjacent to a carbonyl group (α cleavage), a thiamin-dependent process (Chapter 14). However, α cleavage would require the prior oxidation (hydroxylation) of the methyl group of acetate. Although many biological hydroxylation reactions occur, they are rarely used in the major pathways of rapid catabolism. Perhaps this is because the overall yield of energy obtainable via hydroxylation is less than that gained from dehydrogenation and use of an electron transport chain.[78]

The solution to the chemical problem of oxidizing acetyl groups efficiently is one very commonly found in nature; a catalytic cycle. Although direct cleavage is impossible, the two-carbon acetyl group of acetyl-CoA *can* undergo a Claisen condensation with a second compound that contains a carbonyl group. The condensation product has more than two carbon atoms, and a β cleavage to yield $CO_2$ is now possible. Since the cycle is designed to oxidize acetyl units we can regard acetyl-CoA as the **primary substrate** for the cycle. The carbonyl compound with which it condenses can be described as the **regenerating substrate**. To complete the catalytic cycle it is necessary that two carbon atoms be removed as $CO_2$ from the compound formed by condensation of the two substrates and that the remaining molecule be reconvertible to the original regenerating substrate. The reader may wish to play a

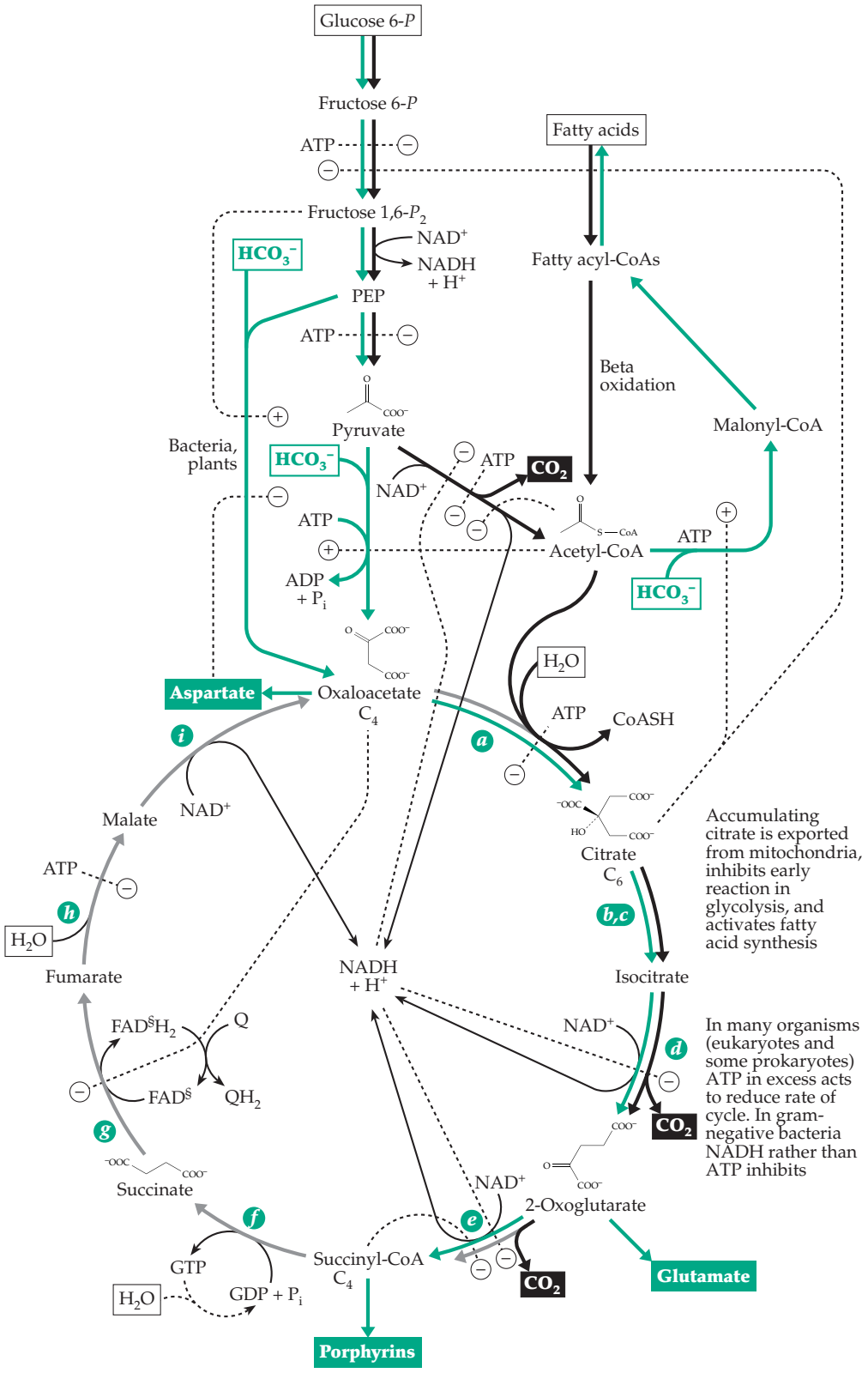



**Figure 17-4** The Krebs citric acid cycle. Some of its controlling interactions and its relationship to glycolysis. See also Figure 10-6. Positive and negative regulatory influences, whether arising by allosteric effects or via covalent modification, are indicated by ⊕ or ⊖. Some biosynthetic reaction pathways related to the cycle are shown in green. Steps are lettered to correspond to the numbering in Fig. 10-6, which shows more complete structural formulas. Three molecules of $H_2O$ (boxed) enter the cycle at each turn, providing hydrogen atoms for generation of NADH + $H^+$ and reduced ubiquinone ($QH_2$). The covalently attached FAD is designated $FAD^§$.

game by devising suitable sequences of reactions for an acetyl-oxidizing cycle and finding the simplest possible regenerating substrate. Ask yourself whether nature could have used anything simpler than **oxaloacetate**, the molecule actually employed in the citric acid cycle.

The first step in the citric acid cycle (step *a*, Fig. 17-4) is the condensation of acetyl-CoA with oxaloacetate to form citrate. The synthase that catalyzes this condensation also removes the CoA by hydrolysis after it has served its function of activating a methyl hydrogen. This hydrolysis also helps to drive the cycle by virtue of the high group transfer potential of the thioester linkage that is cleaved. Before the citrate can be degraded through β cleavage, the hydroxyl group must be moved from its tertiary position to an adjacent carbon where, as a secondary alcohol, it can be oxidized to a carbonyl group. This is accomplished through steps *b* and *c*, both catalyzed by the enzyme aconitase (Eq. 13-17). Isocitrate can then be oxidized to the β-oxoacid **oxalosuccinate**, which does not leave the enzyme surface but undergoes decarboxylation while still bound (step *d*; also see Eq. 13-45).

The second carbon to be removed from citrate is released as $CO_2$ through catalysis by the thiamin diphosphate dependent **oxidative decarboxylation** of **2-oxoglutarate** (α-ketoglutarate; Chapter 15). To complete the cycle the four-carbon succinyl unit of succinyl-CoA must be converted back to oxaloacetate through a pathway requiring two more oxidation steps: Succinyl-CoA is converted to free succinate (step *f*) followed by a β oxidation sequence (steps *g*–*i*; Figs. 10-6 and 17-4). Steps *e* and *f* accomplish a substrate-level phosphorylation (Fig. 15-16). Succinyl-CoA is an unstable thioester with a high group transfer potential. Therefore, step *f* could be accomplished by simple hydrolysis. However, this would be energetically wasteful. The cleavage of succinyl-CoA is coupled to synthesis of ATP in *E. coli* and higher plants and to GTP in mammals. Some of the succinyl-CoA formed in mitochondria is used in other ways, e.g., as in Eq. 17-6 and for biosynthesis of porphyrins.

## 2. Synthesis of the Regenerating Substrate Oxaloacetate

The primary substrate of the citric acid cycle is acetyl-CoA. Despite many references in the biochemical literature to substrates "entering" the cycle as oxaloacetate (or as one of the immediate precursors succinate, fumarate, or malate), *these compounds are not consumed* by the cycle but are completely regenerated; hence the term *regenerating substrate*, which can be applied to any of these four substances. A prerequisite for the operation of a catalytic cycle is that a regenerating substrate be readily available and that its concentration be increased if necessary to accommodate a more rapid rate of reaction of the cycle. Oxaloacetate can normally be formed in any amount needed for operation of the citric acid cycle from **PEP** or from **pyruvate**, both of these compounds being available from metabolism of sugars.

In bacteria and green plants **PEP carboxylase** (Eq. 13-53), a highly regulated enzyme, is responsible for synthesizing oxaloacetate. In animal tissues **pyruvate carboxylase** (Eq. 14-3) plays the same role. The latter enzyme is almost inactive in the absence of the allosteric effector acetyl-CoA. For this reason, it went undetected for many years. In the presence of high concentrations of acetyl-CoA the enzyme is fully activated and provides for synthesis of a high enough concentration of oxaloacetate to permit the cycle to function. Even so, the oxaloacetate concentration in mitochondria is low, only 0.1 to 0.4 x $10^{-6}$ M (10–40 molecules per mitochondrion), and is relatively constant.[65,79]

## 3. Common Features of Catalytic Cycles

The citric acid cycle is not only one of the most important metabolic cycles in aerobic organisms, including bacteria, protozoa, fungi, higher plants, and animals, but also *a typical catalytic cycle*. Other cycles also have one or more primary substrates and at least one regenerating substrate. Associated with every catalytic cycle there must be a metabolic pathway that provides for synthesis of the regenerating substrate. Although it usually needs to operate only slowly to replenish regenerating substrate lost in side reactions, the pathway also provides *a mechanism for the net biosynthesis of any desired quantity of any intermediate in the cycle*. Cells draw off from the citric acid cycle considerable amounts of oxaloacetate, 2-oxoglutarate, and succinyl-CoA for synthesis of other compounds. For example, aspartate and glutamate are formed directly from oxaloacetate and 2-oxoglutarate, respectively, by transamination (Eq. 14-25).[79a,b] Citrate itself is exported from mitochondria and used for synthesis of fatty acids. It is often stated that the citric acid cycle functions in biosynthesis, but when intermediates in the cycle are drawn off for synthesis the complete cycle does not operate. Rather, *the pathway for synthesis of the regenerating substrate, together with some of the enzymes of the cycle, is used to construct a biosynthetic pathway.*

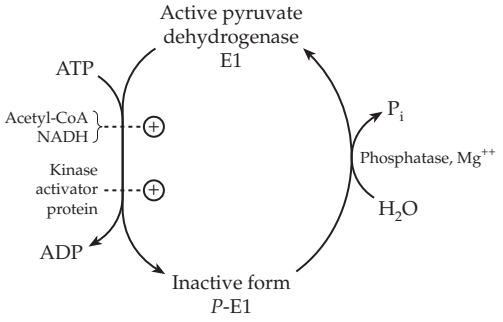
The word **amphibolic** is often applied to those metabolic sequences that are part of a catabolic cycle and at the same time are involved in a biosynthetic (anabolic) pathway. Another term, **anaplerotic**, is sometimes used to describe pathways for the synthesis of regenerating substrates. This word, which was suggested by H. L. Kornberg, comes from a Greek root meaning "filling up."[80]

## 4. Control of the Cycle

What factors determine the rate of oxidation by the citric acid cycle? As with most other important pathways of metabolism, several control mechanisms operate and different steps may become rate limiting under different conditions.[81] Factors influencing the flux through the cycle include (1) the rate of generation of acetyl groups, (2) the availability of oxaloacetate, and (3) the rate of reoxidation of NADH to $NAD^+$ in the electron transport chain. As indicated in Fig. 17-4, acetyl-CoA is a positive effector for conversion of pyruvate to oxaloacetate. Thus, acetyl-CoA "turns on" the formation of a substance required for its own further metabolism. However, when no pyruvate is available operation of the cycle may be impaired by lack of oxaloacetate. This may be the case when liver metabolizes high concentrations of ethanol. The latter is oxidized to acetate but it cannot provide oxaloacetate. Accumulating NADH reduces pyruvate to lactate, further interfering with formation of oxaloacetate.[82] In some individuals the accumulating acetyl units cannot all be oxidized in the cycle and instead are converted to the ketone bodies (Section A,4). A similar problem arises during metabolism of fatty acids by diabetic individuals with inadequate insulin. The accelerated breakdown of fatty acids in the liver overwhelms the system and results in ketosis, even though the oxaloacetate concentration remains normal.[83]

The rates of the oxidative steps in the citric acid cycle are limited by the rate of reoxidation of NADH and reduced ubiquinone in the electron transport chain which may sometimes be restricted by the availability of $O_2$. However, in aerobic organisms this rate is usually determined by the concentration of ADP and/or $P_i$ available for conversion to ATP in the oxidative phosphorylation process (Chapter 18). If catabolism supplies an excess of ATP over that needed to meet the cell's energy needs, the concentration of ADP falls to a low level, cutting off phosphorylation. At the same time, ATP is present in high concentration and acts as a feedback inhibitor for the catabolism of carbohydrates and fats. This inhibition is exerted at many points, a few of which are indicated in Fig. 17-4. Important sites of inhibition are the **pyruvate dehydrogenase complex**,[84–85a] which converts pyruvate into acetyl-CoA; **isocitrate dehydrogenase**,[86,86a] which converts isocitrate into 2-oxoglutarate; and **2-oxoglutarate dehydrogenase**.[87] The enzyme **citrate synthase**, which catalyzes the first reaction of the cycle, is also inhibited by ATP.[88,89]

Mitochondrial pyruvate dehydrogenase, which contains a 60-subunit icosohedral core of dihydrolipoyltransacylase (Fig. 15-14), is associated with three molecules of a two-subunit kinase as well as six molecules of a structural **binding protein** which contains a lipoyl group that can be reduced and acetylated by other subunits of the core protein. The binding protein is apparently essential to the functioning of the dehydrogenase complex but not through its lipoyl group.[90,91] The specific pyruvate dehydrogenase kinase is thought to be one of the most important regulatory proteins involved in controlling energy metabolism in most organisms.[92–92b] Phosphorylation of up to three specific serine hydroxyl groups in the thiamin-containing decarboxylase subunit (designated E1) converts the enzyme into an inactive form (Eq. 17-9). A specific phosphatase reverses the inhibition. The kinase is most active on enzymes whose core lipoyl (E2) subunits are reduced and acetylated, a condition favored by high ratios of [acetyl-CoA] to free [CoASH] and of [NADH] to [$NAD^+$]. Since the kinase inactivates the enzyme the effect is to decrease the pyruvate dehydrogenase action when the system becomes saturated and NADH and acetyl-CoA accumulate. Conversely, a high [pyruvate] inhibits the kinase and increases the action of the dehydrogenase complex. This system also permits various external signals to be felt. For example, insulin has a pronounced stimulatory effect on mitochondrial energy.[65,92,93] One way in which this may be accomplished is through stimulation of the pyruvate dehydrogenase phosphatase, as indicated in Eq. 17-9. A **kinase activator protein** (Eq. 17-9) may also respond to various external stimuli and may be inhibited by insulin.[92]




(17-9)

The activities of 2-oxoglutarate dehydrogenase,[94] and to a lesser extent of pyruvate and isocitrate dehydrogenases, are increased by increases in the free $Ca^{2+}$ concentration.[87] Calcium ions stimulate the phosphatase that dephosphorylates the deactivated phosphorylated pyruvate dehydrogenase and activate the other two dehydrogenases allosterically, increasing the affinities for the substrates.[87] Phosphorylation of the $NAD^+$-dependent isocitrate dehydrogenase also decreases its activity. In *E. coli* the isocitrate dehydrogenase kinase and a protein phosphatase exist as a bifunctional protein able to both deactivate the dehydrogenase and restore its activity.[86] For this organism, the decrease in activity forces substrate into the glyoxylate pathway (Section J,4) instead of the citric acid cycle.

## BOX 17-C USE OF ISOTOPIC TRACERS IN STUDY OF THE TRICARBOXYLIC ACID CYCLE

The first use of isotopic labeling in the study of the citric acid cycle and one of the first in the history of biochemistry was carried out by Harland G. Wood and C. H. Werkman in 1941.[a,b] The aim was to study the fermentation of glycerol by propionic acid bacteria, a process that was not obviously related to the citric acid cycle. Some succinate was also formed in

$$\text{Glycerol} \rightarrow \text{propionate}^- + H^+ + H_2O$$
$$\Delta G' = -69 \text{ kJ/mol}$$

the fermentation, and on the basis of simple measurements of the fermentation balance reported in 1938 it was suggested that $CO_2$ was incorporated into oxaloacetate, which was then reduced to succinate. As we now know, this is indeed an essential step in the propionic acid fermentation (Section F,3). At the time $^{14}C$ was not available but the mass spectrometer, newly developed by A. O. Nier, permitted the use of the stable $^{13}C$ as a tracer. Wood and Werkman constructed a thermal diffusion column and used it to prepare bicarbonate enriched in $^{13}C$ and also built a mass spectrometer. By 1941 it was established unequivocally that carbon dioxide was incorporated into succinate by the bacteria.[c]

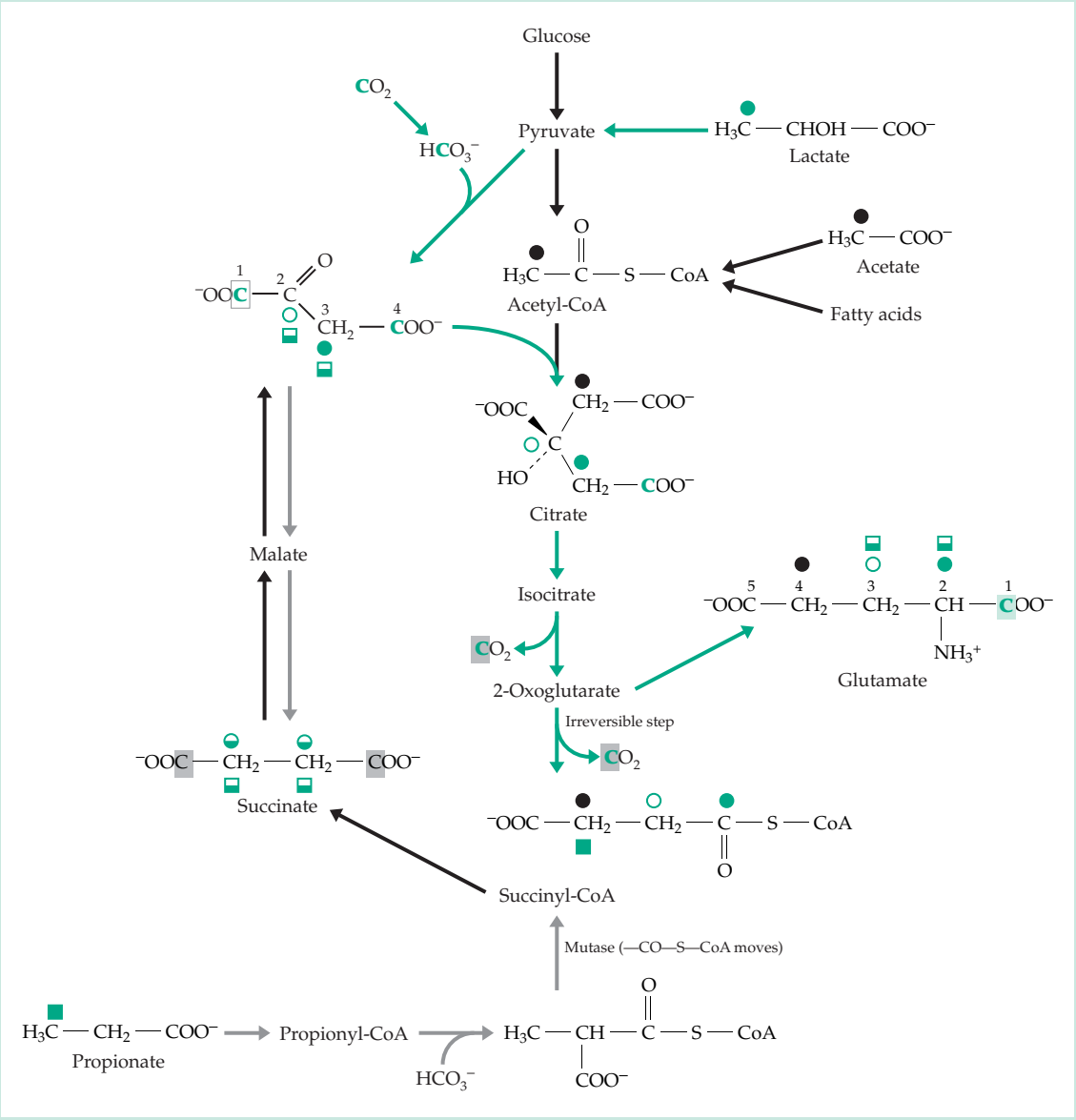
To test the idea that animal tissues could also incorporate $CO_2$ into succinate Wood examined the metabolism of a pigeon liver preparation to which malonate had been added to block succinate dehydrogenase (Box 10-B). Surprisingly, the accumulating succinate, which arose from oxalocetate via citrate, isocitrate, and 2-oxoglutarate (traced by green arrows in accompanying scheme), contained no $^{13}C$. Soon, however, it was shown that $CO_2$ was incorporated into the carboxyl group of 2-oxoglutarate that is adjacent to the carbonyl group. That carboxyl is lost in conversion to succinate (Fig. 10-6) explains the lack of $^{13}C$ in succinate. It is of historical interest that these observations were incorrectly interpreted by many of the biochemists of the time. They agreed that *citrate could not be a member of the tricarboxylic acid cycle*. Since citrate is a symmetric compound it was thought that any $^{13}C$ incorporated into citrate would be present in equal amounts in both terminal carboxyl groups. This would necessarily result in incorporation of $^{13}C$ into succinate. It was not until 1948 that Ogston popularized the concept that by binding with substrates at three points, enzymes were capable of asymmetric attack upon symmetric substrates.[d] In other words, an enzyme could synthesize citrate with the carbon atoms from acetyl-CoA occupying one of the two $-CH_2COOH$ groups surrounding the prochiral center. Later, the complete stereochemistry of the citric acid cycle was elucidated through the use of a variety of isotopic labels (see p. 704). Some of the results are indicated by the asterisks and daggers in the structures in Fig. 10-6.

The operation of the citric acid cycle in living cells, organs, and whole animals has also been observed using NMR and mass spectroscopy with $^{13}C$-containing compounds. For example, a heart can be perfused with a suitable oxygenated perfusion fluid[e] containing various $^{13}C$-enriched substrates such as [U-$^{13}C$]fatty acids, [2-$^{13}C$]acetate, [3-$^{13}C$]L-lactate, or [2,3-$^{13}C$]propionate.[e–k] NMR spectroscopy allows direct and repeated observation of the $^{13}C$ nuclei from a given substrate and its entry into a variety of metabolic pathways. Because of the high dispersion of chemical shift values for $^{13}C$ the NMR resonance for the isotope can be seen at each position within a single compound.

A compound that is especially easy to observe is glutamate. This amino acid, most of which is found in the cytoplasm, is nevertheless in relatively rapid equilibrium with 2-oxoglutarate of the citric acid cycle in the mitochondria. The accompanying scheme shows where isotopic carbon from certain compounds will be located *when it first enters* the citric acid cycle and traces some of the labels into glutamate. For example, uniformly enriched fatty acids will introduce label into the two atoms of the *pro-S* arm of citrate and into 4- and 5-positions of glutamate whereas [2-$^{13}C$]acetate will introduce label only into the C4 position as marked by ● in the scheme. In the NMR spectrum a singlet resonance at 32.4 ppm will be observed. However, as successive turns of the citric acid cycle occur the isotope will appear in increasing amounts in the adjacent 3-position of glutamate. They will be recognized readily by the appearance of a multiplet. The initial singlet will be flanked by a pair of peaks that arise from spin–spin coupling with the adjacent 3-$^{13}C$ of the [2,3-$^{13}C$]isotopomer (see accompanying figure). After longer periods of time the central resonance will weaken and the outer pair strengthen as the recycling occurs.

Metabolism with [U-$^{13}C$]fatty acids gives a labeling pattern similar to that with [2,3-$^{13}C$]acetate and it has been deduced that heart muscle normally metabolizes principally fatty acids for energy. What will happen to the glutamate C4 resonance if [3-$^{13}C$]lactate is added to the perfusion solution? It will enter both acetyl-CoA and oxaloacetate as indicated by ● in the following scheme. That will also intro-duce $^{13}C$ at C3 of glutamate. By looking at spectra at short times the relative amounts of lactate being oxidized via the cycle and that being converted

**BOX 17-C (continued)**

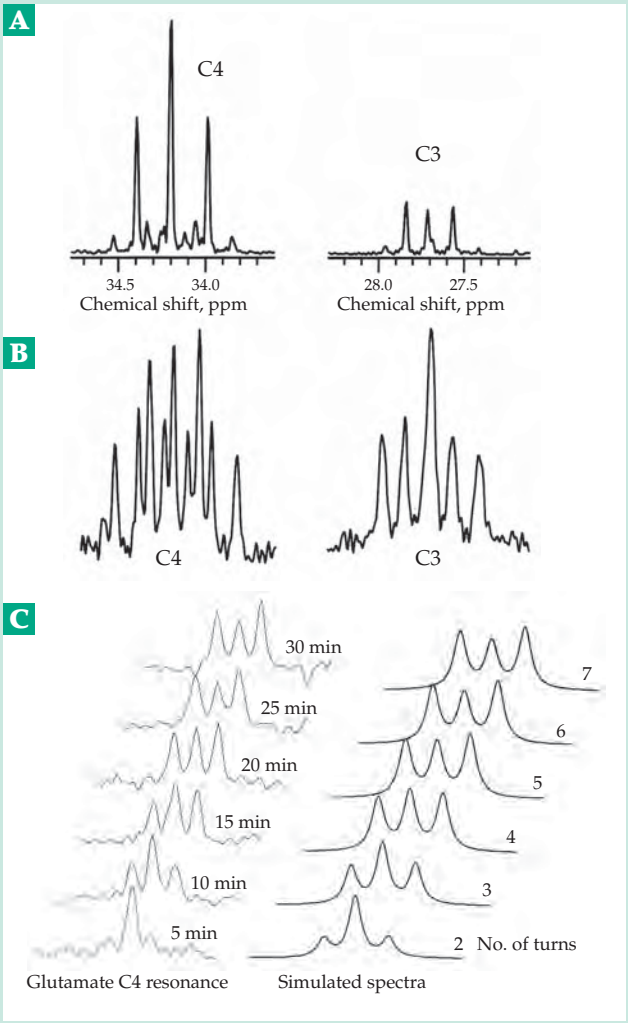



biosynthetically (anaplerotically) to glutamate can be estimated. There is a complication that has long been recognized. Oxaloacetate can be converted by exchange processes to succinate. Since succinate is symmetric the effect is to put 50% of the label into each of the central atoms of succinate (◒ in scheme). The exchange will then transfer label back into the C2 position of oxaloacetate (○) and through citric acid cycle reactions into C3 of glutamate. Now the C4 NMR resonance will contain an additional pair of peaks arising from spin–spin coupling with C2 but which will have a different coupling constant than that for coupling to C3.

If uniformly labeled [U-$^{13}$C]acetate is introduced the additional isotopomers, [3,4 - $^{13}$C]glutamate and [3,4,5 - $^{13}$C]glutamate, will be formed as will others with $^{13}$C in the C1 and C5 positions but which will not affect the C4 resonance. A total of nine lines will be seen as illustrated in curve *a* of the accompanying figure. We see that the multiplet patterns arising from mass isotopomers are complex, but they can be predicted accurately with a computer program.[f] Isotopomers of succinate have also been analyzed.[g]

It is also of interest to introduce $^{13}$C from propionate labeled in various positions. One of these is illustrated in the scheme. In this case the appearance of multiplets arising from [3,4-$^{13}$C] glutamate verifies the existence of end-to-end scrambling of the isotope in succinate. However, is the scrambling complete or are some molecules

## BOX 17-C USE OF ISOTOPIC TRACERS IN STUDY OF THE TRICARBOXYLIC ACID CYCLE (cont.)




A. $^{13}$C-NMR spectrum of extracts of Langendorff-perfused rat hearts perfused for 5 min with [1,2$^{13}$C] acetate, [3$^{13}$C]-lactate and glucose. Only the glutamate C4 (left) and C3 (right) resonances are shown. B. Spectrum after perfusion for 30 min. From Malloy *et al*.[r] C. The glutamate C4 resonance of an intact Langendorff-perfused rat heart supplied with 2 mM [2-$^{13}$C]acetate showing evolution of the multiplet as a function of time after introducing the label. The right panel shows glutamate C4 resonances generated by a computer simulation after turnover of citric acid cycle pools the indicated number of time. From Jeffrey *et al*.[f]

efficiently "channeled" through enzyme–enzyme complexes in such a way as to avoid scrambling? As shown in the scheme, full scrambling would give equal labeling of C2 and C3 of oxaloacetate and of glutamate. Experimentally greater labeling was seen at C3 than at C2 during the first few turns of the cycle suggesting that some channeling does occur.[e]

Isotopomer analysis can also be conducted by mass spectroscopy, which is more sensitive than NMR, using $^{13}$C[h,k,l] or $^{2}$H labeling.[j] Making use of a technique like that employed by Knoop (Box 10-A), a "chemical biopsy" can be performed on animals or on human beings, who may ingest gram quantities of sodium phenylacetate without harm. The phenylacetate is converted to an amide with glutamine (phenylacetylglutamine) which is excreted in the urine, from which it can easily be recovered for analysis.[l–n] This provides a non-invasive way of studying the operation of the citric acid cycle in the human body. Direct measurement on animal brains[o,p] and on human limbs or brain has also been accomplished by NMR spectroscopy[q] and may become more routine as instrumentation is improved.


[a] Wood, H. G. (1972) in *The Molecular Basis of Biological Transport* (Woessner, J. F., and Huijing, F., eds), pp. 1–54, Academic Press, New York
[b] Krampitz, L. O. (1988) *Trends Biochem. Sci.* **13**, 152–155
[c] Wood, H. G., Werkman, C. H., Hemingway, A., and Nier, A. O. (1941) *J. Biol. Chem.* **139**, 377–381
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[e] Sherry, A. D., Sumegi, B., Miller, B., Cottam, G. L., Gavva, S., Jones, J. G., and Malloy, C. R. (1994) *Biochemistry* **33**, 6268–6275
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[h] Des Rosiers, C., Di Donato, L., Comte, B., Laplante, A., Marcoux, C., David, F., Fernandez, C. A., and Brunengraber, H. (1995) *J. Biol. Chem.* **270**, 10027–10036
[i] Sherry, A. D., and Malloy, C. R. (1996) *Cell Biochem. Funct.* **14**, 259–268
[j] Yudkoff, M., Nelson, D., Daikhin, Y., and Erecinska, M. (1994) *J. Biol. Chem.* **269**, 27414–27420
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[m] Magnusson, I., Schumann, W. C., Bartsch, G. E., Chandramouli, V., Kumaran, K., Wahren, J., and Landau, B. R. (1991) *J. Biol. Chem.* **266**, 6975–6984
[n] Chervitz, S. A., and Falke, J. J. (1995) *J. Biol. Chem.* **270**, 24043–24053
[o] Hyder, F., Chase, J. R., Behar, K. L., Mason, G. F., Siddeek, M., Rothman, D. L., and Shulman, R. G. (1996) *Proc. Natl. Acad. Sci. U.S.A.* **93**, 7612–7617
[p] Cerdan, S., Künnecke, B., and Seelig, J. (1990) *J. Biol. Chem.* **265**, 12916–12926
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[r] Malloy, C. R., Thompson, J. R., Jeffery, F. M. H., and Sherry, A. D. (1990) *Biochemistry* **29**, 6756–6761

Acting to counteract any drop in ATP level, accumulating ADP acts as a positive effector for isocitrate dehydrogenases.

Another way in which the phosphorylation state of the adenylate system can regulate the cycle depends upon the need for GDP in step *f* of the cycle (Fig. 17-4). Within mitochondria, GTP is used largely to reconvert AMP to ADP. Consequently, formation of GDP is promoted by AMP, a compound that arises in mitochondria from the utilization of ATP for activation of fatty acids (Eq. 13-44) and activation of amino acids for protein synthesis (Eq. 17-36).

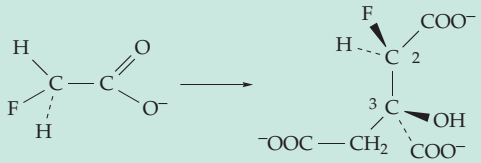
In *E. coli* and some other bacteria ATP does not inhibit citrate synthase but NADH does; the control is via the redox potential of the $NAD^+$ system rather than by the level of phosphorylation of the adenine nucleotide system.[95] Succinic dehydrogenase may be regulated by the redox state of ubiquinone (Chapter 15). Another mechanism of regulation may be the formation of specific protein–protein complexes between enzymes catalyzing reactions of the cycle.[96–97a] This may permit one enzyme to efficiently have a product of its action transferred to the enzyme catalyzing the next step in the cycle.

## 5. Catabolism of Intermediates of the Citric Acid Cycle

Acetyl-CoA is the only substrate that can be completely oxidized to $CO_2$ by the reaction of the citric acid cycle alone. Nevertheless, cells must sometimes

## BOX 17-D FLUOROACETATE AND "LETHAL SYNTHESIS"

Among the most deadly of simple compounds is sodium fluoroacetate. The $LD_{50}$ (the dose lethal for 50% of animals receiving it) is only 0.2 mg/kg for rats, over tenfold less than that of the nerve poison diisopropylphosphofluoridate (Chapter 12).[a,b] Popular, but controversial, as the rodent poison "1080," fluoroacetate is also found in the leaves of several poisonous plants in Africa, Australia, and South America. Surprisingly, difluoroacetate $HCF_2–COO^-$ is nontoxic and biochemical studies reveal that monofluoroacetate has no toxic effect on cells until it is converted metabolically in a "lethal synthesis" to 2*R*,3*R*-2-fluorocitrate, which is a competitive inhibitor of aconitase (aconitate hydratase, Eq. 13-17).[b–g] This fact was difficult to understand since citrate formed by the reaction of fluorooxaloacetate and acetyl-CoA has only weak inhibitory activity toward the same enzyme. Yet, it is the fluorocitrate formed from fluorooxaloacetate that contains a fluorine atom at a site that is attacked by aconitase in the citric acid cycle.




2*R*,3*R* 2-Fluoroacetate

The small van der Waals radius of fluorine (0.135 nm), comparable to that of hydrogen (0.12 nm), is often cited as the basis for the ability of fluoro compounds to "deceive" enzymes. However, the high electronegativity and ability to enter into hydrogen bonds may make F more comparable to –OH in metabolic effects. In the case of fluorocitrate it was proposed that the inhibitory isomer binds in the "wrong way" to aconitase in such a manner that the fluorine atom is coordinated with the ferric ion at the catalytic center.[c] However, 2*R*,3*R*-2-fluorocitrate is a simple competitive inhibitor of aconitase but an irreversible poison. It is especially toxic to nerves and also appears to affect mitochondrial membranes. Therefore, this poison may affect some other target, such as a citrate transporter.[d] Fluoroacetate is only one of many known naturally occurring fluorine compounds.[c]

Another example of lethal synthesis is seen in the use of 5-fluorouracil in cancer therapy (Box 15-E). In this compound and in many other fluorine-containing inhibitors the F atom replaces the H atom that is normally removed as $H^+$ in the enzymatic reaction. The corresponding $F^+$ cannot be formed.[h] Because of the high electronegativity of fluorine a C–F bond is polarized: $C^{\delta+}–F^{\delta-}$. This may have very large effects on reactivity at adjacent positions. For example, the reactivity of 2-fluoroglycosyl groups toward glycosyl transfer is decreased by several orders of magnitude (p. 597).


[a] Gibble, G. W. (1973) *J. Chem. Educ.* **50**, 460–462
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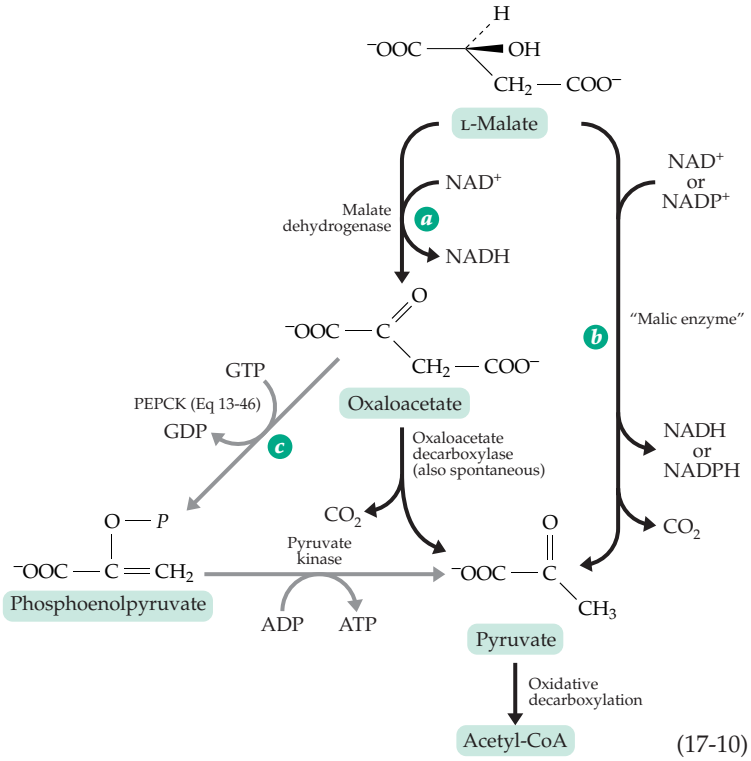
oxidize large amounts of one of the compounds found in the citric acid cycle to $CO_2$.[98,99] For example, bacteria subsisting on succinate as a carbon source must oxidize it for energy as well as convert some of it to carbohydrates, lipids, and other materials. Complete combustion of *any citric acid cycle intermediate* can be accomplished by conversion to malate followed by oxidation of malate to oxaloacetate (Eq. 17-10, step *a*) and decarboxylation (β cleavage) to pyruvate, or (Eq. 17-10, step *b*) oxidation and decarboxylation of malate by the **malic enzyme** (Eq. 13-45) without free oxaloacetate as an intermediate. Pathway *b* is probably the most important. It is catalyzed by two different malic enzymes present in animal mitochondria. One is specific for $NADP^+$ while the other reacts with $NAD^+$ as well.[100,101] They both have complex regulatory properties. For example, the less specific $NAD^+$-utilizing enzyme is allosterically inhibited by ATP but is activated by fumarate, succinate, or isocitrate.[100] Thus, accumulation of citric acid cycle intermediates "turns on" the malic enzyme, allowing the excess to leave the cycle and reenter as acetyl groups. Since the Michaelis constant for malate is high, this will not happen unless malate accumulates, signaling a need for acetyl-CoA. The $NADP^+$-dependent enzyme is activated by a high concentration of free CoA and is inhibited by NADH. Perhaps when glycolysis becomes slow the free CoA level rises and turns on malate oxidation.[101] On the other hand, rapid glycolysis increases the NADH concentration which inhibits the malic enzyme. The result is a buildup of the oxaloacetate concentration and an increase in activity of the citric acid cycle. The malic enzymes are also present in the cytoplasm, where one of them functions as part of an NADPH-generating cycle (Eq. 17-46).




(17-10)

## D. Oxidative Pathways Related to the Citric Acid Cycle

In this section we will consider some other catalytic cycles as well as some noncyclic pathways of oxidation of one- and two-carbon substrates that are utilized by microorganisms.

### 1. The γ-Aminobutyrate Cycle

A modification of the citric acid cycle which involves glutamate and gamma (γ) aminobutyrate (GABA) has an important function in the brain (Fig. 17-5). Both glutamate and γ-aminobutyrate occur in high concentrations in brain (10 and 0.8 mM, respectively). Both are important neurotransmitters, γ-aminobutyrate being a principal neuronal inhibitory substance[102,103] (Chapter 30). In the γ-aminobutyrate cycle acetyl-CoA and oxaloacetate are converted into citrate (step *a*) in the usual way and the citrate is then converted into 2-oxoglutarate. The latter is transformed to L-glutamate either by direct amination (*b*) or by transamination (*c*), the amino donor being γ-aminobutyrate.

γ-Aminobutyrate is formed by decarboxylation of glutamate (Fig. 17-5, step *d*)[104] and is catabolized via transamination (step *e*)[105] to succinic semialdehyde, which is oxidized to succinate[106] and oxaloacetate. The two transamination steps in the pathways may be linked, as indicated in Fig. 17-5, to form a complete cycle that parallels the citric acid cycle but in which 2-oxoglutarate is oxidized to succinate via glutamate and γ-aminobutyrate. No thiamin diphosphate is required, but 2-oxoglutarate is reductively aminated to glutamate. The cycle is sometimes called the **γ-aminobutyrate shunt**, and it plays a significant role in the overall oxidative processes of brain tissue. This pathway is also prominent in green plants.[107–109] For example, under anaerobic conditions the radish *Raphanus sativus* accumulates large amounts of γ-aminobutyrate.[110] Most animal tissues contain very little γ-aminobutyrate, although it has been found in the oviducts of rats at concentrations that exceed those in the brain.[111]

### 2. The Dicarboxylic Acid Cycle

Some bacteria can subsist solely on glycolate, glycine, or oxalate, all of which

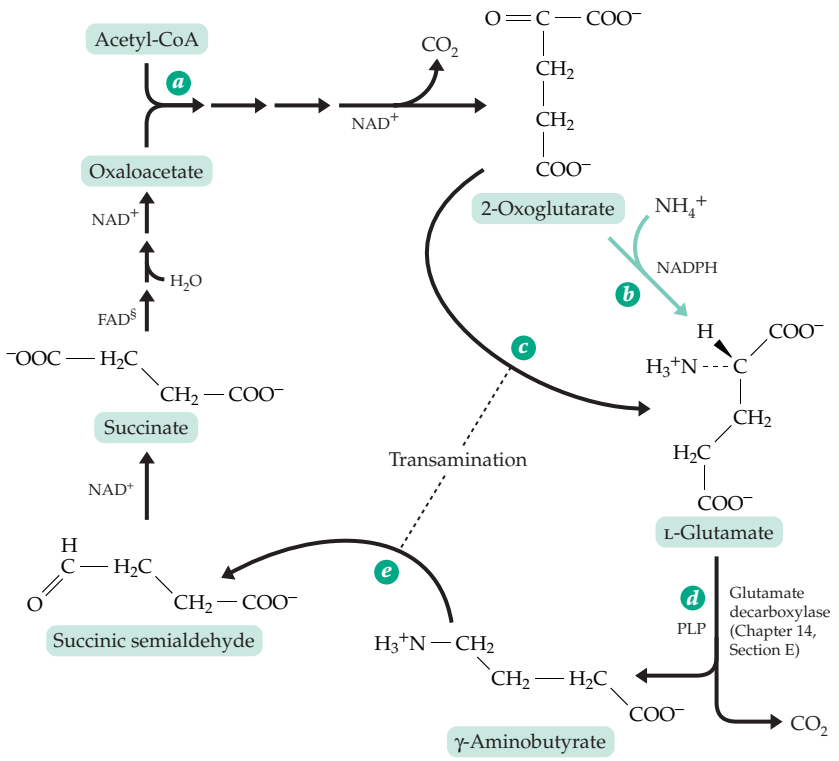



**Figure 17-5** Reactions of the γ-aminobutyrate (GABA) cycle.

are converted to glyoxylate (Eq. 17-11). Glyoxylate is oxidized to $CO_2$ and water to provide energy to the bacteria and is also utilized for biosynthetic purposes. The energy-yielding process is found in the **dicarboxylic acid cycle** (Fig. 17-6), which catalyzes the complete oxidation of glyoxylate. Four hydrogen atoms are removed with generation of two molecules of NADH which can be oxidized by the respiratory chain to provide energy.[112,113] In the dicarboxylic acid cycle glyoxylate is the principal substrate and acetyl-CoA is the regenerating substrate rather than the principal substrate as it is for the citric acid cycle.

The logic of the dicarboxylic acid cycle is simple. Acetyl-CoA contains a potentially free carboxyl group. After the acetyl group of acetyl-CoA has been condensed with glyoxylate and the resulting hydroxyl group has been oxidized, the free carboxyl group appears in oxaloacetate in a position β to the carbonyl group. The carboxyl

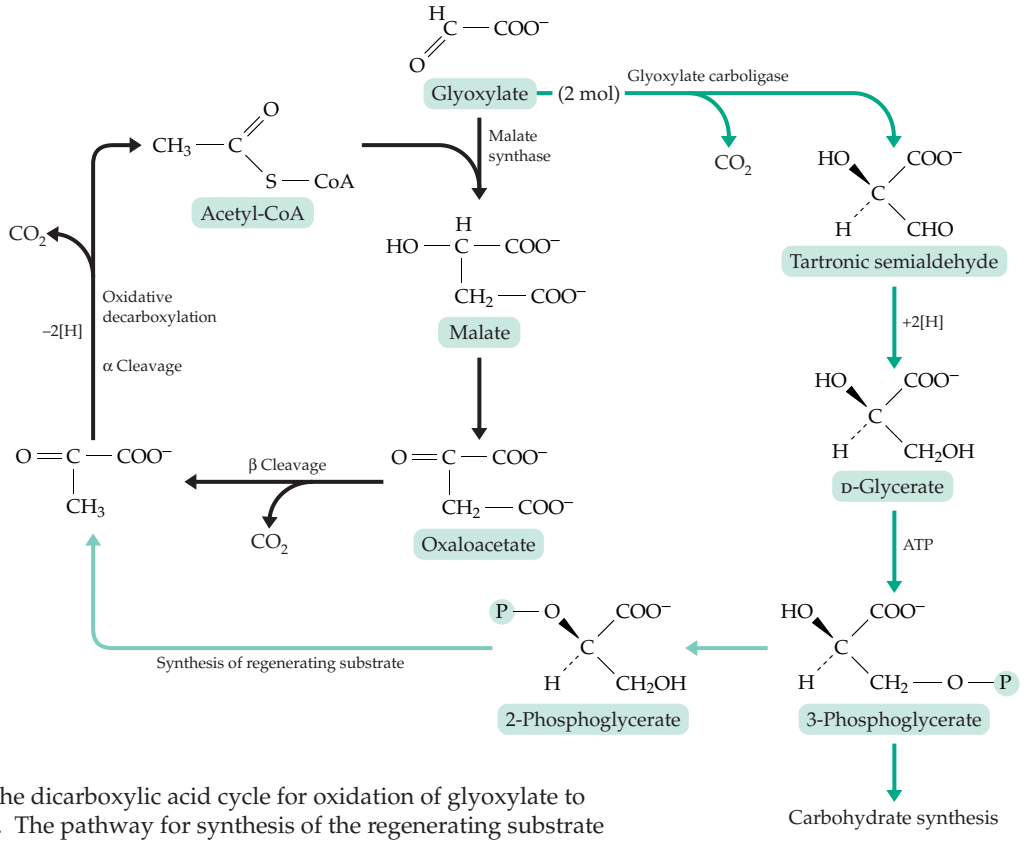
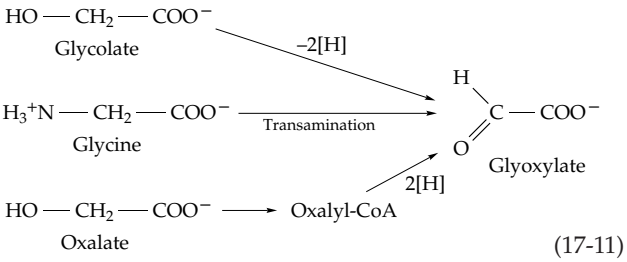



**Figure 17-6** The dicarboxylic acid cycle for oxidation of glyoxylate to carbon dioxide. The pathway for synthesis of the regenerating substrate is indicated by green lines. This pathway is also needed for synthesis of carbohydrates and all other cell constituents.




(17-11)

donated by the glyoxylate is still in the α position. A consecutive β cleavage and an oxidative α cleavage release the two carboxyl groups as carbon dioxide to reform the regenerating substrate. The cycle is simple and efficient. Like the citric acid cycle, it depends upon thiamin diphosphate, without which the α cleavage would be impossible. Comparing the citric acid cycle (Fig. 17-2) with the simpler dicarboxylic acid cycle, we see that in the former the initial condensation product citrate contains a hydroxyl group attached to a tertiary carbon atom. With no adjacent hydrogen it is impossible to oxidize it directly to the carbonyl group which is essential for subsequent chain cleavage; hence the dependence on aconitase to shift the OH to an adjacent carbon. Both cycles involve oxidation of a hydroxy acid to a ketone followed by β cleavage and oxidative α cleavage. In the citric acid cycle additional oxidation steps are needed to convert succinate back to oxaloacetate, corresponding to the fact that the citric acid cycle deals with a more reduced substrate than does the dicarboxylic acid cycle.

The synthetic pathway for the regenerating substrate of the dicarboxylic acid cycle is quite complex. Two molecules of glyoxylate undergo α condensation with decarboxylation by glyoxylate carboligase[114] (see also Chapter 14, Section D) to form **tartronic semialdehyde**. The latter is reduced to D-glycerate, which is phosphorylated to 3-phosphoglycerate and 2-phosphoglycerate. Since the phosphoglycerates are carbohydrate precursors, this **glycerate pathway** provides the organisms with a means for synthesis of carbohydrates and other complex materials from glyoxylate alone. At the same time, 2-phosphoglycerate can be converted to pyruvate and the pyruvate, by oxidative decarboxylation, to the regenerating substrate acetyl-CoA.

## E. Catabolism of Sugars

In most sugars each carbon atom bears an oxygen atom which facilitates chemical attack by oxidation at any point in the carbon chain. Every sugar contains a potentially free aldehyde or ketone group, and the carbonyl function can be moved readily to adjacent positions by isomerases. Consequently, aldol cleavage is also possible at many points. For these reasons, the metabolism of carbohydrates is complex and varied. A sugar chain can be cut in several places giving rise to a variety of metabolic pathways. However, in the energy economy of most organisms, including human beings, the **Embden–Meyerhof–Parnas** or **glycolysis pathway** by which hexoses are converted to pyruvate (Fig. 17-7) stands out above all others. We have already considered this pathway, which is also outlined in Figs. 10-2 and 10-3. Some history and additional important details follow.

### 1. The Glycolysis Pathway

The discovery of glycolysis followed directly the early observations of Buchner and of Harden and Young on fermentation of sugar by yeast juice (p. 767). Another line of research, the study of muscle, soon converged with the investigations of alcoholic fermentation. Physiologists were interested in the process by which an isolated muscle could obtain energy for contraction in the absence of oxygen. It was shown by A. V. Hill that glycogen was converted to lactate to supply the energy, and Otto Meyerhof later demonstrated that the chemical reactions were related to those of alcoholic fermentation. The establishment of the structures and functions of the pyridine nucleotides in 1934 (Chapter 15) coincided with important studies by G. Embden in Frankfurt and of J. K. Parnas in Poland. The sequence of reactions in glycolysis soon became clear. All of the 15 enzymes catalyzing the individual steps in the sequence have been isolated and crystallized and are being studied in detail.[115]

***Formation of pyruvate.*** The conversion of glucose to pyruvate requires ten enzymes (Fig. 17-7), and the sequence can be divided into four stages: preparation for chain cleavage (reactions *1*–*3*), cleavage and equilibration of triose phosphates (reactions *4* and *5*), oxidative generation of ATP (reactions *6* and *7*), and conversion of 3-phosphoglycerate to pyruvate (reactions *8*–*10*).

In preparation for chain cleavage, free glucose is phosphorylated to glucose 6-phosphate by ATP under the action of hexokinase (reaction *1*). Glucose 6-phosphate can also arise by cleavage of a glucosyl unit from glycogen by the consecutive action of glycogen phosphorylase (reaction 1*a*) and phosphoglucomutase, which transfers a phospho group from the oxygen at C-1 to that at C-6 (reaction 1*b*) (see also Eq. 12-39 and associated discussion of the mechanism of this enzyme). Why do cells attach phospho groups to sugars to initiate metabolism of the sugars? Four reasons can be given:

(a) The phospho group constitutes an electrically charged handle for binding the sugar phosphate to enzymes.

(b) There is a kinetic advantage in initiating a reaction sequence with a highly irreversible reaction

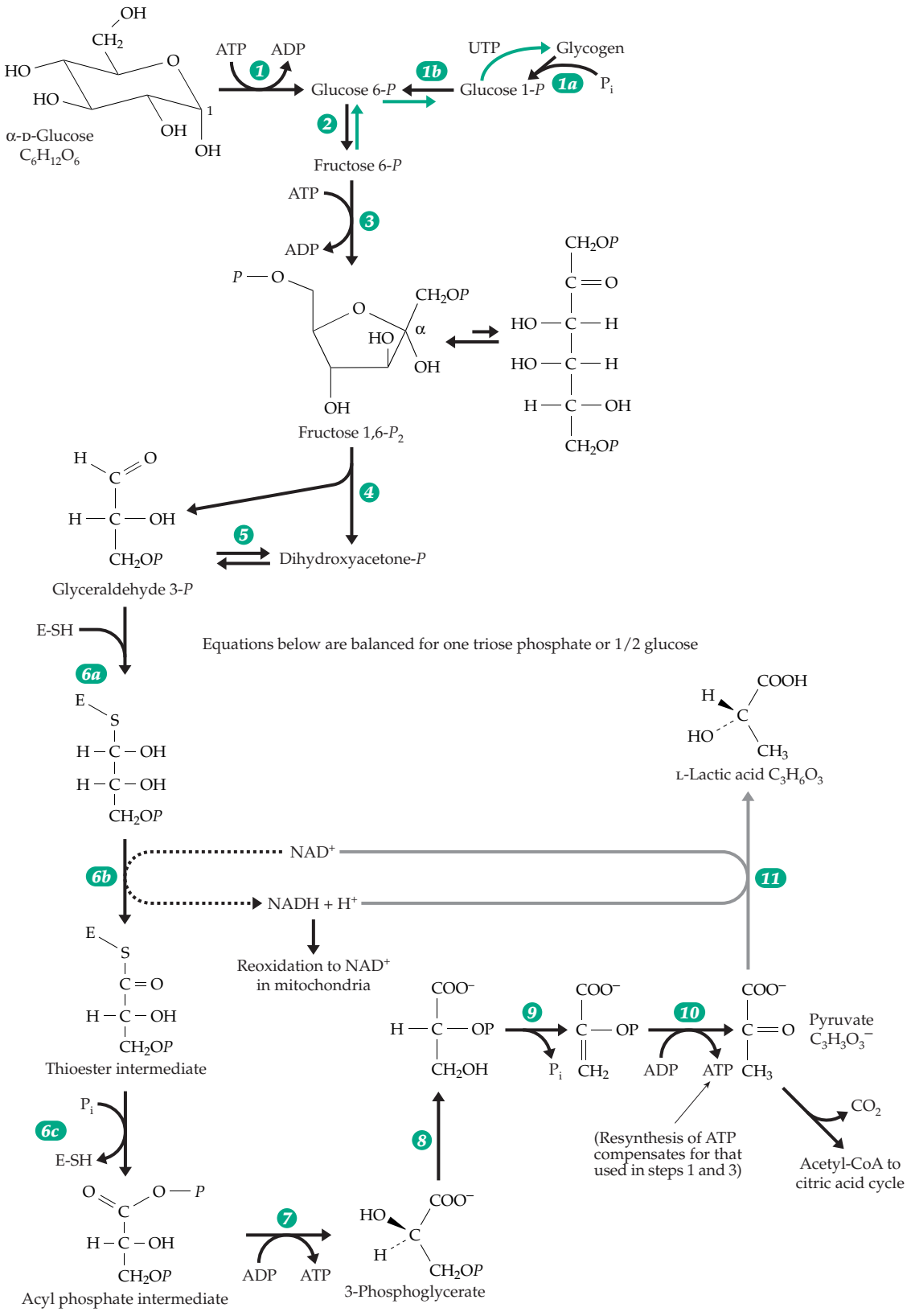



**Figure 17-7** Outline of the glycolysis pathway by which hexoses are broken down to pyruvate. The ten enzymes needed to convert D-glucose to pyruvate are numbered. The pathway from glycogen using glycogen phosphorylase is also included, as is the reduction of pyruvate to lactate (step *11*). Steps *6a*–*7*, which are involved in ATP synthesis via thioester and acyl phosphate intermediates, are emphasized. See also Figures 10-2 and 10-3, which contain some additional information.

such as the phosphorylation of glucose.

(c) Phosphate esters are unable to diffuse out of cells easily and be lost.

(d) There is at least a possibility that the phospho groups may function in catalysis.

Reaction *2* of Fig. 17-7 is a simple isomerization that moves the carbonyl group to C-2 so that β cleavage to two three-carbon fragments can occur. Before cleavage a second phosphorylation (reaction *3*) takes place to form fructose 1,6-bisphosphate. This ensures that when fructose bisphosphate is cleaved by aldolase each of the two halves will have a phosphate handle. This second priming reaction (reaction *3*) is the first step in the series that is unique to glycolysis. The catalyst for the reaction, **phosphofructokinase**, is carefully controlled, as discussed in Chapter 11 (see Fig. 11-2).

Fructose bisphosphate is cleaved by action of an aldolase (reaction *4*) to give glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. These two triose phosphates are then equilibrated by triose phosphate isomerase (reaction *5*; see also Chapter 13). As a result, both halves of the hexose can be metabolized further via glyceraldehyde 3-*P* to pyruvate. The oxidation of glyceraldehyde 3-*P* to the corresponding carboxylic acid, 3-phosphoglyceric acid (Fig. 17-7, reactions *6* and *7*), is coupled to synthesis of a molecule of ATP from ADP and $P_i$. This means that two molecules of ATP are formed per hexose cleaved, and that two molecules of $NAD^+$ are converted to NADH in the process.

The conversion of 3-phosphoglycerate to pyruvate begins with transfer of a phospho group from the C-3 to the C-2 oxygen (reaction *8*) and is followed by dehydration through an α, β elimination catalyzed by **enolase** (reaction *9*). The product, phosphoenolpyruvate (PEP), has a high group transfer potential. Its phospho group can be transferred easily to ADP via the action of the enzyme **pyruvate kinase**, to leave the enol of pyruvic acid which is spontaneously converted to the much more stable pyruvate ion (see Eq. 7-59). Because two molecules of PEP are formed from each glucose molecule, the process provides for the recovery of the two molecules of ATP that were expended in the initial formation of fructose 1,6-bisphosphate from glucose. Several isoenzyme forms exist in mammals. Most of these are allosterically activated by fructose 1,6-bisphosphate.[115a,b] However, the enzyme from trypanosomes is activated by fructose 2,6-$P_2$.[115c]

***The further metabolism of pyruvate.*** In the aerobic metabolism that is characteristic of most tissues of our bodies, pyruvate is oxidatively decarboxylated to acetyl-CoA, which can then be completely oxidized in the citric acid cycle (Fig. 17-4). The NADH produced in reaction *6* of Fig. 17-7, as well as in the oxidative decarboxylation of pyruvate and in subsequent reactions of the citric acid cycle, is reoxidized in the electron transport chain of the mitochondria as described in detail in Chapter 18 (see Fig. 18-5). An important alternative fate of pyruvate is to enter into fermentation reactions. For example, the enzyme lactate dehydrogenase (Fig. 17-7, reaction *11*) catalyzes reduction by NADH of pyruvate to L-lactate, or, for some bacteria, to D-lactate. This reaction can be coupled to the NADH-producing reaction *6* to give a balanced process by which glucose is fermented to lactic acid in the absence of oxygen (see also Eq. 10-3). In a similar process, yeast cells decarboxylate pyruvate (α cleavage) to acetaldehyde which is reduced to ethanol using the NADH produced in reaction *6*. These fermentation reactions are summarized in Fig. 10-3 and, along with many others, are discussed further in Section F of this chapter.

## 2. Generation of ATP by Substrate Oxidation

The formation of ATP from ADP and $P_i$ is a vital process for all cells. It is usually referred to as "phosphorylation" and includes **oxidative phosphorylation** associated with the passage of electrons through an electron transport chain—usually in mitochondria; **photosynthetic phosphorylation**, a similar process occurring in chloroplasts under the influence of light; and **substrate-level phosphorylation**. Only the last is fully understood chemically. The dehydrogenation of glyceraldehyde 3-*P* and the accompanying ATP formation (reactions *6* and *7*, Fig. 17-7; Fig. 15-6) is the best known example of substrate-level phosphorylation and is tremendously important for yeasts and other microorganisms that live anaerobically. They depend upon this one reaction for their entire supply of energy. The conversion of glucose either to lactate or to ethanol and $CO_2$ is accompanied by a net synthesis of only two molecules of ATP and it is most logical to view these as arising from oxidation of glyceraldehyde 3-*P*. The formation of ATP from PEP and ADP in reaction *10* of Fig. 17-7 can be regarded as the recapturing of ATP "spent" in the priming reactions of steps *1* and *3*. With a gain of only two molecules of ATP for each molecule of hexose fermented, it is not surprising that yeast must ferment enormous amounts of sugar to meet its energy needs.

Each glucose unit of glycogen stored in our bodies can be converted to pyruvate with an apparent net gain of *three* molecules of ATP. However, two molecules of ATP were needed for the initial synthesis of each hexose unit of glycogen (Fig. 12-2). Therefore, the overall net yield for fermentation of stored polysaccharide is still only two ATP per hexose. The fermentation of glycogen accounts for the very rapid generation of lactic acid during intense muscular activity. However, in most circumstances within aerobic tissues reoxidation of NADH occurs via the electron transport chain of mitochondria with a much higher yield of ATP. Substrate-

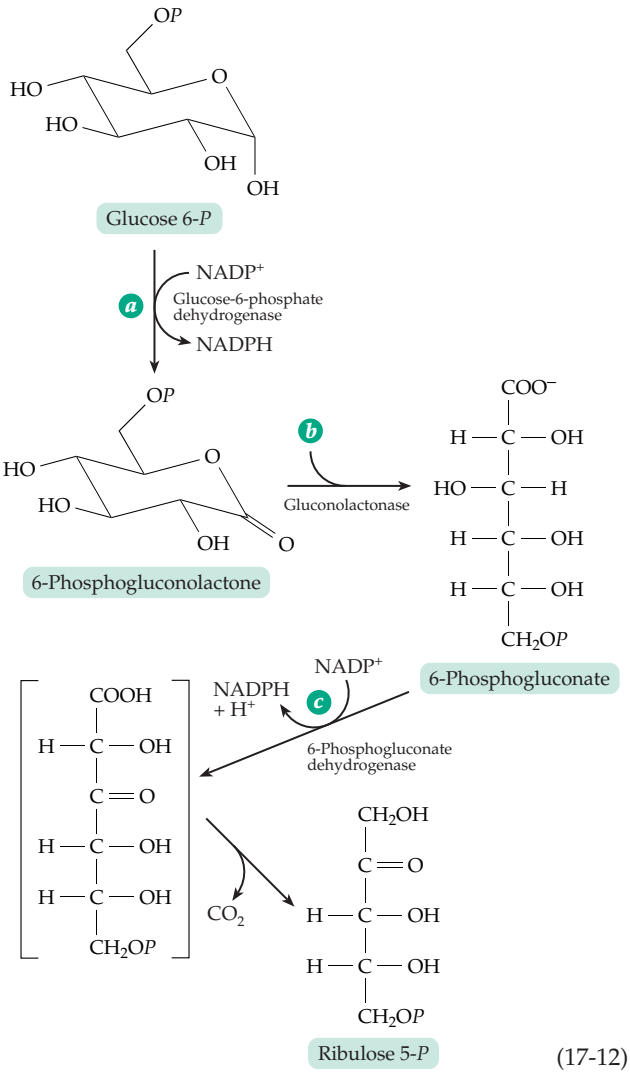
level phosphorylation can also follow oxidative decarboxylation of an α-oxoacid. For example, in the citric acid cycle GTP is formed following oxidative decarboxylation of 2-oxoglutarate (Fig. 17-4, steps *e* and *f*).

## 3. The Pentose Phosphate Pathways

A second way of cleaving glucose 6-phosphate utilizes sequences involving the five-carbon pentose sugars. They are referred to as **pentose phosphate pathways**, the phosphogluconate pathway, or the hexose monophosphate shunt. Historically, the evidence for such routes dates from the experiments of Warburg on the oxidation of glucose 6-*P* to 6-phosphogluconate (Chapter 15). For many years the oxidation remained an enzymatic reaction without a defined pathway. However, it was assumed to be part of an alternative method of degradation of glucose. Supporting evidence was found in the observation that tissues continue to respire in the presence of a high concentration of fluoride ion, a known inhibitor of the enolase reaction and capable of almost completely blocking glycolysis. Some tissues, e.g., liver, are especially active in respiration through this alternative pathway, whose details were elucidated by Horecker and associates.[116,117] We now know that the pentose phosphate pathways are multiple as well as multipurpose. They function in catabolism and also, when operating in the reverse direction, as a **reductive pentose phosphate pathway** that lies at the heart of the sugar-forming reactions of photosynthesis.

The oxidative pentose pathway provides *a means for cutting the chain of a sugar molecule one carbon at a time*, with the carbon removed appearing as $CO_2$. The enzymes required can be grouped into three distinct systems, all of which are found in the cytosol of animal cells: (i) a dehydrogenation–decarboxylation system, (ii) an isomerizing system, and (iii) a sugar rearrangement system. The dehydrogenation–decarboxylation system cleaves glucose 6-*P* to $CO_2$ and the pentose phosphate, ribulose 5-*P* (Eq. 17-12). Three enzymes are required, the first being glucose 6-*P* dehydrogenase[117a] (Eq. 17-12, step *a*; see also Eq. 15-10). The immediate product, a lactone, undergoes spontaneous hydrolysis. However, the action of **gluconolactonase** (Eq. 17-12, step *b*) causes a more rapid ring opening. A second dehydrogenation is catalyzed by **6-phosphogluconate dehydrogenase** (Eq. 17-12, step *c*),[117b] and this reaction is immediately followed by a β decarboxylation catalyzed by the same enzyme (as in Eq. 13-45). The value of $\Delta G°$ for oxidation of glucose 6-*P* to ribulose 5-*P* by $NADP^+$ according to Eq. 17-12 is $-30.8$ kJ mol$^{-1}$, a negative enough value to drive the [NADPH]/[$NADP^+$] ratio to an equilibrium value of over 2000 at a $CO_2$ tension of 0.05 atm.

The isomerizing system, consisting of two enzymes,

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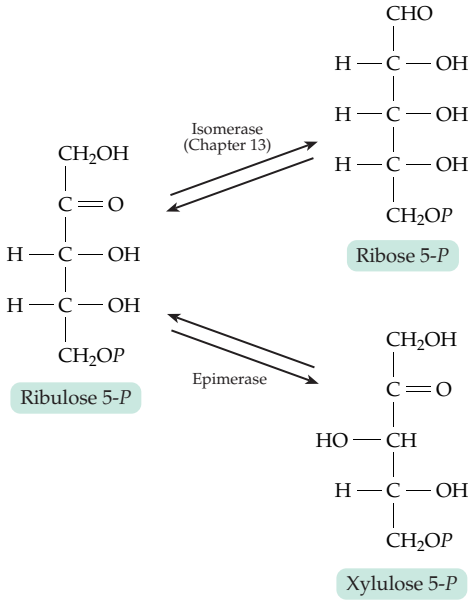
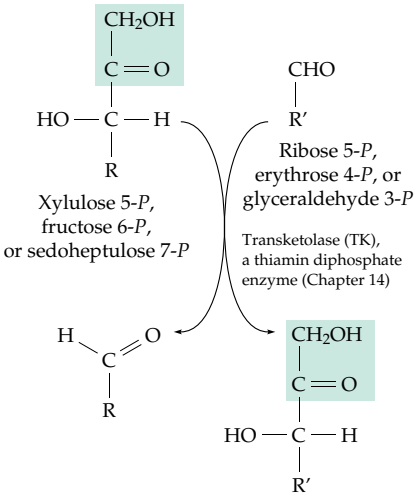
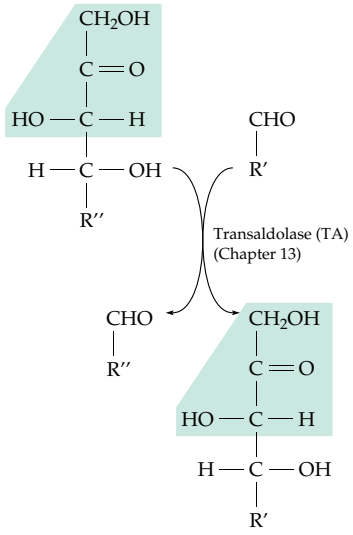



(17-13)




(17-14)




(17-15)

interconverts three pentose phosphates (Eq. 17-13). As a consequence the three compounds exist as an equilibrium mixture. Both xylulose 5-*P* and ribose 5-*P* are needed for further reactions in the pathways.

The ingenious sugar rearrangement system uses two enzymes, **transketolase** and **transaldolase**. Both catalyze chain cleavage and transfer reactions (Eqs. 17-14 and 17-15) that involve the same group of substrates. These enzymes use the two basic types of C–C bond cleavage, adjacent to a carbonyl group (α) and one carbon removed from a carbonyl group (β). Both types are needed in the pentose phosphate pathways just as they are in the citric acid cycle. The enzymes of the pentose phosphate pathway are found in the cytoplasm of both animal and plant cells.[117c] Mammalian cells appear to have an additional set that is active in the endoplasmic reticulum and plants have another set in the chloroplasts.[117c]

***An oxidative pentose phosphate cycle.*** Putting the three enzyme systems together, we can form a cycle that oxidizes hexose phosphates. Three carbon atoms are chopped off one at a time (Fig. 17-8A) leaving a three-carbon triose phosphate as the product. Since the dehydrogenation system works only on glucose 6-*P*, a part of the sugar rearrangement system must be utilized between each of the three oxidation steps. Notice that a $C_5$ unit (ribose 5-*P*) is used in the first reaction with transketolase but is regenerated at the end of the sequence. This $C_5$ unit is the regenerating substrate for the cycle. As indicated by the dashed arrows, it is formed readily in any quantity needed by oxidation of glucose 6-*P*. Before the $C_5$ unit that is formed in each oxidation step can be processed by the sugar rearrangement reactions, it must be isomerized[117c,118,118a,b] from ribulose 5-*P* to xylulose 5-*P*; before the $C_5$ unit, produced at the end of the sequence in Fig. 17-8, can be reutilized as a regenerating substrate, it must be isomerized to ribose 5-*P*. Thus, the cycle is quite complex. The same $C_5$ substrates appear at several points in Fig. 17-8A and substrates from different parts of the cycle become scrambled and the pathway does not degrade all the hexose molecules in a uniform manner. For this reason, Zubay described the pentose phosphate pathways as a "swamp."[119]

The oxidative pentose phosphate cycle is often presented as a means for complete oxidation of hexoses to $CO_2$. For this to happen the $C_3$ unit indicated as the product in Fig. 17-8A must be converted (through the action of aldolase, a phosphatase, and hexose phosphate isomerase) back to one-half of a molecule of glucose-6-*P* which can enter the cycle at the beginning. On the other hand, alternative ways of degrading the $C_3$ product glyceraldehyde-*P* are available. For example, using glycolytic enzymes, it can be oxidized to pyruvate and to $CO_2$ via the citric acid cycle.

As a general rule, $NAD^+$ is associated with catabolic reactions and it is somewhat unusual to find $NADP^+$ acting as an oxidant. However, in mammals the enzymes of the pentose phosphate pathway are specific for $NADP^+$. The reason is thought to lie in the need of NADPH for biosynthesis (Section I). On this basis, the occurrence of the pentose phosphate pathway in tissues having an unusually active biosynthetic function (liver and mammary gland) is understandable. In these tissues the cycle may operate as indicated in Fig. 17-8A with the $C_3$ product also being used in biosynthesis. Furthermore, any of the products from $C_4$ to $C_7$ may be withdrawn in any desired amounts without disrupting the smooth operation of the cycle. For example, the $C_4$ intermediate **erythrose 4-*P*** is required in synthesis of aromatic amino acids by bacteria and plants (but not in animals). **Ribose 5-*P*** is needed for formation of several amino acids and of nucleic acids by all organisms. In some circumstances the formation of ribose 5-*P* may be the only essential function for the pentose phosphate pathway.[120]

Several studies of the metabolism of isotopically labeled glucose[121–122a] have been in accord with

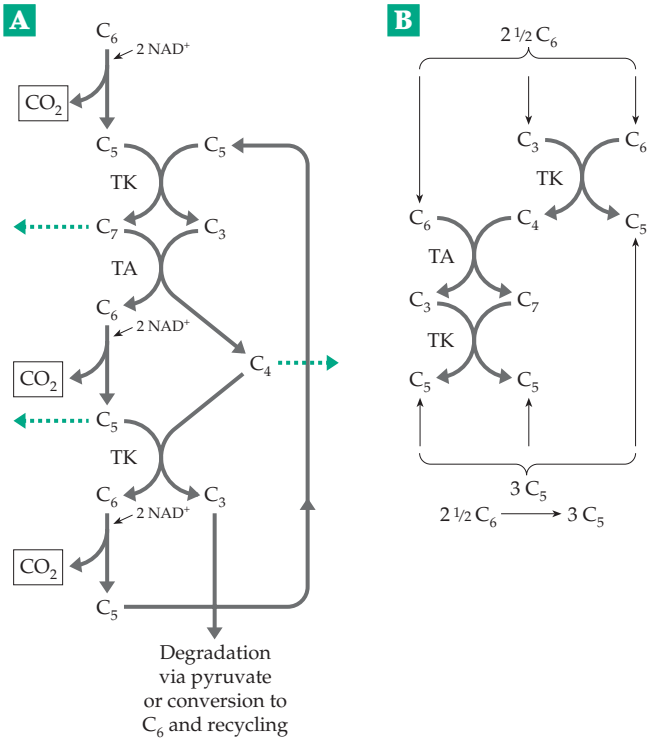



**Figure 17-8** The pentose phosphate pathways. (A) Oxidation of a hexose ($C_6$) to three molecules of $CO_2$ and a three-carbon fragment with the option of removing $C_3$, $C_4$, $C_5$, and $C_7$ units for biosynthesis (dashed arrows). (B) Nonoxidative pentose pathways: $2\,1/2\,C_6 \rightarrow 3\,C_5$ or $2\,C_6 \rightarrow 3\,C_4$ or $3\,1/2\,C_6 \rightarrow 3\,C_7$.

operation of the pentose phosphate pathway as is depicted in Fig. 17-8. However, Williams and associates proposed a modification in the sugar rearrangement sequence in liver[123–126] to include the formation of arabinose 5-*P* (from ribose 5-*P*), an octulose bisphosphate, and an octulose 8-monophosphate. Many investigators argue that these additional reactions are of minor significance.[121,122,127] The measured concentrations of pentose phosphate pathway intermediates in rat livers are close to those predicated for a near-equilibrium state from equilibrium constants measured for the individual steps of Fig. 17-8.[128] Most of the concentrations are in the 4- to 10-μM range but the level of erythrose 4-*P*, which is predicted to be ~ 0.2 μM, is too low to measure.

In contrast to animals, the resurrection plant *Craterostigma plantaginenm* accumulates large amounts of a 2-oxo-octulose. This plant is one of a small group of angiosperms that can withstand severe dehydration and are able to rehydrate and resume normal metabolism within a few hours. During desiccation much of the octulose is converted into sucrose. The plant has extra transketolase genes which may be essential for this rapid interconversion of sugars.[129]

***Nonoxidative pentose phosphate pathways.*** The sugar rearrangement system together with the glycolytic enzymes that convert glucose 6-*P* to glyceraldehyde 3-*P* can function to transform hexose phosphates into pentose phosphates (Fig. 17-8B; Eq. 17-16) which may be utilized for nucleic acid synthesis in erythrocytes and other cells.[130,131]

$$2\,1/2\;\; C_6 \rightarrow 3\,C_5 \qquad (17\text{-}16)$$

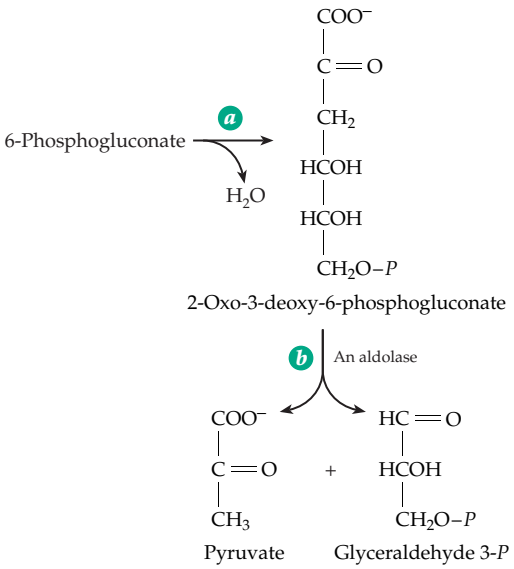
The reader can easily show that the same enzymes will catalyze the net conversion of hexose phosphate to erythrose 4-phosphate or to sedoheptulose 7-phosphate (Eq. 17-17):

$$2\,C_6 \rightarrow 3\,C_4;\;\; 3\,1/2\,C_6 \rightarrow 3\,C_7 \qquad (17\text{-}17)$$

An investigation of metabolism of the red lipid-forming yeast *Rhodotorula gracilis* (which lacks phosphofructokinase and is thus unable to break down sugars through the glycolytic pathway) indicated that 20% of the glucose is *oxidized* through the pentose phosphate pathways while 80% is *altered* by the nonoxidative pentose phosphate pathway.[100] However, it is not clear how the $C_3$ unit used in the nonoxidative pathway (Fig. 17-8B) is formed if glycolysis is blocked. A number of fermentations are also based on the pentose phosphate pathways (Section F,5).

## 4. The Entner–Doudoroff Pathway

An additional way of cleaving a six-carbon sugar chain provides the basis for the **Entner–Doudoroff pathway** which is used by *Zymomonas lindneri* and many other species of bacteria. Glucose 6-*P* is oxidized first to 6-phosphogluconate, which is converted by dehydration to a 2-oxo-3-deoxy derivative (Eq. 17-18,




(17-18)

step *a*). The resulting 2-oxo-3-deoxy sugar is cleaved by an aldolase (Eq. 17-18, step *b*) to pyruvate and glyceraldehyde 3-*P*, which are then metabolized in standard ways.

## F. Fermentation: "Life without Oxygen"

Pasteur recognized in 1860 that fermentation was not a spontaneous process but a result of life in the near absence of air.[132] He realized that yeasts decompose much more sugar under anaerobic conditions than they do aerobically, and that the anaerobic fermentation was essential to the life of these organisms. In addition to the alcoholic fermentation of yeast, there are many other fermentations which have been attractive subjects for biochemical study. If life evolved at a time when no oxygen was available, the most primitive organisms must have used fermentations. They may be the oldest as well as the simplest ways in which cells obtain energy. The enzymes of the glycolysis pathway are found in the small genomes of *Mycoplasma*, *Haemophila*, and *Methanococcus*.[133,134]

Fermentation is also a vital process in the human body. Our muscles usually receive enough oxygen to oxidize pyruvate and to obtain ATP through aerobic metabolism, but there are circumstances in which the oxygen supply is inadequate. During extreme exertion, after most oxygen is consumed, muscle cells produce lactate by fermentation. White muscle of fish and fowl has little aerobic metabolism and normally yields L-lactic acid as a principal end product. Likewise, a variety of tissues within the human body, including the transparent lens and cornea of our eyes, are poorly supplied with blood and depend upon fermentation of glucose to lactic acid. Red blood cells and skin and sometimes adipose tissue are also major producers of lactic acid.[135] Of the ~115 g of lactic acid present in a 70-kg human body, about 29% comes from erythrocytes, 29% from skin, 17% from the brain, and 16% from skeletal muscle.[136] Because lactic acid lowers the pH of cells it must be removed efficiently.

Some of the lactic acid formed in muscle and most of the lactate formed in less aerobic tissues (e.g., adipose tissue)[136a] enters the bloodstream, which normally contains 1–2 mM lactate,[136] and is carried to the liver where it is reoxidized to pyruvate. Part of the pyruvate is then oxidized via the citric acid cycle while a larger part is reconverted to glucose (Section J,5). This glucose may be released into the bloodstream and returned to the muscles. The overall process is known as the **Cori cycle**. Lactic acid accumulates in muscle after vigorous exercise. It is exported to the liver slowly, but if mild exercise continues the lactate may be largely oxidized within muscle via the tricarboxylic acid cycle. Recent NMR studies have shown that lactic acid is formed rapidly during muscular contraction, even when exercise is mild.[136b] During the initial 15 ms of contraction the ATP utilized is regenerated from creatine phosphate (Eq. 6-67). During the remainder of the contraction (up to ~100 ms) glycogen is converted to lactic acid to provide ATP and to replenish the creatine phosphate. In the resting period following contraction most of the lactate is either dehydrogenated to pyruvate and oxidized in mitochondria or exported to other tissues. The glycogen stores in muscle are renewed by synthesis from blood glucose. Lactic acid is a convenient energy carrier and a precursor for gluconeogenesis which can be transferred between tissues easily.[136c] Cancer cells often take advantage of this opportunity to grow rapidly using fermentation of glucose to lactic acid as a source of energy.[136d]

Alcoholic fermentation allows roots of some plants to survive short periods of flooding. Ethanol does not acidify the tissues as does lactic acid, avoiding possible damage from low pH.[137,138] Goldfish can also use the ethanolic fermentation for short times, excreting the ethanol.[139]

### 1. Fermentations Based on the Embden–Meyerhof Pathway

***Homolactic and alcoholic fermentations.*** The reactions by which glucose can be converted to lactate and, by yeast cells, to ethanol and $CO_2$ (Figs. 10-3 and 17-7) illustrate several features common to all fermentations. The NADH produced in the oxidation step is reoxidized in a reaction by which substrate is reduced to the final end product. The NAD alternates between oxidized and reduced forms. This coupling of oxidation steps with reduction steps in exact equivalence is characteristic of all true (anaerobic) fermentations. The formation of ATP from ADP and $P_i$ by substrate-level phosphorylation is also common to all fermentations. The stoichiometry is often nearly exact and simple. For example, according to the reaction of Eq. 17-19, which is outlined step-by-step in Fig. 17-7, a net total of two moles of ATP is formed per mole of glucose fermented.

***Energy relationships.*** If we disregard the synthesis of ATP, the equations for the lactic acid and ethanol fermentations are given by Eqs. 17-19 and 17-20.

$$\text{Glucose} \rightarrow 2\ \text{lactate}^- + 2\ \text{H}^+$$
$$\Delta G'\ (\text{pH } 7) = -196\ \text{kJ mol}^{-1}\ (-46.8\ \text{kcal mol}^{-1}) \qquad (17\text{-}19)$$

$$\text{Glucose} \rightarrow 2\ \text{CO}_2 + 2\ \text{ethanol}$$
$$\Delta G^\circ = -235\ \text{kJ mol}^{-1} \qquad (17\text{-}20)$$

The Gibbs energy changes are negative and of sufficient magnitude that the reactions will unquestionably go to completion. However, the synthesis of two molecules of ATP from inorganic phosphate and ADP, a reaction

(Eq. 17-21) for which $\Delta G'$ is substantially positive, is coupled to the fermentation.

$$ADP^{3-} + HPO_4^{2-} + H^+ \rightarrow ATP^{4-} + H_2O$$
$$\Delta G' \text{ (pH 7)} = +34.5 \text{ kJ mol}^{-1} \text{ (Table 6-5)} \quad (17\text{-}21)$$

To obtain the net Gibbs energy change for the complete reaction we must add 2 x 34.5 = +69.0 kJ to the values of $\Delta G'$ for Eqs. 17-19 and 17-20. When this is done we see that the net Gibbs energy changes are still highly negative, that the reactions will proceed to completion, and that these fermentations can serve as an usable energy source for organisms.

Biochemists sometimes divide $\Delta G$ for the ATP synthesis in a coupled reaction sequence (in this case +69 kJ) by the overall Gibbs energy decrease for the coupled process (196 or 235 kJ mol$^{-1}$) to obtain an "efficiency." In the present case the efficiency would be 35% and 29% for coupling of Eq. 17-21 (for 2 mol of ATP) to Eqs. 17-19 and 17-20, respectively. According to this calculation, nature is approximately one-third efficient in the utilization of available metabolic Gibbs energy for ATP synthesis. However, it is important to realize that this calculation of efficiency has no exact thermodynamic meaning. Furthermore, the utilization of ATP formed by a cell for various purposes is far from 100% efficient.

Why are the Gibbs energy decreases for Eqs. 17-19 and 17-20 so large? No overall oxidation takes place; there is only a rearrangement of the existing bonds between atoms of the substrate. Why does this rearrangement of bonds lead to a substantial negative $\Delta G$? An answer is suggested by an examination of the numbers of each type of bond in the substrate and in the products. During the conversion from glucose to two molecules of lactate one C–C bond, one C–O bond, and one O–H bond are lost and one C–H bond and one C=O are gained. If we add up the bond energies for these bonds (Table 6-6) we find that the difference ($\Delta H$) between substrate and products amounts to only about 20 kJ/mol. However, lactic acid contains a carboxyl group, and carboxyl groups have a special stability as a result of resonance. The extra resonance energy of a carboxyl group (Table 6-6) is ~117 kJ (28 kcal) per mole or 234 kJ/mol for two carboxyl groups. This is approximately the same as the Gibbs energy change (Eq. 17-19) for fermentation of glucose to lactate. Thus, the energy available results largely from the rearrangement of bonds by which the carboxyl groups of lactate are formed. Likewise, the resonance stabilization of $CO_2$ is given by Pauling as 151 kJ/mol, again of just the right magnitude to explain $\Delta G$ in alcoholic fermentations (Eq. 17-20).

On this basis we can state as a general rule that fermentations can occur when substrates consisting of largely singly bonded atoms and groups, such as the carbonyl groups that are not highly stabilized by resonance, are converted to products containing carboxyl groups or to $CO_2$. If we assume an efficiency of ~30%, the energy available will be about sufficient for synthesis of one ATP molecule for each carboxyl group or $CO_2$ created. Bear in mind that generation of ATP also depends upon availability of a mechanism. It is of interest that most synthesis of ATP is linked directly to the chemical processes by which carboxyl groups or $CO_2$ molecules are created in a fermentation process. The most important single reaction is the oxidation of the aldehyde group of glyceraldehyde 3-*P* to the carboxyl group of 3-phosphoglycerate (steps *6a – 6c* and *7* in Fig. 17-7; see also Fig. 15-6).

Compare the fermentation of glucose with the complete oxidation of the sugar to carbon dioxide and water (Eq. 17-22), a process which yeast cells (as well as our own cells) carry out in the presence of air. The overall Gibbs energy change is over 10 times greater than that for fermentation, a fact that permits the cell

$$\text{Glucose} + 6\,O_2 \rightarrow 6\,CO_2 + 6\,H_2O$$
$$\Delta G' = -2872 \text{ kJ } (-686.5 \text{ kcal}) \text{ mol}^{-1} \quad (17\text{-}22)$$

to form an enormously greater quantity of ATP. The net gain in ATP synthesis, accompanying Eq. 17-22, is usually about 38 mol of ATP—19 times more than is available from fermentation of glucose. Thus, the explanation of Pasteur's observation that yeast decomposes much less sugar in the presence of air than in its absence is clear. Also, we can understand why a cell, living anaerobically, must metabolize a very large amount of substrate to grow. (Recall from Chapter 6 that ~1 mol of ATP energy is needed to produce 10 g of cells.)

***Variations of the alcoholic and homolactic fermentations.*** The course of a fermentation is often affected drastically by changes in conditions. Many variations can be visualized by reference to Fig. 17-9, which shows a number of available metabolic sequences. We have already discussed the conversion of glucose to triose phosphate and via reaction pathway *a* to pyruvate, via reaction *c* to lactate, and via reaction *d* to ethanol.

If bisulfite is added to a fermenting culture of yeast, the acetaldehyde formed through reaction *d* is trapped as the bisulfite adduct blocking the reduction of acetaldehyde to ethanol, an essential part of the fermentation. Yeast cells accommodate this change by using the accumulating NADH to reduce half of the triose-*P* to glycerol through pathway *b*. Two enzymes are needed, a dehydrogenase and a phosphatase, to hydrolytically cleave off the phosphate. The balanced reaction is given by Eq. 17-23:

$$\text{Glucose} \rightarrow \text{glycerol} + \text{acetaldehyde (trapped)} + CO_2$$
$$\Delta G' \text{ (pH 7)} = -105 \text{ kJ mol}^{-1} \quad (17\text{-}23)$$

In this reaction only one molecule of $CO_2$ is produced

but the overall Gibbs energy change is still adequate to make the reaction highly spontaneous. However (referring to Fig. 17-9), we see that the net synthesis of ATP is now apparently zero. The fermentation apparently does not permit cell growth. Nevertheless, it has been used industrially for production of glycerol.

Reduction of dihydroxyacetone phosphate to glycerol phosphate also occurs in insect flight muscle and apparently operates as an alternative to lactic acid formation in that tissue. There is no net gain of ATP in the conversion of free glucose to glycerol phosphate and pyruvate, but using stored glycogen in muscle as the starting material, the dismutation of triose-*P* to glycerol-*P* and pyruvate provides one ATP per glucose unit rapidly during the vigorous contraction of the powerful insect flight muscle. During the slower recovery phase, glycerol-*P* is thought to be reoxidized after entering the mitochondria of these highly aerobic cells. Thus, the transport of glycerol-*P* into mitochondria serves as a means for transporting reducing equivalents derived from reoxidation of NADH into the mitochondria. Indeed, the significance of glycerol-*P* to muscle metabolism may be more related to this function than to the rapid formation of ATP (see Chapter 18).

Why does the glycolysis sequence begin with phosphorylation of glucose by ATP? The phospho groups probably provide convenient handles and doubtless assist in substrate recognition. There may be a kinetic advantage but also a danger. Unless there is adequate regulation the "turbo design," in which ATP is used at the outset to drive glycolysis, may lead to accumulation of phosphorylated intermediates and to inadequate concentrations of ATP and inorganic phosphate.[139a,b] Yeast cells guard against this problem by synthesizing trehalose 6-phosphate, which acts as a feedback inhibitor of hexokinase.[139a] Trypanosomes utilize a different type of control. The enzymes that convert glucose into 3-phosphoglycerate are present in membrane-bounded organelles called **glycosomes**. Phosphoglycerate is exported from them into the cytosol where glycolysis is completed.[139b] Since inorganic phosphate is essential for ATP formation, if the $P_i$ concentration falls too low the rate of fermentation by yeast juice is greatly decreased, an observation made by Harden and Young[139c] in 1906.

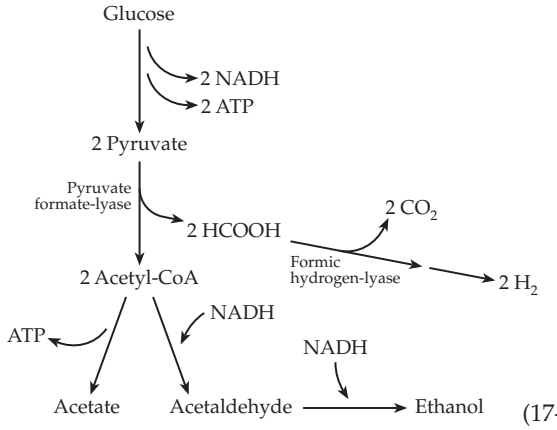
## 2. The Mixed Acid Fermentation

Enterobacteria, including *E. coli*, convert glucose to ethanol and acetic acid and either formic acid or $CO_2$ and $H_2$ derived from it. The stoichiometry is variable but the fermentation can be described in an idealized form as follows:

$$\text{Glucose} + H_2O \rightarrow \text{ethanol} + \text{acetate}^- + H^+ + 2\,H_2 + 2\,CO_2$$

$$\Delta G'\ (\text{pH } 7) = -225\ \text{kJ mol}^{-1} \qquad (17\text{-}24)$$

The details of the process and the oxidation–reduction balance can be pictured as in Eq. 17-25. Pyruvate is cleaved by the pyruvate formate–lyase reaction (Eq. 15-37) to acetyl-CoA and formic acid. Half of the acetyl-CoA is cleaved to acetate via acetyl-*P* with generation of ATP, while the other half is reduced in two steps to ethanol using the two molecules of NADH produced in the initial oxidation of triose phosphate (Eq. 17-25). The overall energy yield is three molecules of ATP per glucose. The "efficiency" is thus (3 x 34.5) ÷ 225 = 46%. Some of the glucose is converted to D-lactic and to succinic acids (pathway *f*, Fig. 17-9); hence the name **mixed acid fermentation**. Table 17-1 gives typical yields of the mixed acid fermentation of *E. coli*. Among the four major products are acetate, ethanol, $H_2$, and $CO_2$, as shown in Eq. 17-25. However, at high pH formate accumulated instead of $CO_2$.




(17-25)

**TABLE 17-1**
**Products of the Mixed Acid Fermentation by *E. coli* at Low and High Values of pH[a]**

| Product (Millimole formed from 100 mmol of glucose) | pH 6.2 | pH 7.8 |
|---|---|---|
| Acetate | 36 | 39 |
| Ethanol | 50 | 51 |
| $H_2$ | 70 | 0.3 |
| $CO_2$ | 88 | 1.7 |
| Formate | 2.4 | 86 |
| Lactate | 79 | 70 |
| Succinate | 11 | 15 |
| Glycerol | 1.4 | 0.3 |
| Acetoin | 0.1 | 0.2 |
| Butanediol | 0.3 | 0.2 |

[a] From Tempest, D. W. and Neijssel, O. M.[140] Based on data of Blackwood.[141]

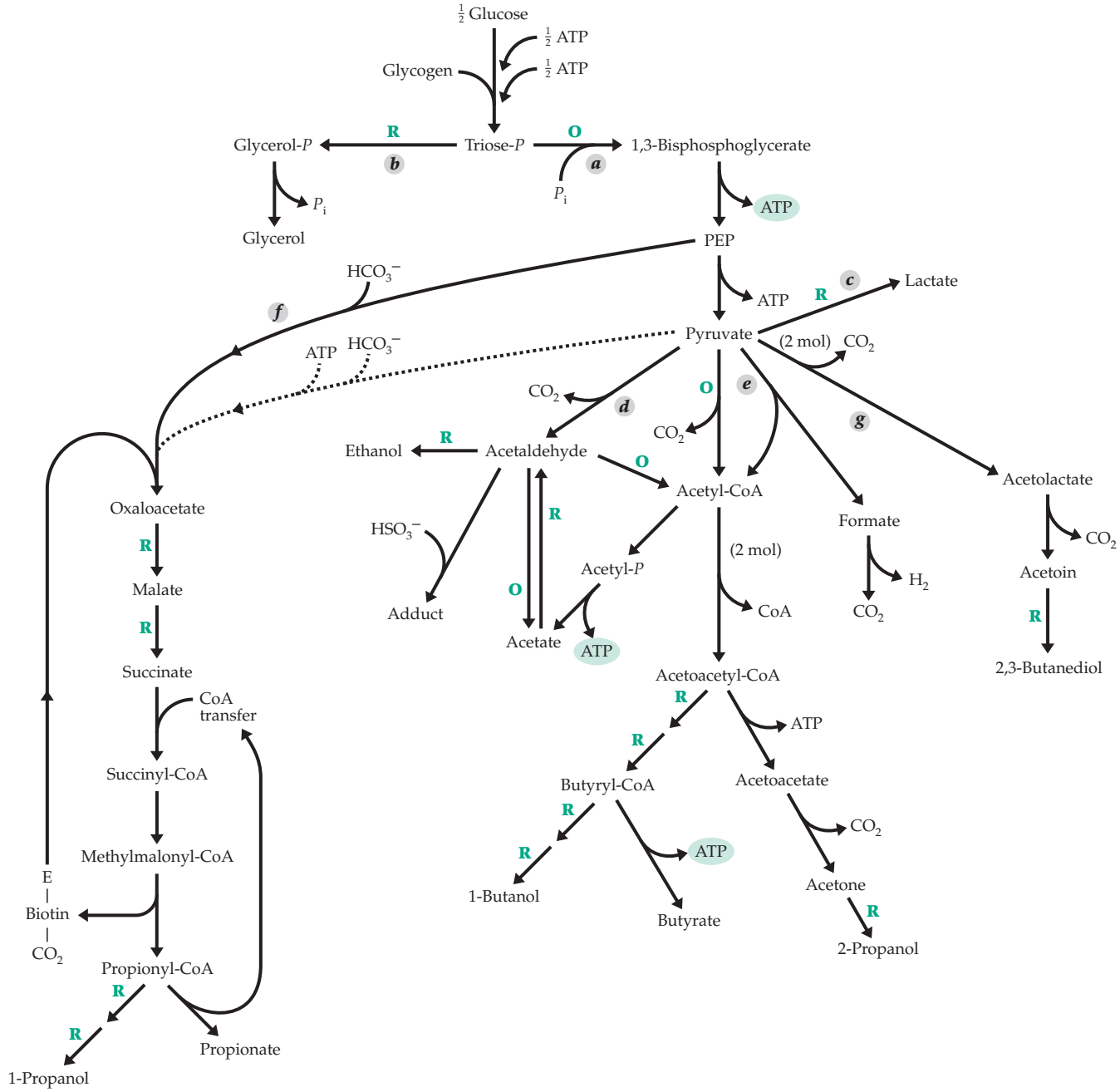


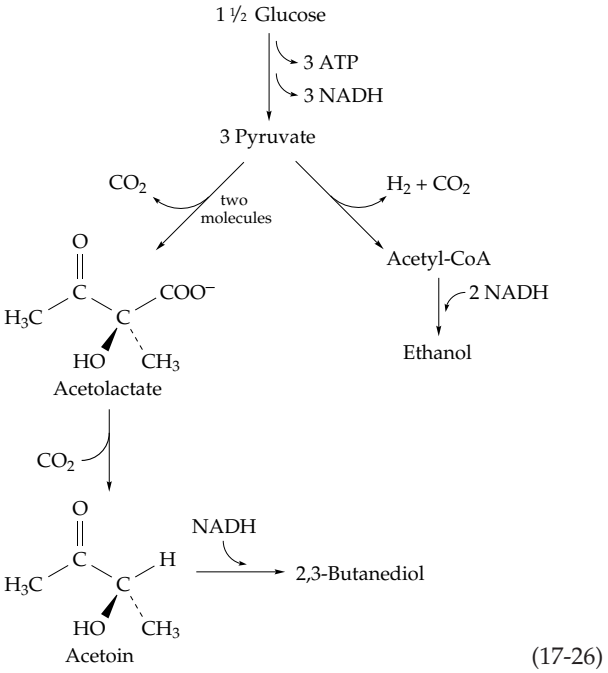

**Figure 17-9** Reaction sequences in fermentation based on the Embden–Meyerhof–Parnas pathway. Oxidation steps (producing NADH + $H^+$) are marked "O"; reduction steps (using NADH + $H^+$) are marked "R."

Over one-third of the glucose was fermented to lactate in both cases.

In some mixed acid fermentations (e.g., that of *Shigella*) formic acid accumulates, but in other cases (e.g., with *E. coli* at pH 6) it is converted to $CO_2$ and $H_2$ (Eq. 17-25). The equilibration of formic acid with $CO_2$ and hydrogen is catalyzed by the **formic hydrogen–lyase** system which consists of two iron–sulfur enzymes. The selenium-containing **formate dehydrogenase** (Eq. 16-63) catalyzes oxidation of formate to $CO_2$ by $NAD^+$, while a membrane-bound **hydrogenase** (Eq. 16-48) equilibrates NADH + $H^+$ with $NAD^+ + H_2$. Hydrogenase also serves to release $H_2$ from excess NADH. Krebs pointed out that an excess of NADH may arise because growth of cells requires biosynthesis of many components such as amino acids. When glucose is the sole source of carbon, biosynthetic reactions involve an excess of oxidation steps over reduction steps.[142] The excess of reducing equivalents may be released as $H_2$ or

may be used to form highly reduced products such as succinate.

Among such genera as *Aerobacter* and *Serratia* part of the pyruvate formed is condensed with decarboxylation to form ***S* acetolactate**,[143] which is decarboxylated to acetoin (Eq. 17-26; pathway *g* of Fig. 17-9). The acetoin is reduced with NADH to **2,3-butanediol**, while a third molecule of pyruvate is converted to ethanol, hydrogen, and $CO_2$ (Eq. 17-26). The reaction provides the basis for industrial production of butanediol, which can be dehydrated nonenzymatically to butadiene.




(17-26)

Mixed acid fermentations are not limited to bacteria. For example, trichomonads, parasitic flagellated protozoa, have no mitochondria. They export pyruvate into the bloodstreams of their hosts and also contain particles called **hydrogenosomes** which can convert pyruvate to acetate, succinate, $CO_2$, and $H_2$.[144] Hydrogenosomes are bounded by double membranes and have a common evolutionary relationship with both mitochondria and bacteria. The enzyme that catalyzes pyruvate cleavage in hydrogenosomes apparently does not contain lipoate and may be related to the pyruvate–ferredoxin oxidoreductase of clostridia (Eq. 15-35). The hydrogenosomes also contain an active hydrogenase.

Many invertebrate animals are true facultative anaerobes, able to survive for long periods, sometimes indefinitely, without oxygen.[145–147] Among these are *Ascaris* (Fig. 1-14), oysters, and other molluscs. Succinate and alanine are among the main end products of anaerobic metabolism. The former may arise by a mixed acid fermentation that also produces pyruvate. The pyruvate is converted to acetate to balance the fermentation in *Ascaris lumbricoides*, which is in effect an obligate anaerobe. However, in molluscs the pyruvate may undergo transamination with glutamate to form alanine and 2-oxoglutarate; the oxoglutarate may be oxidatively decarboxylated to succinate. The reactions depend upon the availability of a store of glutamate or of other amino acids, such as arginine, that can give rise to glutamate.

## 3. The Propionic Acid Fermentation

Propionic (propanoic) acid-producing bacteria are numerous in the digestive tract of ruminants. Within the rumen some bacteria digest cellulose to form glucose, which is then converted to lactate and other products. The propionic acid bacteria can convert either glucose or lactate into propionic and acetic acids which are absorbed into the bloodstream of the host. Usually some succinic acid is also formed.

The basis of the propionic acid fermentation is conversion of pyruvate to oxaloacetate by carboxylation and the further conversion through succinate and succinyl-CoA to methylmalonyl-CoA and propionyl-CoA, reactions which are almost the exact reverse of those for the oxidation of propionate in the animal body (Fig. 17-3, pathway *d*). However, whereas the carboxylation of pyruvate to oxaloacetate in the animal body requires ATP, the propionic acid bacteria save one equivalent of ATP by using a carboxyltransferase (p. 725). This enzyme donates a carboxyl group from a preformed carboxybiotin compound generated in the decarboxylation of methylmalonyl-CoA in the next to final step of the reaction sequence (Fig. 17-10). A second molecule of ATP is saved by linking directly the conversion of succinate to succinyl-CoA to the cleavage of propionyl-CoA to propionate through the use of a CoA transferase (Eq. 12-50). To provide for oxidation–reduction balance, two-thirds of the glucose goes to propionate and one-third to acetate (Eq. 17-27):

$$1\tfrac{1}{2}\,\text{Glucose} \rightarrow 2\ \text{propionate}^- + \text{acetate}^- + 3\ H^+ + CO_2 + H_2O$$
$$\Delta G'\ (\text{pH } 7) = -465\ \text{kJ per } 1\tfrac{1}{2}\ \text{mol of glucose} \qquad (17\text{-}27)$$

More carboxyl groups and $CO_2$ molecules are formed in this fermentation ($2^2/_3$ per glucose molecule) than in the regular lactic acid fermentation. The yield of ATP (also $2\ ^2/_3$ mol/mol of glucose fermented) is correspondingly greater and $\Delta G'$ is more negative.

Using the same mechanism (Fig. 17-10), propionic acid bacteria are also able to ferment lactate, the product of fermentation by other bacteria, to propionate and acetate (Eq. 17-28). The net gain is one molecule of ATP. This reaction probably accounts for the niche

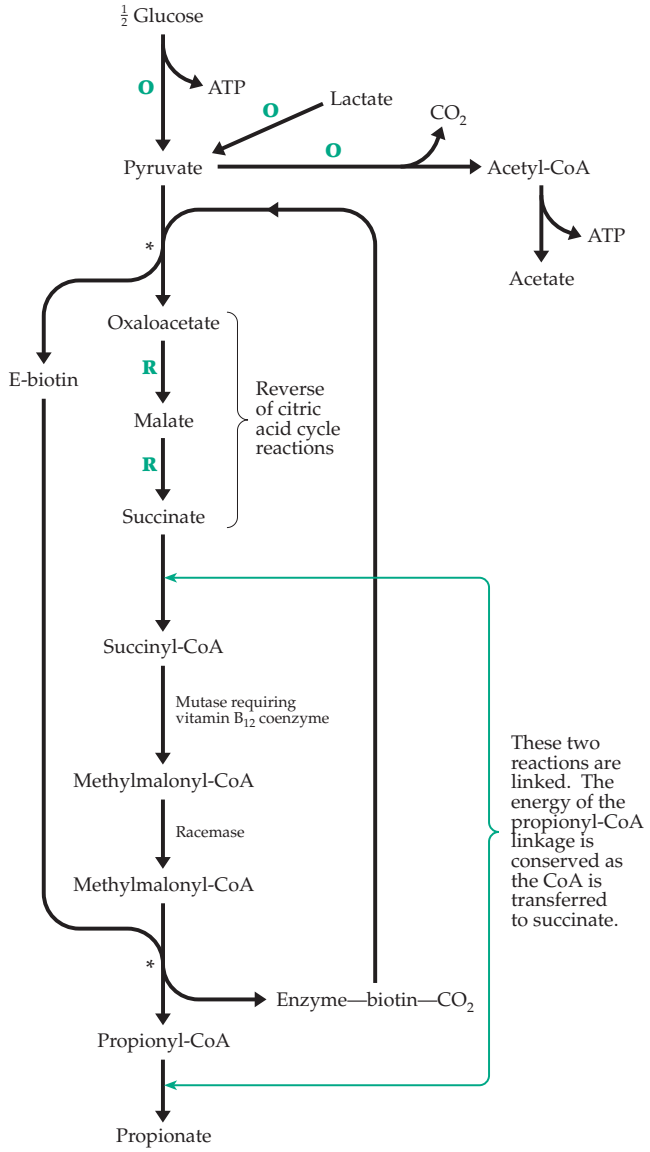



**Figure 17-10** Propionic acid fermentation of *Propionobacteria* and *Veillonella*. Oxidation steps are designated by the symbol "O" and reduction steps by "R." The two coupled reactions marked by asterisks are catalyzed by carboxyltransferase.

$$3\ \text{Lactate}^- \rightarrow 2\ \text{propionate}^- + \text{acetate}^- + H_2O + CO_2$$
$$\Delta G'\ (\text{pH } 7) = -171\ \text{kJ per 3 mol of lactate} \qquad (17\text{-}28)$$

in the ecology of the animal rumen that is occupied by propionic acid bacteria.

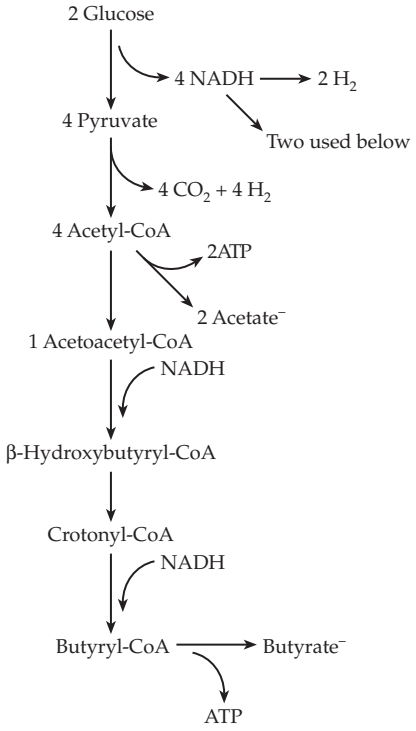
## 4. Butyric Acid and Butanol-Forming Fermentations

A variety of fermentations are carried out by bacteria of the genus *Clostridium* and by the rumen organisms *Eubacterium (Butyribacterium)* and *Butyrivibrio*.

For example, glucose may be converted to butyric and acetic acids together with $CO_2$ and $H_2$ (Eqs. 17-29 and 17-30).

$$2\ \text{Glucose} + 2\ H_2O \rightarrow \text{butyrate}^- + 2\ \text{acetate}^- + 4\ CO_2 + 6\ H_2 + 3\ H^+$$
$$\Delta G'\ (\text{pH } 7) = -479\ \text{kJ per 2 mol of glucose} \qquad (17\text{-}29)$$

The yield of ATP (3½ mol/mol of glucose) is the highest we have discussed giving an efficiency of 50%. Another fermentation yields butanol, isopropanol, ethanol, and acetone.

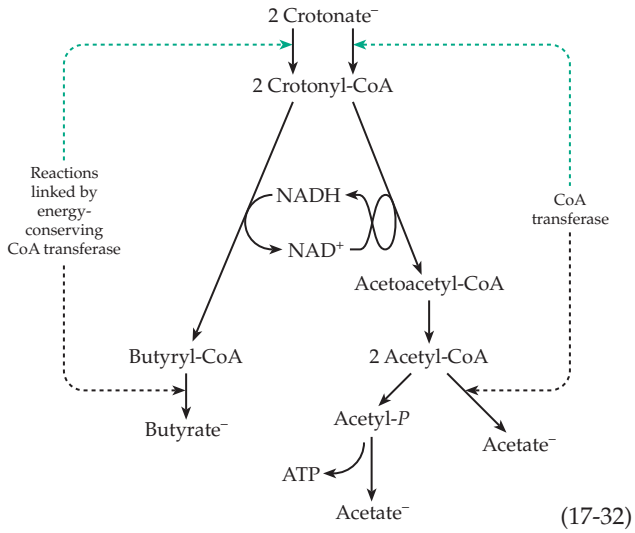



(17-30)

The fermentation of Eq. 17-31 is catalyzed by *Clostridium kluyveri*. The value of $-\Delta G'$ is one of the lowest that we have considered but is still enough to provide easily for the synthesis of one molecule of ATP.

$$2\ \underset{\text{Crotonate}}{CH_3CH{=}CH{-}COO^-} + 2\ H_2O \rightarrow \text{butyrate}^- + 2\ \text{acetate}^- + H^+$$
$$\Delta G'\ (\text{pH } 7) = -105\ \text{kJ mol}^{-1} \qquad (17\text{-}31)$$

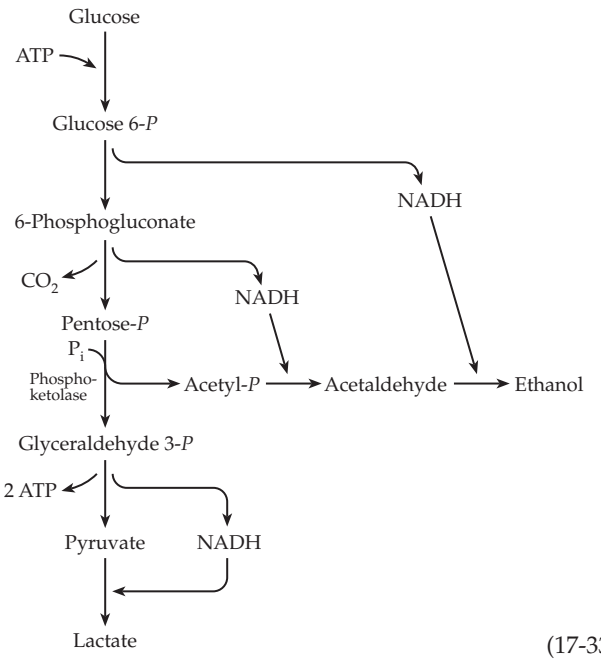
The energy of the butyryl-CoA linkage and of one of the acetyl-CoA linkages is conserved and utilized in the initial formation of crotonyl-CoA (Eq. 17-32). That leaves one acetyl-CoA which can be converted via acetyl-*P* to acetate with formation of ATP.




(17-32)

## 5. Fermentations Based on the Phosphogluconate and Pentose Phosphate Pathways

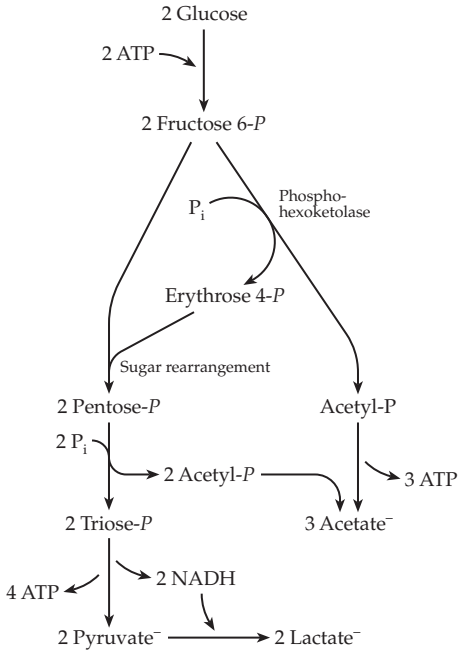
Some lactic acid bacteria of the genus *Lactobacillus*, as well as *Leuconostoc mesenteroides* and *Zymomonas mobilis*, carry out the **heterolactic** fermentation (Eq. 17-33) which is based on the reactions of the pentose phosphate pathway. These organisms lack aldolase, the key enzyme necessary for cleavage of fructose 1,6-bisphosphate to the triose phosphates. Glucose is converted to ribulose 5-*P* using the oxidative reactions of the pentose phosphate pathway. The ribulose-phosphate is cleaved by phosphoketolase (Eq. 14-23) to acetyl-phosphate and glyceraldehyde 3-phosphate, which are converted to ethanol and lactate, respectively. The overall yield is only one ATP per glucose fermented.




(17-33)

This is generated in the substrate level oxidative phosphorylation catalyzed by phosphoketolase. Metabolic engineering of *Zymomonas* was accomplished by transferring from other bacteria two operons that provide for assimilation of xylose and a complete set of enzymes for the pentose phosphate pathway. The engineered bacteria are able to convert pentose phosphates nonoxidatively (see Fig. 17-8) into glyceraldehyde 3-phosphate, which is converted to ethanol in high yield and with much greater synthesis of ATP than according to Eq. 17-33.[148]

A variation of the heterolactic fermentation is used by *Bifidobacterium* (Eq. 17-34).[149] Phosphoketolase and a **phosphohexoketolase**, which cleaves fructose 6-*P* to erythrose 4-*P* and acetyl-*P*, are required, as are the enzymes of the sugar rearrangement system (Section E,3). The net yield of ATP is 2 ½ molecules per molecule of glucose.




(17-34)

## G. Biosynthesis

In this section and sections H – K the general principles and strategy of synthesis of the many carbon compounds found in living things will be considered. Since green plants and autotrophic bacteria are able to assemble all of their needed carbon compounds from $CO_2$, let us first examine the mechanisms by which this is accomplished. We will also need to ask how some organisms are able to subsist on such simple compounds as methane, formate, or acetate.

### 1. Metabolic Loops and Biosynthetic Families

As was pointed out in Chapter 10, routes of biosynthesis (anabolism) often closely parallel pathways of biodegradation (catabolism). Thus, catabolism begins with hydrolytic breakdown of polymeric molecules; the resulting monomers are then cleaved into small two- and three-carbon fragments. Biosynthesis begins with formation of monomeric units from small pieces followed by assembly of the monomers into polymers. The mechanisms of the individual reactions of biosynthesis and biodegradation are also often closely parallel. However, in most instances, there are clear-cut differences. A first principle of biosynthesis is that *biosynthetic pathways, although related to catabolic pathways, differ from them in distinct ways and are often catalyzed by completely different sets of enzymes*.

The sum of the pathways of biosynthesis and biodegradation form a continuous loop – a series of reactions that take place concurrently and often within the same part of a cell. Metabolic loops often begin in the central pathways of carbohydrate metabolism with three- or four-carbon compounds such as phosphoglycerate, pyruvate, and oxaloacetate. After loss of some atoms as $CO_2$ the remainder of the compound rejoins the "mainstream" of metabolism by entering a catabolic pathway leading to acetyl-CoA and oxidation in the citric acid cycle. Not all of the loops are closed within a given species. For example, *human beings are unable to synthesize the vitamins and the "essential amino acids."* We depend upon other organisms to make these compounds, but we do degrade them. Some metabolites, such as uric acid, are excreted by humans and are further catabolized by bacteria. From a chemical viewpoint the whole of nature can be regarded as an enormously complex set of branching and interconnecting metabolic cycles. Thus, the synthetic pathways used by autotrophs are all parts of metabolic loops terminating in oxidation back to $CO_2$.

It is often not possible to state at what point in a metabolic loop biosynthesis has been completed and biodegradation begins. An end product X that serves one need of a cell may be a precursor to another cell component Y which is then degraded to complete the loop. The reactions that convert X to Y can be regarded as either biosynthetic (for Y) or catabolic (for X).

### 2. Key Intermediates and Biosynthetic Families

In examining routes of biosynthesis it is helpful to identify some key intermediates. One of these is **3-phosphoglycerate**. This compound is a primary product of photosynthesis and may reasonably be regarded as the starting material from which all other carbon compounds in nature are formed. Phosphoglycerate, in most organisms, is readily interconvertible with both **glucose** and **phosphoenolpyruvate (PEP)**. Any of these three compounds can serve as the precursor for synthesis of other organic materials. A first stage in biosynthesis consists of those reactions by which 3-phosphoglycerate or PEP arise, whether it be from $CO_2$, formate, acetate, lipids, or polysaccharides.

The further biosynthetic pathways from 3-phosphoglycerate to the myriad amino acids, nucleotides, lipids, and miscellaneous compounds found in cells are complex and numerous. However, the basic features are relatively simple. Figure 17-11 indicates the origins of many substances including the 20 amino acids present in proteins, nucleotides, and lipids. Among the additional key biosynthetic precursors that can be identified from this chart are **glucose 6-phosphate**, **pyruvate**, **oxaloacetate**, **acetyl-CoA**, **2-oxoglutarate**, and **succinyl-CoA**.

The amino acid **serine** originates almost directly from 3-phosphoglycerate. **Aspartate** arises from oxaloacetate and **glutamate** from 2-oxoglutarate. These three amino acids each are converted to "families" of other compounds.[150] A little attention paid to establishing correct family relationships will make the study of biochemistry easier. Besides the serine, aspartate, and glutamate–oxoglutarate families, a fourth large family originates directly from pyruvate and a fifth (mostly lipids) from acetyl-CoA. The aromatic amino acids are formed from erythrose 4-*P* and PEP via the key intermediate **chorismic acid** (Box 9-E; Fig. 25-1). Other families of compounds arise from glucose 6-*P* and from the **pentose phosphates**. These groups have been set off roughly by the boxes outlined in green in Fig. 17-11.

## H. Harnessing the Energy of ATP for Biosynthesis

In the past it seemed reasonable to think that some biosynthetic pathways involved exact reversal of catabolic pathways. For example, it was observed that glycogen phosphorylase catalyzed elongation of glycogen branches by transfer of glycosyl groups from glucose 1-phosphate. Likewise, the enzymes needed for the β oxidation of fatty acid derivatives, when isolated from mitochondria, catalyze formation of fatty acyl-CoA derivatives from acetyl-CoA and a reducing agent such as NADH. However, reactant concentrations within cells are rarely appropriate for reversal of a catabolic sequence. For catabolic sequences the Gibbs energy change is usually distinctly negative and reversal requires high concentrations of end products. However, the latter are often removed promptly from the cells. For example, NADH produced in degradation of fatty acids is oxidized to $NAD^+$ and is therefore never available in sufficient concentrations to reverse the β oxidation sequence.

*Nature's answer to the problem of reversing a catabolic pathway lies in the coupling of cleavage of ATP to the biosynthetic reaction.* The concept was introduced in Chapter 10, in which one sequence for linking hydrolysis of ATP to biosynthesis was discussed. However, living cells employ several different methods of harnessing the Gibbs energy of hydrolysis of ATP to drive biosynthetic processes. Many otherwise strange aspects of metabolism become clear if it is recognized that they provide a means for coupling ATP cleavage to biosynthesis. A few of the most important of these coupling mechanisms are summarized in this section.

## 1. Group Activation

Consider the formation of an ester (or of an amide) from a free carboxylic acid and an alcohol (or amine) by elimination of a molecule of water (Eq. 17-35). The reaction is thermodynamically unfavorable with values of $\Delta G'$ (pH 7) ranging from ~ +10 to 30 kJ $mol^{-1}$ depending on conditions and structures of the specific compounds. Long ago, organic chemists learned that such reactions can be made to proceed by careful removal of the water that is generated (Eq. 17-35).

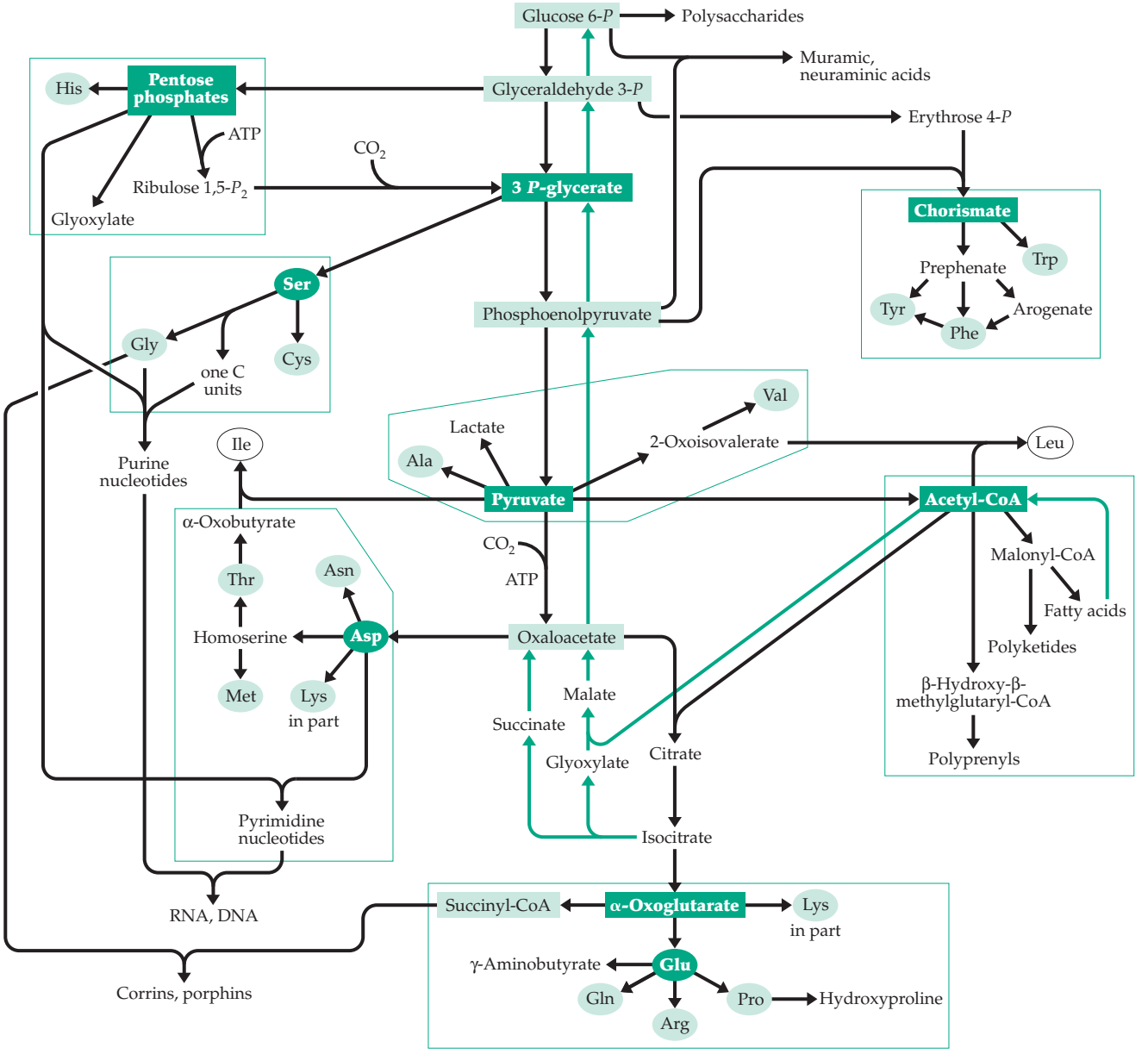
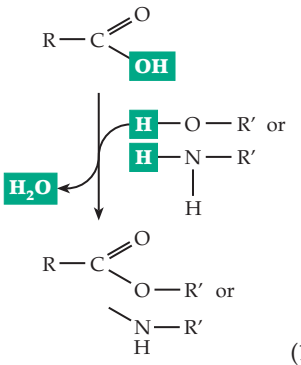
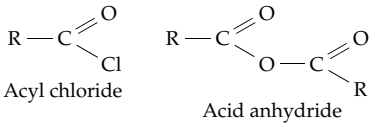



**Figure 17-11** Some major biosynthetic pathways. Some key intermediates are enclosed in boxes and the 20 common amino acids of proteins are encircled. Key intermediates for each family are in shaded boxes or elipses. Green lines trace the reactions of the glyoxylate pathway and of glucogenesis.




(17-35)

However, it is often better to "activate" the carboxylic acid by conversion to an acyl chloride or an anhydride:




Nucleophilic attack on the carbonyl group of such a compound results in displacement of a good leaving group, $Cl^-$ or $R{-}COO^-$. Nature has followed the same approach in forming from carboxylic acids **acyl phosphates** or **acyl-CoA** derivatives.

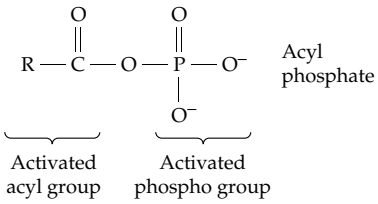
The virtue of these "activated" acyl compounds in biosynthetic reactions was considered in Chapter 12 and Table 15-1. Just as a carboxylic acid can be converted to an active acyl derivative, so other groups can be activated. ATP and other compounds with phospho groups of high group transfer potential are **active phospho** compounds. Sulfate is converted to a phosphosulfate anhydride, an **active sulfo** derivative. Sugars are converted to compounds such as glucose 1-*P* or sucrose, which contain **active glycosyl** groups. The group transfer potentials of the latter, though not as great as that of the phospho groups of ATP, are still high enough to make glucose 1-phosphate and sucrose effective glycosylating reagents. Table 17-2 lists several of the more important activated groups.

Group activation usually takes place at the expense of ATP cleavage.

## TABLE 17-2
## "Activated" Groups Used in Biosynthesis

| Group | Typical activated forms |
|---|---|
| Phospho | Pyrophosphate; Guanidine phosphate; Enol phosphate; Acyl phosphate |
| Sulfo | in PAPS (3′-Phosphoadenosine-5′-phosphosulfate) |
| Acyl | Thioester; Acyl phosphate |
| Glycosyl | Glycosyl phosphate; Glucosyl; Fructosyl; Sucrose |
| Enoyl | Enol phosphate |
| Carbamoyl | Carbamoyl phosphate |
| Alkyl | *S*-Adenosylmethionine |

As pointed out in Chapter 12, acyl phosphates play a central role in metabolism by virtue of the fact that they contain both an activated acyl group and an activated phospho group. The high group transfer potential can be conserved in subsequent reactions *in either one group or the other* (but not in both). Thus, displacement on P by an oxygen of ADP will regenerate ATP and attack on C by an – SH will give a thioester.

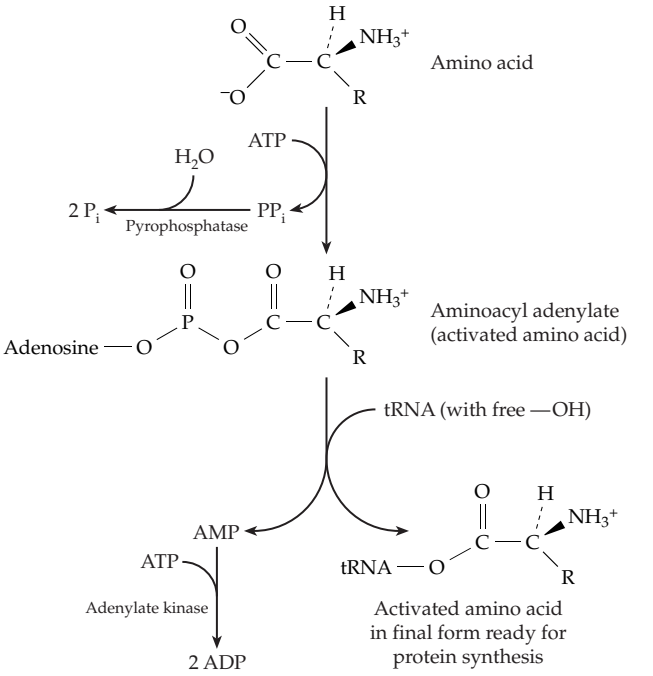



Several of the other compounds in Table 17-2 can also be split in two ways to yield different activated groups, e.g., the phosphosulfate anhydride, enoyl phosphate, and carbamoyl phosphate. It is probably only through intermediates of this type that cleavage of ATP can be coupled to synthesis of activated groups. Such **common intermediates** are essential to the synthesis of ATP by substrate-level phosphorylation (Fig. 15-6).

## 2. Hydrolysis of Pyrophosphate

The splitting of inorganic pyrophosphate ($PP_i$) into two inorganic phosphate ions is catalyzed by **pyrophosphatases** (p. 636)[150a,b] that apparently occur universally. Their function appears to be simply to remove the product $PP_i$ from reactions that produce it, shifting the equilibrium toward formation of a desired compound. An example is the formation of **aminoacyl-tRNA** molecules needed for protein synthesis. As shown in Eq. 17-36, the process requires the use of two ATP molecules to activate one amino acid. While the "spending" of two ATPs for the addition of one monomer unit to a polymer does not appear necessary from a thermodynamic viewpoint, it is frequently observed, and there is no doubt that hydrolysis of $PP_i$ ensures that the reaction will go virtually to completion. Transfer RNAs tend to become saturated with amino acids according to Eq. 17-36 even if the concentration of free amino acid in the cytoplasm is low. On the other hand, kinetic considerations may be involved. Perhaps the biosynthetic sequence would move too slowly if it were not for the extra boost given by the removal of $PP_i$. Part of the explanation for the complexity may depend on control mechanisms which are only incompletely understood.

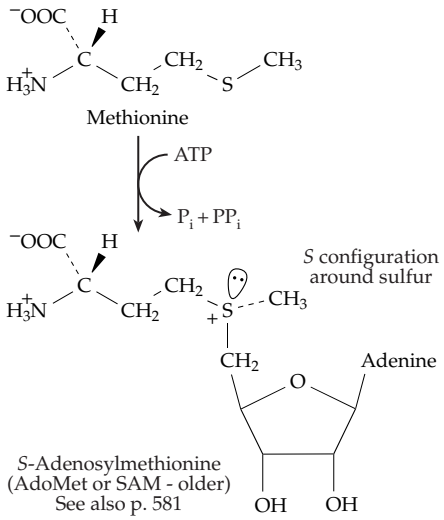
In some metabolic reactions pyrophosphate esters are formed by consecutive transfer of the terminal phospho groups of two ATP molecules onto a hydroxyl




Net: 2 ATP + amino acid + tRNA ⟶ aminoacyl tRNA + 2 ADP + 2 $P_i$ (17-36)

group. Such esters often react with elimination of $PP_i$, e.g., in polymerization of prenyl units (reaction type 6B, Table 10-1; Fig. 22-1). Again, hydrolysis to $P_i$ follows. Thus, *cleavage of pyrophosphate is a second very general method for coupling ATP cleavage to synthetic reactions*.

Although pyrophosphatases are ubiquitous, there are organisms in which $PP_i$ is conserved by the cell and replaces ATP in several glycolytic reactions. These include *Propionibacterium*,[151,152] sulfate-reducing bacteria,[153] the photosynthetic *Rhodospirillum*, and the parasitic *Entamoeba histolytica*.[152,154] In the latter the internal concentration of $PP_i$ is about 0.2 mM. Green plants also accumulate $PP_i$ at concentrations of up to 0.2 mM.[155] Apparently, pyrophosphate is not always hydrolyzed immediately. Another mystery of metabolism is the accumulation of inorganic **polyphosphate** in chains of tens to many hundreds of phospho groups linked, as in pyrophosphate, by phosphoanhydride bonds. These polyphosphates are present in many bacteria, including *E. coli*, and also in fungi, plants, and animals.[156–156b] They constitute a store of energy as well as of phosphate. Various other functions have also been proposed. A polyphosphate kinase transfers a terminal phospho group from polyphosphate chains onto ADP to form ATP. This source of metabolic energy is evidently essential to the ability of *Pseudomonas aeruginosa* to form biofilms.[156a] Both endophosphatases and exophosphatases, of uncertain function, can degrade the chains hydrolytically. An exophosphatase from *E. coli* can completely hydrolyze polyphosphate chains of 1000 units processively without release of intermediates.[156b]

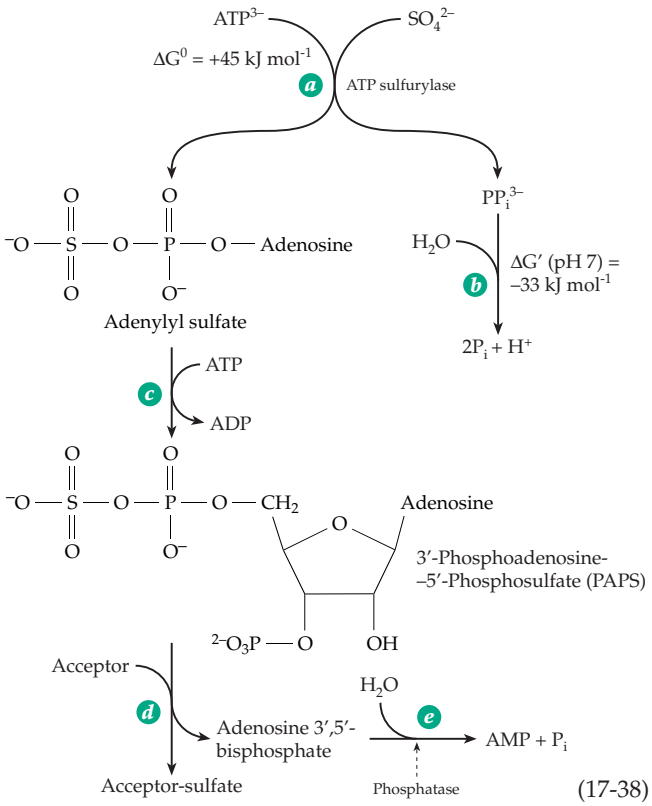



(17-37)

In a few instances group activation is coupled to cleavage of ATP at C-5' presumably with formation of bound tripolyphosphate ($PPP_i$). The latter is hydrolyzed to $P_i$ and $PP_i$ and ultimately to *three* molecules of $P_i$. An example is the formation of *S*-adenosylmethionine[157] shown in Eq. 17-37. The reaction is a displacement on the 5'-methylene group of ATP by the sulfur atom of methionine. While the initial product may be enzyme-bound $PPP_i$, it is $P_i$ and $PP_i$ that are released from the enzyme, the $P_i$ arising from the terminal phosphorus ($P_\gamma$) of ATP.[157] The *S*-adenosylmethionine formed has the *S* configuration around the sulfur.[158]

## 3. Coupling by Phosphorylation and Subsequent Cleavage by a Phosphatase

A third general method for coupling the hydrolysis of ATP to drive a synthetic sequence is to transfer the terminal phospho group from ATP to a hydroxyl group *somewhere* on a substrate. Then, after the substrate has undergone a synthetic reaction, the phosphate is removed by action of a phosphatase. For example, in the activation of sulfate (Eq. 17-38),[159] the overall standard Gibbs energy change for steps *a* (catalyzed by **ATP sulfurylase**[160,161]) and *b* is distinctly positive (+12 kJ $mol^{-1}$). The equilibrium concentration of adenylyl sulfate formed in this group activation process is extremely low. Nature's solution to this problem is to spend another molecule of ATP to phosphorylate the 3' – OH of adenosine phosphosulfate. As the latter is formed, it is converted to 3'-phosphoadenosine-5'-phosphosulfate (Eq. 17-38, step *c*) by a kinase, which is often part of a bifunctional enzyme that also contains the active site of ATP sulfurylase.[162–163a] Since the equilibrium in this step lies far toward the right, the product accumulates in a substantial concentration (up to 1 mM in cell-free systems)




(17-38)

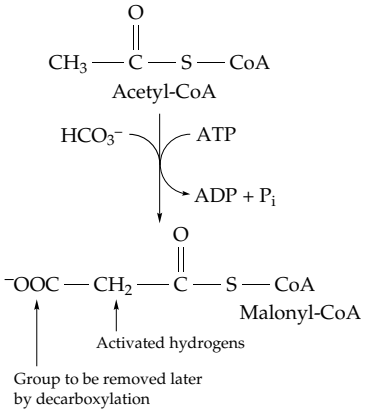
and serves as the active sulfo group donor in formation of sulfate esters. The reaction cycle is completed by two more reactions. In Eq. 17-38, step *d*, the sulfo group is transferred to an acceptor, and in step *e* the extra phosphate group is removed from adenosine 3',5'-bisphosphate by a specific phosphatase. Since the reconversion of AMP to ADP requires expenditure of still a third high-energy linkage of ATP, the overall process makes use of three high-energy phosphate linkages for formation of one sulfate ester.

An analogous use of ATP is found in photosynthetic reduction of carbon dioxide in which ATP phosphorylates ribulose 5-*P* to ribulose bisphosphate and the phosphate groups are removed later by phosphatase action on fructose bisphosphate and sedoheptulose bisphosphate (Section J,2). Phosphatases involved in synthetic pathways usually have a high substrate specificity and are to be distinguished from nonspecific phosphatases which are essentially digestive enzymes (Chapter 12).

## 4. Carboxylation and Decarboxylation: Synthesis of Fatty Acids

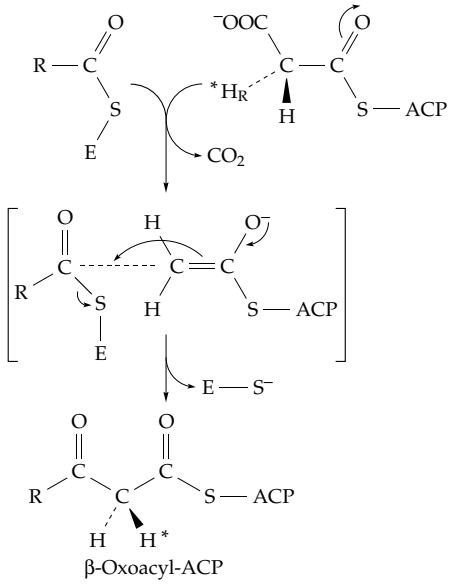
A fourth way in which cleavage of ATP can be coupled to biosynthesis was recognized in about 1958 when Wakil and coworkers discovered that synthesis of fatty acids in animal cytoplasm is stimulated by carbon dioxide. However, when $^{14}CO_2$ was used in

the experiment no radioactivity appeared in the fatty acids formed. Rather, it was found that acetyl-CoA was carboxylated to **malonyl-CoA** in an ATP- and biotin-requiring process (Eq. 17-39; see also Chapter 13). The carboxyl group formed in this reaction is later converted back to $CO_2$ in a decarboxylation (Fig. 17-12).



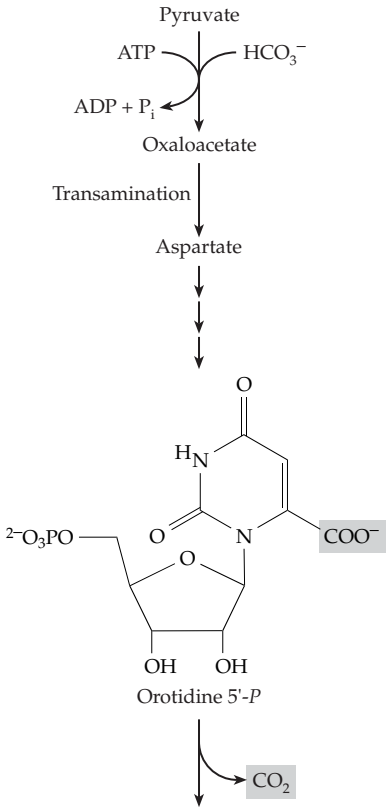

(17-39)

We know now that in most bacteria and green plants both an acetyl group of acetyl-CoA and a malonyl group of malonyl-CoA are transferred (steps *a* and *d* of Fig. 17-12) to the sulfur atoms of the phosphopantetheine groups of a low-molecular-weight **acyl carrier protein** (ACP; Chapter 14). The malonyl group of the malonyl-ACP is then condensed (step *f* of Fig. 17-12) with an acetyl group, which has been transferred from acetyl-ACP onto a thiol group of the enzyme (E in Eq. 17-40). The enolate anion indicated in this equation is generated by decarboxylation of the malonyl-ACP. It is this decarboxylation that drives the reaction to completion and, in effect, links C–C bond formation to the cleavage of the ATP required for the carboxylation step. A related sequence involving multifunctional proteins is used by animals and fungi[164] (Section J,6).




(17-40)

Carboxylation followed by a later decarboxylation is an important pattern in other biosynthetic pathways, too. Sometimes the decarboxylation follows the carboxylation by many steps. For example, pyruvate (or PEP) is converted to uridylic acid (Eq. 17-41; details are shown in Fig. 25-14):




(17-41)

## I. Reducing Agents for Biosynthesis

Still another difference between biosynthesis of fatty acids and oxidation (in mammals) is that the former has an absolute requirement for NADPH (Fig. 17-12) while the latter requires $NAD^+$ and flavoproteins (Fig. 17-1). This fact, together with many other observations, has led to the generalization that *biosynthetic reduction reactions usually require NADPH rather than NADH*. Many measurements have shown that in the cytosol of eukaryotic cells the ratio [NADPH]/[$NADP^+$] is high, whereas the ratio [NADH]/[$NAD^+$] is low. Thus, the $NAD^+$/NADH system is kept highly oxidized, in line with the role of $NAD^+$ as a principal biochemical oxidant, while the $NADP^+$/NADPH system is kept reduced.

The use of NADPH in step *g* of Fig. 17-12 ensures that significant amounts of the β-oxoacyl-ACP derivative are reduced to the alcohol. Another difference between β oxidation and biosynthesis is that the alcohol formed in this reduction step in the biosynthetic process

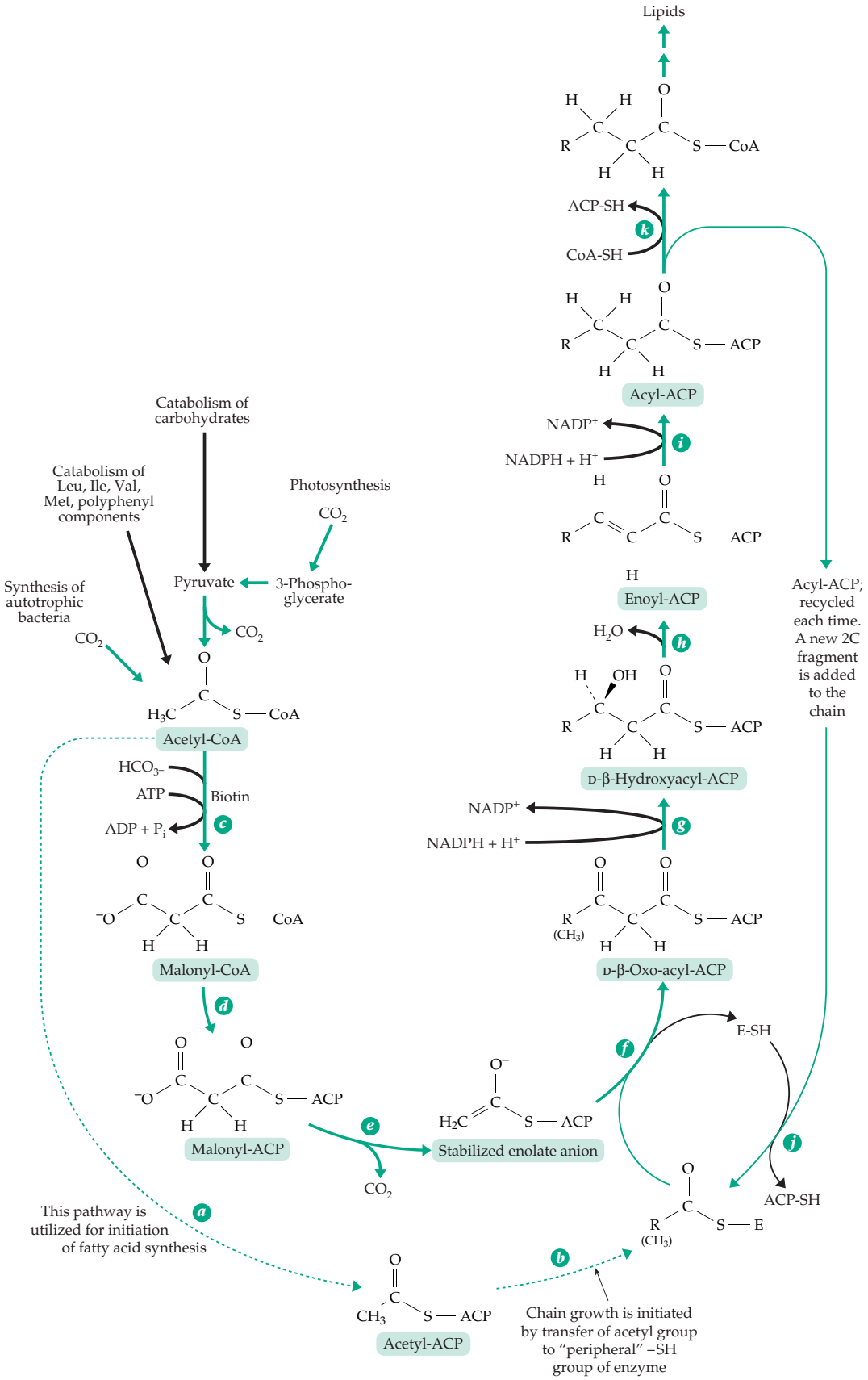



**Figure 17-12** The reactions of cytoplasmic biosynthesis of saturated fatty acids. Compare with pathway of β oxidation (Fig. 17-1).

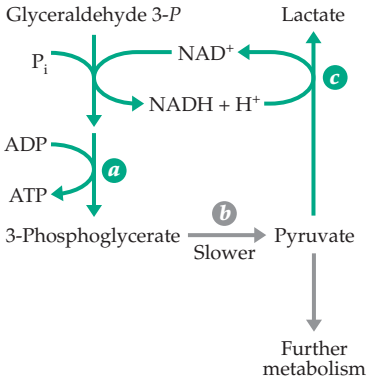
has the D configuration while the corresponding alcohol in β oxidation has the L configuration.

## 1. Reversing an Oxidative Step with a Strong Reducing Agent

The second reduction step in biosynthesis of fatty acids in the rat liver (step *i*) also required NADPH. The corresponding step in β oxidation utilizes FAD, but NADPH is a stronger reducing agent than $FADH_2$. Therefore, use of a reduced pyridine nucleotide again provides a thermodynamic advantage in pushing the reaction in the biosynthetic direction. Interesting variations have been observed among different species. For example, fatty acid synthesis in the rat requires only NADPH, but the multienzyme complexes from *Mycobacterium phlei*, *Euglena gracilis*, and the yeast *Saccharomyces cerevisiae* all give much better synthesis with a mixture of NADPH and NADH than with NADPH alone.[165] Apparently, NADPH is required in step *g* and NADH in step *i*. This seems reasonable because the equilibrium in step *i* lies far toward the product formation, and NADH at a very low concentration could carry out the reduction.

## 2. Regulation of the State of Reduction of the NAD and NADP Systems

The ratio $[NAD^+]/[NADH]$ appears to be maintained at a relatively constant value and in equilibrium with a series of different reduced and oxidized substrate pairs. Thus, it was observed that in the cytoplasm of rat liver cells, the dehydrogenations catalyzed by lactate dehydrogenase, *sn*-glycerol 3-phosphate dehydrogenase, and malate dehydrogenase are all at equilibrium with the same ratio of $[NAD^+]/[NADH]$.[166] In one experiment rat livers were removed and frozen in less than 8 s by "freeze-clamping" (Section L,2) and the concentrations of different components of the cytoplasm determined[167]; the ratio $[NAD^+]/[NADH]$ was found to be 634, while the ratio of [lactate]/[pyruvate] was 14.2. From these values an apparent equilibrium constant for reaction *c* of Eq. 17-42 was calculated as $K_c' = 9.0 \times 10^3$. The known equilibrium constant for the reaction (from *in vitro* experiments) is $8.8 \times 10^3$ (Eq. 17-43). In a similar way it was shown that several other dehydrogenation reactions are nearly at equilibrium. This conclusion has been confirmed more recently by NMR observations.[168]




(17-42)

$$K_c'\,(\text{pH } 7,\ 38°\text{C}) = \frac{[\text{lactate}]}{[\text{pyruvate}]} \times \frac{[NAD^+]}{[NADH]} = 8.8 \times 10^3 \qquad (17\text{-}43)$$

Now consider Eq. 17-42, step *a*, the ADP- and $P_i$-requiring oxidation of glyceraldehyde 3-phosphate (Fig. 15-6). Experimental measurements indicated that this reaction is also at equilibrium in the cytoplasm. In one series of experiments the measured phosphorylation state ratio $[ATP]/[ADP][P_i]$ was 709, while the ratio [3-phosphoglycerate]/[glyceraldehyde 3-phosphate] was 55.5. The overall equilibrium constant for Eq. 17-42*a* is given by Eq. 17-44. That calculated from known equilibrium constants is 60.

$$K_a'\,(\text{pH } 7,\ 38°\text{C}) = \frac{[ATP]}{[ADP][P_i]} \times \frac{[\text{3-phosphoglycerate}]}{[\text{glyceraldehyde phosphate}]} \times \frac{[NADH]}{[NAD^+]} = 709 \times 55.5 \times 1/634 = 62 \qquad (17\text{-}44)$$

From these data Krebs and Veech concluded that the oxidation state of the NAD system is determined largely by the phosphorylation state ratio of the adenylate system.[169] If the ATP level is high the equilibrium in Eq. 17-42*a* will be reached at a higher $[NAD^+]/[NADH]$ ratio and lactate may be oxidized to pyruvate to adjust the [lactate]/[pyruvate] ratio.

It is important not to confuse the reactions of Eq. 17-42 as they occur in an aerobic cell with the tightly coupled pair of redox reactions in the homolactate fermentation (Fig. 10-3; Eq. 17-19). The reactions of steps *a* and *c* of Eq. 17-42 are essentially at equilibrium, but the reaction of step *b* may be relatively slow. Furthermore, pyruvate is utilized in many other metabolic pathways and ATP is hydrolyzed and converted to ADP through innumerable processes taking place within the cell. Reduced NAD does not cycle between the two enzymes in a stoichiometric way and the "reducing equivalents" of NADH formed are, in large measure, transferred to the mitochondria. The proper view of the reactions of Eq. 17-42 is that the redox pairs represent a kind of **redox buffer system** that poises the $NAD^+/NADH$ couple at a ratio appropriate for its metabolic function.

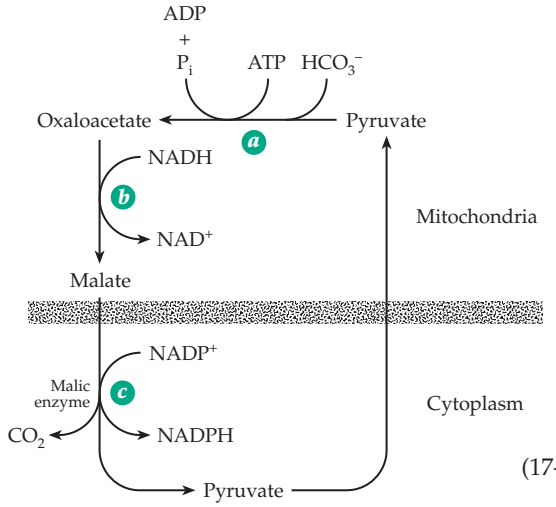
Somewhat surprisingly, within the mitochondria the ratio [$NAD^+$]/[NADH] is 100 times lower than in the cytoplasm. Even though mitochondria are the site of oxidation of NADH to $NAD^+$, the intense catabolic activity occurring in the β oxidation pathway and the citric acid cycle ensure extremely rapid production of NADH. Furthermore, the reduction state of NAD is apparently buffered by the low potential of the β-hydroxybutyrate–acetoacetate couple (Chapter 18, Section C,2). Mitochondrial pyridine nucleotides also appear to be at equilibrium with glutamate dehydrogenase.[169]

How is the cytoplasmic [NADPH]/[$NADP^+$] ratio maintained at a value higher than that of [NADH]/[$NAD^+$]? Part of the answer is from operation of the pentose phosphate pathway (Section E,3). The reactions of Eq. 17-12, if they attained equilibrium, would give a ratio of cytosolic [NADPH]/[$NADP^+$] > 2000 at 0.05 atm $CO_2$. Compare this with the ratio 1/634 for [NADH/[$NAD^+$] deduced from the observation on the reactions of Eq. 17-42.

Consider also the following **transhydrogenation** reaction (Eq. 17-45):

$$NADH + NADP^+ \rightarrow NAD^+ + NADPH \quad (17\text{-}45)$$

There are soluble enzymes that catalyze this reaction for which *K* equals ~ 1. Within mitochondria an energy-linked system (Chapter 18) involving the membrane shifts the equilibrium to favor NADPH. However, within the cytoplasm, the reaction of Eq. 17-45 is driven by coupling ATP cleavage to the transhydrogenation via carboxylation followed by eventual decarboxylation. One cycle that accomplishes this is given in Eq. 17-46. The first step (step *a*) is ATP-dependent carboxylation of pyruvate to oxaloacetate, a reaction that occurs only within mitochondria (Eq. 14-3). Oxaloacetate can be reduced by malate dehydrogenase using NADH (Eq. 17-46, step *b*), and the resulting malate can be exported from the mitochondria. In the cytoplasm the malate is oxidized to pyruvate, with decarboxylation, by the malic enzyme (Eq. 13-45). The malic enzyme (Eq. 17-46, step *c*) is specific for $NADP^+$, is very active, and also appears to operate at or near equilibrium within the cytoplasm. On this basis, using known equilibrium constants, it is easy to show that the ratio [NADPH]/[$NADP^+$] will be ~ $10^5$ times higher at equilibrium than the ratio [NADH]/[$NAD^+$].[169,170]




(17-46)

Since NADPH is continuously used in biosynthetic reactions, and is thereby reconverted to $NADP^+$, the cycle of Eq. 17-46 must operate continuously. As in Eq. 17-42, a true equilibrium does not exist but steps *b* and *c* are both essentially at equilibrium. These equilibria, together with those of Eq. 17-42 for the NAD system, ensure the correct redox potential of both pyridine nucleotide coenzymes in the cytoplasm.

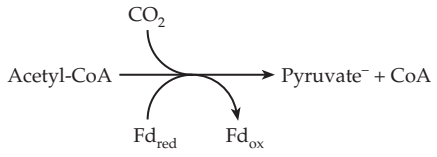
Malate is not the only form in which $C_4$ compounds are exported from mitochondria. Much oxaloacetate is combined with acetyl-CoA to form citrate; the latter leaves the mitochondria and is cleaved by the ATP-dependent citrate-cleaving enzymes (Eq. 13-39). This, in effect, exports both acetyl-CoA (needed for lipid synthesis) and oxaloacetate which is reduced to malate within the cytoplasm. Alternatively, oxaloacetate may be transaminated to aspartate. The aspartate, after leaving the mitochondria, may be converted in another transamination reaction back to oxaloacetate. All of these are part of the nonequilibrium process by which $C_4$ compounds diffuse out of the mitochondria before completing the reaction sequence of Eq. 17-46 and entering into other metabolic processes. Note that the reaction of Eq. 17-46 leads to the *export* of reducing equivalents from mitochondria, the opposite of the process catalyzed by the malate–aspartate shuttle which is discussed in Chapter 18 (Fig. 18-18). The two processes are presumably active under different conditions.

While the difference in the redox potential of the two pyridine nucleotide systems is clear-cut in mammalian tissues, in *E. coli* the apparent potentials of the two systems are more nearly the same.[171]

## 3. Reduced Ferredoxin in Reductive Biosynthesis

Both the $NAD^+$ and $NADP^+$ systems have standard electrode potentials $E°'$ (pH 7) of −0.32 V. However, because of the differences in concentration ratios, the $NAD^+$ system operates at a less negative potential (−0.24 V) and the $NADP^+$ system at a more negative potential (−0.38 V) within the cytoplasm of eukaryotes. In green plants and in many bacteria a still more powerful reducing agent is available in the form of reduced ferredoxin. The value of $E°'$ (pH 7) for clostridial ferredoxin is −0.41 V, corresponding to a Gibbs energy change for the two-electron reduction of a substrate ~18 kJ $mol^{-1}$ more negative than the corresponding

value of $\Delta G'$ for reduction by NADPH. Using reduced ferredoxin (Fd) some photosynthetic bacteria and anaerobic bacteria are able to carry out reductions that are virtually impossible with the pyridine nucleotide system. For example, pyruvate and 2-oxoglutarate can be formed from acetyl-CoA (Eq. 15-35) and succinyl-CoA, respectively (Eq. 17-47).[172–173a] In our bodies the reaction of Eq. 17-47, with $NAD^+$ as the oxidant, goes only in the opposite direction and is essentially irreversible.




(17-47)

## J. Constructing the Monomer Units

Now let us consider the synthesis of the monomeric units from which biopolymers are made. How can simple one-carbon compounds such as $CO_2$ and formic acid be incorporated into complex carbon compounds? How can carbon chains grow in length or be shortened? How are branched chains and rings formed?

### 1. Carbonyl Groups in Chain Formation and Cleavage

Except for some vitamin $B_{12}$-dependent reactions, the cleavage or formation of carbon–carbon bonds usually depends upon the participation of carbonyl groups. For this reason, carbonyl groups have a central mechanistic role in biosynthesis. The activation of hydrogen atoms β to carbonyl groups permits β condensations to occur during biosynthesis. Aldol or Claisen condensations require the participation of two carbonyl compounds. Carbonyl compounds are also essential to thiamin diphosphate-dependent condensations and the aldehyde pyridoxal phosphate is needed for most C–C bond cleavage or formation within amino acids.

Because of the importance of carbonyl groups to the mechanism of condensation reactions, much of the assembly of either straight-chain or branched-carbon skeletons takes place between compounds in which the average oxidation state of the carbon atoms is similar to that in carbohydrates (or in formaldehyde, $H_2CO$). The diversity of chemical reactions possible with compounds at this state of oxidation is a maximum, a fact that may explain why carbohydrates and closely related substances are major biosynthetic precursors and why the average state of oxidation of the carbon in most living things is similar to that in carbohydrates.[174] This fact may also be related to the presumed occurrence of formaldehyde as a principal component of the earth's atmosphere in the past and to the ability of formaldehyde to condense to form carbohydrates.

In Fig. 17-13 several biochemicals have been arranged according to the oxidation state of carbon. Most of the important biosynthetic intermediates lie within ± 2 electrons per carbon atom of the oxidation state of carbohydrates. As the chain length grows, they tend to fall even closer. It is extremely difficult to move through enzymatic processes between 2C, 3C, and 4C compounds (i.e., vertically in Fig. 17-13) except at the oxidation level of carbohydrates or somewhat to its right, at a slightly higher oxidation level. On the other hand, it is often possible to move horizontally with ease using oxidation–reduction reactions. Thus, fatty acids are assembled from acetate units, which lie at the same oxidation state as carbohydrates and, after assembly, are reduced.

Among compounds of the same overall oxidation state, e.g., acetic acid and sugars, the oxidation states of individual carbon atoms can be quite different. Thus, in a sugar every carbon atom can be regarded as immediately derived from formaldehyde, but in acetic acid one end has been oxidized to a carboxyl group and the other has been reduced to a methyl group. Such internal oxidation–reduction reactions play an important role in the chemical manipulations necessary to assemble the carbon skeletons needed by a cell. Decarboxylation is a feature of many biosynthetic routes. Referring again to Fig. 17-13, notice that many of the biosynthetic intermediates such as pyruvate, oxoglutarate, and oxaloacetate are more oxidized than the carbohydrate level. However, their decarboxylation products, which become incorporated into the compounds being synthesized, are closer to the oxidation level of carbohydrates.

### 2. Starting with $CO_2$

There are three known pathways by which autotrophic organisms can use $CO_2$ to synthesize triose phosphates or 3-phosphoglycerate, three-carbon compounds from which all other biochemical substances can be formed.[175–177] The first of these is the **reductive tricarboxylic cycle.** This is a reversal of the oxidative citric acid cycle in which reduced ferredoxin is used as a reductant in the reaction of Eq. 17-47 to incorporate $CO_2$ into pyruvate. Succinyl-CoA can react with $CO_2$ in the same type of reaction to form 2-oxoglutarate, accomplishing the reversal of the only irreversible step in the citric acid cycle. Using these reactions photosynthetic bacteria and some anaerobes that can generate a high ratio of reduced to oxidized ferredoxin carry out the reductive tricarboxylic acid cycle. Together with

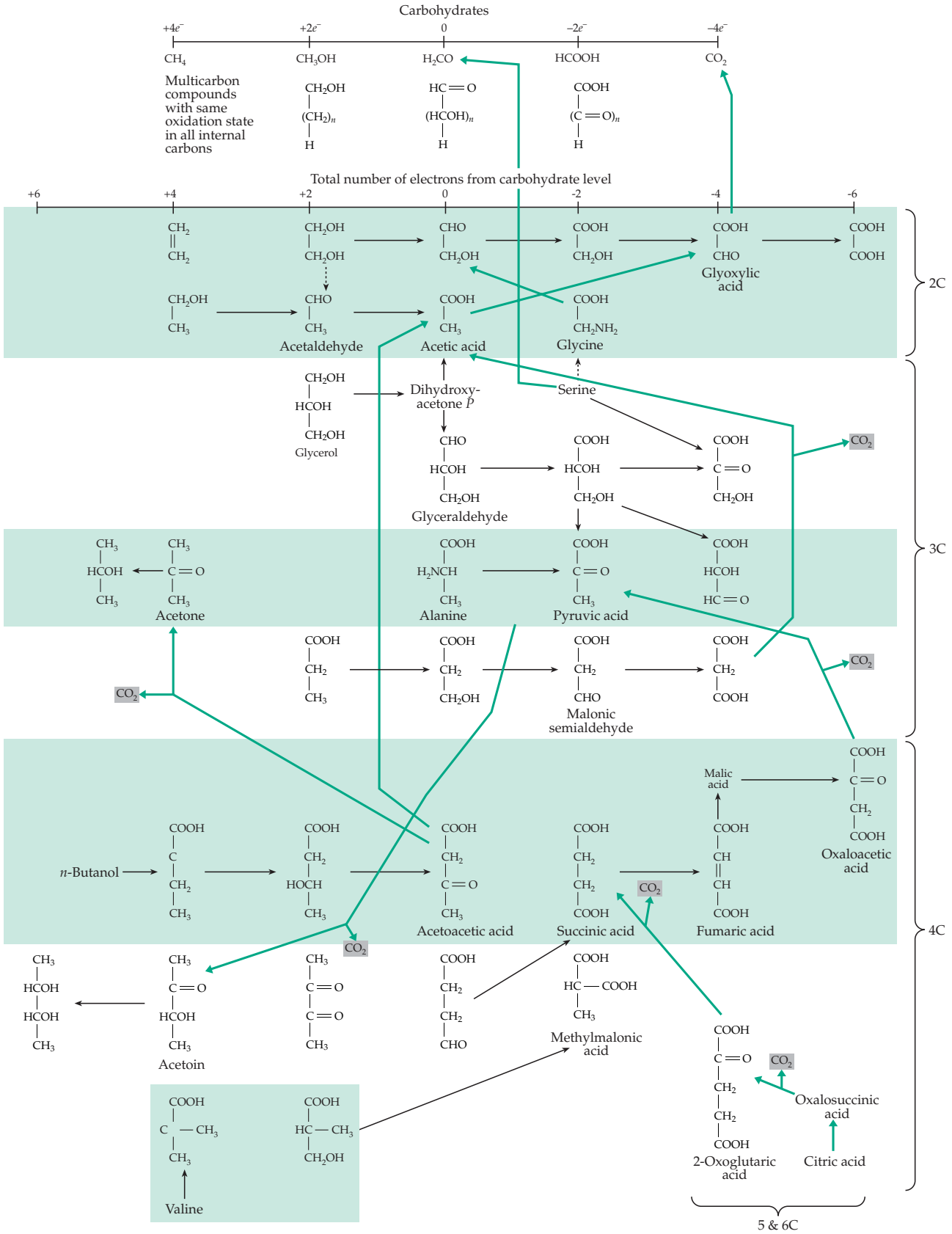



**Figure 17-13** Some biochemical compounds arranged in order of average oxidation state of the carbon atoms and by carbon-chain lengths. Black horizontal arrows mark some biological interconversions among compounds with the same chain length, while green lines show changes in chain length and are often accompanied by decarboxylation.

Eq. 17-47, the cycle provides for the complete synthesis of pyruvate from $CO_2$.[178,179]

A quantitatively much more important pathway of $CO_2$ fixation is the **reductive pentose phosphate pathway** (ribulose bisphosphate cycle or **Calvin–Benson cycle**; Fig. 17-14). This sequence of reactions, which takes place in the chloroplasts of green plants and also in many chemiautotrophic bacteria, is essentially a way of reversing the oxidative pentose phosphate pathway (Fig. 17-8). The latter accomplishes the complete oxidation of glucose or of glucose 1-phosphate by $NADP^+$ (Eq. 17-48):

$$\begin{aligned}\text{Glucose 1-}P^{2-} + ATP^{4-} + 8\ H_2O + 12\ NADP^+ \rightarrow \\ 6\ CO_2 + 12\ NADPH + ADP^{3-} + 2HPO_4^{2-} + 13\ H^+ \\ \Delta G'\ (\text{pH } 7) = -299\ \text{kJ mol}^{-1}\end{aligned} \qquad (17\text{-}48)$$

It would be almost impossible for a green plant to fix $CO_2$ using photochemically generated NADPH by an exact reversal of Eq. 17-48 because of the high positive Gibbs energy change. To solve this thermodynamic problem the reductive pentose phosphate pathway has been modified in a way that couples ATP cleavage to the synthesis.

The **reductive carboxylation** system is shown within the green shaded box of Fig. 17-14. Ribulose 5-phosphate is the starting compound and in the first step one molecule of ATP is expended to form **ribulose 1,5-bisphosphate**. The latter is carboxylated and cleaved to two molecules of 3-phosphoglycerate. This reaction was discussed in Chapter 13. The reductive step (step *c*) of the system employs NADPH together with ATP. Except for the use of the NADP system instead of the NAD system, it is exactly the reverse of the glyceraldehyde phosphate dehydrogenase reaction of glycolysis. Looking at the first three steps of Fig. 17-14 it is clear that in the reductive pentose phosphate pathway three molecules of ATP are utilized for each $CO_2$ incorporated. On the other hand, in the oxidative direction *no* ATP is generated by the operation of the pentose phosphate pathway.

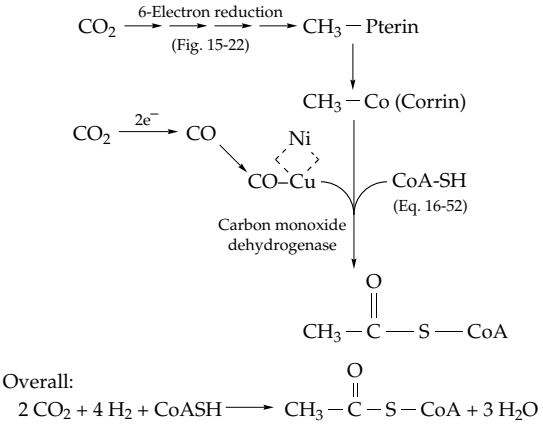
The reactions enclosed within the shaded box of Fig. 17-14 do not give the whole story about the coupling mechanism. A phospho group was transferred from ATP in step *a* and to complete the hydrolysis it must be removed in some future step. This is indicated in a general way in Fig. 17-14 by the reaction steps *d*, *e*, and *f*. Step *f* represents the action of specific phosphatases that remove phospho groups from the seven-carbon sedoheptulose bisphosphate and from fructose bisphosphate. In either case the resulting ketose monophosphate reacts with an aldose (via transketolase, step *g*) to regenerate ribulose 5-phosphate, the $CO_2$ acceptor. The overall reductive pentose phosphate cycle (Fig. 17-14B) is easy to understand as a reversal of the oxidative pentose phosphate pathway in which the oxidative decarboxylation system of Eq. 17-12 is replaced by the reductive carboxylation system of Fig. 17-14A. The scheme as written in Fig. 17-14B shows the incorporation of three molecules of $CO_2$. The reductive carboxylation system operates three times with a net production of one molecule of triose phosphate. As with other biosynthetic cycles, any amount of any of the intermediate metabolites may be withdrawn into various biosynthetic pathways without disruption of the flow through the cycle.

The overall reaction of carbon dioxide reduction in the Calvin–Benson cycle (Fig. 17-14) becomes

$$\begin{aligned}6\ CO_2 + 12\ NADPH + 18\ ATP^{4-} + 11\ H_2O \rightarrow \\ \text{glucose-1-}P^{2-} + 12\ NADP^+ + 18\ ADP^{3-} \\ 17\ HPO_4^{2-} + 6\ H^+\end{aligned} \qquad (17\text{-}49)$$

The Gibbs energy change $\Delta G'$ (pH 7) is now −357 kJ $mol^{-1}$ instead of the +299 kJ $mol^{-1}$ required to reverse the reaction of Eq. 17-48.

The third pathway for reduction of $CO_2$ to acetyl-CoA is utilized by acetogenic bacteria, by methanogens, and probably by sulfate-reducing bacteria.[179–181] This **acetyl-CoA pathway** (or **Wood–Ljungdahl pathway**) involves reduction by $H_2$ of one of the two molecules of $CO_2$ to the methyl group of methyltetrahydromethanopterin in methanogens and of methyltetra-hydrofolate in acetogens. The pathway utilized by methanogens is illustrated in Fig. 15-22.[182–184] A similar process utilizing $H_2$ as the reductant is employed by acetogens.[179,185–188a] In both cases a methyl corrinoid is formed and its methyl group is condensed with a molecule of carbon monoxide bound to a copper ion in a Ni–Cu cluster.[189a,b] The resulting acetyl group is transferred to a molecule of coenzyme A as illustrated in Eq. 16-52.[189] The bound CO is formed by reduction of $CO_2$, again using $H_2$ as the reductant.[190] The overall reaction for acetyl-CoA synthesis is given by Eq. 17-50. Conversion of acetyl-CoA to pyruvate via Eq. 17-47 leads into the glucogenic pathway.




(17-50)

An alternative pathway by which some acetogenic bacteria form acetate is via reversal of the glycine decarboxylase reaction of Fig. 15-20. Methylene-THF is formed by reduction of $CO_2$, and together with $NH_3$ and $CO_2$ a lipoamide group of the enzyme and PLP forms glycine. The latter reacts with a second methylene-THF to form serine, which can be deaminated to pyruvate and assimilated. Methanogens may use similar pathways but ones that involve methanopterin (Fig. 15-17).[191]

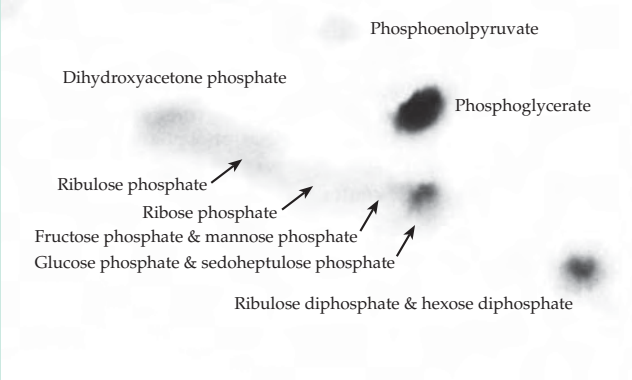
### 3. Biosynthesis from Other Single-Carbon Compounds

Various bacteria and fungi are able to subsist on such one-carbon compounds as methane, methanol, methylamine, formaldehyde, and formate.[192–197] Energy is obtained by oxidation to $CO_2$. **Methylotrophic bacteria** initiate oxidation of methane by hydroxylation (Chapter 18) and dehydrogenate the resulting methanol or exogenous methanol using the PPQ cofactor (Eq. 15-51).[198] Further dehydrogenation to formate and of formate to $CO_2$ via formate dehydrogenase (Eq. 16-63) completes the process. Some methylotrophic bacteria incorporate $CO_2$ for biosynthetic purposes via the ribulose bisphosphate (Calvin–Benson) cycle but many use pathways that begin with formaldehyde (or methylene-THF). Others employ variations of the reductive pentose phosphate pathway to convert formaldehyde to triose phosphate. In one of these, the **ribulose monophosphate cycle** or Quayle cycle,[192,193] ribulose 5-*P* undergoes an aldol condensation with formaldehyde to give a 3-oxo-hexulose 6-phosphate (Eq. 17-51, step *a*). The latter is isomerized to fructose 6-*P* (Eq. 17-51, step *b*). If this equation is applied to the

## BOX 17-E $^{14}C$ AND THE CALVIN–BENSON CYCLE

The chemical nature of photosynthesis had intrigued chemists for decades but little was learned about the details until radioactive $^{14}C$ became available. Discovered in 1940 by Ruben and Kamen, the isotope was available in quantity by 1946 as a product of nuclear reactors. Initial studies of photosynthesis had been conducted by Ruben and Kamen using $^{11}C$ but $^{14}C$ made rapid progress possible. In 1946 Melvin Calvin and Andrew A. Benson began their studies that elucidated the mechanism of incorporation of $CO_2$ into organic materials.

A key development was two-dimensional paper chromatography with radioautography (Box 3-C). A suspension of the alga *Chlorella* (Fig. 1-11) was allowed to photosynthesize in air. At a certain time, a portion of $H^{14}CO_3$ was injected into the system, and after a few seconds of photosynthesis with $^{14}C$ present the suspension of algae was run into hot methanol to denature proteins and to stop the reaction. The soluble materials extracted from the algal cells were concentrated and chromatographed; radioautographs were then prepared. It was found that after 10 s of photosynthesis in the presence of $^{14}CO_2$, the algae contained a dozen or more $^{14}C$ labeled compounds. These included malic acid, aspartic acid, phosphoenolpyruvate, alanine, triose phosphates, and other sugar phosphates and diphosphates. However, *during the first five seconds a single compound, 3-phosphoglycerate, contained most of the radioactivity*.[a,b] This finding suggested that a two-carbon regenerating substrate might be carboxylated by $^{14}CO_2$ to phosphoglycerate. Search for this two-carbon compound was unsuccessful, but Benson, in Calvin's laboratory, soon identified ribulose bisphosphate,[c] which kinetic studies proved to be the true regenerating substrate.[c,d] Its carboxylation and cleavage[e] represent the first step in what has come to be known as the Calvin–Benson cycle (Fig. 17-14).[f]




Chromatogram of extract of the alga *Scenedesmus* after photosynthesis in the presence of $^{14}CO_2$ for 10 s. Courtesy of J. A. Bassham. The origin of the chromatogram is at the lower right corner.


[a] Benson, A. A., Bassham, J. A., Calvin, M., Goodale, T. C., Haas, V. A., and Stepka, W. (1950) *J. Am. Chem. Soc.* **72**, 1710–1718
[b] Benson, A. A. (1951) *J. Am. Chem. Soc.* **73**, 2971–2972
[c] Benson, A. A., Kawaguchi, S., Hayes, P., and Calvin, M. (1952) *J. Am. Chem. Soc.* **74**, 4477–4482
[d] Bassham, J. A., Benson, A. A., Kay, L. D., Harris, A. Z., Wilson, A. T., and Calvin, M. (1954) *J. Am. Chem. Soc.* **76**, 1760–1770
[e] Calvin, M., and Bassham, J. A. (1962) *The Photosynthesis of Carbon Compounds*, Benjamin, New York
[f] Fuller, R. C. (1999) *Photosynth Res.* **62**, 1–29

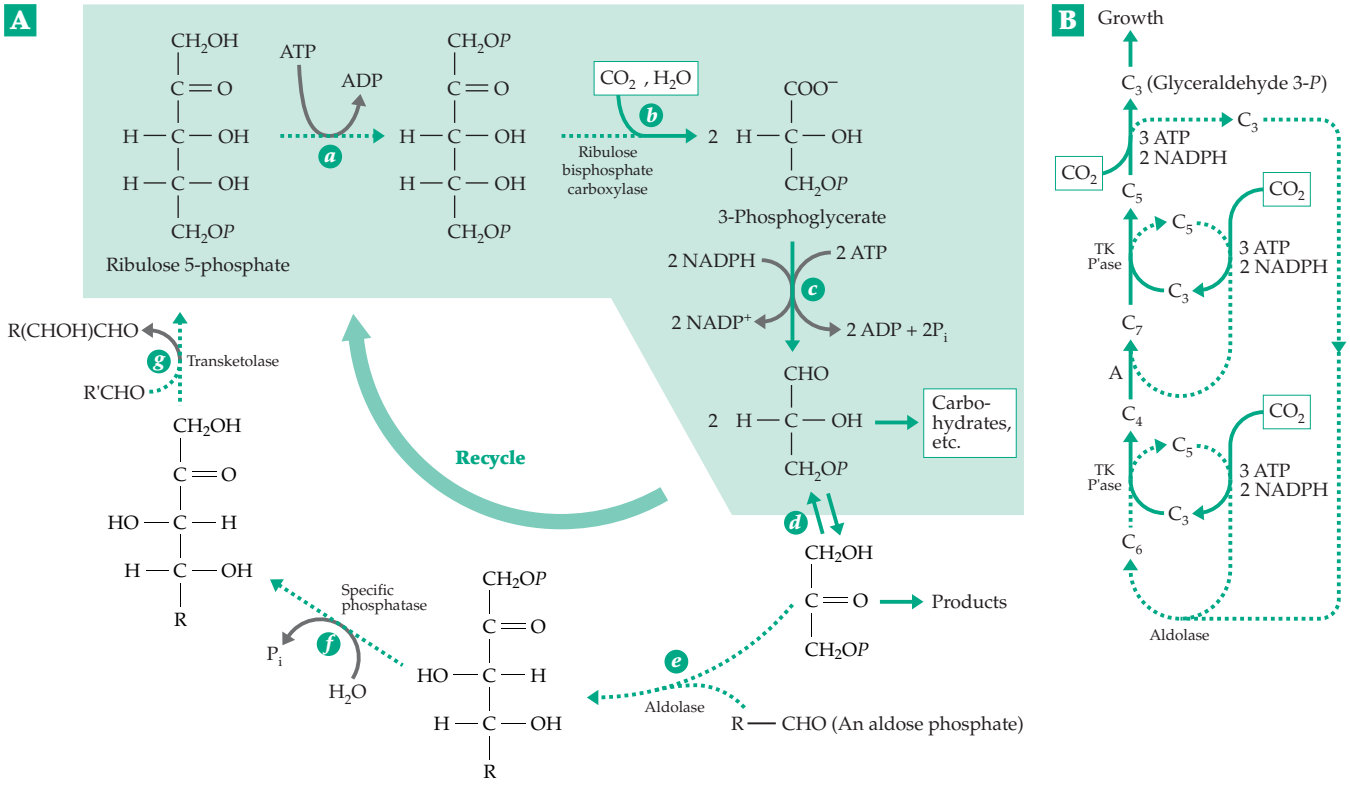



**Figure 17-14** (A) The reductive carboxylation system used in reductive pentose phosphate pathway (Calvin–Benson cycle). The essential reactions of this system are enclosed within the dashed box. Typical subsequent reactions follow. The phosphatase action completes the phosphorylation–dephosphorylation cycle. (B) The reductive pentose phosphate cycle arranged to show the combining of three $CO_2$ molecules to form one molecule of triose phosphate. Abbreviations are RCS, reductive carboxylation system (from above); A, aldolase, Pase, specific phosphatase; and TK, transketolase.

three $C_5$ sugars three molecules of fructose 6-phosphate will be formed. One of these can be phosphorylated by ATP to fructose 1,6-bisphosphate, which can be cleaved by aldolase. One of the resulting triose phosphates can then be removed for biosynthesis and the second, together with the other two molecules of fructose 6-*P*, can be recycled through the sugar rearrangement sequence of Fig. 17-8B to regenerate the three ribulose 5-*P* molecules that serve as the regenerating substrate.

In bacteria, which lack formate dehydrogenase, formaldehyde can be oxidized to $CO_2$ to provide energy beginning with the reactions of Eq. 17-51. The resulting fructose 6-*P* is isomerized to glucose 6-*P*, which is then dehydrogenated via Eq. 17-12 to form $CO_2$ and the regenerating substrate ribulose 5-phosphate.

A number of pseudomonads and other bacteria convert $C_1$ compounds to acetate via tetrahydrofolic acid-bound intermediates and $CO_2$ using the **serine pathway**[179,192,193] shown in Fig. 17-15. This is a cyclic process for converting one molecule of formaldehyde (bound to tetrahydrofolate) plus one of $CO_2$ into acetate. The regenerating substrate is **glyoxylate**. Before condensation with the "active formaldehyde" of methylene THF, the glyoxylate undergoes transamination to glycine (Fig. 17-15, step *a*). The glycine plus formaldehyde forms serine (step *b*), which is then transaminated to hydroxypyruvate, again using step *a*. Glyoxylate plus formaldehyde could have been joined in a thiamin-dependent condensation. However, as in the γ-aminobutyrate shunt (Fig. 17-5), the coupled transamination step of Fig 17-15 permits use of PLP-dependent C–C bond formation.

Conversion of hydroxypyruvate to PEP (Fig. 17-15) involves reduction by NADH and phosphorylation by ATP to form 3-phosphoglycerate, which is converted to PEP as in glycolysis. The conversion of malate to acetate and glyoxylate via malyl-CoA and isocitrate lyase (Eq. 13-40) forms the product acetate and regenerates glyoxylate. As with other metabolic cycles, various intermediates, such as PEP, can be withdrawn for biosynthesis. However, there must be an independent route of synthesis of the regenerating substrate glyoxylate. One way in which this can be accomplished is to form glycine via the reversal of the glycine decarboxylase pathway as is indicated by the shaded green lines in Fig. 17-15.

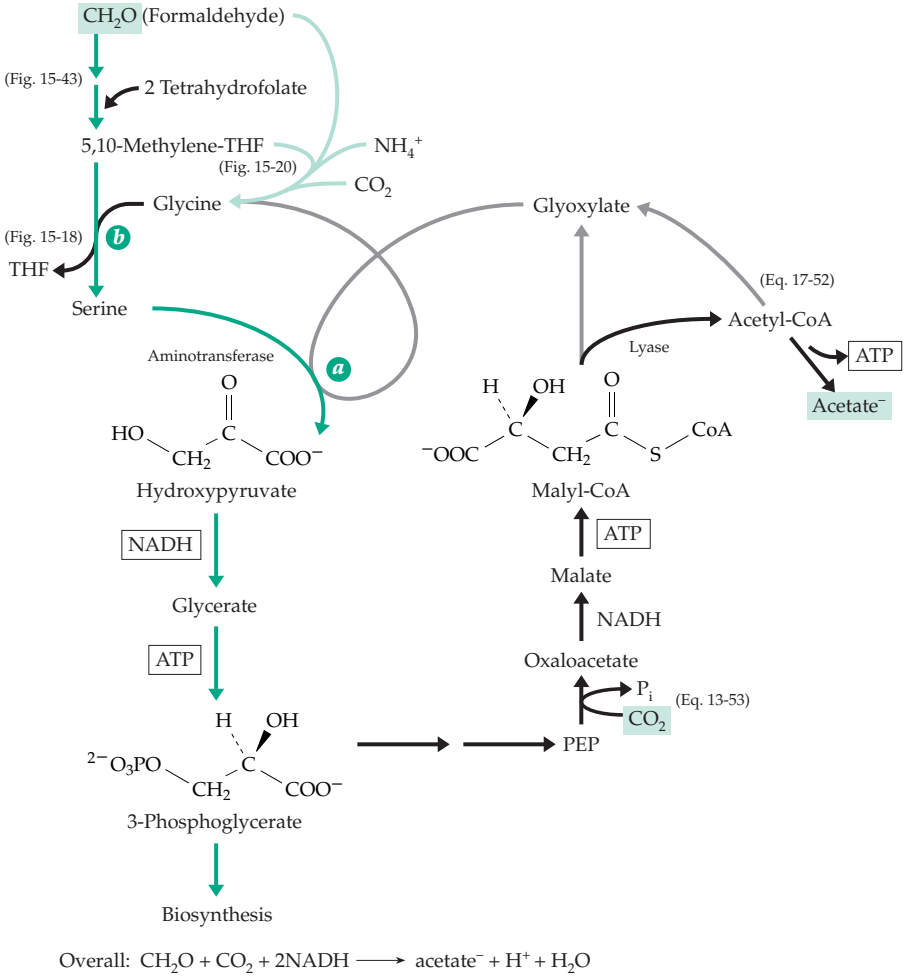
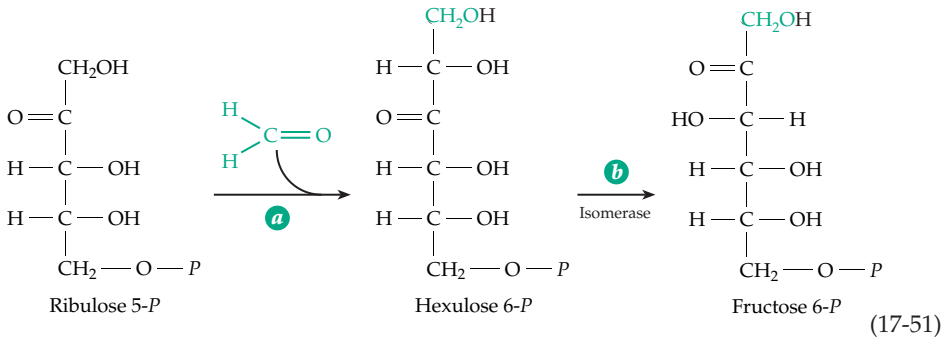



**Figure 17-15** One of the serine pathways for assimilation of one-carbon compounds.




(17-51)

## 4. The Glyoxylate Pathways

The reductive carboxylation of acetyl-CoA to pyruvate (Eq. 17-47) occurs only in a few types of bacteria. For most species, from microorganisms to animals, the oxidative decarboxylation of pyruvate to acetyl-CoA is irreversible. This fact has many important consequences. For example, carbohydrate is readily converted to fat; because of the irreversibility of this process, excess calories lead to the deposition of fats. However, in animals fat cannot be used to generate most of the biosynthetic intermediates needed for formation of carbohydrates and proteins because those intermediates originate largely from $C_3$ units.

This limitation on the conversion of $C_2$ acetyl units to $C_3$ metabolites is overcome in many organisms by

the **glyoxylate cycle** (Fig. 17-16), which converts *two* acetyl units into one $C_4$ unit. The cycle provides a way for organisms, such as *E. coli*,[111,199] *Saccharomyces*,[200] *Tetrahymena*, and the nematode *Caenorhabditis*,[201] to subsist on acetate as a sole or major carbon source. It is especially prominent in plants that store large amounts of fat in their seeds (**oil seeds**). In the germinating oil seed the glyoxylate cycle allows fat to be converted rapidly to sucrose, cellulose, and other carbohydrates needed for growth.

A key enzyme in the glyoxylate cycle is **isocitrate lyase**, which cleaves isocitrate (Eq. 13-40) to succinate and glyoxylate. The latter is condensed with a second acetyl group by the action of **malate synthase** (Eq. 13-38). The L-malate formed in this reaction is dehydrogenated to the regenerating substrate oxaloacetate. Some of the reaction steps are those of the citric acid cycle and it appears that in bacteria there is no spatial separation of the citric acid cycle and glyoxylate pathway. However, in plants the enzymes of the glyoxylate cycle are present in specialized peroxisomes known as **glyoxysomes**.[60] The glyoxysomes also contain the enzymes for β oxidation of fatty acids, allowing for efficient conversion of fatty acids to **succinate**. This compound is exported from the glyoxysomes and enters the mitochondria where it undergoes β oxidation to oxaloacetate. The latter can be converted by PEP carboxylase (Eq. 13-53) or by PEP carboxykinase (Eq. 13-46) to PEP.

An **acetyl-CoA-glyoxylate** cycle, which catalyzes oxidation of acetyl groups to glyoxylate, can also be constructed from isocitrate lyase and citric acid cycle

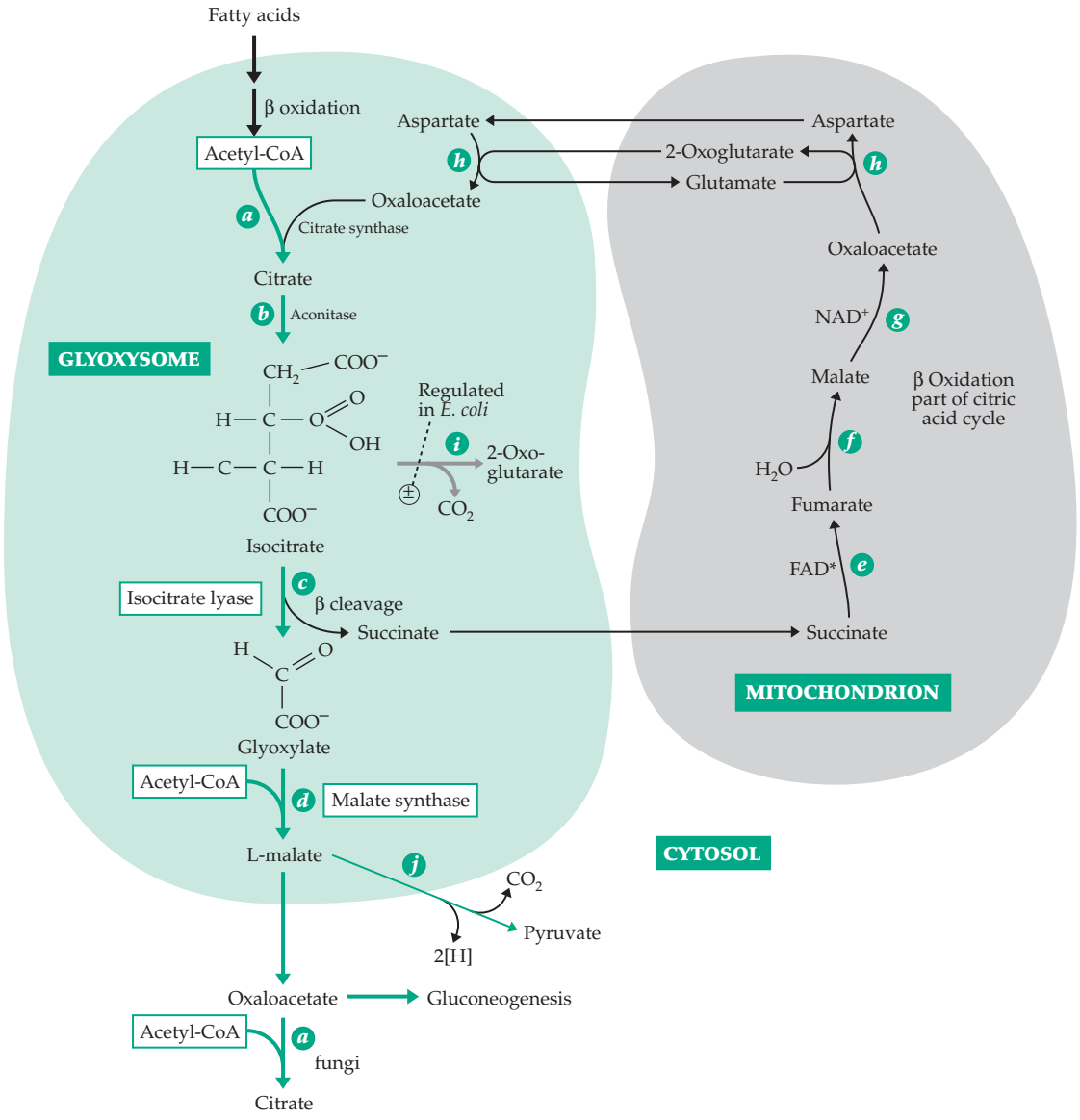
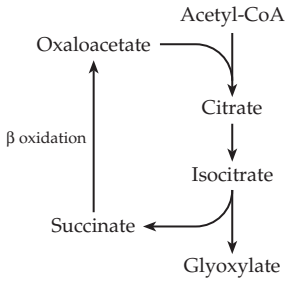



**Figure 17-16** The glyoxylate pathway. The green line traces the pathway of labeled carbon from fatty acids or acetyl-CoA into malate and other products.

enzymes. Glyoxylate is taken out of the cycle as the product and succinate is recycled (Eq. 17-52). The independent pathway for synthesis of the regenerating substrate oxaloacetate is condensation of glyoxylate with acetyl-CoA (malate synthetase) to form malate and oxidation of the latter to oxaloacetate as in the main cycle of Fig. 17-16.




(17-52)

## 5. Biosynthesis of Glucose from Three-Carbon Compounds

Now let us consider the further conversion of PEP and of the triose phosphates to **glucose 1-phosphate**, the key intermediate in biosynthesis of other sugars and polysaccharides. The conversion of PEP to glucose 1-*P* represents a reversal of part of the glycolysis sequence. It is convenient to discuss this along with **gluconeogenesis**, the reversal of the complete glycolysis sequence from lactic acid. This is an essential part of the Cori cycle (Section F) in our own bodies, and the same process may be used to convert pyruvate derived from deamination of alanine or serine (Chapter 24) into carbohydrates.

Just as with the pentose phosphate cycle, an exact reversal of the glycolysis sequence (Eq. 17-53) is precluded on thermodynamic grounds. Even at very high values of the phosphorylation state ratio $R_p$, the reaction:

$$2\ \text{Lactate}^- + 3\ \text{ATP}^{4-} + 2\ \text{H}_2\text{O} \rightarrow \text{C}_6\text{H}_{10}\text{O}_5\ (\text{glycogen}) + 3\ \text{ADP}^{3-} + 3\ \text{HPO}_4^{2-} + \text{H}^+$$

$$\Delta G'\ (\text{pH } 7) = +107\ \text{kJ per glycosyl unit} \qquad (17\text{-}53)$$

would be unlikely to go to completion. The actual pathways used for gluconeogenesis (Fig. 17-17, green lines) differ from those of glycolysis (black lines) in three significant ways. First, while glycogen breakdown is initiated by the reaction with inorganic phosphate catalyzed by phosphorylase (Fig. 17-17, step *a*), the biosynthetic sequence from glucose 1-*P*, via uridine diphosphate glucose (Fig. 17-17, step *b*; see also Eq. 17-56), is coupled to cleavage of ATP. Second, in the catabolic process (glycolysis) fructose 6-*P* is converted to fructose 1,6-$P_2$ through the action of a kinase (Fig. 17-17, step *c*), which is then cleaved by aldolase. The resulting triose phosphate is degraded to PEP. In glucogenesis a phosphatase is used to form fructose *P* from fructose $P_2$ (Fig. 17-17, step *d*). Third, during gly-colysis PEP is converted to pyruvate by a kinase with generation of ATP (Fig. 17-17, step *e*). During glucogenesis pyruvate is converted to PEP indirectly via oxaloacetate (Fig. 17-17, steps *f* and *g*) using pyruvate carboxylase (Eq. 14-3) and PEP carboxykinase (Eq. 13-46). This is another example of the coupling of ATP cleavage through a carboxylation–decarboxylation sequence. The net effect is to use two molecules of ATP (actually one ATP and one GTP) rather than *one* to convert pyruvate to PEP.

The overall reaction for reversal of glycolysis to form glycogen (Eq. 17-54) now has a comfortably negative standard Gibbs energy change as a result of coupling the cleavage of 7 ATP to the reaction.

$$2\ \text{Lactate}^- + 7\ \text{ATP}^{4-} + 6\ \text{H}_2\text{O} \rightarrow \text{glycogen} + 7\ \text{ADP}^{3-} + 7\ \text{HPO}_4^{2-} + 5\ \text{H}^+$$

$$\Delta G'\ (\text{pH } 7) = -31\ \text{kJ mol}^{-1}\ \text{per glycosyl unit} \qquad (17\text{-}54)$$

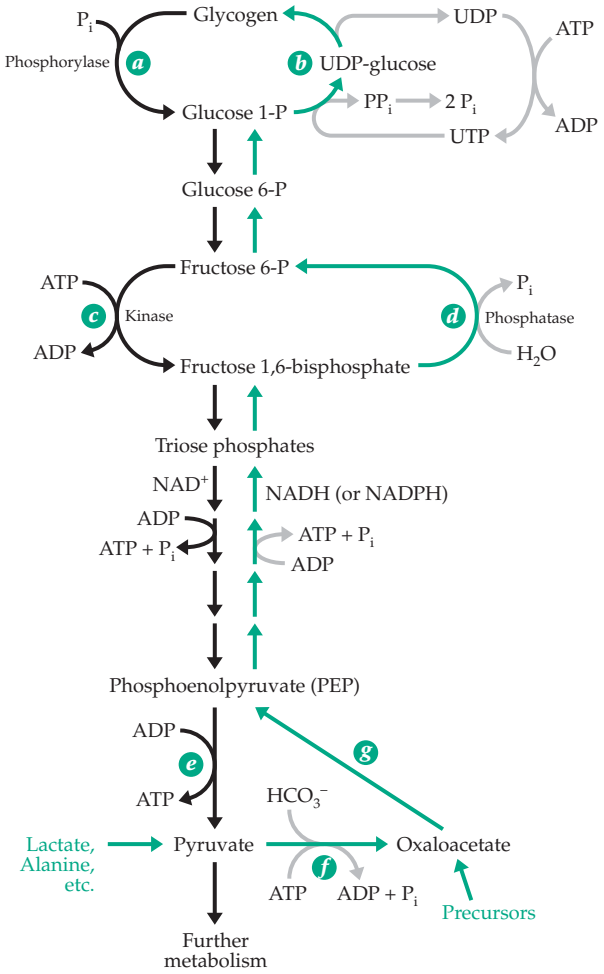
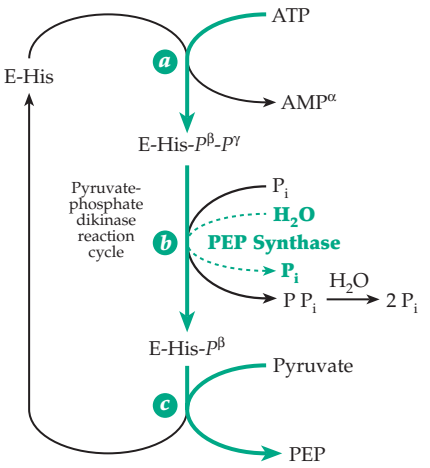



**Figure 17-17** Comparison of glycolytic pathway (left) with pathway of gluconeogenesis (right, green arrows).

Two enzymes that are able to convert pyruvate directly to PEP are found in some bacteria and plants. In each case, as in the animal enzyme system discussed in the preceding paragraph, the conversion involves expenditure of two high-energy linkages of ATP. The **PEP synthase** of *E. coli* first transfers a pyrophospho group from ATP onto an imidazole group of histidine in the enzyme (Eq. 17-55). A phospho group is hydrolyzed from this intermediate (dashed green line in Eq. 17-55, step *b*), ensuring that sufficient intermediate E–His–*P* is present. The latter reacts with pyruvate to form PEP.[202,203] **Pyruvate–phosphate dikinase** is a similar enzyme first identified in tropical grasses and known to play an important role in the $CO_2$ concentrating system of the so-called "$C_4$ plants" (Chapter 23).[204] The same enzyme participates in gluconeogenesis in *Acetobacter*. The reaction cycle for this enzyme is also portrayed in Eq. 17-55. In this case $P_i$, rather than water, is the attacking nucleophile in Eq. 17-55 and $PP_i$ is a product. The latter is probably hydrolyzed by pyrophosphatase action, the end result being an overall reaction that is the same as with PEP synthase. Kinetic and positional isotope exchange studies suggest that the $P_i$ must be bound to pyruvate–phosphate dikinase before the bound ATP can react with the imidazole group.[202] Likewise, AMP doesn't dissociate until $P_i$ has reacted to form $PP_i$.




(17-55)

## 6. Building Hydrocarbon Chains with Two-Carbon Units

Fatty acid chains are taken apart two carbon atoms at a time by β oxidation. Biosynthesis of fatty acids reverses this process by using the two-carbon acetyl unit of acetyl-CoA as a starting material. The coupling of ATP cleavage to this process by a carboxylation–decarboxylation sequence, the role of acyl carrier protein (Section H,4), and the use of NADPH as a reductant (Section I) have been discussed and are summarized in Fig. 17-12, which gives the complete sequence of reactions for fatty acid biosynthesis. Why does β oxidation require CoA derivatives while biosynthesis requires the more complex acyl carrier protein (ACP)? The reason may involve control. ACP is a complex handle able to hold the growing fatty acid chain and to guide it from one enzyme to the next. In *E. coli* the various enzymes catalyzing the reactions of Fig. 17-12 are found in the cytosol and behave as independent proteins. The same is true for fatty acid synthases of higher plants which resemble those of bacteria.[205,205a]

It is thought that the ACP molecule lies at the center of the complex and that the growing fatty acid chain on the end of the phosphopantetheine prosthetic group moves from one subunit to the other.[164,206] The process is started by a **primer** which is usually acetyl-CoA in *E. coli*. Its acyl group is transferred first to the central molecule of ACP (step *a*, Fig. 17-12) and then to a "peripheral" thiol group, probably that of a cysteine side chain on a separate protein subunit (step *b*, Fig. 17-12). Next, a malonyl group is transferred (step *d*) from malonyl-CoA to the free thiol group on the ACP. The condensation (steps *e* and *f*) occurs with the freeing of the peripheral thiol group. The latter does not come into use again until the β-oxoacyl group formed has undergone the complete sequence of reduction reactions (steps *g*–*i*). Then the growing chain is again transferred to the peripheral –SH (step *j*) and a new malonyl unit is introduced on the central ACP.

After the chain reaches a length of 12 carbon atoms, the acyl group tends to be transferred off to a CoA molecule (step *k*) rather than to pass around the cycle again. Thus, chain growth is terminated. This tendency systematically increases as the chain grows longer.

In higher animals as well as in *Mycobacterium*,[207] yeast,[208] and *Euglena*, the **fatty acid synthase** consists of only one or two multifunctional proteins. The synthase from animal tissues has seven catalytic activities in a single 263-kDa 2500-residue protein.[209] The protein consists of a series of domains that contain the various catalytic activities needed for the entire synthetic sequence. One domain contains an ACP-like site with a bound 4'-phosphopantetheine as well as a cysteine side chain in the second acylation site. This synthase produces free fatty acids, principally the $C_{16}$ palmitate. The final step is cleavage of the acyl-CoA by a thioesterase, one of the seven enzymatic activities of the synthase. See Chapter 21 for further discussion.

## 7. The Oxoacid Chain Elongation Process

As mentioned in Section 4, glyoxylate can be converted to oxaloacetate by condensation with acetyl-CoA (Fig. 17-16) and the oxaloacetate can be decarboxylated to pyruvate. This sequence of reactions resembles that of the conversion of oxaloacetate to 2-oxoglutarate in the citric acid cycle (Fig. 17-4). *Both*

*are examples of a frequently used general chain elongation process for α-oxo acids*. This sequence, which is illustrated in Fig. 17-18, has four steps: (1) condensation of the α-oxo acid with an acetyl group, (2) isomerization by dehydration and rehydration (catalyzed by aconitase in the case of the citric acid cycle), (3) dehydrogenation, and (4) β decarboxylation. In many cases steps 3 and 4 are combined as a single enzymatic reaction. The isomerization of the intermediate hydroxy acid in step 2 is required because the hydroxyl group, which is attached to a tertiary carbon bearing no hydrogen, must be moved to the adjacent carbon atom before oxidation to a ketone can take place. However, in the case of glyoxylate, isomerization is not necessary because R = H.

It may be protested that the reaction of the citric acid cycle by which oxaloacetate is converted to oxoglutarate does not follow exactly the pattern of Fig. 17-18. The carbon dioxide removed in the decarboxylation step does not come from the part of the molecule donated by the acetyl group but from that formed from oxaloacetate. However, the end result is the same. Furthermore, there are two known citrate-forming enzymes with different stereospecificities (Chapter 13), one of which leads to a biosynthetic pathway strictly according to the sequence of Fig. 17-18.

At the bottom of Fig. 17-18 several stages of the α-oxo acid elongation process are arranged in tandem. We see that glyoxylate (a product of the acetyl-CoA–glyoxylate cycle) can be built up systematically to

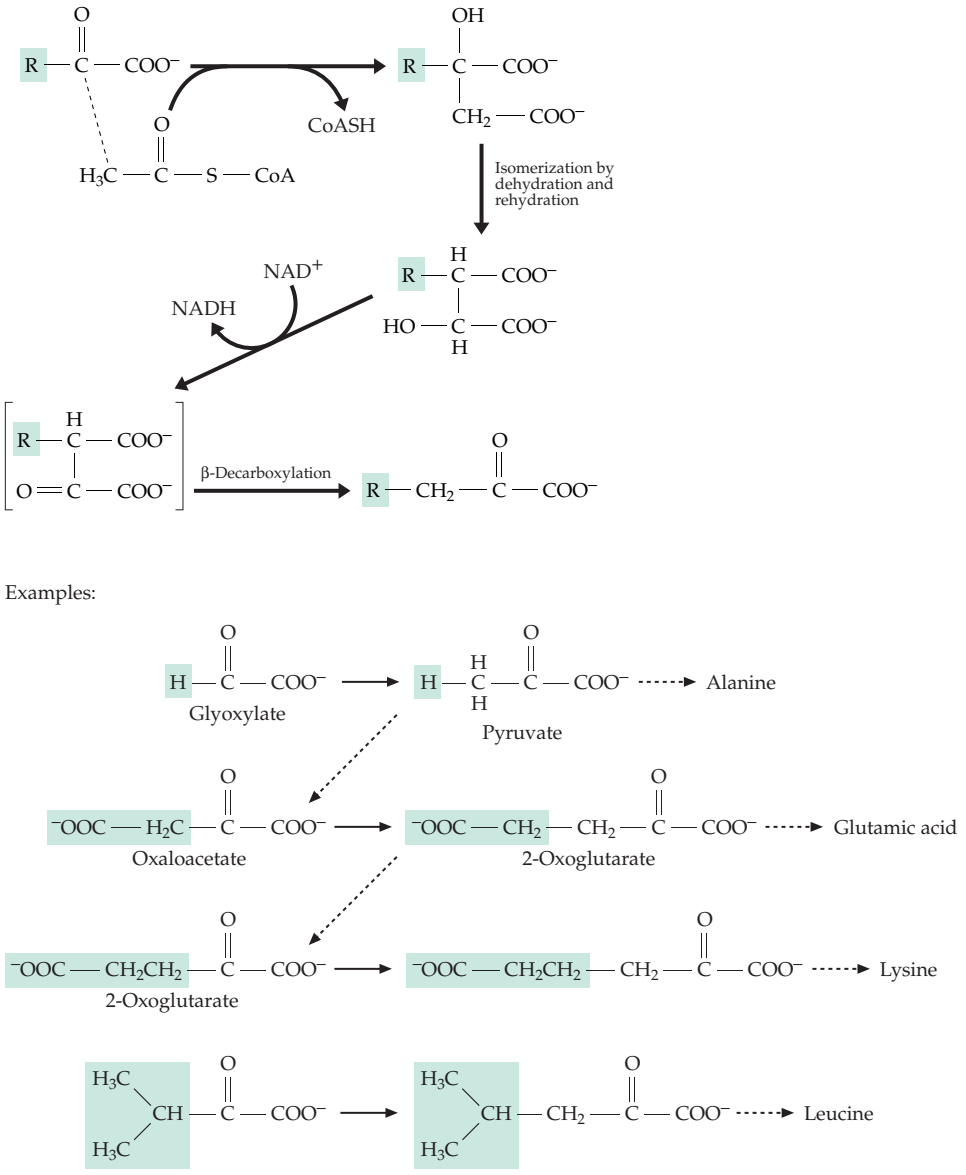
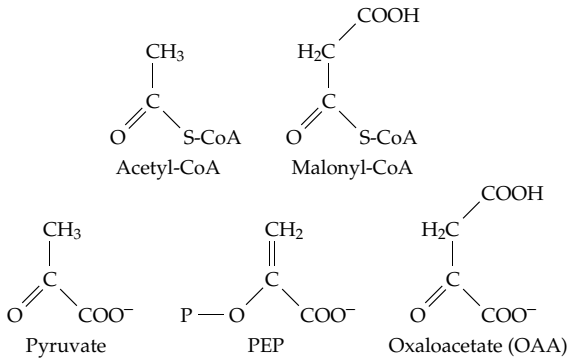



**Figure 17-18** The oxoacid chain elongation process.

pyruvate, oxaloacetate, 2-oxoglutarate, and 2-oxoadipate (a precursor of lysine) using this one reaction sequence. Methanogens elongate 2-oxoadipate by one and two carbon atoms using the same sequence to give 7- and 8-carbon dicarboxylates.[210]

## 8. Decarboxylation as a Driving Force in Biosynthesis

Consider the relationship of the following prominent biosynthetic intermediates one to another:




Utilization of acetyl-CoA for the synthesis of long-chain fatty acids occurs via carboxylation to malonyl-CoA. *We can think of the malonyl group as a β-carboxylated acetyl group*. During synthesis of a fatty acid the carboxyl group is lost, and only the acetyl group is ultimately incorporated into the fatty acid. In a similar way *pyruvate can be thought of as an α-carboxylated acetaldehyde and oxaloacetate as an α- and β-dicarboxylated acetaldehyde*. During biosynthetic reactions these three- and four-carbon compounds also often undergo decarboxylation. Thus, they both can be regarded as "activated acetaldehyde units." Phosphoenolpyruvate is an α-carboxylated phosphoenol form of acetaldehyde and undergoes both decarboxylation and dephosphorylation before contributing a two-carbon unit to the final product.

It is of interest to compare two chain elongation processes by which two-carbon units are combined. In the synthesis of fatty acids the acetyl units are condensed and then are reduced to form straight hydrocarbon chains. In the oxo-acid chain elongation mechanism, the acetyl unit is introduced but is later decarboxylated. Thus, the chain is increased in length by one carbon atom at a time. These two mechanisms account for a great deal of the biosynthesis by chain extension. However, there are other variations. For example, glycine (a carboxylated methylamine), under the influence of pyridoxal phosphate and with accompanying decarboxylation, condenses with succinyl-CoA (Eq. 14-32) to extend the carbon chain and at the same time to introduce an amino group. Likewise, serine (a carboxylated ethanolamine) condenses with palmitoyl-CoA in biosynthesis of sphingosine (as in Eq. 14-32). Phosphatidylserine is decarboxylated to phosphatidylethanolamine in the final synthetic step for that phospholipid (Fig. 21-5).

## 9. Stabilization and Termination of Chain Growth by Ring Formation

Biochemical substances frequently undergo cyclization to form stable five- and six-atom ring structures. The three-carbon glyceraldehyde phosphate exists in solution primarily as the free aldehyde (and its covalent hydrate) but glucose 6-phosphate exists largely as the cyclic hemiacetal. In this ring form no carbonyl group is present and further chain elongation is inhibited. When the hemiacetal of glucose 6-*P* is enzymatically isomerized to glucose 1-*P* the ring is firmly locked. Glucose 1-*P*, in turn, serves as the biosynthetic precursor of polysaccharides and related compounds, in all of which the sugar rings are stable. Ring formation can occur in lipid biosynthesis, too. Among the **polyketides** (Chapter 21), polyprenyl compounds (Chapter 22), and aromatic amino acids (Chapter 25) are many substances in which ring formation has occurred by ester or aldol condensations followed by reduction and elimination processes. This is a typical sequence for biosynthesis of highly stable aromatic rings.

## 10. Branched Carbon Chains

Branched carbon skeletons are formed by standard reaction types but sometimes with addition of rearrangement steps. Compare the biosynthetic routes to three different branched five-carbon units (Fig. 17-19) The first is the use of a **propionyl group** to initiate formation of a branched-chain fatty acid. Propionyl-CoA is carboxylated to methylmalonyl-CoA, whose acyl group is transferred to the acyl carrier protein before condensation. Decarboxylation and reduction yields an acyl-CoA derivative with a methyl group in the 3-position.

The second five-carbon branched unit, in which the branch is one carbon further down the chain, is an intermediate in the biosynthesis of **polyprenyl** (isoprenoid) compounds and steroids. Three two-carbon units are used as the starting material with decarboxylation of one unit. Two acetyl units are first condensed to form acetoacetyl-CoA. Then a third acetyl unit, which has been transferred from acetyl-CoA onto an SH group of the enzyme, is combined with the acetoacetyl-CoA through an ester condensation. The thioester linkage to the enzyme is hydrolyzed to free the product **3-hydroxy-3-methylglutaryl-CoA** (HMG-CoA). This sequence is illustrated in Eq. 17-5. The thioester group of HMG-CoA is reduced to the

alcohol **mevalonic acid**, a direct precursor to isopentenyl pyrophosphate, from which the polyprenyl compounds are formed (Fig. 22-1).

The third type of carbon-branched unit is 2-oxoisovalerate, from which valine is formed by transamination. The starting units are two molecules of pyruvate which combine in a thiamin diphosphate-dependent α condensation with decarboxylation. The resulting α-acetolactate contains a branched chain but is quite unsuitable for formation of an α amino acid. A rearrangement moves the methyl group to the β position (Fig. 24-17), and elimination of water from the diol forms the enol of the desired α-oxo acid (Fig. 17-19). The precursor of isoleucine is formed in an analogous way by condensation, with decarboxylation of one molecule of pyruvate with one of 2-oxobutyrate.

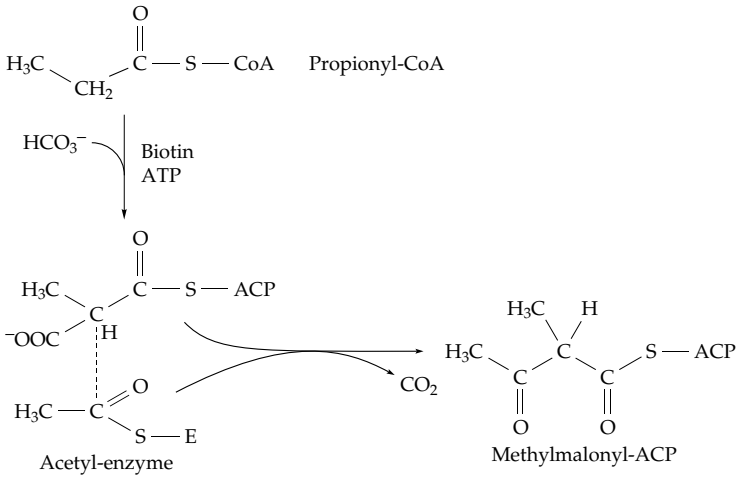
## K. Biosynthesis and Modification of Polymers

There are three chemical problems associated with the assembly of a protein, nucleic acid, or other biopolymer. The first is *to overcome thermodynamic barriers*. The second is *to control the rate of synthesis*, and the third is *to establish the pattern or sequence in which the monomer units are linked together*. Let us look briefly at how these three problems are dealt with by living cells.

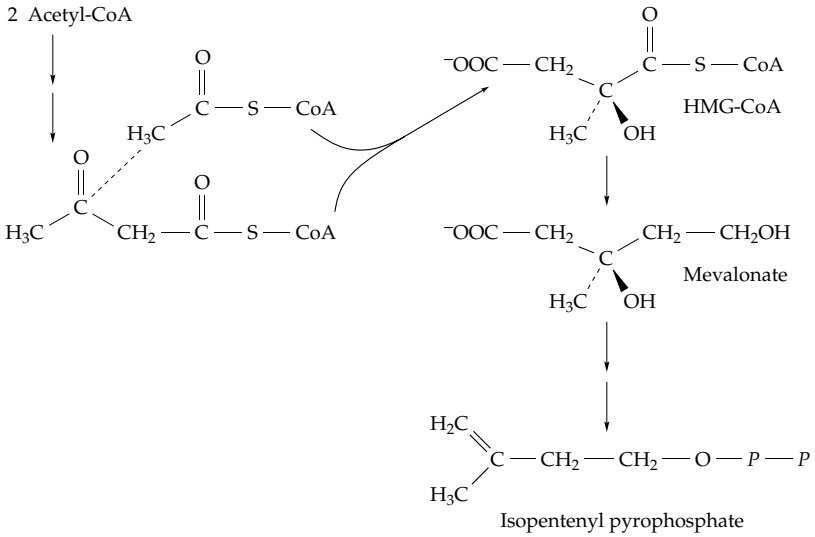
### 1. Peptides and Proteins

Activation of amino acids for incorporation into oligopeptides and proteins can occur via two routes of acyl activation. In the first of these an **acyl phosphate** (or acyl adenylate) is formed and reacts with an amino group to form a peptide linkage (Eq. 13-4). The tripeptide **glutathione** is formed in two steps of this type (Box 11-B). In the second method of activation **aminoacyl**

**1.** Starter piece for branched-chain fatty acids




**2.** Polyisoprenoid compounds




**3.** Branched-chain amino acids

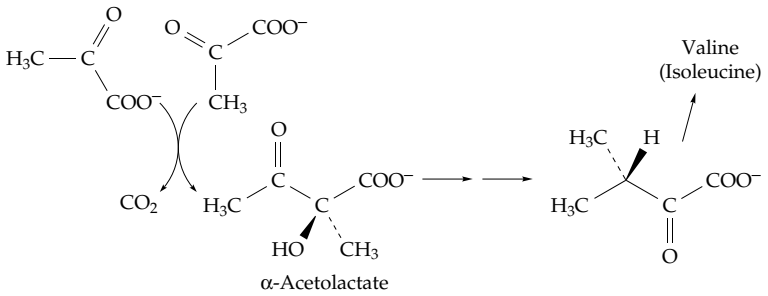



**Figure 17-19** Biosynthetic origins of three five-carbon branched structural units. Notice that decarboxylation is involved in driving each sequence.

**adenylates** are formed. They transfer their activated aminoacyl groups onto specific tRNA molecules during synthesis of proteins (Eq. 17-36). In other cases activated aminoacyl groups are transferred onto –SH groups to form intermediate **thioesters**. An example is the synthesis of the antibiotic **gramicidin S** formed by *Bacillus brevis*. The antibiotic is a cyclic decapeptide with the following five-amino-acid sequence repeated twice in the ringlike molecule[211]:

$$(\text{–D-Phe–L-Pro–L-Val–L-Orn–L-Leu–})_2$$

The soluble enzyme system responsible for its synthesis contains a large 280-kDa protein that not only activates the amino acids as aminoacyl adenylates and transfers them to thiol groups of 4'-phosphopantetheine groups covalently attached to the enzyme but also serves as a template for joining the amino acids in proper sequence.[211–214] Four amino acids—proline, valine, ornithine (Orn), and leucine—are all bound. A second enzyme (of mass 100 kDa) is needed for activation of phenylalanine. It is apparently the activated phenylalanine (which at some point in the process is isomerized from L- to D-phenylalanine) that initiates polymer formation in a manner analogous to that of fatty acid elongation (Fig. 17-12). Initiation occurs when the amino group of the activated phenylalanine (on the second enzyme) attacks the acyl group of the aminoacyl thioester by which the activated proline is held. Next, the freed imino group of proline attacks the activated valine, etc., to form the pentapeptide. Then two pentapeptides are joined and cyclized to give the antibiotic. The sequence is absolutely specific, and it is remarkable that this relatively small enzyme system is able to carry out each step in the proper sequence. Many other peptide antibiotics, such as the bacitracins, tyrocidines,[215] and enniatins, are synthesized in a similar way,[213,216,217] as are depsipeptides and the immunosuppresant cyclosporin. A virtually identical pattern is observed for formation of **polyketides**,[218,219] whose chemistry is considered in Chapter 21.

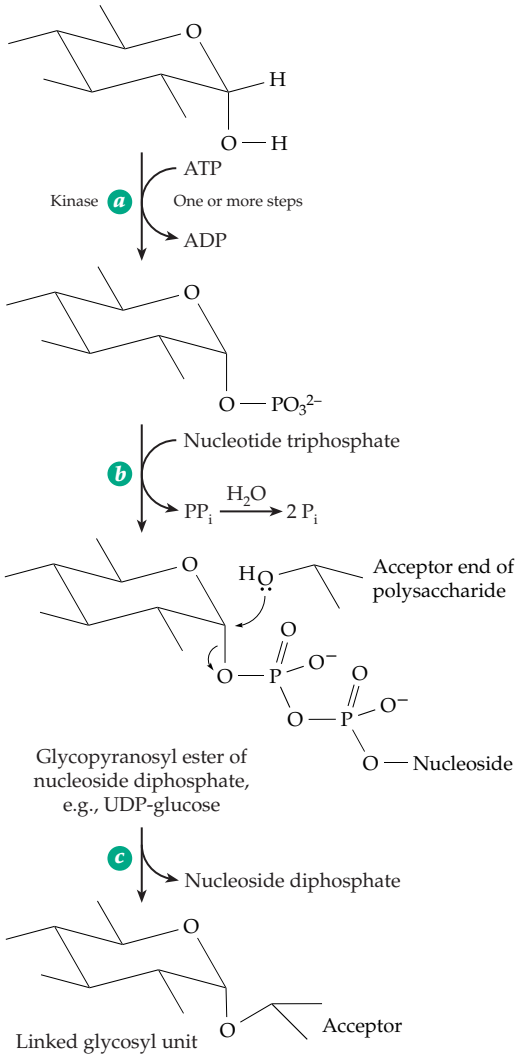
While peptide antibiotics are synthesized according to enzyme-controlled polymerization patterns, both proteins and nucleic acids are made by **template mechanisms**. The sequence of their monomer units is determined by genetically encoded information. A key reaction in the formation of proteins is the transfer of activated aminoacyl groups to molecules of tRNA (Eq. 17-36). The tRNAs act as carriers or adapters as explained in detail in Chapter 29. Each **aminoacyl-tRNA synthetase** must recognize the correct tRNA and attach the correct amino acid to it. The tRNA then carries the activated amino acid to a ribosome, where it is placed, at the correct moment, in the active site. **Peptidyltransferase**, using a transacylation reaction, in an *insertion mechanism* transfers the C terminus of the growing peptide chain onto the amino group of the new amino acid to give a tRNA-bound peptide one unit longer than before.

## 2. Polysaccharides

Incorporation of a sugar monomer into a polysaccharide also involves cleavage of two high-energy phosphate linkages of ATP. However, the activation process has its own distinctive pattern (Eq. 17-56). Usually a sugar is first phosphorylated by a kinase or a kinase plus a phosphomutase (Eq. 17-56, step *a*). Then a nucleoside triphosphate (NuTP) reacts under the influence of a second enzyme with elimination of pyrophosphate and formation of a **glycopyranosyl ester** of the nucleoside diphosphate, more often known as a **sugar nucleotide** (Eq. 17-56, step *b*). The inorganic pyrophosphate is hydrolyzed by pyrophosphatase while the sugar nucleotide donates the activated glycosyl group for polymerization (Eq. 17-56, step *c*). In this step the glycosyl group is transferred with displacement of the nucleoside diphosphate. Thus, the overall process involves first the cleavage of ATP to ADP and $P_i$, and then the cleavage of a nucleoside triphosphate to a nucleoside diphosphate plus $P_i$. The nucleoside triphosphate in Eq. 17-56, step *b* is sometimes ATP, in which case the overall result is the splitting of two molecules of ATP to ADP. However, as detailed in Chapter 20, the whole series of nucleotide "handles" serve to carry various activated glycosyl units.

What determines the pattern of incorporation of sugar units into polysaccharides? Homopolysaccharides, like cellulose and the linear amylose form of starch, contain only one monosaccharide component in only one type of linkage. A single synthetase enzyme can add unit after unit of an activated sugar (UDP glucose or other sugar nucleotide) to the growing end. However, at least two enzymes are needed to assemble a branched molecule such as that of the glycogen molecule. One is the synthetase; the second is a **branching enzyme**, a transglycosylase. After the chain ends attain a length of about ten monosaccharide units the branching enzyme attacks a glycosidic linkage somewhere in the chain. Acting much like a hydrolase, it forms a glycosyl enzyme (or a stabilized carbocation) intermediate. The enzyme does not release the severed chain fragment but transfers it to another nearby site on the branched polymer. In the synthesis of glycogen, the chain fragment is joined to a free 6-hydroxyl group of the glycogen, creating a new branch attached by an α-1,6-linkage.

Other carbohydrate polymers consist of **repeating oligosaccharide units**. Thus, in hyaluronan units of glucuronic acid and N-acetyl-D-glucosamine alternate (Fig. 4-11). The "O antigens" of bacterial cell coats (p. 180) contain repeating subunits made up of a "block" of four or five different sugars. In these and

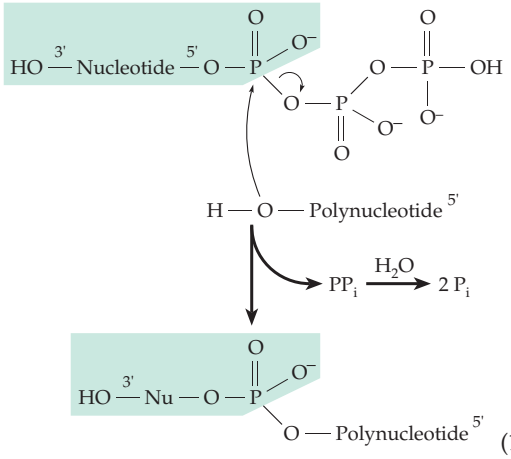



(17-56)

many other cases the pattern of polymerization is established by the specificities of individual enzymes. An enzyme capable of joining an activated glucosyl unit to a growing polysaccharide will do so only if the proper structure has been built up to that point. In cases where a block of sugar units is transferred it is usually *inserted* at the nonreducing end of the polymer, which may be covalently attached to a protein. Notice that the insertion mode of chain growth exists for lipids, polysaccharides, and proteins.

## 3. Nucleic Acids

The activated nucleotides are the nucleoside 5'-triphosphates. The ribonucleotides ATP, GTP, UTP, and CTP are needed for RNA synthesis and the 2'-deoxyribonucleotide triphosphates, dATP, dTTP, dGTP, and dCTP for DNA synthesis. In every case, the addition of activated monomer units to a growing polynucleotide chain is catalyzed by an enzyme that




(17-57)

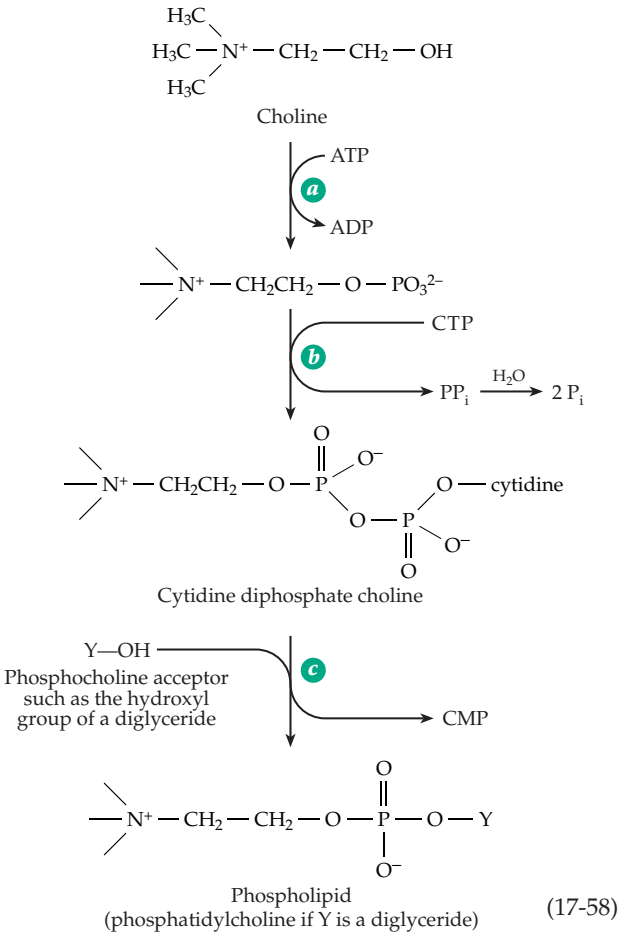
binds to the template nucleic acid. The choice of the proper nucleotide unit to place next in the growing strand is determined by the nucleotide already in place in the complementary strand, a matter that is dealt with in Chapters 27 and 28. The chemistry is a simple displacement of pyrophosphate (Eq. 17-57). The 3'-hydroxyl of the polynucleotide attacks the phosphorus atom of the activated nucleoside triphosphate. Thus, *nucleotide chains always grow from the 5' end, with new units being added at the 3' end*.

## 4. Phospholipids and Phosphate–Sugar Alcohol Polymers

Choline and ethanolamine are activated in much the same way as are sugars. For example, choline can be phosphorylated using ATP (Eq. 17-58, step *a*) and the phosphocholine formed can be further converted (Eq. 17-58, step *b*) to **cytidine diphosphate choline**. Phosphocholine is transferred from the latter onto a suitable acceptor to form the final product (Eq. 17-58, step *c*). The polymerization pattern differs from that for polysaccharide synthesis. When the sugar nucleotides react, the entire nucleoside diphosphate is eliminated (Eq. 17-56), but CDP-choline and CDP-ethanolamine react with elimination of CMP (Eq. 17-58, step *c*), leaving one phospho group in the final product. The same thing is true in the synthesis of the bacterial teichoic acids (Chapter 8). Either CDP-glycerol or CDP-ribitol is formed first and polymerization takes place with elimination of CMP to form the alternating phosphate–sugar alcohol polymer.[220]

## 5. Irreversible Modification and Catabolism of Polymers

While polymers are being synthesized continuously by cells, they are also being modified and torn down. Nothing within a cell is static. As discussed in Chapters




(17-58)

10 and 29, everything turns over at a slower or faster rate. Hydrolases attack all of the polymers of which cells are composed, and active catabolic reactions degrade the monomers formed. Membrane surfaces are also altered, for example, by hydroxylation and glycosylation of both glycoproteins and lipid head groups. It is impossible to list all of the known modification reactions of biopolymers. They include hydrolysis, methylation, acylation, isopentenylation, phosphorylation, sulfation, and hydroxylation. Precursor molecules are cut and trimmed and often modified further to form functional proteins or nucleic acids. Phosphotransferase reactions splice RNA transcripts to form mRNA and a host of alterations convert precursors into mature tRNA molecules (Chapter 28). Even DNA, which remains relatively unaltered, undergoes a barrage of chemical attacks. Only because of the presence of an array of repair enzymes (Chapter 27) does our DNA remain nearly unchanged so that faithful copies can be provided to each cell in our bodies and can be passed on to new generations.

## L. Regulation of Biosynthesis

A simplified view of metabolism is to consider a cell as a "bag of enzymes." Indeed, much of metabolism can be explained by the action of several thousand enzymes promoting specific reactions of their substrates. These reactions are based upon the natural chemical reactivities of the substrates. However, the enzymes, through the specificity of their actions and through association with each other,[96,221–223] channel the reactions into a selected series of metabolic pathways. The reactions are often organized as cycles which are inherently stable. We have seen that biosynthesis often involves ATP-dependent reductive reactions. *It is these reductive processes that produce the less reactive nonpolar lipid groupings and amino acid side chains so essential to the assembly of insoluble intracellular structures.* Oligomeric proteins, membranes, microtubules, and filaments are all the natural result of aggregation caused largely by hydrophobic interactions with electrostatic forces and hydrogen bonding providing specificity. A major part of metabolism is the creation of complex molecules that aggregate spontaneously to generate structure. This structure includes the lipid-rich cytoplasmic membranes which, together with embedded carrier proteins, control the entry of substances into cells. Clearly, the cell is now much more than a bag of enzymes, containing several compartments, each of which contains its own array of enzymes and other components. Metabolite concentrations may vary greatly from one compartment to another.

The reactions that modify lipids and glycoproteins provide a driving force that assists in moving membrane materials generated internally into the outer surface of cells. Other processes, including the breakdown by lysosomal enzymes, help to recycle membrane materials. Oxidative attack on hydrophobic materials such as the sterols and the fatty acids of membrane lipids results in their conversion into more soluble substances which can be degraded and completely oxidized. The flow of matter within cells tends to occur in metabolic loops and some of these loops lead to formation of membranes and organelles and to their turnover. This flow of matter, which is responsible for growth and development of cells, is driven both by hydrolysis of ATP coupled to biosynthesis and by irreversible degradative alterations of polymers and lipid materials. It also provides for transient formation and breakup of complexes of macromolecules, which may be very large, in response to varying metabolic needs.

Anything that affects the rate of a reaction involved in either biosynthesis or degradation of any component of the cell will affect the overall picture in some way. Thus, every chemical reaction that contributes to a quantitatively significant extent to metabolism has

some controlling influence. Since molecules interact with each other in so many ways, reactions of metabolic control are innumerable. Small molecules act on macromolecules as effectors that influence conformation and reactivity. Enzymes act on each other to break covalent bonds, to oxidize, and to crosslink. Transferases add phospho, glycosyl, methyl, and other groups to various sites. The resulting alterations often affect catalytic activities. The number of such interactions significant to metabolic control within an organism may be in the millions. Small wonder that biochemical journals are filled with a confusing number of postulated control mechanisms.

Despite this complexity, some regulatory mechanisms stand out clearly. The control of enzyme synthesis through feedback repression and the rapid control of activity by feedback inhibition (Chapter 11) have been considered previously. Under some circumstances, in which there is a constant growth rate, these controls may be sufficient to ensure the harmonious and proportional increase of all constitutents of a cell. Such may be the case for bacteria during logarithmic growth (Box 9-B) or for a mammalian embryo growing rapidly and drawing all its nutrients from the relatively constant supply in the maternal blood.

Contrast the situation in an adult. Little growth takes place, but the metabolism must vary with time and physiological state. The body must make drastic readjustments from normal feeding to a starvation situation and from resting to heavy exercise. The metabolism needed for rapid exertion is different from that needed for sustained work. A fatty diet requires different metabolism than a high-carbohydrate diet. The necessary control mechanisms must be rapid and sensitive.

## 1. Glycogen and Blood Glucose

Two special features of glucose metabolism in animals are dominant.[224] The first is the storage of glycogen for use in providing muscular energy rapidly. This is a relatively short-term matter but the rate of glycolysis can be intense: The entire glycogen content of muscle could be exhausted in only 20 s of anaerobic fermentation or in 3.5 min of oxidative metabolism.[225] There must be a way to turn on glycolysis quickly and to turn it off when it is no longer needed. At the same time, it must be possible to reconvert lactate to glucose or glycogen (gluconeogenesis). The glycogen stores of the muscle must be repleted from glucose of the blood. If insufficient glucose is available from the diet or from the glycogen stores of the liver, it must be synthesized from amino acids.

The second special feature of glucose metabolism is that certain tissues, including brain, blood cells, kidney medulla, and testis, ordinarily obtain most of their energy through oxidation of glucose.[226,227] For this reason, the glucose level of blood cannot be allowed to drop much below the normal 5 mM. The mechanism of regulation of the blood glucose level is complex and incompletely understood. A series of hormones are involved.

***Insulin.*** This 51-residue cross-linked polypeptide (Fig. 7-17) is synthesized in the pancreatic islets of Langerhans, a tissue specialized for synthesis and secretion into the bloodstream of a series of small peptide hormones. One type of islet cells, the β cells, forms primarily insulin which is secreted in response to high (> 5mm) blood [glucose].[228] Insulin has a wide range of effects on metabolism,[228a] which are discussed in Chapter 11, Section G. Most of these effects are thought to arise from binding to insulin receptors (Figs. 11-11 and 11-12) and are mediated by cascades such as that pictured in Fig. 11-13.[229–232c] The end result is to increase or decrease activities of a large number of enzymes as is indicated in Table 17-3. Some of those are also shown in Fig. 17-20, which indicates interactions with the tricarboxylic acid cycle and lipid metabolism. Binding of insulin to the extracellular domain of its dimeric receptor induces a conformational change that activates the intracellular tyrosine kinase domains of the two subunits. Recent studies suggest that in the activated receptor the two transmembrane helices and the internal tyrosine kinase domains move closer together, inducing the essential autophosphophosphorylation.[232b] The kinase domain of the phosphorylated receptor, in turn, phosphorylates several additional proteins, the most important of which seem to be the insulin receptor substrates IRS-1 and IRS-2. Both appear to be essential in different tissues.[232d,e] Phosphorylated forms of these proteins initiate a confusing variety of signaling cascades.[232f–i]

One of the most immediate effects of insulin is to stimulate an increased rate of uptake of glucose by muscle and adipocytes (fat cells) and other insulin-sensitive tissues. This uptake is accomplished largely by movement of the glucose transporter GLUT4 (Chapter 8) from internal "sequestered" storage vesicles located near the cell membrane into functioning positions in the membranes.[232f,j–m] Activation of this translocation process apparently involves IRS-1 and phosphatidylinositol (PI) 3-kinase, which generates PI-3,4,5-$P_3$ (Fig. 11-9).[232c,n,o] The latter induces the translocation. However, the mechanism remains obscure. The process may also require a second signaling pathway which involves action of the insulin receptor kinase on an adapter protein known as **CAP**, a transmembrane caveolar protein **flotillin**, and a third protein **Cbl**, a known cellular protooncogene. Phosphorylated Cbl forms a complex with CAP and flotillin in a "lipid raft" which induces the exocytosis of the sequestered GLUT4 molecules.[232o,p]