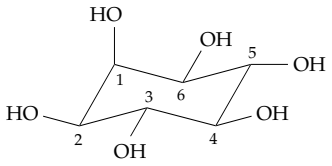
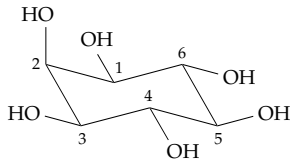
TABLE 17-3
Some Effects of Insulin on Enzymes

Name of Enzyme	Type of Regulation
A. Activity increased	
Enzymes of glycolysis	
Glucokinase	Transcription induced
Phosphofructokinase	via 2,6-fructose P_2
Pyruvate kinase	Dephosphorylation
6-Phosphofructo-2-kinase	Dephosphorylation
Enzymes of glycogen synthesis	
Glucokinase	Transcription
Glycogen synthase (muscle)	Dephosphorylation
Enzymes of lipid synthesis	
Pyruvate dehydrogenase (adipose)	Dephosphorylation (Eq. 17-9)
Acetyl-CoA carboxylase	Dephosphorylation
ATP-citrate lyase	Phosphorylation
Fatty acid synthase	
Lipoprotein lipase	
Hydroxymethylglutaryl-CoA reductase	
B. Activity decreased	
Enzymes of gluconeogenesis	
Pyruvate carboxylase	
PEP carboxykinase	Transcription inhibited
Fructose 1,6-bisphosphate	
Glucose 6-phosphatase	
Enzymes of lipolysis	
Triglyceride lipase	
(hormone-sensitive lipase)	Dephosphorylation
Enzymes of glycogenolysis	
Glycogen phosphorylase	
C. Other proteins affected by insulin	
Glucose transporter GLUT4	Redistribution
Ribosomal protein S6	Phosphorylation by $p90^{rsk}$
IGF-II receptor	Redistribution
Transferrin receptor	Redistribution
Calmodulin	Phosphorylation

A clue to another possible unrecognized mechanism of action for insulin comes from the observation that urine of patients with non-insulin-dependent diabetes contains an unusual isomer of inositol, D-*chiro*-inositol.[233,234]



D-*chiro*-Inositol



myo-Inositol
(numbered as D)

Plasma of such individuals contains an antagonist of insulin action, an inositol phosphoglycan containing *myo*-inositol as a cyclic 1,2-phosphate ester and galactosamine and mannose in a 1:1:3 ratio.[235] This appears to be related to the glycosyl phosphatidylinositol (GPI) membrane anchors (Fig. 8-13). It has been suggested that such a glycan, perhaps containing *chiro*-inositol, is released in response to insulin and serves as a second messenger for insulin.[235–236a] This hypothesis remains unproved.[237] However, insulin does greatly stimulate a GPI-specific phospholipase C, at least in yeast.[237a] Another uncertainty surrounds the possible cooperation of chromium (Chapter 16) in the action of insulin.

How do the insulin-secreting pancreatic β cells sense a high blood glucose concentration? Two specialized proteins appear to be involved. The sugar transporter **GLUT2** allows the glucose in blood to equilibrate with the free glucose in the β cells,[237b] while **glucokinase** (hexokinase IV or D) apparently serves as the glucose sensor.[228,238] Despite the fact that glucokinase is a monomer, it displays a cooperative behavior toward glucose binding, having a low affinity at low [glucose] and a high affinity at high [glucose]. Mutant mice lacking the glucokinase gene develop early onset diabetes which is mild in heterozygotes but severe and fatal within a week of birth for homozygotes.[239,240] These facts alone do not explain how the sensor works and there are doubtless other components to the signaling system.

A current theory is that the increased rate of glucose catabolism in the β cells when blood [glucose] is high leads to a high ratio of [ATP]/[ADP] which induces closure of ATP-sensitive K^+ channels and opening of voltage-gated Ca^{2+} channels.[241] This could explain the increase in [Ca^{2+}] within β cells which has been associated with secretion of insulin[242,243] and which is thought to induce the exocytosis in insulin storage granules. The internal [Ca^{2+}] in pancreatic islet cells is observed to oscillate in a characteristic way that is synchronized with insulin secretion.[243]

Glucagon. This 29-residue peptide is the principal hormone that counteracts the action of insulin. Glucagon acts primarily on liver cells (hepatocytes) and adipose tissue and is secreted by the α cells of the islets of Langerhans in the pancreas, the same tissue whose β cells produce insulin, if the blood glucose concentration falls much below 2 mM.[244–250] Like the insulin-secreting β cells, the pancreatic α cells contain glucokinase, which may be involved in sensing the drop in glucose concentration. However, the carrier GLUT2 is not present and there is scant information on the sensing mechanism.[248]

Glucagon promotes an increase in the blood glucose level by stimulating breakdown of liver glycogen, by inhibiting its synthesis, and by stimulating gluconeogenesis. All of these effects are mediated by cyclic AMP through cAMP-activated protein kinase (Fig. 11-4) and through fructose 2,6-P_2 (Fig. 11-4 and next section). Glucagon also has a strong effect in promoting the release of glucose into the bloodstream. **Adrenaline** has similar effects, again mediated by cAMP. However, glucagon affects the liver, while adrenaline affects many tissues. **Glucocorticoids** such as cortisol (Chapter 22) also promote gluconeogenesis and the accumulation of glycogen in the liver through their action on gene transcription.

The release of glucose from the glycogen stores in the liver is mediated by **glucose 6-phosphatase**, which is apparently embedded within the membranes of the endoplasmic reticulum. A labile enzyme, it consists of a 357-residue catalytic subunit,[251,252] which may be associated with other subunits that participate in transport.[252,253] A deficiency of this enzyme causes the very severe type 1a **glycogen storage disease** (see Box 20-D).[251,253] Only hepatocytes have significant glucose 6-phosphatase activity.

2. Phosphofructo-1-Kinase in the Regulation of Glycolysis

The metabolic interconversions of glucose 1-*P*, glucose 6-*P*, and fructose 6-*P* are thought to be at or near equilibrium within most cells. However, the phosphorylation by ATP of fructose 6-*P* to fructose 1,6-P_2 catalyzed by phosphofructose-1-kinase (Fig. 11-2, step *b*; Fig. 17-17, top center) is usually far from equilibrium. This fact was established by comparing the mass action ratio [fructose 1,6-P_2] [ADP]/[fructose 6-P] [ATP] measured within tissues with the known equilibrium constant for the reaction. At equilibrium this mass action ratio should be equal to the equilibrium constant (Section I,2). The experimental techniques for determining the four metabolite concentrations that are needed for evaluation of the mass action ratio in tissues are of interest. The tissues must be frozen very rapidly. This can be done by compressing them between large liquid nitrogen-cooled aluminum clamps. For details see Newsholme and Start,[225] pp. 30 – 32. Tissues can be cooled to – 80°C in less than 0.1 s in this manner. The frozen tissue is then powdered, treated with a frozen protein denaturant such as perchloric acid, and analyzed. From data obtained in this way, a mass action ratio of 0.03 was found for the phosphofructo-1-kinase reaction in heart muscle.[225] This is much lower than the equilibrium constant of over 3000 calculated from the value of $\Delta G'$ (pH 7) = −20.1 kJ mol^{-1}. Thus, like other biochemical reactions that are nearly irreversible thermodynamically, this reaction is far from equilibrium in tissues.

The effects of ATP, AMP, and fructose 2,6-bisphosphate on phosphofructokinase have been discussed in Chapter 11, Section C. Fructose 2,6-P_2 is a potent allosteric activator of phosphofructokinase and a strong competitive inhibitor of fructose 1,6-bisphosphatase (Fig. 11-2). It is formed from fructose 6-*P* and ATP by the 90-kDa bifunctional phosphofructo-2-kinase/fructose 2,6-bisphosphatase. Thus, the same protein forms and destroys this allosteric effector. Since the bifunctional enzyme is present in very small amounts, the rate of ATP destruction from the substrate cycling is small.

Glucagon causes the concentration of *liver* fructose 2,6-P_2 to drop precipitously from its normal value. This, in turn, causes a rapid drop in glycolysis rate and shifts metabolism toward gluconeogenesis. At the same time, liver glycogen breakdown is inceased and glucose is released into the bloodstream more rapidly. The effect on fructose 2,6-P_2 is mediated by a cAMP-dependent protein kinase which phosphorylates the bifunctional kinase/phosphatase in the liver.[254] This modification greatly reduces the kinase activity and strongly activates the phosphatase, thereby destroying the fructose 2,6-P_2. The changes in activity appear to be largely a result of changes in the appropriate K_m values which are increased for fructose 6-*P* and decreased for fructose 2,6-P_2.[255]

3. Gluconeogenesis

If a large amount of lactate enters the liver, it is oxidized to pyruvate which enters the mitochondria. There, part of it is oxidized through the tricarboxylic acid cycle. However, if [ATP] is high, pyruvate dehydrogenase is inactivated by phosphorylation (Eq. 17-9) and the amount of pyruvate converted to oxaloacetate and malate (Eq. 17-46) may increase. Malate may leave the mitochondrion to be reoxidized to oxaloacetate, which is then converted to PEP and on to glycogen (heavy green arrows in Fig. 17-20). When [ATP] is high, phosphofructokinase is also blocked, but the fructose 1,6-bisphosphatase, which hydrolyzes one phosphate group from fructose 1,6-P_2 (Fig. 11-2, step *d*), is active. If the glucose content of blood is low, the glucose 6-*P* in the liver is hydrolyzed and free glucose is secreted. Otherwise, most of the glucose 6-*P* is converted to glycogen. Muscle is almost devoid of glucose 6-phosphatase, the export of glucose not being a normal activity of that tissue.

Gluconeogenesis in liver is strongly promoted by glucagon and adrenaline. The effects, mediated by cAMP, include stimulation of fructose 1,6-bisphosphatase and inhibition of phosphofructo-1-kinase, both caused by the drop in the level of fructose 2,6-P_2.[254,256] The conversion of pyruvate to PEP via oxaloacetate is also promoted by glucagon. This occurs primarily by stimulation of pyruvate carboxylase (Eq. 14-3).[257,258] However, it has been suggested that the most important mechanism by which glucagon enhances gluconeogenesis is through stimulation of mitochondrial respiration, which in turn may promote gluconeogenesis.[257]

The conversion of oxaloacetate to PEP by PEP-carboxykinase (PEPCK, Eq. 14-43; Fig. 17-20) is another control point in gluconeogenesis. Insulin inhibits gluconeogenesis by decreasing transcription of the mRNA for this enzyme.[259–261a] Glucagon and cAMP stimulate its transcription. The activity of PEP carboxykinase[262] is also enhanced by Mn^{2+} and by very low concentrations of Fe^{2+}. However, the enzyme is readily inactivated by Fe^{2+} and oxygen.[263] Any regulatory significance is uncertain.

Although the regulation of gluconeogenesis in the liver may appear to be well understood, some data indicate that the process can occur efficiently in the presence of high average concentrations of fructose 2,6-P_2. A possible explanation is that liver consists of several types of cells, which may contain differing concentrations of this inhibitor of gluconeogenesis.[264] However, mass spectroscopic studies suggest that glucose metabolism is similar throughout the liver.[265]

4. Substrate Cycles

The joint actions of phosphofructokinase and fructose 1,6-bisphosphatase (Fig. 11-2, steps *b* and *c*; see also Fig. 17-20) create a substrate cycle of the type discussed in Chapter 11, Section F. Such cycles apparently accomplish nothing but the cleavage of ATP to ADP and P_i (ATPase activity). There are many cycles of this type in metabolism and the fact that they do not ordinarily cause a disastrously rapid loss of ATP is a consequence of the tight control of the metabolic pathways involved. In general, only one of the two enzymes of Fig. 11-2, steps *b* and *c*, is fully activated at any time. Depending upon the metabolic state of the cell, degradation may occur with little biosynthesis or biosynthesis with little degradation. Other obvious substrate cycles involve the conversion of glucose to glucose 6-*P* and hydrolysis of the latter back to glucose (Fig. 17-20, upper left-hand corner), the synthesis and breakdown of glycogen (upper right), and the conversion of PEP to pyruvate and the reconversion of the latter to PEP via oxaloacetate and malate (partially within the mitochondria).

While one might suppose that cells always keep substrate cycling to a bare minimum, experimental measurements on tissues *in vivo* have indicated surprisingly high rates for the fructose 1,6-bisphosphatase–phosphofructokinase cycle in mammalian tissues when glycolytic flux rates are low and also for the pyruvate → oxaloacetate → PEP → pyruvate cycle.[266] As pointed out in Chapter 11, by maintaining a low rate of substrate cycling under conditions in which the carbon flux is low (in either the glycolytic or gluconeogenic direction) the system is more sensitive to allosteric effectors than it would be otherwise. However, when the flux through the glycolysis pathway is high the relative amount of cycling is much less and the amount of ATP formed approaches the theoretical 2.0 per glucose.[267]

Substrate cycles generate heat, a property that is apparently put to good use by cold bumblebees whose thoracic temperature must reach at least 30°C before they can fly. The insects apparently use the fructose bisphosphatase–phosphofructokinase substrate cycle (Fig. 11-2, steps *b* and *c*) to warm their flight muscles.[268] It probably helps to keep us warm, too.

5. Nuclear Magnetic Resonance, Isotopomer Analysis, and Modeling of Metabolism

As as been pointed out in Boxes 3-C and 17-C, the use of ^{13}C and other isotopic tracers together with NMR and mass spectroscopy have provided powerful tools for understanding the complex interrelationships among the various interlocking pathways of metabolism. In Box 17-C the application of ^{13}C NMR to the

citric acid cycle was described. Similar approaches have been used to provide direct measurement of the glucose concentration in human brain (1.0 ± 0.1 mM; 4.7 ± 0.3 mM in plasma)[226] and to study gluconeogenesis[269–271] as well as fermentation.[271a] Similar investigations have been made using mass spectroscopy.[272] The metabolism of acetate through the glycoylate pathway in yeast has been observed by ^{13}C NMR.[200] Data obtained from such experiments are being used in attempts to model metabolism and to understand how flux rates through the various pathways are altered in response to varying conditions.[65,273–276]

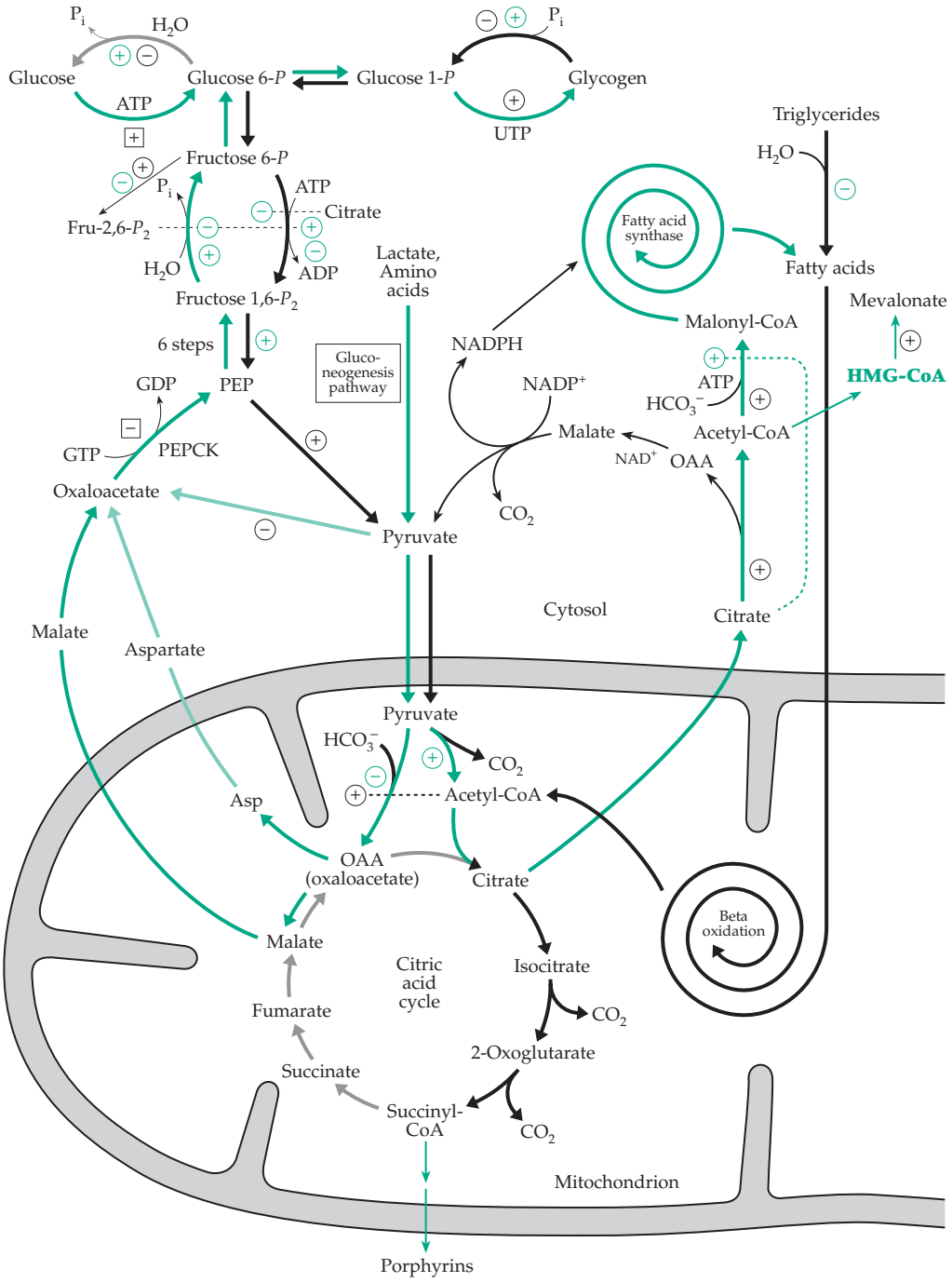


Figure 17-20 The interlocking pathways of glycolysis, gluconeogenesis, and fatty acid oxidation and synthesis with indications of some aspects of control in hepatic tissues. (⟶) Reactions of glycolysis, fatty acid degradation, and oxidation by the citric acid cycle. (⟶) Biosynthetic pathways. Some effects of insulin via indirect action on enzymes ⊕, ⊖, or on transcription ⊞, ⊟. Effects of glucagon ⊕, ⊖.

6. The Fasting State

During prolonged fasting, glycogen supplies are depleted throughout the body and fats become the principal fuels. Both glucose and pyruvate are in short supply. While the hydrolysis of lipids provides some glycerol (which is phosphorylated and oxidized to dihydroxyacetone-*P*), the quantity of glucose precursors formed in this way is limited. Since the animal body cannot reconvert acetyl-CoA to pyruvate, there is a continuing need for both glucose and pyruvate. The former is needed for biosynthetic processes, and the latter is a precursor of oxaloacetate, the regenerating substrate of the citric acid cycle. For this reason, during fasting the body readjusts its metabolism. As much as 75% of the glucose need of the brain can gradually be replaced by ketone bodies derived from the breakdown of fats (Section A,4).[277] Glucocorticoids (e.g., cortisol; Chapter 22) are released from the adrenal glands. By inducing enzyme synthesis, these hormones increase the amounts of a variety of enzymes within the cells of target organs such as the liver. Glucocorticoids also appear to increase the sensitivity of cell responses to cAMP and hence to hormones such as glucagon.[268]

The overall effects of glucocorticoids include an increased release of glucose from the liver (increased activity of glucose 6-phosphatase), an elevated blood glucose and liver glycogen, and a decreased synthesis of mucopolysaccharides. The reincorporation of amino acids released by protein degradation is inhibited and synthesis of enzymes degrading amino acids is enhanced. Among these enzymes are tyrosine and alanine aminotransferases, enzymes that initiate amino acid degradation which gives rise to the glucogenic precursors fumarate and pyruvate.

The inability of the animal body to form the glucose

BOX 17-F LACTIC ACIDEMIA AND OTHER DEFICIENCIES IN CARBOHYDRATE METABOLISM

The lactate concentration in blood can rise from its normal value of 1–2 mM to as much as 22 mM after very severe exercise such as sprinting, but it gradually returns to normal, requiring up to 6–8 h, less if mild exercise is continued. However, continuously high lactic acid levels are observed when enzymes of the gluconeogenic pathway are deficient or when oxidation of pyruvate is partially blocked.[a,b] Severe and often lethal deficiencies of the four key gluconeogenic enzymes pyruvate carboxylase, PEP carboxykinase, fructose 1,6-bisphosphatase, and glucose 6-phosphatase are known.[b] Pyruvate carboxylase deficiency may be caused by a defective carboxylase protein, by an absence of the enzyme that attaches biotin covalently to the three mitochondrial biotin-containing carboxylases (Chapter 14, Section C), or by defective transport of biotin from the gut into the blood. The latter types of deficiency can be treated successfully with 10 mg biotin per day.

Deficiency of pyruvate dehydrogenase is the most frequent cause of lactic acidemia.[a,c] Since this enzyme has several components (Fig. 15-15), a number of forms of the disease have been observed. Patients are benefitted somewhat by a high-fat, low-carbohydrate diet. Transient lactic acidemia may result from infections or from heart failure. One treatment is to administer dichloroacetate, which stimulates increased activity of pyruvate dehydrogenase, while action is also taken to correct the underlying illness.[d] Another problem arises if a lactate transporter is defective so that lactic acid accumulates in muscles.[e]

A different problem results from deficiency of enzymes of glycolysis such as phosphofructokinase (see Box 20-D), phosphoglycerate mutase, and pyruvate kinase. Lack of one isoenzyme of phosphoglycerate mutase in muscle leads to intolerance to strenuous exercise.[f] A deficiency in pyruvate kinase is one of the most common defects of glycolysis in erythrocytes and leads to a shortened erythrocyte lifetime and hereditary hemolytic anemia.[g]

Deficiency of the first enzyme of the pentose phosphate pathway, glucose 6-phosphate dehydrogenase, is widespread.[h] Its geographical distribution suggests that, like the sickle-cell trait, it confers some resistance to malaria. A partial deficiency of 6-phosphogluconolactonase (Eq. 17-12, step *b*) has also been detected within a family and may have contributed to the observed hemolytic anemia.[i]

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[a] Robinson, B. H. (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. 1479–1499, McGraw-Hill, New York

[b] Robinson, B. H. (1982) *Trends Biochem. Sci.* **7**, 151–153

[c] McCartney, R. G., Sanderson, S. J., and Lindsay, J. G. (1997) *Biochemistry* **36**, 6819–6826

[d] Stacpoole, P. W., 17 other authors, and Dichloroacetate-Lactic Acidosis Study Group (1992) *N. Engl. J. Med.* **327**, 1564–1569

[e] Fishbein, W. N. (1986) *Science* **234**, 1254–1256

[f] DiMauro, S., Mirando, A. F., Khan, S., Gitlin, K., and Friedman, R. (1981) *Science* **212**, 1277–1279

[g] Tanaka, K. R., and Paglia, D. E. (1995) in *The Metabolic and Molecular Bases of Inherited Disease* (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., eds), pp. 3485–3511, McGraw-Hill, New York

[h] Pandolfi, P. P., Sonati, F., Rivi, R., Mason, P., Grosveld, F., and Luzzatto, L. (1995) *EMBO J.* **14**, 5209–5215

[i] Beutler, E., Kuhl, W., and Gelbart, J. (1985) *Proc. Natl. Acad. Sci. U.S.A.* **82**, 3876–3878

precursors pyruvate or oxaloacetate from acetyl units is sometimes a cause of severe metabolic problems. Ketosis, which was discussed in Section A,4, develops when too much acetyl-CoA is produced and not efficiently oxidized in the citric acid cycle. Ketosis occurs during starvation, with fevers, and in insulin-dependent diabetes (see also Box 17-G). In cattle, whose metabolism is based much more on acetate than is ours, spontaneously developing ketosis is a frequent problem.

7. Lipogenesis

A high-carbohydrate meal leads to an elevated blood glucose concentration. The glycogen reserves within cells are filled. The ATP level rises, blocking the citric acid cycle, and citrate is exported from mitochondria (Fig. 17-20). Outside the mitochondria citrate is cleaved by the ATP-requiring citrate lyase (Eq. 14-37) to acetyl-CoA and oxaloacetate. The oxaloacetate can be reduced to malate and the latter oxidized with $NADP^+$ to pyruvate (Eq. 17-46), which can again enter the mitochondrion. In this manner acetyl groups are exported from the mitochondrion as acetyl-CoA which can be carboxylated, under the activating influence of citrate, to form malonyl-CoA, the precursor of fatty acids. The NADPH formed from oxidation of the malate provides part of the reducing equivalents needed for fatty acid synthesis. Additional NADPH is available from the pentose phosphate pathway. Thus, excess carbohydrate is readily converted into fat by our bodies. These reactions doubtless occur to some extent in most cells, but they are quantitatively

BOX 17-G DIABETES MELLITUS

The most prevalent metabolic problem affecting human beings is diabetes mellitus.[a–c] Out of a million people about 400 develop **type I** (or juvenile-onset) **insulin-dependent diabetes mellitus** (IDDM) between the ages of 8 and 12. Another 33,000 (over 3%) develop diabetes by age 40–50, and by the late 70s over 7% are affected. A propensity toward diabetes is partially hereditary, and recessive susceptibility genes are present in a high proportion of the population. The severity of the disease varies greatly. About half of the type I patients can be treated by diet alone, while the other half must receive regular insulin injections because of the atrophy of the insulin-producing cells of the pancreas. Type I diabetes sometimes develops very rapidly with only a few days of ravenous hunger and unquenchable thirst before the onset of ketoacidosis. Without proper care death can follow quickly. This suggested that a virus infection might cause the observed death of the insulin-secreting β cells of the pancreatic islets. However, the disease appears to be a direct result of an autoimmune response (Chapter 31). Antibodies directed against such proteins as insulin, glutamate decarboxylase,[d,e] and a tyrosine phosphatase[f] of the patient's own body are present in the blood. There may also be a direct attack on the β cells by T cells of the immune system (see Chapter 31).[g,h] The events that trigger such autoimmune attacks are not clear, but there is a strong correlation with susceptibility genes, in both human beings[i,j] and mice.[k,l]

Adults seldom develop type I diabetes but often suffer from **type II** or **non-insulin-dependent diabetes mellitus** (NIDDM). This is not a single disease but a syndrome with many causes. There is usually a marked decrease in sensitivity to insulin (referred to as **insulin resistance**) and poor uptake and utilization of glucose by muscles.[m] In rare cases this is a result of a mutation in the gene for the insulin molecule precursors (Eq. 10-8) or in the gene regulatory regions of the DNA.[n,o] Splicing of the mRNA[p] may be faulty or there may be defects in the structure or in the mechanisms of activation of the insulin receptors (Figs. 11-11 and 11-12).[q] The number of receptors may be too low or they may be degraded too fast to be effective. About 15% of persons with NIDDM have mutations in the insulin substrate protein IRS-1 (Chapter 11, Section G) but the significance is not clear.[m,r] Likewise, the causes of the loss of sensitivity of insulin receptors as well as other aspects of insulin resistance are still poorly understood.[s] In addition, prolonged high glucose concentrations result in decreased insulin synthesis or secretion, both of which are also complex processes. After synthesis the insulin hexamer is stored as granules of the hexamer $(insulin)_6Zn_2$ (Fig. 7-18) in vesicles at low pH. For secretion to occur the vesicles must first dock at membrane sites and undergo exocytosis. The insulin dissolves, releasing the Zn^{2+}, and acts in the monomeric form.[t] Because the mechanisms of action of insulin are still not fully understood, it is difficult to interpret the results of the many studies of diabetes mellitus.

A striking symptom of diabetes is the high blood glucose level which may range from 8 to 60 mM. Lower values are more typical for mild diabetes because when the glucose concentration exceeds the renal threshold of ~8 mM the excess is secreted into the urine. Defective utilization of glucose seems to be tied to a failure of glucose to exert proper

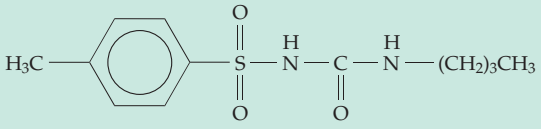
BOX 17-G DIABETES MELLITUS (continued)

feedback control. The result is that gluconeogenesis is increased with corresponding breakdown of proteins and amino acids. The liver glycogen is depleted and excess nitrogen from protein degradation appears in the urine. In IDDM diabetes the products of fatty acid degradation accumulate, leading to ketosis. The volume of urine is excessive and tissues are dehydrated.

Although the acute problems of diabetes, such as coma induced by ketoacidosis, can usually be avoided, it has not been possible to prevent long-term complications that include cataract formation and damage to the retina and kidneys. Most diabetics eventually become blind and half die within 15–20 years. Many individuals with NIDDM develop insulin-dependent diabetes in later life as a result of damage to the pancreatic β cells. The high glucose level in blood appears to be a major cause of these problems. The aldehyde form of glucose reacts with amino groups of proteins to form Schiff bases which undergo the Amadori rearrangement to form ketoamines (Eq. 4-8). The resulting modified proteins tend to form abnormal disulfide crosslinkages. Crosslinked aggregates of lens proteins may be a cause of cataract. The accumulating glucose-modified proteins may also induce autoimmune responses that lead to the long-term damage to kidneys and other organs. Another problem results from reduction of glucose to sorbitol (Box 20-A). Accumulation of sorbitol in the lens may cause osmotic swelling, another factor in the development of cataracts.[w,x] Excessive secretion of the 37-residue polypeptide **amylin**, which is synthesized in the β cells along with insulin, is another frequent complication of diabetes.[u,v] Amylin precipitates readily within islet cells to form **amyloid deposits** which are characteristic of NIDDM.

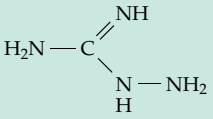
For many persons with diabetes regular injections of insulin are essential. Insulin was discovered in 1921 in Toronto by Banting and Best, with a controversial role being played by Professor J. J. R. Macleod, who shared the Nobel prize with Banting in 1923.[y,z] In 1922 the first young patients received pancreatic extracts and a new, prolonged life.[z–bb] Persons with IDDM are still dependent upon daily injections of insulin, but attempts are being made to treat the condition with transplanted cells from human cadavers.[cc] Animal insulins are suitable for most patients, but allergic reactions sometimes make **human insulin** essential. The human hormone, which differs from bovine insulin in three positions (Thr in human vs Ala in bovine at positions 8 of the A chain and 30 of the B chain and Ile vs Val at position 10 of the A chain), is now produced in bacteria using recombinant DNA. Nonenzymatic laboratory synthesis of insulin has also been achieved, but it is difficult to place the disulfide crosslinks in the proper positions. New approaches mimic the natural synthesis, in which the crosslinking takes place in proinsulin (Fig. 10-7).

NIDDM is strongly associated with obesity,[dd] and dieting and exercise often provide adequate control of blood glucose. Sulfonylurea drugs such as the following induce an increase in the number of insulin receptors formed and are also widely used in treatment of the condition.[ee,ff] These drugs bind to and inhibit ATP-sensitive K^+ channels in the β cell membranes. A defect in this sulfonylurea receptor has been associated with excessive insulin secretion



1-Butyl-*p*-tolylsulfonylurea

in infants.[17] New types of drugs are being tested.[gg–kk] These include inhibitors of aldose reductase,[ii] which forms sorbitol; compounds such as aminoguanidine, which inhibit formation of advanced products of glycation and newly discovered fungal metabolites that activate insulin receptors.[jj]



Aminoguanidine

[a] Atkinson, M. A., and Maclaren, N. K. (1990) *Sci. Am.* **263**(Jul), 62–71

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BOX 17-G (continued)

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most important in the liver, in fat cells of adipose tissue, and in mammary glands. The process is also facilitated by insulin, which promotes the activation of pyruvate dehydrogenase (Eq. 17-9) and of fatty acid synthase of adipocytes.[277a] Activity of fatty acid synthase seems to be regulated by the rate of transcription of its gene, which is controlled by a transcription factor designated either as **adipocyte determination and differentiation factor-1** (**ADD-1**) or **sterol regulatory element-binding protein-1c** (**SREBP-1c**). This protein (ADD-1/SREBP-1c) may be a general mediator of insulin action.[277b] The nuclear DNA-binding protein known as **peroxisome proliferator-activated receptor gamma** (**PPAR$_\gamma$**) is also involved in the control of insulin action, a conclusion based directly on discovery of mutations in persons with type II diabetes.[277c] A newly discovered hormone **resistin**, secreted by adipocytes, may also play a role.[277d] Another adipocyte hormone, **leptin**, impairs insulin action.[277e] Recent evidence suggests that both insulin and leptin may have direct effects on the brain which also influence blood glucose levels.[277f] Malonyl-CoA, which may also play a role in insulin secretion,[278,279] inhibits carnitine palmitoyltransferase I (CPT I; Fig. 17-2), slowing fatty acid catabolism.[280]

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Study Questions

1. Write out a complete step-by-step mechanism for the reactions by which citrate can be synthesized from pyruvate and then exported from mitochondria for use in the biosynthesis of fatty acids. Include a chemically reasonable mechanism for the action of ATP–citrate lyase, which catalyzes the following reaction:

   ATP + citrate + CoA-SH → Acetyl-CoA + oxaloacetate + ADP + P_i

   Show how this reaction can be incorporated into an ATP-driven cyclic pathway for generating NADPH from NADH.

2. Show which parts (if any) of the citric acid cycle are utilized in each of the following reactions and what, if any, additional enzymes are needed in each case.
   a. Oxidation of acetyl-CoA to CO_2
   b. Catabolism of glutamate to CO_2
   c. Biosynthesis of glutamate from pyruvate
   d. Formation of propionate from pyruvate

3. Here is a possible metabolic reaction for a fungus.

   L-Leucine + 2-oxoglutarate^{2-} + 2 $^1/_2$ O_2 → L-glutamate$^-$ + citrate^{3-} + H_2O + 2H^+
   $\Delta G^{\circ\prime}$ (pH 7) = −1026 kJ/mol

   Suggest a metabolic pathway for this reaction. Is it thermodynamically feasible?

4. It has been suggested that in *Escherichia coli* pyruvate may act as the regenerating substrate for a catalytic cycle by which glyoxylate, OHC–COO^-, is oxidized to CO_2. Key enzymes in this cycle are thought to be a 2-oxo-4-hydroxyglutarate aldolase and 2-oxoglutarate dehydrogenase. Propose a detailed pathway for this cycle.

5. Some bacteria use a "dicarboxylic acid cycle" to oxidize glyoxylate OHC–COO^- to CO_2. The regenerating substrate for this cycle is acetyl-CoA. It is synthesized from glyoxylate by a complex pathway that begins with conversion of two molecules of glyoxylate to tartronic semialdehyde: $^-$OOC–CHOH–CHO. The latter is then dehydrogenated to D-glycerate.

   Write out a detailed scheme for the dicarboxylate cycle. Also indicate how glucose and other cell constituents can be formed from intermediates created in this biosynthetic pathway.

6. Some bacteria that lack the usual aldolase produce ethanol and lactic acid in a 1:1 molar ratio via the "heterolactic fermentation." Glucose is converted to ribulose 5-phosphate via the pentose phosphate pathway enzymes. A thiamin diphosphate-dependent "phosphoketolase" cleaves xylulose 5-phosphate in the presence of inorganic phosphate to acetyl phosphate and glyceraldehyde 3-phosphate.

   Propose a mechanism for the phosphoketolase reaction and write a balanced set of equations for the fermentation.

7. Bacteria of the genera *Aerobacter* and *Serratia* ferment glucose according to the following equation:

   1 $^1/_2$ Glucose → 2,3-butanediol + 3 CO_2 + H_2 + ethanol

   Write out a detailed pathway for the reactions. Use the pyruvate formate–lyase reaction. What yield of ATP do you expect per molecule of glucose fermented?

8. Some Clostridia ferment glucose as follows:

   2 Glucose + 2 H_2O → butyrate$^-$ + 2 acetate$^-$ + 4 CO_2 + 6 H_2 + 3 H^+
   $\Delta G^{\circ\prime}$ (pH 7) = −479 kJ/mol of butyrate formed

   Write out detailed pathways. How much ATP do you think can be formed per glucose molecule fermented?

9. Propionic acid bacteria use the following fermentation:

   1 $^1/_2$ Glucose → 2 propionate + acetate$^-$ + 3 H^+ + CO_2 + H_2O
   $\Delta G^{\circ\prime}$ = −464 kJ/mol of acetate formed

   Write out a detailed pathway for the reactions. How much ATP can be formed per molecule of glucose?

10. Consider the following reaction which can occur in the animal body:

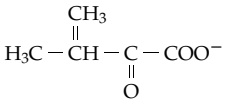
    2 Lactate$^-$ ($C_3H_5O_3^-$) + CoASH + 2 NAD^+ → butyryl-CoA (C_4H_7O–S–CoA) + 2 CO_2 + 2 NADH + H_2O
    $\Delta G^{\circ\prime}$ (pH 7) ~ 70 kJ/mol of butyryl-CoA formed

    Outline the sequence of reactions involved in this

transformation. Do you think that any ATP will either be used or generated in the reaction? Explain.

11. Leucine, an essential dietary constituent for human beings, is synthesized in many bacteria and plants using pyruvate as a starting material. Outline this pathway of metabolism and illustrate the chemical reaction mechanisms involved in each step.

12. Write a step-by-step sequence for all of the chemical reactions involved in the biosynthesis of L-leucine from 2-oxoisovalerate:



Notice that this compound contains one carbon atom less than leucine. Start by condensing 2-oxoisovalerate with acetyl-CoA in a reaction similar to that of citrate synthase. Use structural formulas. Show all intermediate structures and indicate what coenzymes are needed. Use curved arrows to indicate the flow of electrons in each step.

13. Some fungi synthesize lysine from 2-oxoglutarate by elongating the chain using a carbon atom derived from acetyl-CoA to form the 6-carbon 2-oxoadipate. The latter is converted by an ATP-dependent reduction to the ε-aldehyde. Write out reasonable mechanisms for the conversion of 2-oxoglutarate to the aldehyde. The latter is converted on to L-lysine by a non–PLP-dependent transamination via saccharopine (Chapter 24).

14. Outline the pathway for biosynthesis of L-leucine from glucose and NH_4^+ in autotrophic organisms. In addition, outline the pathways for degradation of leucine to CO_2, water, and NH_4^+ in the human body. For this overall pathway or "metabolic loop," mark the locations (one or more) at which each of the following processes occurs.
    a. Synthesis of a thioester by dehydrogenation
    b. Substrate-level phosphorylation
    c. Thiamin-dependent α condensation
    d. Oxoacid chain-elongation process
    e. Transamination
    f. Oxidative decarboxylation of an α-oxoacid
    g. Partial β oxidation
    h. Thiolytic cleavage
    i. Claisen condensation
    j. Biotin-dependent carboxylation

15. A photosynthesizing plant is exposed to $^{14}CO_2$. On which carbon atoms will the label first appear in glucose?

16. The Calvin–Benson cycle and the pentose phosphate pathway (Eq. 17-12) have many features in common but run in opposite directions. Since the synthesis of glucose from CO_2 requires energy, the energy expenditure for the two processes will obviously differ. Describe the points in each pathway where a Gibbs energy difference is used to drive the reaction in the desired direction.

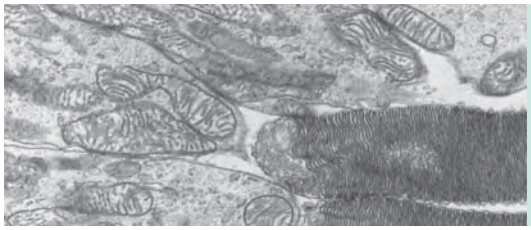
17. Draw the structure of ribulose 1(^{14}C), 5-bisphosphate. Enter an asterisk (*) next to carbon 1 to show that this position is ^{14}C-labeled.

    Draw the structures of the products of the ribulose bisphosphate carboxylase reaction, indicating the radioactive carbon position with an asterisk.

18. A wood-rotting fungus is able to convert glucose to oxalate approximately according to the following equation:

    Glucose + 5 O_2 → 2 Oxalic acid ($C_2O_4H_2$) + 2 CO_2 + 4 H_2O

    Propose a mechanism. See Munir *et al.*[281] for details.

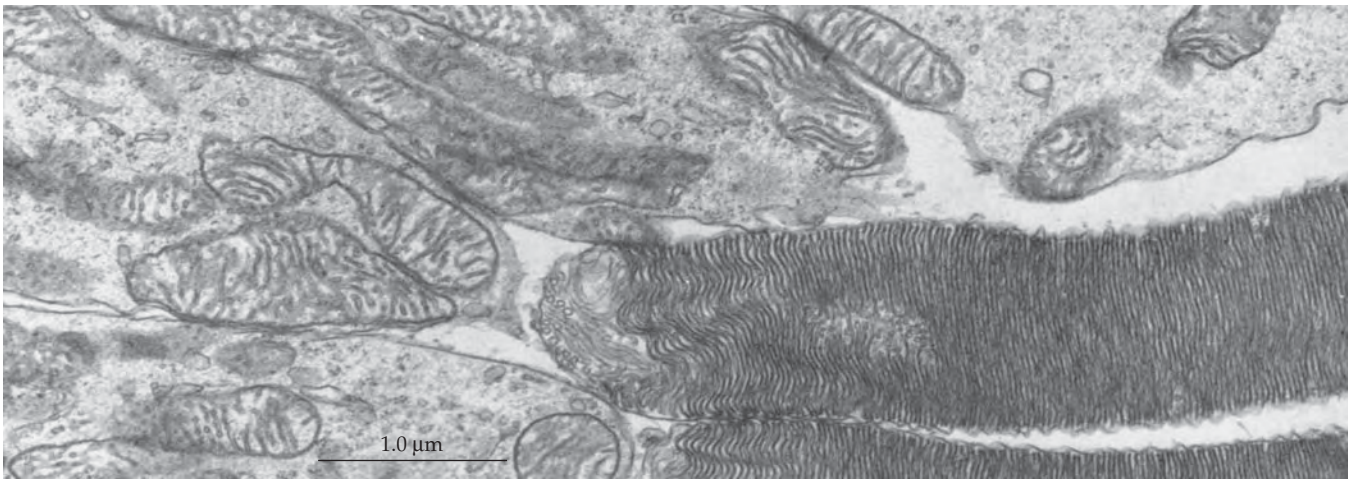


Electrons flowing through the electron transport chains in the membranes of the mitochondria, (at the left) in this thin section through the retina of a kangaroo rat (*Dipodomys ordi*) generates ATP. The ATP provides power for the synthesis and functioning of the stacked photoreceptor disks seen at the right. The outer segment of each rod cell (See Fig. 23-40), which may be 15–20 mm in length, consists of these disks, whose membranes contain the photosensitive protein pigment rhodopsin. Absorption of light initiates an electrical excitation which is sent to the brain. Micrograph from Porter and Bonneville (1973) Fine Structure of Cells and Tissues, Lea and Febiger, Philadelphia, Pennsylvania. Courtesy of Mary Bonneville.

Contents

Electron Transport, Oxidative Phosphorylation, and Hydroxylation

18



In this chapter we will look at the processes by which reduced carriers such as NADH and $FADH_2$ are oxidized within cells. Most familiar to us, because it is used in the human body, is **aerobic respiration**. Hydrogen atoms of NADH, $FADH_2$, and other reduced carriers appear to be transferred through a chain of additional carriers of increasingly positive reduction potential and are finally combined with O_2 to form H_2O. In fact, the hydrogen nuclei move freely as protons (or sometimes as H^- ions); it is the *electrons* that are deliberately transferred. For this reason, the chain of carriers is often called the **electron transport chain**. It is also referred to as the **respiratory chain**.

Because far more energy is available to cells from oxidation of NADH and $FADH_2$ than can be obtained by fermentation, the chemistry of the electron transport chain and of the associated reactions of ATP synthesis assumes great importance. A central question becomes "How is ATP generated by flow of electrons through this series of carriers"? Not only is most of the ATP formed in aerobic and in some anaerobic organisms made by this process of **oxidative phosphorylation**, but the solar energy captured during photosynthesis is used to form ATP in a similar manner. The mechanism of ATP generation may also be intimately tied to the function of membranes in the transport of ions. In a converse manner, the mechanism of oxidative phosphorylation may be related to the utilization of ATP in providing energy for the contraction of muscles.

In some organisms, especially bacteria, energy may be obtained through oxidation of H_2, H_2S, CO, or Fe^{2+} rather than of the hydrogen atoms removed from organic substrates. Furthermore, some bacteria use **anaerobic respiration** in which NO_3^-, SO_4^{2-}, or CO_2 act as oxidants either of reduced carriers or of reduced inorganic substances. In the present chapter, we will consider these energy-yielding processes as well as the chemistry of reactions of oxygen that lead to incorporation of atoms from O_2 into organic compounds.

The oxidative processes of cells have been hard to study, largely because the enzymes responsible are located in or on cell membranes. In bacteria the sites of electron transport and oxidative phosphorylation are on the inside of the plasma membrane or on membranes of mesosomes. In eukaryotes they are found in the inner membranes of the mitochondria and, to a lesser extent, in the endoplasmic reticulum. For this reason we should probably start with a closer look at mitochondria, the "power plants of the cell."

A. The Architecture of the Mitochondrion

Mitochondria are present in all eukaryotic cells that use oxygen in respiration, but the number per cell and the form and size vary.[1–4] Certain tiny trypanosomes have just *one* mitochondrion but some oocytes have as many as 3×10^5. Mammalian cells typically contain several hundred mitochondria and liver cells[5] more than 1000. Mammalian sperm cells may contain 50–75 mitochondria,[6] but in some organisms only one very large helical mitochondrion, formed by the fusion of many individual mitochondria, wraps around the base of the tail. Typical mitochondria appear to be about the size of cells of *E. coli*. However, study of ultrathin serial sections of a single yeast cell by electron microscopy has shown that, under some growth conditions, all of the mitochondria are interconnected.[7]

In every case a mitochondrion is enclosed by two

concentric membranes, an *outer* and an *inner* membrane, each ~5–7 nm thick (Figs. 18-1, 18-2). The inner membrane is folded to form the **cristae**. The number of cristae, the form of the cristae, and the relative amount of the internal **matrix** space are variable. In liver there is little inner membrane and a large matrix space, while in heart mitochondria there are more folds and a higher rate of oxidative phosphorylation. The enzymes catalyzing the tricarboxylic acid cycle are also unusually active in heart mitochondria. A typical heart mitochondrion has a volume of 0.55 μm^3; for every cubic micrometer of mitochondrial volume there are 89 μm^2 of inner mitochondrial membranes.[9]

Mitchondria can swell and contract, and forms other than that usually seen in osmium-fixed electron micrographs have been described. In some mitochondria the cristae are swollen, the matrix volume is much reduced, and the **intermembrane space** between the membranes is increased. Rapidly respiring mitochondria fixed for electron microscopy exhibit forms that have been referred to as "energized" and "energized-twisted."[10] The micrograph (Fig. 18-1) and drawing (Fig. 18-2) both show a significant amount of intermembrane space. However, electron micrographs of mitochondria from rapidly frozen aerobic tissues show almost none.[11] Recent studies by electron microscopic tomography show cristae with complex tubular structures. The accepted simple picture of mitochondrial structure (Fig. 18-1) is undergoing revision.[12–12b] The isolated mitochondria that biochemists have studied may be fragments of an interlinked **mitochondrial reticulum** that weaves its way through the cell.[12b] However, this reticulum may not be static but may break and reform. The accepted view that the mitochondrial matrix space is continuous with the internal space in the cristae is also the subject of doubts. Perhaps they are two different compartments.[12a]

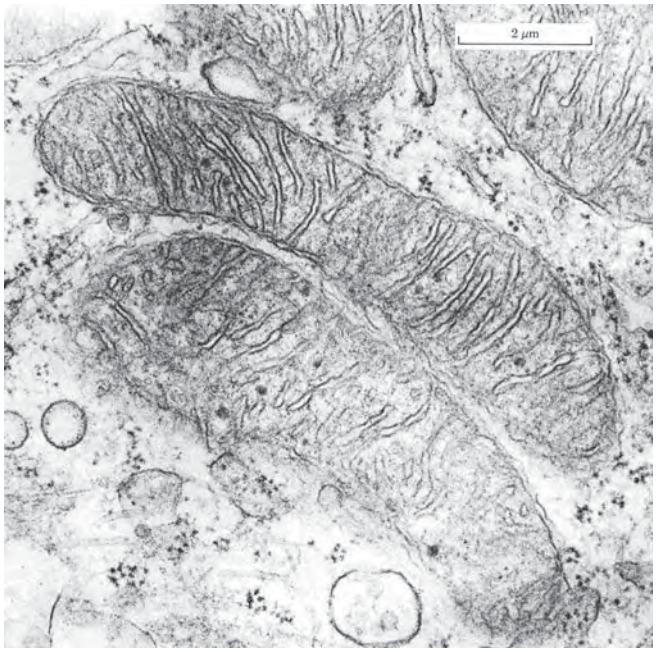


Figure 18-1 Thin section of mitochondria of a cultured kidney cell from a chicken embryo. The small, dark, dense granules within the mitochondria are probably calcium phosphate. Courtesy of Judie Walton.

1. The Mitochondrial Membranes and Compartments

The outer membranes of mitochondria can be removed from the inner membranes by osmotic rupture.[13] Analyses on separated membrane fractions show that *the outer membrane* is less dense (density ~1.1 g / cm^3) than the inner (density ~1.2 g / cm^3). It is highly permeable to most substances of molecular mass 10 kDa or less because of the presence of pores of ~2 nm diameter. These are formed by **mitochondrial porins**,[14–17] which are similar to the outer membrane porins of gram-negative bacteria (Fig. 8-20). The ratio of phospholipid to protein (~0.82 on a weight basis) is much higher than in the inner membrane. Extraction of the phospholipids by acetone destroys the membrane. Of the lipids present, there is a low content of cardiolipin, a high content of phosphatidylinositol and cholesterol, and no ubiquinone.

The inner membrane is impermeable to many substances. Neutral molecules of <150 Da can penetrate the membranes, but the permeability for all other materials including small ions such as H^+, K^+, Na^+, and Cl^- is tightly controlled. The ratio of phospholipid to protein in the inner membrane is ~0.27, and cardiolipin makes up ~20% of the phospholipid present. Cholesterol is absent. Ubiquinone and other components of the respiratory chain are all found in the inner membrane. Proteins account for 75% of the mass of the membrane.

Another characteristic of the inner mitochondrial membrane is the presence of projections on the inside surface, which faces the mitochondrial matrix. See Fig. 18-14. These spherical 85-kDa particles, discovered by Fernandez Moran in 1962 and attached to the membrane through a "stalk", display ATP-hydrolyzing (ATPase) activity. The latter was a clue that the knobs might participate in the *synthesis* of ATP during oxidative phosphorylation. In fact, they are now recognized as a complex of proteins called **coupling factor 1** (F_1) or **ATP synthase**.

In addition to bacterialike mitochondrial ribosomes and small circular molecules of DNA, mitochondria may contain variable numbers of dense granules of calcium phosphate, either $Ca_3(PO_4)_2$ or hydroxylapatite (Fig. 8-34),[4,18] as well as of phospholipoprotein.[4]

Quantitatively the major constituents of the matrix space are a large number of proteins. These account for about 56% by weight of the matrix material and exist in a state closer to that in a protein crystal than in a true solution.[19–20a] Mitochondrial membranes also contain proteins, both tightly bound relatively non-polar intrinsic proteins and extrinsic proteins bound to the membrane surfaces. The other mitochondrial compartment, the intermembrane space, may normally be very small but it is still "home" for a few enzymes.

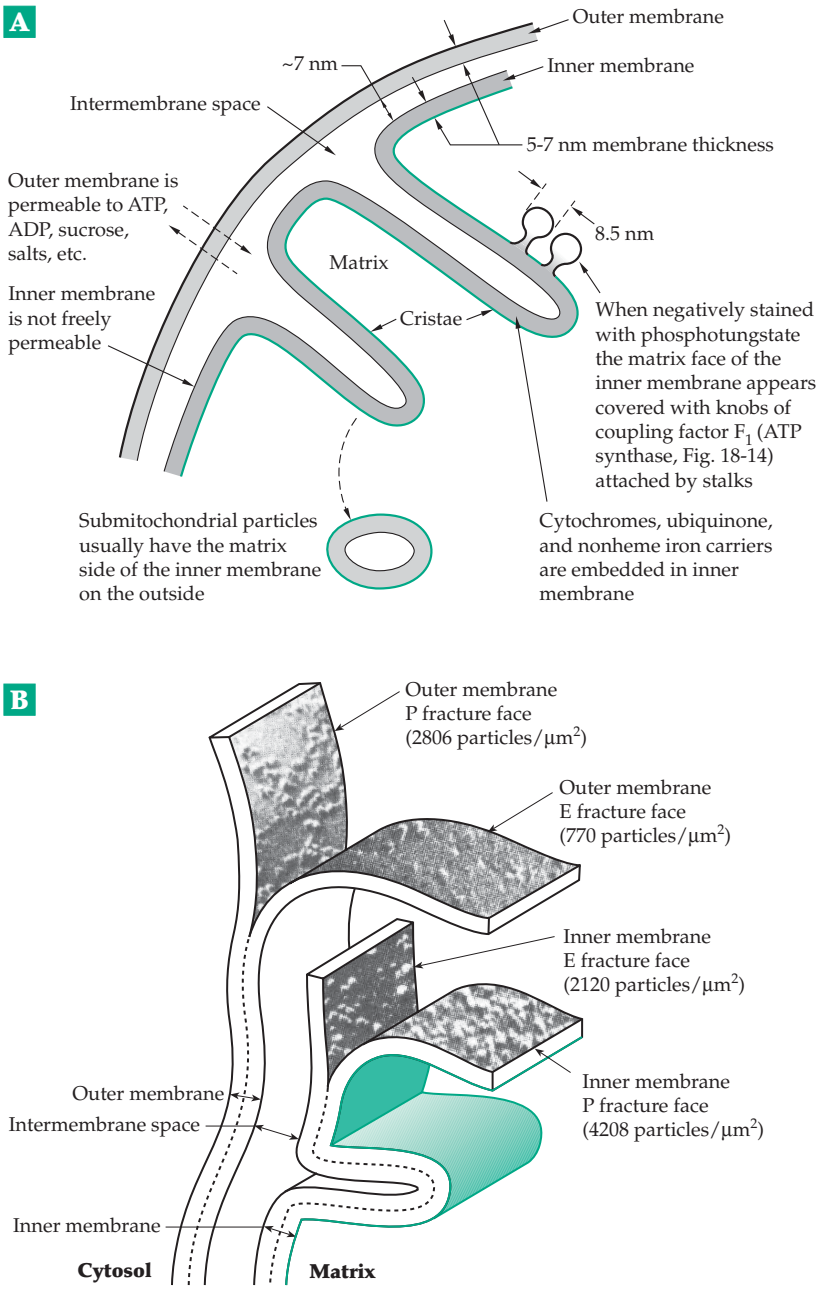


Figure 18-2 (A) Schematic diagram of mitochondrial structure. (B) Model showing organization of particles in mitochondrial membranes revealed by freeze-fracture electron microscopy. The characteristic structural features seen in the four half-membrane faces (EF and PF) that arise as a result of fracturing of the outer and inner membranes are shown. The four smooth membrane surfaces (ES and PS) are revealed by etching. From Packer.[8]

2. The Chemical Activities of Mitochondria

Mention of mitochondria usually brings to the mind of the biochemist the **citric acid cycle,** the **β oxidation pathway** of fatty acid metabolism, and **oxidative phosphorylation**. In addition to these major processes, many other chemical events also occur. Mitochondria concentrate Ca^{2+} ions and control the entrance and exit of Na^+, K^+, dicarboxylates, amino acids, ADP, P_i and ATP, and many other substances.[16] Thus, they exert regulatory functions both on catabolic and biosynthetic sequences. The glycine decarboxylase system (Fig. 15-20) is found in the mitochondrial matrix and is especially active in plant mitochondria (Fig. 23-37). Several cytochrome P450-dependent hydroxylation reactions, important to the biosynthesis and catabolism of steroid hormones and to the metabolism of vitamin D, take place within mitochondria. Mitochondria make only a few of their own proteins but take in several hundred other proteins from the cytoplasm as they grow and multiply.

Where within the mitochondria are specific enzymes localized? One approach to this question is to see how easily the enzymes can be dissociated from mitochondria. Some enzymes come out readily under hypotonic conditions. Some are released only upon sonic oscillation, suggesting that they are inside the matrix space. Others, including the cytochromes and the flavoproteins that act upon succinate and NADH, are so firmly embedded in the inner mitochondrial membranes that they can be dissociated only through the use of non-denaturing detergents.

Because the enzymes of the citric acid cycle[20a] (with the exception of succinate dehydrogenase) and β oxidation are present in the matrix, the reduced electron carriers must approach the inner membrane from

the matrix side (the M side). Thus, the embedded enzymes designed to oxidize NADH, succinate, and other reduced substrates must be accessible from the matrix side. However, *sn*-glycerol 3-phosphate dehydrogenase, a flavoprotein, is accessible from the "outside" of the cytoplasmic (C side) of the inner membrane.[21] Fluorescent antibodies to cytochrome *c* bind only to the cytoplasmic (intermembrane) side of the inner membrane, but antibodies to cytochrome oxidase label both sides, which suggested that this protein complex spans the membrane.[22] However, oxidation of cytochrome *c* by cytochrome oxidase occurs only on the cytoplasmic surface.[22] Antibodies to the ATP synthase that makes up the knobs bind strictly to the matrix side.

The outer mitochondrial membrane contains monoamine oxidase, cytochrome b_5, fatty acyl-CoA synthase, and enzymes of cardiolipin synthesis[22a] as well as other proteins. Cardiolipin (diphosphatidylglycerol; Fig. 21-4) is found only in the inner mitochondrial membrane and in bacteria. It is functionally important for several mitochondrial enzymes including cytochrome oxidase and cytochome bc_1.[22a–c] It is also essential to photosynthetic membranes for which an exact role in an interaction between the lipid membrane and the associated protein has been revealed by crystallography.[22d] In other respects the composition of the inner mitochondrial membrane resembles that of the membranes of the endoplasmic reticulum. Isoenzyme III of adenylate kinase, a key enzyme involved in equilibrating ATP and AMP with ADP (Eq. 6-65), is one of the enzymes present in the intermembrane space. A number of other kinases, as well as sulfite oxidase, are also present between the membranes.[4]

As mentioned in Box 6-D, mitochondria sometimes take up calcium ions. The normal total concentration of Ca^{2+} is ~1 mM and that of free Ca^{2+} may be only ~0.1 μM.[22e,f] However, under some circumstances mitochondria accumulate large amounts of calcium, perhaps acting as a Ca^{2+} buffer.[22g,f] The so called ryanodine receptors (Fig. 19-21), prominent in the endoplasmic reticulum, have also been found in heart mitochondria, suggesting a function in control of calcium oscillations.[22i,j] On the other hand, accumulation of calcium by mitochondria may be pathological and the activation of Ca^{2+}-dependent proteases may be an initial step in apoptosis.[22h,22k]

TABLE 18-1
Catalog of Mitochondrial Genes[a]

Name and symbol	*Homo sapiens*	*Reclinomonas americana*	*Saccharomyces cerevisiae*	*Arabidopsis thaliana*
Ribosomal RNA				
s rRNA (small, 12s)	1	1	1	1
l rRNA (large, 16s)	1	1	1	1
5 S RNA		1		1
Transfer RNAs	22[b]	26	24	22
NADH dehydrogenase				
Subunits ND1–6, ND4L	7	12	0	9
Cytochrome *b*	1	1	1	1
Cytochrome oxidase				
Subunits I, II, III	3	3	3	3
ATP synthase				
Subunits 6, 8, others	2	5	3	4
Total protein coding genes	13	62	8	27
Total genes	37	92	35	53
Size of DNA (kbp)	16.596	69	75	367

[a] Data from Palmer, J. D. (1997) *Nature (London)* **387**, 454–455.[1]
[b] One for each amino acid of the genetic code but two each for serine and leucine.

3. The Mitochondrial Genome

Each mitochondrion contains several molecules of DNA (**mtDNA**), usually in a closed, circular form, as well as the ribosomes, tRNA molecules, and enzymes needed for protein synthesis.[1,23–26] With rare exceptions almost all of the mitochondrial DNA in a human cell is inherited from the mother.[6,26a] The size of the DNA circles varies from 16–19 kb in animals[27] to over 200 kb in many higher plants. Complete sequences of many mitochondrial DNAs are known.[28,28a] Among these are the 16,569 bp human mtDNA,[29] the 16,338 bp bovine mtDNA, the 16,896 bp mtDNA of the wallaroo *Macropus robustus*,[30] and the 17,533 bp mtDNA of the amphibian *Xenopus laevis*.[31,32] The sea urchin *Paracentotus lividus* has a smaller 15,697 bp genome. However, the order of the genes in this and other invertebrate mtDNA is different from that in mammalian mitochondria.[33] Protozoal mtDNAs vary in size from ~5900 bp for the

parasitic malaria organism *Plasmodium falciparum*[34,35] to 41,591 bp for *Acanthamoeba castellani*[36] and 69,034 bp for the fresh water flagellate *Reclinomonas americana*.[26,37]

All of the mammalian mtDNAs are organized as shown in Fig. 18-3. The two strands of the DNA can be separated by virtue of their differing densities. The heavy (H) strand has a 5'→3' polarity in a counterclockwise direction in the map of Fig. 18-3, while the light (L) strand has a clockwise polarity. From the sequences 13 genes for specific proteins, 2 genes for ribosomal RNA molecules, and 22 genes for transfer RNAs have been identified. The genes are listed in Table 18-1 and have also been marked on the map in Fig. 18-3. The map also shows the tRNA genes, labeled with standard one-letter amino acid abbreviations, and the directions of transcription. Most of the protein genes are on the H-strand. One small region, the D-loop, contains an origin of replication and control signals for transcription (see Chapters 27 and 28).

The genes in mammalian mtDNA are closely packed with almost no nucleotides between them. However, the 19.5-kb mtDNA of *Drosophila* contains an (A+T)-rich region, which varies among species.[38] In the much larger 78-kb genome of yeast *Saccharomyces cerevisiae* many (A+T)-rich spacer regions are present.[39] The yeast mitochondrial genome also contains genes for several additional proteins. Mitochondria of *Reclinomonas americana* contain 97 genes, including those for 5S RNA, the RNA of ribonuclease P as well as a variety of protein coding genes. Perhaps this organism is primitive, resembling the original progenitor of eukaryotic life.[26] The mtDNA of trypanosomes is present in the large mitochondrion or kinetoplast as 40–50 "maxicircles" ~20–35 kb in size, together with 5000–10,000 "minicircles", each of 645–2500 bp (see Fig. 5-16). The latter encode **guide RNA** for use in RNA editing (Chapter 28). The large mitochondrial DNAs of higher plants, e.g., *Arabidopsis* (Table 18-1), also contain additional protein genes as well as large segments of DNA between the genes. The genome of the turnip (*Brassica campestris*) exists both as a 218-kb **master chromosome** and smaller 83- and 135-kb incomplete chromosomes, a pattern existing for most land plants.[40–42] The muskmelon contains 2500 kb of mitochondrial DNA (mtDNA). On the other hand, most mtDNA of the liverwort *Marchantia polymorpha* consists of 186-kb linear duplexes.[42a]

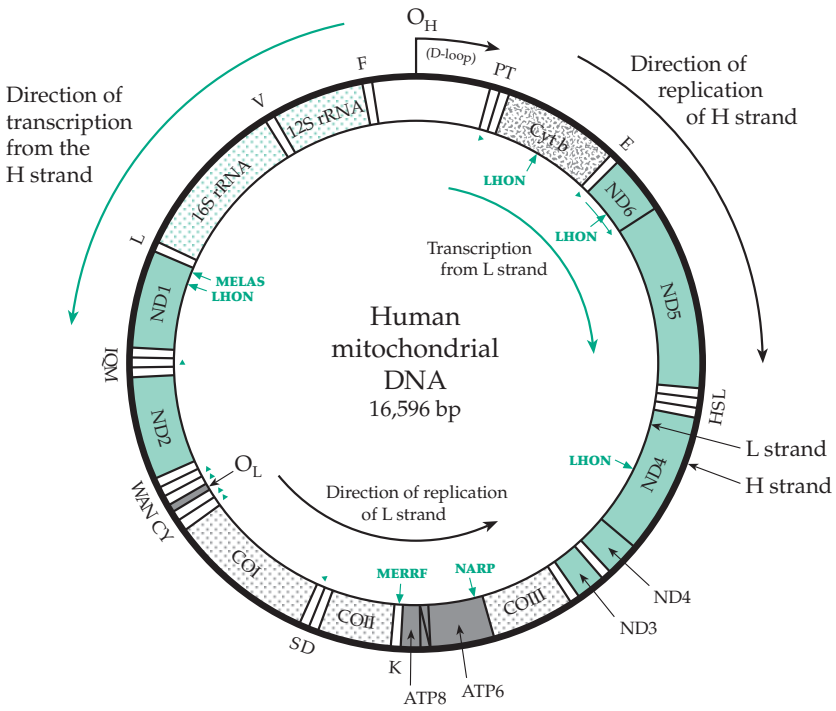


Figure 18-3 Genomic map of mammalian mitochondrial DNA. The stippled areas represent tRNA genes which are designated by the single-letter amino acid code; polarity is counterclockwise except for those marked by green arrow heads. All protein-coding genes are encoded on the H strand (with counterclockwise polarity), with the exception of ND6, which is encoded on the L strand. COI, COII, and COIII: cytochrome oxidase subunits I, II, and III; Cyt *b*: cytochrome *b*; ND: NADH dehydrogenase; ATP:ATP synthase. O_H and O_L: the origins of H and L strand replication, respectively. After Wallace[46] and Shoffner and Wallace.[45] Positions of a few of many known mutations that cause serious diseases are marked using abbrevations defined in Box 18-B.

Why does mtDNA contain *any* protein genes, or why does mtDNA even exist? It seems remarkable that the cells of our bodies make the 100 or so extra proteins (encoded in the nucleus) needed for replication, transcription, amino acid activation, and mitochondrial ribosome formation and bring these into the mitochondria for the sole purpose of permitting the synthesis there of 13 proteins. The explanation is not evident. What are the 13 proteins?

TABLE 18-2
Alterations in the Genetic Code in the DNA of Animal Mitochondria

Codons	Nuclear DNA[a]	Mitochondrial DNA
AGA, ACG	Arg	Termination
AUA	Ile	Met
UGA	Termination	Trp

[a] See Table 5-5 for the other "standard" codons.

Three are the large functional subunits of cytochrome oxidase, one is cytochrome *b*, and seven are subunits of the NADH dehydrogenase system (Complex I). Two are subunits of ATP synthase. These are all vitally involved in the processes of electron transport and oxidative phosphorylation, but so are other proteins that are imported from the cytoplasm.

One gene in yeast mtDNA is especially puzzling. The *var 1* gene encodes a mitochondrial ribosomal protein, whose sequence varies with the strain of yeast. The gene is also involved in unusual recombinational events.[47] Another unusual aspect of yeast mitochondrial genetics is the frequent appearance of "petite" mutants, which grow on an agar surface as very small colonies. These have lost a large fraction of their mitochondrial DNA and, therefore, the ability to make ATP by oxidative phosphorylation. The remaining petite mtDNA may sometimes become integrated into nuclear DNA.[48] A few eukaryotes that have no aerobic metabolism also have no mitochondria.[49]

4. Growth and Development

Mitochondria arise by division and growth of preexisting mitochondria. Because they synthesize only a few proteins and RNA molecules, they must import many proteins and other materials from the cytoplasm. A mitochondrion contains at least 100 proteins that are encoded by nuclear genes.[50,50a] The mechanisms by which proteins are taken up by mitochondria are complex and varied. Many of the newly synthesized proteins carry, at the N terminus, presequences that contain **mitochondrial targeting signals**[51–53] (Chapter 10). These amino acid sequences often lead the protein to associate with receptor proteins on the outer mitochondrial membrane and subsequently to be taken up by the mitochondria. While the targeting sequences are usually at the N terminus of a polypeptide, they are quite often internal. The N-terminal sequences are usually removed by action of the **mitochondrial processing peptidase** (MPP) in the matrix, but internal targeting sequences are not removed.[52] Targeting of proteins to mitochondria may be assisted by **mRNA binding proteins** that guide appropriate mRNAs into the vicinity of mitochondria or other organelles.[53]

In addition to targeting signals, polypeptides destined for the inner mitochondrial membrane contain additional **topogenic signals** that direct the polypeptide to its destination. These topogenic signals are distinct from the targeting signals, which they sometimes follow. Topogenic signals are usually hydrophobic sequences, which may become transmembrane segments of the protein in its final location.[52,54] The uptake of many proteins by mitochondria requires the electrical potential that is usually present across the inner membrane (Section E). The fact that mitochondrial proteins usually have higher isoelectric points and carry more positive charges at neutral pH favors uptake.[55] In addition, chaperonins assist in

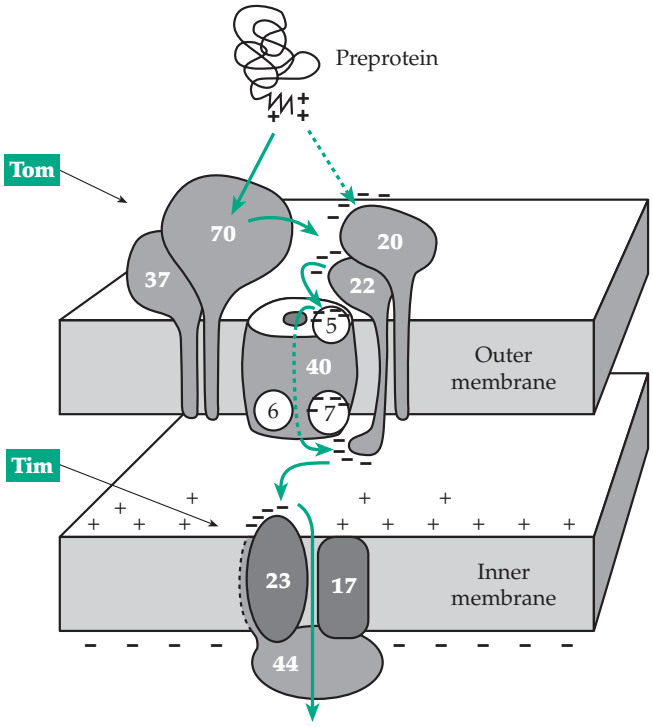


Figure 18-4 Schematic diagram of the protein transport machinery of mitochondrial membranes labeled according to the uniform nomenclature.[57] Subunits of outer membrane receptors and translocase are labeled Tom (translocase of outer membrane) and those of the inner membrane Tim (translocase of inner membrane). They are designated Tom70, etc., according to their sizes in kilodaltons (kDa). Preproteins are recognized by receptor Tom70•Tom 37 and / or by Tom22•Tom20. Clusters of negative charges on many components help guide the preprotein through the uptake pores in one or both membranes.[50,58] See Pfanner *et al.*,[57] Schatz,[50,50a] and Gabriel *et al.*[50b]

unfolding the protein to be taken up, assist in transport of some proteins,[50a] and may help the imported proteins to assemble into oligomeric structures.[51–53,56]

Protein uptake also requires a set of special proteins described as the **translocase of the outer mitochondrial membrane** (**Tom**) and **translocase of the mitochondrial inner membrane** (**Tim**). Subunits that form the receptor targets and transport pores are designated, according to their approximate molecular masses in kD as Tom70, Tim23, etc. (Fig. 18-4).[57] Preproteins are recognized by the receptor complexes Tom70 • Tom37 and / or Tom22 • Tom20 on the mitochondrial surface. They then enter the **general import pore** formed by Tom40, Tom6, and Tom7 with the assistance of a small integral membrane protein Tom5, which has a positively charged C-terminal membrane anchor segment and a negatively charged N-terminal portion that may bind to the positively charged mitochondrial targeting sequences.[50,59] A number of other translocase components, including Tom20 and Tom22 of the outer membrane and Tim23 of the inner membrane, also have acidic extramembranous domains.[58] This suggested an "acid chain" hypothesis according to which the targeting signal interacts consecutively with a series of acidic protein domains that help to guide it across the two membranes.[50a,58,59] A series of small proteins, Tim8, 9, 10, 12, 13, function in yeast mitochondria to mediate the uptake of metabolite transporters. A defect in the human nuclear gene for a protein that resembles Tim8 causes **deafness dystonia**, a recessive X-linked neurodegenerative disorder.[59a,b]

B. Electron Transport Chains

During the 1940s, when it had become clear that formation of ATP in mitochondria was coupled to electron transport, the first attempts to pick the system apart and understand the molecular mechanism began. This effort led to the identification and at least partial characterization of several flavoproteins, iron-sulfur centers, ubiquinones, and cytochromes, most of which have been described in Chapters 15 and 16. It also led to the picture of mitochondrial electron transport shown in Fig. 10-5 and which has been drawn in a modern form in Fig. 18-5.

1. The Composition of the Mitochondrial Electron Transport System

Because of the difficulty of isolating the electron transport chain from the rest of the mitochondrion, it is easiest to measure ratios of components (Table 18-3). Cytochromes a, a_3, b, c_1, and c vary from a 1:1 to a 3:1 ratio while flavins, ubiquinone, and nonheme iron occur in relatively larger amounts. The much larger amount of pyridine nucleotides is involved in carrying electrons from the various soluble dehydrogenases of the matrix to the immobile carriers in the inner membrane, while ubiquinone has a similar function within the lipid bilayer of mitochondrial membranes.

TABLE 18-3
Ratios of Components in the Electron Transport Chain of Mitochondria[a,b]

Electron carrier	Rat liver mitochondria	Beef heart mitochondria
Cytochrome a_3	1.0	1.1
Cytochrome a	1.0	1.1
Cytochrome b	1.0	1.0
Cytochrome c_1	0.63	0.33–0.51
Cytochrome c	0.78	0.66–0.85
Pyridine nucleotides	24	
Flavins	3	1
Ubiquinone	3–6	7
Copper		2.2
Nonheme iron		5.5

[a] From Wainio, W. W. (1970) *The Mammalian Mitochondrial Respiratory Chain*, Academic Press, New York, and references cited therein.
[b] Molecular ratios are given. Those for the cytochromes refer to the relative numbers of heme groups.

What are the molar concentrations of the electron carriers in mitochondrial membranes? In one experiment, cytochrome b was found in rat liver mitochondria to the extent of 0.28 µmol/g of protein. If we take a total mitochondrion as about 22% protein, the average concentration of the cytochrome would be ~0.06 mM. Since all the cytochromes are concentrated in the inner membranes, which may account for 10% or less of the volume of the mitochondrion, the concentration of cytochromes may approach 1 mM in these membranes. This is sufficient to ensure rapid reactions with substrates.

2. The Sequence of Electron Carriers

Many approaches have been used to deduce the sequence of carriers through which electron flow takes place (Fig. 18-5). In the first place, it seemed reasonable to suppose that the carriers should lie in order of increasing oxidation–reduction potential going from left to right of the figure. However, since the redox potentials existing in the mitochondria may be somewhat different from those in isolated enzyme preparations, this need not be strictly true.

The development by Chance of a dual wavelength spectrophotometer permitted easy observation of the state of oxidation or reduction of a given carrier within mitochondria.[60] This technique, together with the study of specific inhibitors (some of which are indicated in Fig. 18-5 and Table 18-4), allowed some electron transport sequences to be assigned. For example, blockage with **rotenone** and **amytal** prevented reduction of the cytochrome system by NADH but allowed reduction by succinate and by other substrates having their own flavoprotein components in the chain. Artificial electron acceptors, some of which are shown in Table 18-5, were used to bypass parts of the chain as indicated in Fig. 18-5.

Submitochondrial particles and complexes. Many methods have been employed to break mitochondrial membranes into submitochondrial particles that retain an ability to catalyze some of the reactions of the chain.[61] For example, the Keilin–Hartree preparation of heart muscle is obtained by homogenizing mitochondria and precipitation at low pH.[62] The resulting particles have a low cytochrome *c* content and do not carry out oxidative phosphorylation.

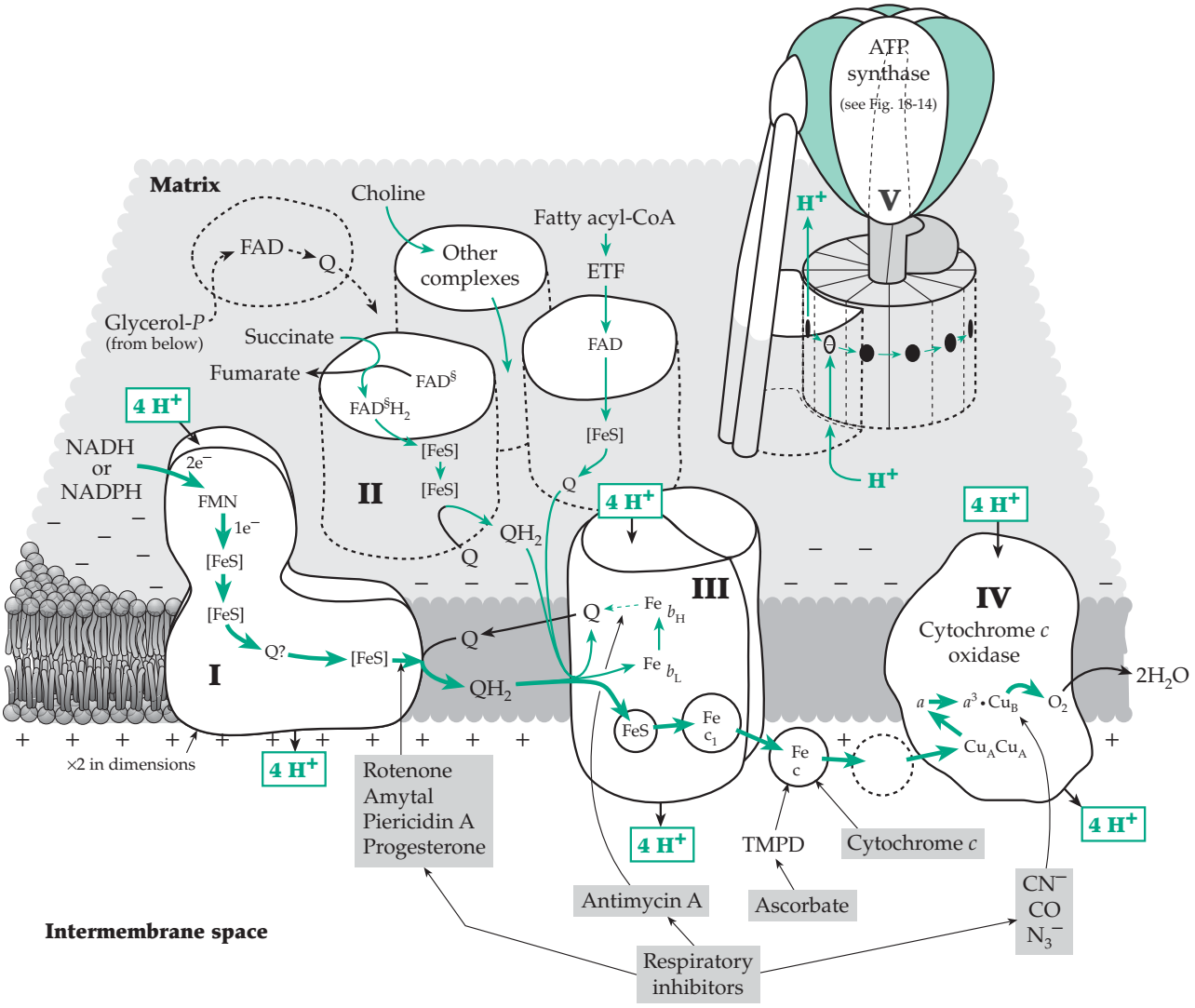
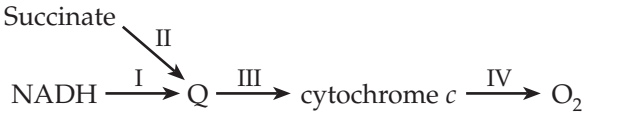


Figure 18-5 A current concept of the electron transport chain of mitochondria. Complexes I, III, and IV pass electrons from NADH or NADPH to O_2, one NADH or two electrons reducing one O to H_2O. This electron transport is coupled to the transfer of about 12 H^+ from the mitochondrial matrix to the intermembrane space. These protons flow back into the matrix through ATP synthase (V), four H^+ driving the synthesis of one ATP. Succinate, fatty acyl-CoA molecules, and other substrates are oxidized via complex II and similar complexes that reduce ubiquinone Q, the reduced form QH_2 carrying electrons to complex III. In some tissues of some organisms, glycerol phosphate is dehydrogenated by a complex that is accessible from the intermembrane space.

However, they do transport electrons and react with O_2. Other electron transport particles have been prepared by sonic oscillation. Under the electron microscope such particles appear to be small membranous vesicles resembling mitochondrial cristae.

Many detergents are strong denaturants of proteins, but some of them disrupt mitochondrial membranes without destroying enzymatic activity. A favorite is **digitonin** (Fig. 22-12), which causes disintegration of the outer membrane. The remaining fragments of inner membrane retain activity for oxidative phosphorylation. Such submitochondrial particles can be fractionated further by chemical treatments. Separate complexes can be obtained by treating the inner membranes with the nondenaturing detergent cholate (Fig. 22-10) and isolating the complexes by differential salt fractionation using ammonium sulfate. The isolated complexes I – IV catalyze reactions of four different portions of the electron transport process[63–65] as indicated in Eq. 18-1:



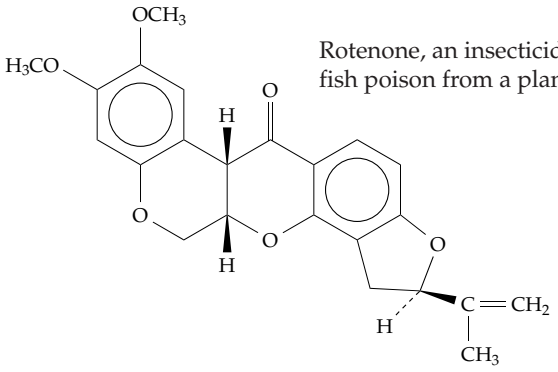
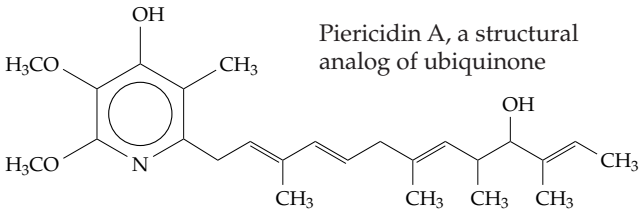
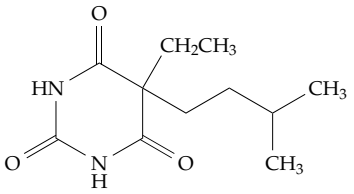
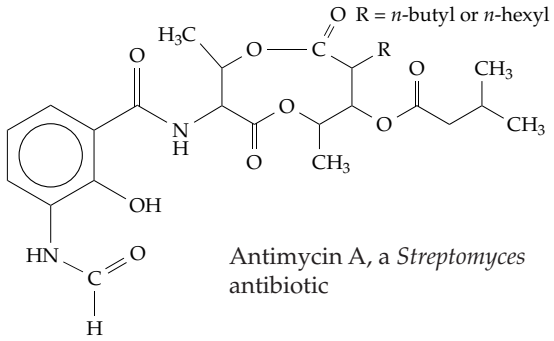
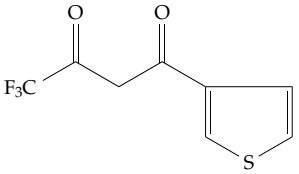
(18-1)

These complexes are usually named as follows: I, **NADH-ubiquinone oxidoreductase**; II, **succinate-ubiquinone oxidoreductase**; III, **ubiquinol-cytochrome *c* oxidoreductase**; IV, **cytochrome *c* oxidase**. The designation **complex V** is sometimes applied to ATP synthase (Fig. 18-14). Chemical analysis of the electron transport complexes verified the probable location of some components in the intact chain. For example, a high iron content was found in both complexes I and II and copper in complex IV.

We now recognize not only that these complexes are discrete structural units but also that they are functional units. Complete X-ray crystallographic structures are available for complexes III and IV and for much of the ATP synthase complex. As is indicated in Fig. 18-5, complexes I – IV are linked by two soluble electron carriers, ubiquinone and cytochrome *c*.

The lipid-soluble ubiquinone (Q) is present in both bacterial and mitochondrial membranes in relatively large amounts compared to other electron carriers (Table 18-2). It seems to be located at a point of convergence of the NADH, succinate, glycerol phosphate, and choline branches of the electron transport chain. Ubiquinone plays a role somewhat like that of NADH, which carries electrons between dehydrogenases in the cytoplasm and from soluble dehydrogenases in the aqueous mitochondrial matrix to flavoproteins embedded in the membrane. Ubiquinone transfers electrons plus protons between proteins within the

TABLE 18-4
Some Well-Known Respiratory Inhibitors[a]

	Rotenone, an insecticide and fish poison from a plant root
	Piericidin A, a structural analog of ubiquinone
	Amytal (amobarbital)
Progesterone	(See Fig. 22-11)
	Antimycin A, a *Streptomyces* antibiotic
	Thenoyltrifluoroacetone (TTB, 4,4,4-trifluoro-1-(2-thienyl)-1,3-butadione
Cyanide	$^-C\equiv N$
Azide	$N\equiv N\equiv N^-$
Carbon monoxide	CO

[a] See Fig. 18-5 for sites of inhibition.

BOX 18-A HISTORICAL NOTES ON RESPIRATION

Animal respiration has been of serious interest to scientists since 1777, when Lavoisier concluded that foods undergo slow combustion within the body, supposedly in the blood. In 1803–1807, Spallanzani established for the first time that the tissues were the actual site of respiration, but his conclusions were largely ignored. In 1884, MacMunn discovered that cells contain the heme pigments, which are now known as **cytochromes**. However, the leading biochemists of the day dismissed the observations as experimental error, and it was not until the present century that serious study of the chemistry of biological oxidations began.[a,b]

Recognition that substrates are oxidized by **dehydrogenation** is usually attributed to H. Wieland. During the years 1912–1922 he showed that synthetic dyes, such as methylene blue, could be substituted for oxygen and would allow respiration of cells in the absence of O_2. Subsequent experiments (see Chapter 15) led to isolation of the soluble pyridine nucleotides and flavoproteins and to development of the concept of an electron transport chain.

Looking at the other end of the respiratory chain, Otto Warburg[c,d] noted in 1908 that all aerobic cells contain iron. Moreover, iron-containing charcoal prepared from blood catalyzed nonenzymatic oxidation of many substances, but iron-free charcoal prepared from cane sugar did not. Cyanide was found to inhibit tissue respiration at low concentrations similar to those needed to inhibit nonenzymatic catalysis by iron salts. On the basis of these investigations, Warburg proposed in 1925 that aerobic cells contain an iron-based *Atmungsferment* (respiration enzyme), which was later called **cytochrome oxidase**. It was inhibited by carbon monoxide.

Knowing that carbon monoxide complexes of hemes are dissociated by light, Warburg and Negelein, in 1928, determined the photochemical **action spectrum** (see Chapter 23) for reversal of the carbon monoxide inhibition of respiration of the yeast *Torula utilis*. The spectrum closely resembled the absorption spectrum of known heme derivatives (Fig. 16-7). Thus, it was proposed that O_2, as well as CO, combines with the iron of the heme group in the Atmungsferment.

Meanwhile, during 1919–1925, David Keilin, while peering through a microscope equipped with a spectroscopic ocular at thoracic muscles of flies and other insects, observed a pigment with four distinct absorption bands. At first he thought it was derived by some modification of hemoglobin, but when he found the same pigment in fresh baker's yeast, he recognized it as an important new substance. In his words:[e]

One day while I was examing a suspension of yeast freshly prepared from a few bits of compressed yeast shaken vigorously with a little water in a test-tube, I failed to find the characteristic four-banded absorption spectrum, but before I had time to remove the suspension from the field of vision of the microspectroscope the four absorption bands suddenly reappeared. This experiment was repeated time after time and always with the same result: the absorption bands disappeared on shaking the suspension with air and reappeared within a few seconds on standing.

I must admit that this first visual perception of an intracellular respiratory process was one of the most impressive spectacles I have witnessed in the course of my work. Now I have no doubt that cytochrome is not only widely distributed in nature and completely independent of haemoglobin, but that it is an intracellular respiratory pigment which is much more important than haemoglobin.

Keilin soon realized that three of the absorption bands, those at 604, 564, and 550 nm (*a*, *b*, and *c*), represented different pigments, while the one at 521 nm was common to all three. Keilin proposed the names cytochromes *a*, *b*, and *c*. The idea of an electron transport or respiratory chain followed[e] quickly as the flavin and pyridine nucleotide coenzymes were recognized to play their role at the dehydrogenase level. Hydrogen removed from substrates by these carriers could be used to oxidize reduced cytochromes. The latter would be oxidized by oxygen under the influence of cytochrome oxidase.

In 1929, Fiske and Subbarow,[d,f–h] curious about the occurrence of purine compounds in muscle extracts, discovered and characterized ATP. It was soon shown (largely through the work of Lundsgaard and Lohman)[f] that hydrolysis of ATP provided energy for muscular contraction. At about the same time, it was learned that synthesis of ATP accompanied glycolysis. That ATP could also be formed as a result of electron transport became clear following an observation of Engelhardt[h,i] in 1930, that methylene blue stimulated ATP synthesis by tissues.

The study of electron transport chains and of oxidative phosphorylation began in earnest after Kennedy and Lehninger,[j] in 1949, showed that mitochondria were the site not only of ATP synthesis but also of the operation of the citric acid cycle and fatty acid oxidation pathways. By 1959, Chance had introduced elegant new techniques of spectrophotometry that led to formulation of the electron

BOX 18-A (continued)

transport chain as follows:

Substrate → pyridine nucleotides → flavoprotein → cyt b → cyt c → cyt a → cyt a_3 → O_2

Since that time, some new components have been added, notably the ubiquinones and iron-sulfur proteins, but the basic form proposed for the chain was correct.

[a] Kalckar, H. M. (1969) *Biological Phosphorylations*, Prentice-Hall, Englewood Cliffs, New Jersey
[b] Kalckar, H. M. (1991) *Ann. Rev. Biochem.* **60**, 1–37
[c] Edsall, J. T. (1979) *Science* **205**, 384–385
[d] Fiske, C. H., and Subbarow, Y. (1929) *Science* **70**, 381–382
[e] Keilin, D. (1966) *The History of Cell Respiration and Cytochrome*, Cambridge Univ. Press, London and New York
[f] Kalckar, H. (1980) *Trends Biochem. Sci.* **5**, 56–57
[g] Schlenk, F. (1987) *Trends Biochem. Sci.* **12**, 367–368
[h] Saraste, M. (1998) *Science* **283**, 1488–1493
[i] Slater, E. C. (1981) *Trends Biochem. Sci.* **6**, 226–227
[j] Talalay, P., and Lane, M. D. (1986) *Trends Biochem. Sci.* **11**, 356–358

membrane bilayer. Membranes also contain **ubiquinone-binding proteins**,[66,67] which probably hold the ubiquinone that is actively involved in electron transport. Perhaps some ubiquinone molecules function as fixed carriers. There is also uncertainty about the number of sites at which ubiquinone functions in the chain.

Mitochondrial electron transport in plants and fungi. Plant mitochondria resemble those of mammals in many ways, but they contain additional dehydrogenases and sometimes utilize alternative pathways of electron transport,[68–73] as do fungi.[74] Mitochondria are impermeable to NADH and NAD^+. Animal mitochondria have shuttle systems (see Fig. 18-16) for bringing the reducing equivalents of NADH into mitochondria and to the NADH dehydrogenase that faces the matrix side of the inner membrane. However, plant mitochondria also have an NADH dehydrogenase on the *outer* surface of the inner membrane (Fig. 18-6). This enzyme transfers electrons to ubiquinone, is not inhibited by rotenone (see Fig. 18-5), and also acts on NADPH. Inside the mitochondria a high-affinity NADH dehydrogenase resembles complex I of animal mitochondria and is inhibited by rotenone.[73] There is also a low-affinity NADH dehydrogenase, which is insensitive to rotenone. Some plant mitochondria respire slowly in the presence of cyanide. They utilize an **alternative oxidase** that replaces complex III and cytochome *c* oxidase and which is not inhibited by antimycin or by cyanide (Fig. 18-6).[68,71,75] It is especially active in thermogenic plant tissues (Box 18-C). A

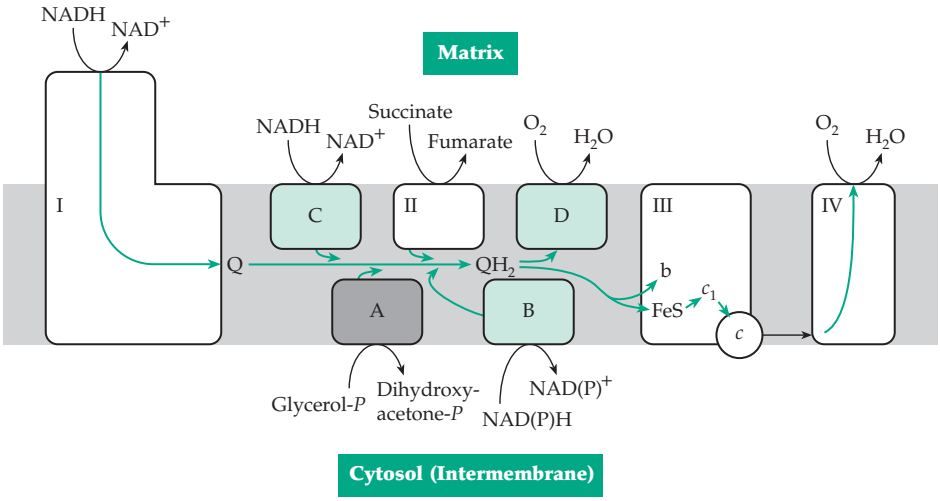


Figure 18-6 Schematic diagram of some mitochondrial dehydrogenase and oxidase complexes of plants and also the glycerol phosphate dehydrogenase of animals, which is embedded in the inner membrane. Complexes I–IV are also shown. (A) The glycerol phosphate dehydrogenase of some animal tissues. It is accessible from the intermembrane space on the cytosolic side. (B) The rotenone-insensitive NAD(P)H dehydrogenase of the external membrane surface of plants. (C) The rotenone-insensitive NADH dehydrogenase facing the matrix side in some plants. (D) The plant alternative oxidase. Ubiquinone, Q. The three green stippled dehydrogenases are not coupled to proton pumps or ATP synthesis. After Hoefnagel *et al.*[73]

BOX 18-B DEFECTS OF MITOCHONDRIAL DNA

A mutation in any of the 13 protein subunits, the 22 tRNAs, or the two rRNAs whose genes are carried in mitochondrial DNA may possibly cause disease. The 13 protein subunits are all involved in electron transport or oxidative phosphorylation. The syndromes resulting from mutations in mtDNA frequently affect oxidative phosphorylation (OXPHOS) causing what are often called "OXPHOS diseases."[a–g] Mitochondrial oxidative phosphorylation also depends upon ~100 proteins encoded in the nucleus. Therefore, OXPHOS diseases may result from defects in either mitochondrial or nuclear genes. The former are distinguished by the fact that they are inherited almost exclusively maternally. Most mitochondrial diseases are rare. However, mtDNA is subject to rapid mutation, and it is possible that accumulating mutants in mtDNA may be an important component of aging.[h–k]

The first recognition of mitochondrial disease came in 1959. A 30-year old Swedish woman was found to have an extremely high basal metabolic rate (180% of normal), a high caloric intake (>3000 kcal / day), and an enormous perspiration rate. She had developed these symptoms at age seven. Examination of her mitochondria revealed that electron transport and oxidative phosphorylation were very loosely coupled. This explains the symptoms. However, the disease (Luft disease) is extremely rare and the underlying cause isn't known.[i] Its recognition did focus attention on mitochondria, and by 1988, 120 different mtDNA defects had been described.[e,i]

Some OXPHOS disorders, including Luft disease, result from mutations in nuclear DNA. A second group arise from point mutations in mtDNA and a third group involve deletions, often very large, in mtDNA. Persons with these deletions survive because they have both mutated and normal mtDNA, a condition of **heteroplasmy** of mtDNA. As these persons age their disease may become more severe because they lose many normal mitochondria.[d,e]

The names of mitochondrial diseases are often complex and usually are described by abbreviations. Here are a few of them: **LHON**, Lebers hereditary optical neuropathy; **MERRF**, myoclonic epilepsy and ragged-red-fiber disease; **MELAS**, mitochondrial encephalomyopathy, lactic acidosis, and strokelike episodes; **NARP**, neurological muscle weakness, ataxia, and retinitis pigmentosa; **Leigh disease — SNE**, subacute necrotizing encephalomyelopathy; **KSS**, Kearns–Sayre syndrome; **CPEO**, chronic progressive external ophthalmoplegia. LHON is a hereditary disease that often leads to sudden blindness from death of the optic nerve especially among males. Any one of several point mutations in subunits ND1, 2, 4, 5, and 6 of NADH dehydrogenase (complex I; Figs. 18-3 and 18-5), cytochrome *b* of complex II, or subunit I of cytochrome oxidase may cause this syndrome. Most frequent is an R340H mutation of the ND4 gene at position 11,778 of mtDNA (Fig. 18-3).[e,l,m] It may interfere with reduction of ubiquinone.[n] Mutations in the ND1 gene at position 3460 and in the ND6 gene at position 14484 or in the cytochrome *b* gene at position 15257 cause the same disease.[l] The most frequent (80–90%) cause of MERRF, which is characterized by epilepsy and by the appearance of ragged red fibers in stained sections of muscle, is an A → G substitution at position 8344 of mtDNA in the TψC loop (Fig. 5-30) of mitochondrial $tRNA^{Lys}$. A similar disease, MELAS, is accompanied by strokes (not seen in MERRF) and is caused in 80% of cases by an A → G substitution in the dihydrouridine loop (Fig. 5-30) of mitochondrial $tRNA^{Leu}$.[o] CPEO, Leigh disease, and KSS often result from large deletions of mtDNA.[p] NARP and related conditions have been associated with an L156R substitution in the ATPase 6 gene of ATP synthase.[q]

Can mitochondrial diseases be treated? Attempts are being made to improve the function of impaired mitochondria by adding large amounts of ubiquinone, vitamin K, thiamin, riboflavin, and succinate to the diet.[e] One report suggests that mitochondrial decay during aging can be reversed by administration of *N*-acetylcarnitine.[k]

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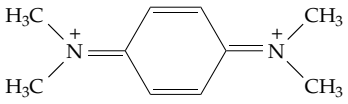
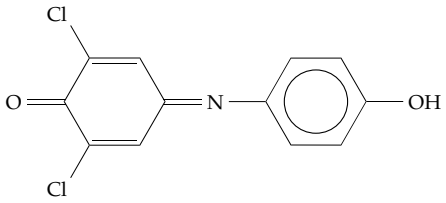
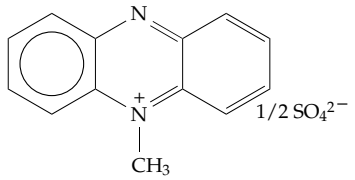
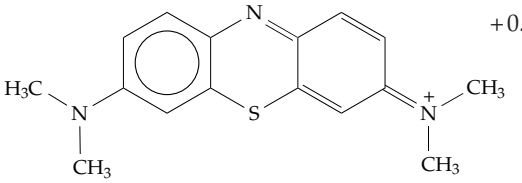
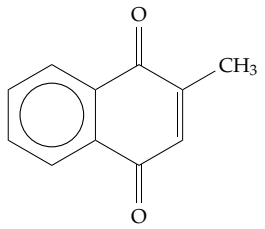
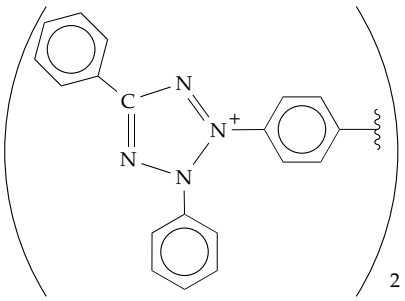
[q] Hartzog, P. E., and Cain, B. D. (1993) *J. Biol. Chem.* **268**, 12250–12252

TABLE 18-5
Some Artificial Electron Acceptors[a,b]

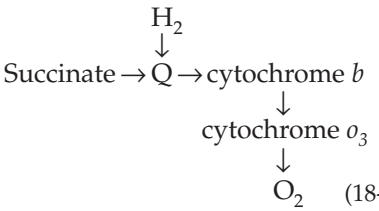
Compound	Structure	E°′(pH 7) 30°C
Ferricyanide	$Fe(CN)_6^{3-}$	+0.36 V (25°C)
Oxidized form of tetramethyl-*p*-phenylenediamine		+0.260 V
2,6-Dichlorophenol-indophenol (DCIP)		+0.217 V
Phenazine methosulfate (PMS)		+0.080 V
Ascorbate	(See Box 18-D)	+0.058 V
Methylene blue		+0.011 V
Menadione		+0.008 V (°25C)
Tetrazolium salts, e.g., "neotetrazolium chloride"		−0.125 V

[a] From Wainio, W. W. (1970) *The Mammalian Mitochondrial Respiratory Chain*, Academic Press, New York, pp. 106–111.
[b] See Fig. 18-5 for sites of action.

similar oxidase is present in trypanosomes.[72] Neither the rotenone-insensitive dehydrogenases nor the alternative oxidases are coupled to synthesis of ATP.

Electron transport chains of bacteria. The bacterial electron transport systems are similar to that of mitochondria but simpler. Bacteria also have a variety of alternative pathways that allow them to adapt to various food sources and environmental conditions.[76,77] The gram-negative soil bacterium *Paracoccus denitrificans*, which has been called "a free-living mitochondrion," has a mammalian-type respiratory system. Its complexes I–IV resemble those of animals and of fungi,[78–79] but *Paracoccus* has fewer subunits in each complex. Complex I of *E. coli* is also similar to that of our own bodies.[79–80] However, other major flavoprotein dehydrogenases in *E. coli* act on D-lactate and *sn*-3-glycerol phosphate.[81] Pyruvate is oxidized by a membrane-bound flavoprotein (Fig. 14-2). All of these enzymes pass electrons to ubiquinone-8 (Q_8).[82] Succinate dehydrogenase of *E. coli* resembles that of mitochondria,[83] and the ubiquinol oxidase of *Paracoccus* resembles complexes III + IV of mitochondria. It can be resolved into a three-subunit bc_1 complex, a three-subunit c_1aa_3 complex, and another 57-kDa peptide.[84] The last contains a 22-kDa cytochrome c_{552}, which is considerably larger than mitochondrial cytochrome *c*.

The cytochrome aa_3 terminal oxidase is produced constitutively, i.e., under all conditions. However, when cells are grown on succinate or H_2 another set of enzymes is produced with the *b*-type cytochrome o_3 as the terminal **quinol oxidase** (Eq. 18-2).[85]



(18-2)

Two terminal quinol oxidase systems, both related to cytochrome *c* oxidase, are utilized by *E. coli* to oxidize ubiquinol-8. When cultured at high oxygen tensions, cytochrome bo_3 (also called cytochrome *bo*) is the major oxidase. It utilizes heme *o* (Fig. 16-5) instead of heme *a*. However, at low oxygen tension, e.g., in the late logarithmic stage of growth, the second oxidase, cytochrome *bd*, is formed.[76,86–88a] It contains two molecules of the chlorin heme *d* (Fig. 16-5), which appear to be involved directly in binding O_2. This terminal oxidase system is present in many bacteria and can utilize either O_2 or nitrite as the oxidant. A simpler electron transport chain appears to be involved in the oxidation of pyruvate by *E. coli*. The flavoprotein pyruvate oxidase passes electrons to Q_8, whose reduced form can pass electrons directly to cytochrome *d*. Incorporation of these two pure protein complexes and ubiquinone-8 into phospholipid vesicles has given an active reconstituted chain.[82] Other bacteria utilize a variety of quinol oxidase systems, which contain various combinations of cytochromes: aa_3, caa_3, *cao*, bo_3, and ba_3.[88b,c]

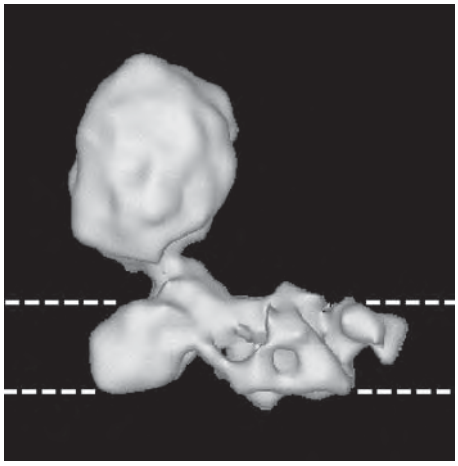


Figure 18-7 Three-dimensional image of bovine NADH-Ubiquinone oxidoreductase (complex I) reconstructed from individual images obtained by electron cyro-microscopy. The resolution is 2.2 nm. The upper portion projects into the mitochondrial matrix while the horizontal part lies within the membrane as indicated. Courtesy of N. Grigorieff.[90]

3. Structures and Functions of the Individual Complexes I – IV and Related Bacterial Assemblies

What are the structures of the individual electron transport complexes? What are the subunit compositions? What cofactors are present? How are electrons transferred? How are protons pumped? We will consider these questions for each of complexes I–IV, as found in both prokaryotes and eukaryotes.[88d,e]

Complex I, NADH-ubiquinone oxidoreductase. Complex I oxidizes NADH, which is generated within the mitochondrial matrix by many dehydrogenases. Among these are the pyruvate, 2-oxoglutarate, malate, and isocitrate dehydrogenases, which function in the tricarboxylic acid cycle; the β-oxoacyl-CoA dehydrogenase of the β oxidation system for fatty acids; and 2-hydroxybutyrate, glutamate, and proline dehydrogenases. All produce NADH, which reacts with the flavoprotein component of complex I. Whether from bacteria,[79] fungal mitochondria,[89] or mammalian mitochondria[89a,90] complex I exists as an L-shaped object, of which each of the two arms is ~23 nm long. One arm projects into the matrix while the other lies largely within the inner mitochondrial membrane (Fig. 18-7). The mitochondrial complex, which has a mass of ~1 MDa, has the same basic structure as the 530-kDa bacterial complex. However, the arms are thicker in the mitochondrial complex. Analysis of the denatured proteins by gel electrophoresis revealed at least 43 peptides.[78,90] Bound to some of these are the electron carriers FMN, Fe_2S_2, and Fe_4S_4 clusters, ubiquinone or other quinones, and perhaps additional unidentified cofactors.[79] Complex I from *E. coli* is smaller, containing only 14 subunits. These are encoded by a cluster of 14 genes, which can be directly related by their sequences to subunits of mitochondrial complex I and also to the corresponding genes of *Paracoccus denitrificans*.[80,91] Complex I of *Neurospora* contains at least 35 subunits.[89] The 14 subunits that are present both in bacteria and in mitochondria probably form the structural core of the complex. The other subunits thicken, strengthen, and rigidify the arms. Some of the "extra" subunits have enzymatic activities that are not directly related to electron transport. Among these are a 10-kDa prokaryotic type acyl carrier protein (ACP), which may be a relic of a bacterial fatty acid synthase, reflecting the endosymbiotic origin of mitochondria.[92] Also present is a 40-kDa NAD(P)H dependent reductase / isomerase, which may be involved in a biosynthetic process, e.g., synthesis of a yet unknown redox group.[79,92]

In all cases, FMN is apparently the immediate acceptor of electrons from NADH. From the results of extrusion of the Fe–S cores (Chapter 16) and EPR measurements it was concluded that there are three tetranuclear (Fe_4S_4) iron–sulfur centers and at least two binuclear (Fe_2S_2) centers[93,94] as well as bound ubiquinone.[95] Chemical analysis of iron and sulfide suggested up to eight Fe–S clusters per FMN, while gene sequences reveal potential sites for formation of six Fe_4S_4 clusters and two Fe_2S_2 clusters.[78] Treatment of complex I with such "chaotropic agents" as 2.5 M urea or 4 M sodium trichloroacetate followed by fractionation with ammonium sulfate[95] gave three fractions:

(1) A soluble NADH dehydrogenase consisting of a 51-kDa peptide that binds both the FMN and also one tetranuclear Fe – S cluster (designated N3) and a 24-kDa peptide that carries a binuclear Fe–S center designated N1b. (2) A 75-kDa peptide bearing two binuclear Fe–S centers, one of which is called N1a and also 47-, 30-, and 13-kDa peptides. One of these carries tetranuclear center N4. (3) A group of insoluble, relatively nonpolar proteins, one of which carries tetranuclear cluster N2. It may be the immediate donor of electrons to a ubiquinone held by a ubiquinone-binding protein designated QP-N. In bacteria seven of these are homologs of the seven NADH dehydrogenase subunits encoded by mtDNA (Fig. 18-3). A 49-kDa subunit of complex I in the yeast *Yarrowia lipolytica* is strikingly similar to the hydrogen reactive subunit of NiFe hydrogenases (Fig. 16-26).[95a] These proteins are thought to lie within the membrane arm and to form ~55 transmembrane α helices. Ubiquinone may also function as a carrier within complex I,[96,97] and there may be a new redox cofactor as well.[79] The following tentative sequence (Eq. 18-3) for electron transfer within complex I (with apparent $E^{\circ'}$ values of carriers) has been suggested. By equilibration with external redox systems, the redox potentials of these centers within the mitochondria have been estimated and are given (in V) in Eq. 18-3. The presence of a

$$\begin{array}{lccccccccc} & & & & & & & & & \text{rotenone} \\ \text{NADH} \rightarrow \text{FMN} \rightarrow & \text{N1a} & \rightarrow & (\text{N3}, & \text{N1b}, & \text{N4}) & \rightarrow & \text{N2} & \rightarrow \text{QP-N} \dashrightarrow & \text{Q} \\ & \text{Fe}_2\text{S}_2 & & \text{Fe}_4\text{S}_4 & \text{Fe}_2\text{S}_2 & \text{Fe}_4\text{S}_4 & & \text{Fe}_4\text{S}_4 & \ominus & \\ -0.32 & & -0.38 & & -0.24 & & -0.21 & \text{V} & & \end{array} \qquad (18\text{-}3)$$

large fraction of the bound ubiquinone as a free radical suggests that the quinone functions as a one-electron acceptor rather than a two-electron acceptor. A characteristic of complex I is inhibition by rotenone or piericidin, both of which block electron transport at the site indicated in Fig. 18-5.

Complex II, succinate-ubiquinone oxidoreductase. Complex II, which carries electrons from succinate to ubiquinone, contains covalently linked 8^{α}-(*N*-histidyl)-FAD (Chapter 15) as well as Fe–S centers and one or more ubiquinone-binding sites. There are four subunits whose structures and properties have been highly conserved among mitochondria and bacteria and also in **fumarate reductases**. The latter function in the opposite direction during anaerobic respiration with fumarate as the terminal oxidant, both in bacteria[98–99a] and in parasitic helminths and other eukaryotes that can survive prolonged anaerobic conditions (Chapter 17, Section F,2).[100] Complex II from *E. coli* consists of 64-, 27-, 14-, and 13-kDa subunits, which are encoded by genes *sdhCDAB* of a single operon.[101–103] The two larger hydrophilic subunits associate to form the readily soluble succinate dehydrogenase. The 64-kDa subunit carries the covalently bound FAD while the 27-kDa subunit carries three Fe–S centers. The two small 13- and 14-kDa subunits form a hydrophobic anchor and contain a ubiquinol-binding site (QD-S)[103] as well as a heme that may bridge the two subunits[102] to form cytochrome b_{556}. The functions of the heme is uncertain. The soluble mammalian succinate dehydrogenase resembles closely that of *E. coli* and contains three Fe – S centers: binuclear S1 of $E^{\circ'}$ 0 V, and tetranuclear S2 and S3 of –0.25 to –0.40 and + 0.065 V, respectively. Center S3 appears to operate between the –2 and –1 states of Eq. 16-17 just as does the cluster in the *Chromatium* high potential iron protein. The function of the very low potential S2 is not certain, but the following sequence of electron transport involving S1 and S3 and the bound ubiquinone QD–S[66] has been proposed (Eq. 18-4).

$$\text{Succinate} \rightarrow (\text{FAD*, S1}) \rightarrow \text{S3} \rightarrow \text{QP–S} \rightarrow \text{Q} \qquad (18\text{-}4)$$

In addition to complexes I and II several other membrane-associated FAD-containing dehydrogenase systems also send electrons to soluble ubiquinone. These include dehydrogenases for choline, *sn*-glycerol 3-phosphate, and the electron-transfer protein (ETF) of the fatty acyl-CoA β oxidation system (Fig. 18-5). The last also accepts electrons from dehydrogenases for sarcosine (*N*-methylglycine), dimethylglycine, and other substrates. The *sn*-glycerol 3-phosphate dehydrogenase is distinguished by its accessibility from the intermembrane (cytosolic) face of the inner mitochondrial membrane (Fig. 18-6).

Complex III (ubiquinol-cytochrome c oxidoreductase or cytochrome bc_1 complex). Mitochondrial complex III is a dimeric complex, each subunit of which contains 11 different subunits with a total molecular mass of ~240 kDa per monomer.[104–107] However, in many bacteria the complex consists of only three subunits, cytochrome *b*, cytochrome c_1, and the high potential (~0.3 V) Rieske iron-sulfur protein, which is discussed in Chapter 16, Section A,7. These three proteins are present in all bc_1 complexes. In eukaryotes the 379-residue cytochrome *b* is mitochondrially encoded. Although there is only one cytochrome *b* gene in the mtDNA, two forms of cytochrome *b* can be seen in absorption spectra: b_H (also called b_{562} or b_K) and lower potential b_L (also called b_{566} or b_T).[107a,b]

X-ray diffraction studies have revealed the complete 11-subunit structure of bovine bc_1 complex[104,106–107] as well as a nearly complete structure of the chicken bc_1 complex (Fig. 18-8).[105] The bovine complex contains 2166 amino acid residues per 248-kDa monomer and

exists in crystals as a 496-kDa dimer and probably functions as a dimer.[106-107] The two hemes of cytochrome *b* are near the two sides of the membrane, and the Fe–S and cytochrome c_1 subunits are on the surface next to the intermembrane space (Fig. 18-8). On the matrix side (bottom in Fig. 18-8A) are two large ~440 residue "core" subunits that resemble subunits of the mitochondrial processing protease. They may be evolutionary relics of that enzyme.[106,108,108a] Mitochondrial cytochrome b_H has an $E°'$ value of +0.050 V, while that of b_L is −0.090 V at pH 7.[109] That of the Rieske Fe–S protein is + 0.28 V.[110]

The sequence of electron transport within complex III has been hard to determine in detail. For reasons discussed in Section C, the "Q-cycle" shown in Fig. 18-9 has been proposed.[111–114a] As is indicated in Fig. 18-9, complex II accepts electrons from QH_2 and passes them consecutively to the Fe–S protein, cytochrome c_1, and the external cytochrome *c*. However, half of the electrons are recycled through the two heme groups of cytochrome *b*, as is indicated in the figure and explained in the legend. The X-ray structure (Fig. 18-8) is consistent with this interpretation. Especially intriguing is the fact that the Fe_2S_2 cluster of the Rieske protein subunit has been observed in two or three different conformations.[105–107,114a-c] In Fig. 18-8C the structures of two conformations are superimposed. The position of the long helix at the right side is unchanged but the globular domain at the top can be tilted up to bring the Fe_2S_2 cluster close to the heme of cytochrome c_1, or down to bring the cluster close to heme b_L. Movement between these two positions is probably part of the catalytic cycle.[115]

The simpler cytochrome bc_1 complexes of bacteria such as *E. coli*,[102] *Paracoccus dentrificans*,[116] and the photosynthetic *Rhodobacter capsulatus*[117] all appear to function in a manner similar to that of the large mitochondrial complex. The bc_1 complex of *Bacillus subtilis* oxidizes reduced **menaquinone** (Fig. 15-24) rather than ubiquinol.[118] In chloroplasts of green plants photochemically reduced **plastoquinone** is oxidized by a similar complex of cytochrome *b*, *c*-type cytochrome *f*, and a Rieske Fe–S protein.[119–120a] This cytochrome b_6f complex delivers electrons to the copper protein plastocyanin (Fig. 23-18).

The electron acceptor for complex III is cytochrome *c*, which, unlike the other cytochromes, is water soluble and easily released from mitochondrial membranes. Nevertheless, it is usually present in a roughly 1:1 ratio with the fixed cytochromes, and it seems unlikely that it is as free to diffuse as are ubiquinone and NAD^+.[121,122] However, a small fraction of the cytochrome *c* may diffuse through the intermembrane space and accept electrons from cytochrome b_5, which is located in the outer membrane.[123] Cytochrome *c* forms a complex with cardiolipin (diphosphatidylglycerol), a characteristic component of the inner mitochondrial membrane.[124]

Complex IV. Cytochrome c oxidase (ubiquinol-cytochrome c oxidoreductase). Complex IV from mammalian mitochondria contains 13 subunits. All of them have been sequenced, and the three-dimensional structure of the complete complex is known (Fig. 18-10).[125–127] The simpler cytochrome *c* oxidase from *Paracoccus denitrificans* is similar but consists of only three subunits. These are homologous in sequence to those of the large subunits I, II, and III of the mitochondrial complex. The three-dimensional structure of the *Paracoccus* complex is also known. Its basic structure is nearly identical to that of the catalytic core of subunits I, II, and III of the mitochondrial complex (Fig. 18-10,A).[128] All three subunits have transmembrane helices. Subunit III seems to be structural in function, while subunits I and II contain the oxidoreductase centers: two hemes *a* (*a* and a_3) and two different copper centers, Cu_A (which contains two Cu^{2+}) and a third Cu^{2+} (Cu_B) which exists in an EPR-silent exchange coupled pair with a_3. Bound Mg^{2+} and Zn^{2+} are also present in the locations indicated in Fig. 18-10.

The Cu_A center has an unusual structure.[130–132] It was thought to be a single atom of copper until the three-dimensional structure revealed a dimetal center, whose structure follows. The Cu_B-cytochrome a_3 center is also unusual. A histidine ring is covalently attached to tyrosine.[133–135a] Like the tyrosine in the active site of galactose oxidase (Figs. 16-29, 16-30), which carries a covalently joined cysteine, that of cytochrome oxidase may be a site of tyrosyl radical formation.[135]

Cytochrome *c* oxidase accepts four electrons, one at a time from cytochrome *c*, and uses them to reduce O_2 to two H_2O. Electrons enter the oxidase via the Cu_A center and from there pass to the cytochrome *a* and on to the cytochrome a_3 – Cu_B center where the reduction of O_2 takes place. A possible sequence of steps in the catalytic cycle is given in Fig. 18-11. Reduction of O_2 to two H_2O requires four electrons and also four protons. An additional four protons are evidently pumped across the membrane for each catalytic cycle.[136–138] The overall reaction is:

$$4\,e^- + 8\,H^+_{IN} + O_2 \longrightarrow 2\,H_2O + 4\,H^+_{OUT} \qquad (18\text{-}5)$$

The reaction of O_2 with cytochrome *c* oxidase to form the oxygenated species A (Fig. 18-11) is very rapid, occurring with apparent lifetime τ (Eq. 9-5) of ~8–10 μs.[139] Study of such rapid reactions has depended upon a flow-flash technique developed by Greenwood and Gibson.[136,140,141] Fully reduced cytochrome oxidase is allowed to react with carbon monoxide, which binds to the iron in cytochrome a_3 just as does O_2. In fact, it was the spectroscopic observation that only half of the

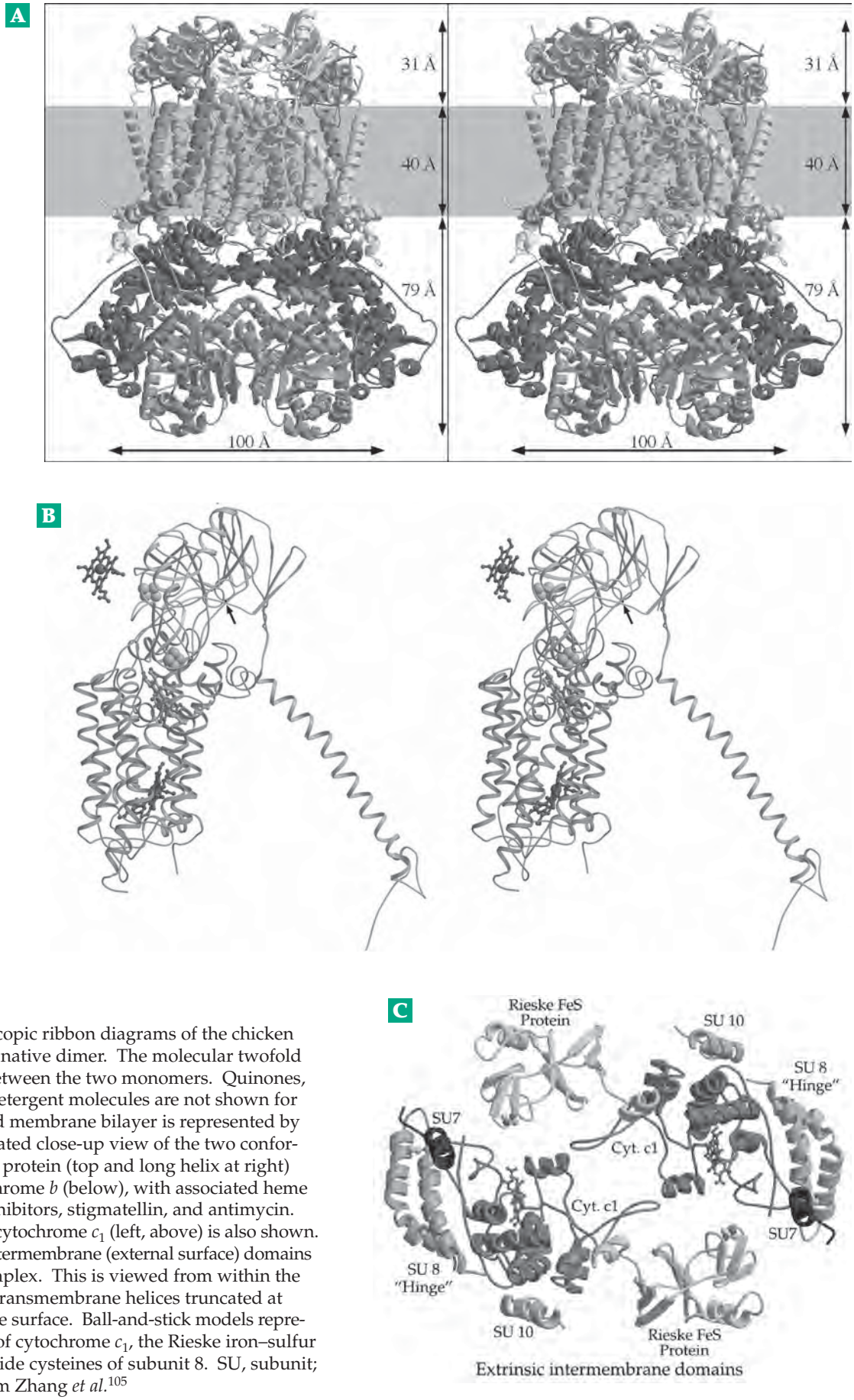


Figure 18-8 Stereoscopic ribbon diagrams of the chicken bc_1 complex (A) The native dimer. The molecular twofold axis runs vertically between the two monomers. Quinones, phospholipids, and detergent molecules are not shown for clarity. The presumed membrane bilayer is represented by a gray band. (B) Isolated close-up view of the two conformations of the Rieske protein (top and long helix at right) in contact with cytochrome *b* (below), with associated heme groups and bound inhibitors, stigmatellin, and antimycin. The isolated heme of cytochrome c_1 (left, above) is also shown. (C) Structure of the intermembrane (external surface) domains of the chicken bc_1 complex. This is viewed from within the membrane, with the transmembrane helices truncated at roughly the membrane surface. Ball-and-stick models represent the heme group of cytochrome c_1, the Rieske iron–sulfur cluster, and the disulfide cysteines of subunit 8. SU, subunit; cyt, cytochrome. From Zhang *et al.*[105]

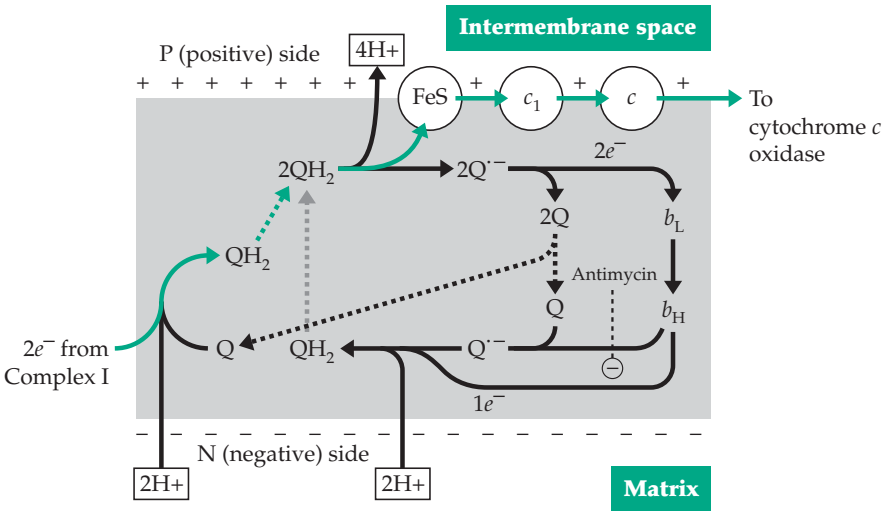
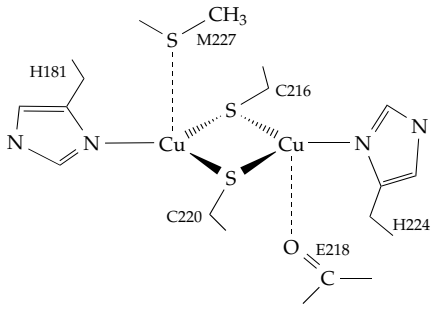
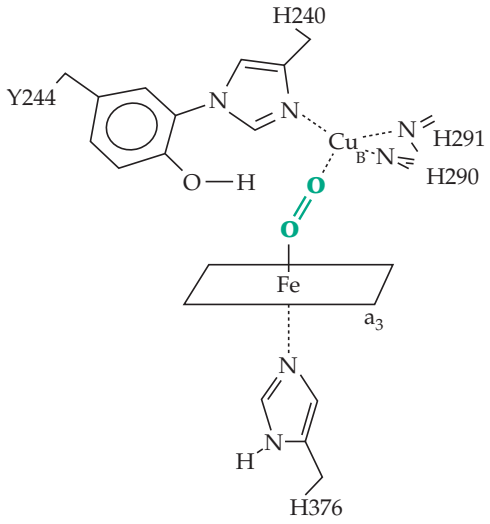


Figure 18-9 Proposed routes of electron transfer in mitochondrial complex III according to Peter Mitchell's Q cycle. Ubiquinone (Q) is reduced to QH_2 by complex I (left side of diagram) using two H^+ taken up from the matrix (leaving negative charges on the inner membrane surface). After diffusing across the bilayer (dashed line) the QH_2 is oxidized in the two steps with release of the two protons per QH_2 on the positive (P) side of the membrane. In the two-step oxidation via anionic radical Q^- one electron flows via the Rieske Fe–S protein and the cytochrome c_1 heme to external cytochrome c. The other electron is transferred to heme b_L of cytochrome b, then across the membrane to heme b_H which now reduces Q to Q^-. A second QH_2 is dehydrogenated in the same fashion and the electron passed through the cytochrome b centers is used to reduce Q^- to QH_2 with uptake from the matrix of 2 H^+. The resulting QH_2 diffuses back across the membrane to function again while the other Q diffuses back to complex I. The net result is pumping of 4 H^+ per 2 e^- passed through the complex. Notice that in the orientation used in this figure the matrix is at the bottom, not the top as in Figs. 18-4 and 18-5.



The Cu_A center of cytochrome oxidase

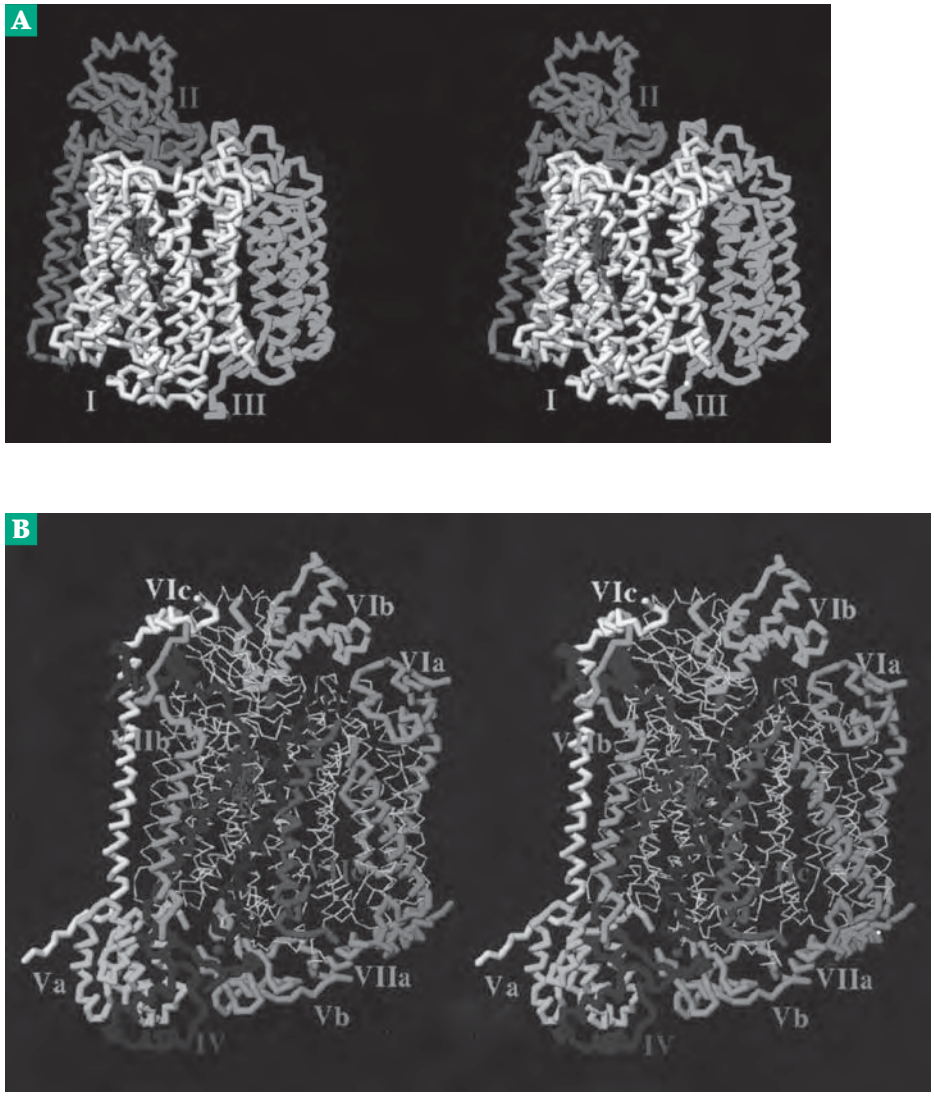


The Cu_B•A_3 center of cytochrome oxidase

cytochrome a combined with CO that led Keilin to designate the reactive component a_3. This CO complex is mixed with O_2-containing buffer and irradiated with a laser pulse to release the CO and allow O_2 to react. The first rapid reaction observed is the binding of O_2 (step c in Fig. 18-11). Formation of a peroxy intermediate from the initial oxygenated form (A in Fig. 18-11) is very fast. The O – O bond of O_2 has already been cleaved in form P (Fig. 18-11), which has until recently been thought to be the peroxy intermediate. In fact, spectroscopic measurements indicate that form P contains an oxo-ferryl ion with the second oxygen of the original O_2 converted to an OH ion and probably coordinated with Cu_B.[136a,136c,142,142a–c] P may also contain an organic radical, perhaps formed from tyrosine 244 as indicated in Fig. 18-11.

A second relaxation time of $\tau = 32–45$ μs has been assigned[139] to the conversion of the peroxide intermediate P to P′. A third relaxation time ($\tau = 100–140$ μs) is associated with the oxidation of Cu_A by a (not shown in Fig. 18-11).[143] This electron transfer step limits the rate of step f of Fig. 18-11. Another reduction step with $\tau \sim 1.2$ ms is apparently associated with electron transfer in step h. This slowest step still allows a first-order reaction rate of ~800 s^{-1}.

When O_2 reacts with cytochrome c oxidase, it may be bound initially to either the a_3 iron or to Cu_B, but in the peroxy intermediate P it may bind to both atoms. Oxyferryl compound F (Fig. 8-11) as well as radical species, can also be formed by treatment of the oxidized



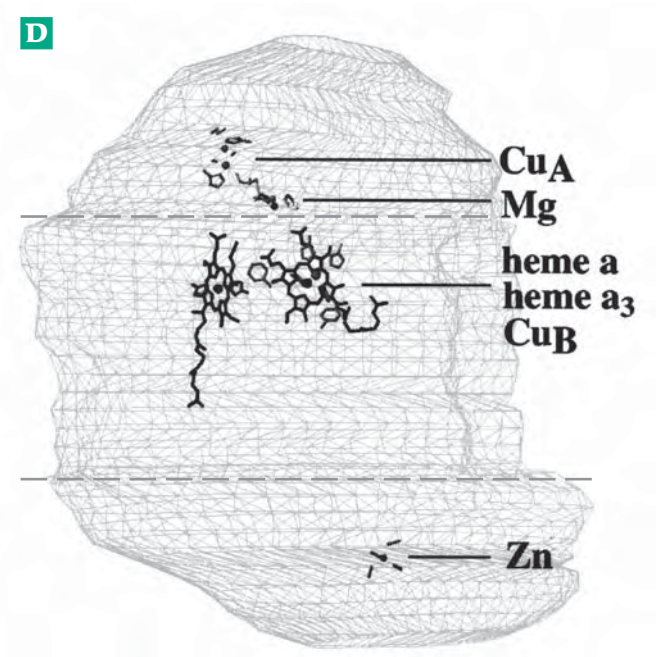


Figure 18-10 Structure of mitochondrial cytochrome *c* oxidase. (A) Stereoscopic C_α backbone trace for one monomeric complex of the core subunits I, II, and III. (B) Stereoscopic view showing all 13 subunits. The complete complex is a dimer of this structure. From Tsukihara *et al.*[125] (C) MolScript ribbon drawing of one monomeric unit. The horizontal lines are drawn at distances of ±1.0 and ± 2.0 nm from the center of the membrane bilayer as estimated from eight phospholipid molecules bound in the structure. From Wallin *et al.*[127] Courtesy of Arne Elofsson. (D) Schematic drawing of the same complex showing positions of the Cu_A dimetal center, bound Mg^{2+}, heme *a*, the bimetal heme a_3 - Cu_B center, and bound Zn^{2+}. The location of an 0.48-nm membrane bilayer is marked. From Tsukihara *et al.*[129] (A), (B), and (D) courtesy of Shinya Yoshikawa.

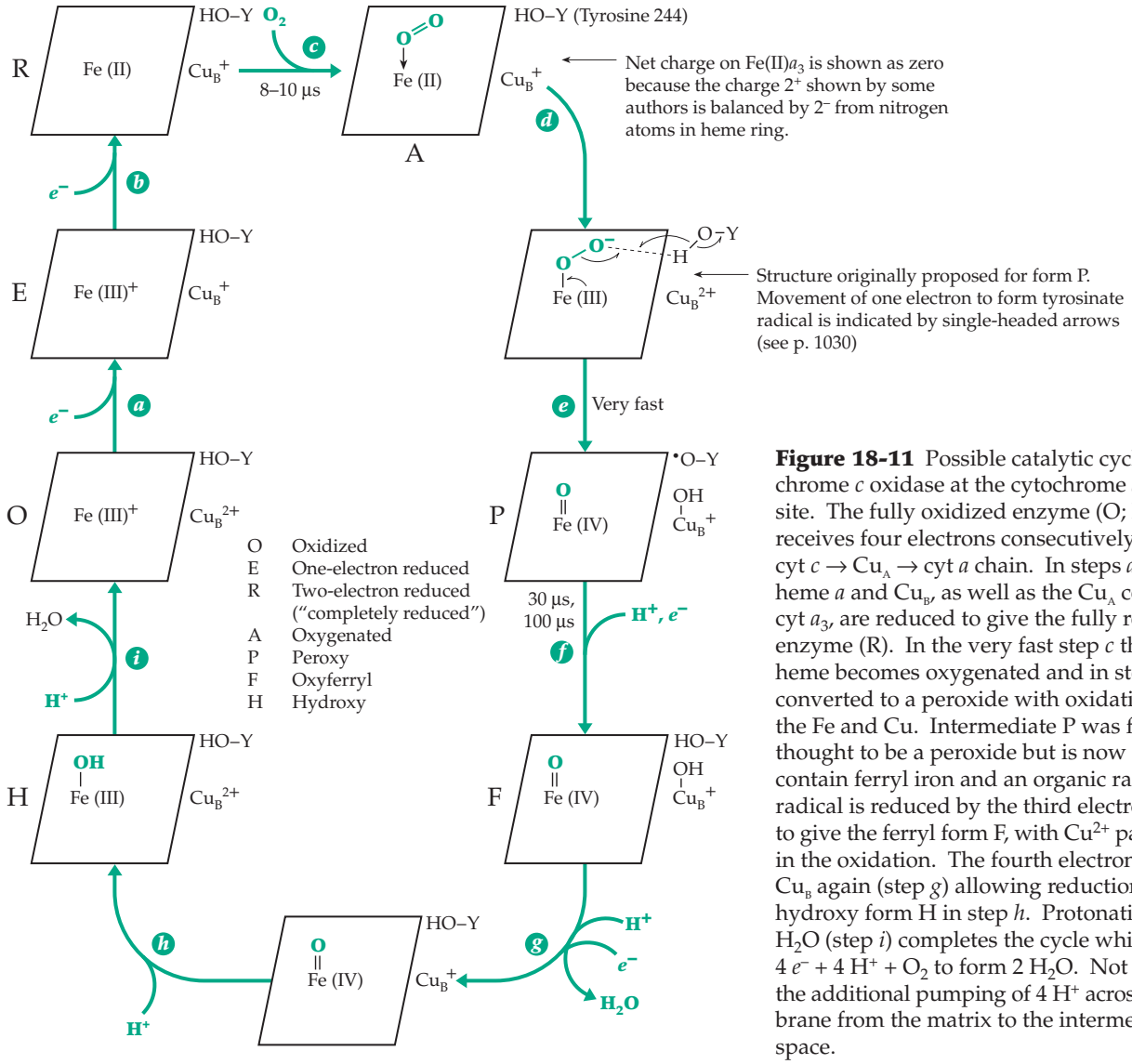


Figure 18-11 Possible catalytic cycle of cytochrome *c* oxidase at the cytochrome $a_3 - Cu_B$ site. The fully oxidized enzyme (O; left center) receives four electrons consecutively from the cyt $c \rightarrow Cu_A \rightarrow$ cyt *a* chain. In steps *a* and *b* both heme *a* and Cu_B, as well as the Cu_A center and cyt a_3, are reduced to give the fully reduced enzyme (R). In the very fast step *c* the cyt a_3 heme becomes oxygenated and in step *d* is converted to a peroxide with oxidation of both the Fe and Cu. Intermediate P was formerly thought to be a peroxide but is now thought to contain ferryl iron and an organic radical. This radical is reduced by the third electron in step *f* to give the ferryl form F, with Cu^{2+} participating in the oxidation. The fourth electron reduces Cu_B again (step *g*) allowing reduction to the hydroxy form H in step *h*. Protonation to form H_2O (step *i*) completes the cycle which utilizes 4 e^- + 4 H^+ + O_2 to form 2 H_2O. Not shown is the additional pumping of 4 H^+ across the membrane from the matrix to the intermembrane space.

enzyme O with hydrogen peroxide.[143a-144] Use of various inhibitors has also been important in studying this enzyme. Cyanide, azide, and sulfide ions, as well as carbon monoxide, are powerful inhibitors. Cyanide specifically binds to the Fe^{3+} form of cytochrome a_3 preventing its reduction,[145] while CO competes with O_2 for its binding site. A much-used reagent that modifies carboxyl groups in proteins, and which inhibits many proton translocating proteins, is dicyclohexyl carbodiimide (Eq. 3-10).[146] The step-by-step flow of electrons through cytochrome *c* oxidase seems quite well defined. However, one of the most important aspects is unclear. How is the pumping of protons across the membrane coupled to electron transport?[137,138,142,147,147a] Many recent studies have employed directed mutation of residues in all four subunits to locate possible proton pathways or channels.[148–152] Most ideas involve movement through hydrogen bonded chains (Eq. 9-94), which may include the carboxylate groups of the bound hemes.[153] Conformational changes may be essential to the gating of proton flow by electron transfers.[143]

The surface of the matrix side of cytochrome oxidase contains histidine and aspartate side chains close together. It has been suggested that they form a proton collecting antenna that contains groups basic enough to extract protons from the buffered matrix and guide them to a proton conduction pathway.[154] Calcium ions also affect proton flow.[153a,b] We will return to this topic in Section C,3 (p. 1040).

C. Oxidative Phosphorylation

During the 1940s when it had become clear that formation of ATP from ADP and inorganic phosphate

was coupled to electron transport in mitochondria, intensive efforts were made to discover the molecular mechanisms. However, nature sometimes strongly resists attempts to pry out her secrets, and the situation which prevailed was aptly summarized by Ephraim Racker: "Anyone who is not confused about oxidative phosphorylation just doesn't understand the situation."[155] The confusion is only now being resolved.

1. The Stoichiometry (P/O Ratio) and Sites of Oxidative Phosphorylation

Synthesis of ATP *in vitro* by tissue homogenates was demonstrated in 1937 by Kalckar, who has written a historical account.[156] In 1941, Ochoa[157] obtained the first reliable measurement of the P/O ratio, the *number of moles of ATP generated per atom of oxygen utilized* in respiration. The P/O ratio is also equal to the number of moles of ATP formed for each pair of electrons passing through an electron transport chain. Ochoa established that for the oxidation of pyruvate to acetyl-CoA and CO_2, with two electrons passed down the mitochondrial electron transport chain, the P/O ratio was ~3. This value has since been confirmed many times.[158–160] However, experimental difficulties in measuring the P/O ratio are numerous.[161] Many errors have been made, even in recent years, and some investigators[162] have contended that this ratio is closer to 2.5 than to 3. One method for measuring the P/O ratio is based on the method of determining the amount of ATP used that is described in the legend to Fig. 15-2.

The experimental observation of a P/O ratio of ~3 for oxidation of pyruvate and other substrates that donate NADH to the electron transport chain led to the concept that there are *three sites for generation of ATP*. It was soon shown that the P/O ratio was only 2 for oxidation of succinate. This suggested that one of the sites (site I) is located between NADH and ubiquinone and precedes the diffusion of QH_2 formed in the succinate pathway to complex III.

In 1949, Lehninger used ascorbate plus tetramethylphenylene-diamine (TMPD, Table 18-4) to introduce electrons into the chain at cytochrome *c*. The sequence ascorbate → TMPD → cytochrome *c* was shown to occur nonenzymatically. Later, it became possible to use cytochrome *c* as an electron donor directly. In either case only one ATP was generated, as would be anticipated if only site III were found to the right of cytochrome *c*. Site I was further localized by Lardy, who used hexacyanoferrate (III) (ferricyanide) as an artificial oxidant to oxidize NADH in the presence of antimycin *a*. Again a P/O ratio of one was observed. Finally, in 1955, Slater showed that passage of electrons from succinate to cytochrome c also gave only one ATP, the one generated at site II. The concept of three sites of ATP formation became generally accepted. However, as we shall see, these sites are actually proton-pumping sites, and there may be more than three of them.

Respiratory control and uncoupling. With proper care relatively undamaged mitochondria can be isolated. Such mitochondria are said to be **tightly coupled**. By this we mean that electrons cannot pass through the electron transport chain without generation of ATP. If the concentration of ADP or of P_i becomes too low, both phosphorylation and respiration cease. This **respiratory control** by ADP and P_i is a property of undamaged mitochondria. It may seem surprising that damaged mitochondria or submitochondrial particles are often able to transfer electrons at a faster rate than do undamaged mitochondria. However, electron transfer in damaged mitochondria occurs *without synthesis of ATP* and with no slowdown as the ADP concentration drops. A related kind of **uncoupling** of electron transport from ATP synthesis is brought about by various lipophilic anions called **uncouplers**, the best known of which is **2,4-dinitrophenol**. Even before the phenomenon of uncoupling was discovered, it had been known that dinitrophenol substantially increased the respiration rates of animals. The compound had even been used (with some fatal results) in weight control pills. The chemical basis of uncoupling will be considered in Section D.

"States" of mitochondria and spectrophotometric observation. Chance and Williams defined five **states** of tightly coupled mitochondria[60,163]; of these, states 3 and 4 are most often mentioned. If no oxidizable substrate or ADP is added the mitochondria have a very low rate of oxygen uptake and are in state 1. If oxidizable substrate and ADP are added rapid O_2 uptake is observed, the rate depending upon the rate of flow of electrons through the electron transport chain. This is state 3. As respiration occurs the coupled phosphorylation converts ADP into ATP, exhausting the ADP. Respiration slows to a very low value and the mitochondria are in state 4. If the substrate is present in excess, addition of more ADP will return the mitochondria to state 3.

Chance and associates employed spectrophotometry on intact mitochondria or submitochondrial particles to investigate both the sequence of carriers and the sites of phosphorylation. Using the dual wavelength spectrophotometer, the light absorption at the absorption maximum (λ_{max}) of a particular component was followed relative to the absorption at some other reference wavelength (λ_{ref}). The principal wavelengths used are given in Table 18-6. From these measurements the state of oxidation or reduction of each one of the carriers could be observed in the various states and in the presence of inhibitors. The

TABLE 18-6
Wavelengths of Light Used to Measure States of Oxidation of Carriers in the Electron Transport Chain of Mitochondria[a]

Carrier	λ_{max} (nm)[b]	λ_{ref} (nm)
NADH	340	374
Flavins	465	510
Cytochromes		
b^{2+}	564(α)	575
	530(β)	
	430(γ)	
c_1^{2+}	534(α)	
	523(β)	
	418(γ)	
c^{2+}	550(α)	540
	521(β)	
	416(γ)	
a^{2+}	605(α)	630(590)
	450(γ)[a]	
a_3^{2+}	600(α)[a]	
	445(γ)	455

[a] After Chance, B. and Williams, G. R. (1955) *J. Biol. Chem*, **217**, 409–427; (1956) Adv. Enzymol. **17**, 409–427.
[b] The wavelengths used for each carrier in dual wavelength spectroscopy appear opposite each other in the two columns. Some positions of other absorption bands of cytochromes are also given.

experiments served to establish that electrons passing down the chain do indeed reside for a certain length of time on particular carriers. That is, in a given state each carrier exists in a defined ratio of oxidized to reduced forms ([ox] / [red]). Such a result would not be seen if the entire chain functioned in a cooperative manner with electrons passing from the beginning to the end in a single reaction. By observing changes in the ratio [ox] / [red] under different conditions, some localization of the three phosphorylation sites could be made. In one experiment antimycin *a* was added to block the chain ahead of cytochrome c_1. Then tightly coupled mitochondria were allowed to go into state 4 by depletion of ADP. Since the concentration of oxygen was high and cytochrome a_3 has a low K_m for O_2 (~3 μM) cytochrome a_3 was in a highly oxidized state. Cytochrome *a* was also observed to be oxidized, while cytochrome c_1 and *c* remained reduced. The presence of this **crossover point** suggested at the time that cytochrome *c* might be at or near one of the "energy conservation sites." Accounts of more recent experiments using the same approach are given by Wilson *et al*.[164]

2. Thermodynamics and Reverse Electron Flow

From Table 6-8 the value of $\Delta G'$ for oxidation of one mole of NADH by oxygen (1 atm) is −219 kJ. At a pressure of ~10^{-2} atm O_2 in tissues the value is −213 kJ. However, when the reaction is coupled to the synthesis of three molecules of ATP ($\Delta G'$ = +34.5 kJ mol^{-1}) the net Gibbs energy change for the overall reaction becomes $\Delta G'$ = −110 kJ mol^{-1}. This is still very negative. However, we must remember that the concentrations of ATP, ADP, and P_i can depart greatly from the 1:1:1 ratio implied by the $\Delta G'$ value.

An interesting experiment is to allow oxidative phosphorylation to proceed until the mitochondria reach state 4 and to measure the **phosphorylation state ratio R_p**, which equals the value of [ATP] / [ADP][P_i] that is attained. This mass action ratio, which has also been called the "phosphorylation ratio" or "phosphorylation potential" (see Chapter 6 and Eq. 6-29), often reaches values greater than 10^4–10^5 M^{-1} in the cytosol.[164] An extrapolated value for a zero rate of ATP hydrolysis of log R_p = 6.9 was estimated. This corresponds (Eq. 6-29) to an increase in group transfer potential (ΔG of hydrolysis of ATP) of 39 kJ/mol. It follows that the overall value of ΔG for oxidation of NADH in the coupled electron transport chain is less negative than is $\Delta G'$. If synthesis of three molecules of ATP is coupled to electron transport, the system should reach an equilibrium when $R_p = 10^{6.4}$ at 25°C, the difference in ΔG and $\Delta G'$ being 3RT ln Rp = 3 x 5.708 x 6.4 = 110 kJ mol^{-1}. This value of Rp is, within experimental error, the same as the maximum value observed.[165] There apparently is an almost true equilibrium among NADH, O_2, and the adenylate system if the P/O ratio is 3.

Within more restricted parts of the chain it is possible to have *reversed electron flow*. Consider the passage of electrons from NADH, partway through the chain, and back out to fumarate, the oxidized form of the succinate–fumarate couple. The Gibbs energy change $\Delta G'$ (pH 7) for oxidation of NADH by fumarate is −67.7 kJ mol^{-1}. In uncoupled mitochondria electron flow would always be from NADH to fumarate. However, in tightly coupled mitochondria, in which ATP is being generated at site I, the overall value of $\Delta G'$ becomes much less negative. If $R_p = 10^4$ M^{-1}, $\Delta G'$ for the coupled process becomes approximately zero (−67.7 + 68 kJ mol^{-1}). Electron flow can easily be reversed so that succinate reduces NAD^+. Such ATP-driven reverse flow occurs under some physiological conditions within mitochondria of living cells, and some anaerobic bacteria generate all of their NADH by reversed electron flow (see Section E).

Another experiment involving equilibration with the electron transport chain is to measure the "observed potential" of a carrier in the chain as a function of the concentrations of ATP, ADP, and P_i. The observed

potential E is obtained by measuring log([ox] / [red]) and applying Eq. 18-6 in which $E°'$ is the known midpoint potential of the couple (Table 6-8) and n is the number of electrons required to reduce one molecule of the carrier. If the system is equilibrated with a "redox buffer" (Chapter 6), E can be fixed at a preselected value. For example, a 1:1 mixture of succinate and fumarate would fix E at +0.03 V while the couple 3-hydroxybutyrate-acetoacetate in a 1:1 ratio would fix it at $E°' = -0.266$ V. Consider the potential of cytochrome b_{562} (b_H), which has an $E°'$ value of 0.030 V. Substituting this in Eq. 18-7 and using $E = -0.266$ V (as obtained by equilibration with 3-hydroxybutyrate–acetoacetate), it is easy to calculate that at equilibrium the ratio [ox] / [red] for cytochrome b_{562} is about 10^{-5}. In other words, in the absence of O_2 this cytochrome will be kept almost completely in the reduced form in an uncoupled mitochondrion.

$$E = \frac{-\Delta G}{nF} = E°' + \frac{0.0592}{n}\log\frac{[\text{ox}]}{[\text{red}]} = \text{observed potential of carrier} \quad (18\text{-}6)$$

TABLE 18-7
Electrode Potentials of Mitochondrial Electron Carriers and Gibbs Energy Changes Associated with Passage of Electrons[a]

	Electron carrier	$E°'$ (pH 7) isolated	$E°'$ (pH 7.2) in mitochondria	ΔG (kJ mol^{-1}) for 2 e^- flow to O_2 at 10^{-2} atm, carriers at pH 7
	NADH / NAD+	–0.320		–213
Group I	Flavoprotein		~ –0.30	
~ –0.30 V	Fe–S protein		~ –0.305	
	β-Hydroxybutyrate–acetoacetate	–0.266		–203
	Lactate–pyruvate	–0.185		–187
	Succinate-fumarate	0.031		–146
Group II	Flavoprotein		~ –0.045	
~ 0 V	Cytochrome b_T		–0.030	
	Cu		0.001	
	Fe–S protein		0.030	
	Cytochrome b_K		0.030	
	Ubiquinone	0.10	0.045	–132
	Cytochrome a_3 + ATP		0.155	
Group III	Cytochrome c_1		0.215	
	Cytochrome c	0.254	0.235	–102
	Cytochrome b_T + ATP		0.245	
	Cytochrome a	0.29	0.210	
	Cu		0.245	
	Fe–S protein		0.28	
Group IV	Cytochrome a_3		0.385	–77
	O_2 (10^{-2} atm)	0.785		0.00
	1 atm	0.815		

[a] Data from Wilson, D. F., Dutton, P. L., Erecinska, M., Lindsay, J. G., and Soto, N. (1972) *Acc. Chem. Res.* **5**, 234–241 and Wilson, D. F., Erecinska, M., and Sutoon, P. L. (1974) *Ann. Rev. Biophys. Bioeng.* **3**, 203–230.

However, if the electron transport between 3-hydroxybutyrate and cytochrome b_{562} is tightly coupled to the synthesis of one molecule of ATP, the observed potential of the carrier will be determined not only by the imposed potential E_i of the equilibrating system but also by the phosphorylation state ratio of the adenylate system (Eq. 18-7). Here $\Delta G'_{ATP}$ is the group transfer potential ($-\Delta G'$ of hydrolysis) of ATP at pH 7 (Table 6-6), and n' is the number of electrons passing through the chain required to synthesize one ATP. In the upper part of the equation n is the number of electrons required to reduce the carrier, namely one in the case of cytochrome b_{562}.

From Eq. 18-7 it is clear that in the presence of a high phosphorylation state ratio a significant fraction of cytochrome b_{562} may remain in the reduced form at equilibrium. Thus, if $R_p = 10^4$, if $E°'$ for cytochrome b_{562} is 0.030 V, if $n' = 2$, and the potential E is fixed at –0.25 V using the hydroxybutyrate–acetoacetate couple, we calculate, from Eq. 18-7, that the ratio [ox] / [red] for cytochrome b_{562} will be 1.75. Now, if R_p is varied the observed potential of the carrier should change as predicted by Eq. 18-7. This variation has been observed.[164] For a tenfold change in R_p the observed potential of cytochrome b_{562} changed by 0.030 V, just that predicted if $n' = 2$. On the other hand, the observed potential of cytochrome c varied by 0.059 V for every tenfold change in the ratio. This is just as expected if $n' = 2$, and if synthesis of two molecules of

$$E(\text{observed}) = E^{\circ\prime} + \frac{0.0592}{n}\log_{10}\frac{[\text{ox}]}{[\text{red}]}$$
$$= E_i + \frac{\Delta G'_{\text{ATP}}}{96.5n'} + \frac{RT}{n'F}\ln\frac{[\text{ATP}]}{[\text{ADP}][\text{P}_i]}$$
$$= E_i + \frac{0.358}{n'} + \frac{0.0592}{n'}\log_{10} R_{\text{P}} \qquad (18\text{-}7)$$

ATP is coupled to the electron transport to cytochrome *c*. Thus, we have experimental evidence that when one-electron carriers such as the cytochromes are involved, the passage of *two electrons* is required to synthesize one molecule of ATP. Furthermore, from experiments of this type it was concluded that the sites of phosphorylation were localized in or related to complexes I, III, and IV.

Another kind of experiment is to equilibrate the electron transport chain with an external redox pair of known potential using *uncoupled* mitochondria. The value of $E^{\circ\prime}$ of a particular carrier can then be measured by observation of the ratio [ox] / [red] and applying Eq. 18-7. While changes in the equilibrating potential E will be reflected by changes in [ox] / [red] the value of $E^{\circ\prime}$ will remain constant. The $E^{\circ\prime}$ values of Fe–S proteins and copper atoms in the electron transport chain have been obtained by equilibrating mitochondria, then rapidly freezing them in liquid nitrogen, and observing the ratios [ox] / [red] by EPR at 77K (Table 18-7).

The values of $E^{\circ\prime}$ of the mitochondrial carriers fall into four **isopotential groups** at ~−0.30, ~0, ~+0.22, and ~+0.39 V (Table 18-7). When tightly coupled mitochondria are allowed to go into state 4 (low ADP, high ATP, O_2 present but low respiration rate), the observed potentials change. That of the lowest isopotential group (which includes NAD^+/ NADH) falls to ~−0.38 V, corresponding to a high state of reduction of the carriers to the left of the first phosphorylation site in Fig. 18-4. Groups 2 and 3 remain close to their midpoint potentials at ~−0.05 and +0.26 V. In this condition the potential difference between each successive group of carriers amounts to ~0.32 V, just enough to balance the formation of one molecule of ATP for each two electrons passed at a ratio $R_p \approx 10^4$ M^{-1} (Eq. 18-7).

Two cytochromes show exceptional behavior and appear twice in Table 18-7. The midpoint potential $E^{\circ\prime}$ of cytochrome b_{566} (b_L) changes from −0.030 V in the absence of ATP to +0.245 V in the presence of a high concentration of ATP. On the other hand, $E^{\circ\prime}$ for cytochrome a_3 drops from +0.385 to 0.155 V in the presence of ATP. These shifts in potential must be related to the coupling of electron transport to phosphorylation.

3. The Mechanism of Oxidative Phosphorylation

It was natural to compare mitochondrial ATP synthesis with substrate-level phosphorylations, in which high-energy intermediates are generated *by the passage of electrons through the substrates*. The best known example is oxidation of the aldehyde group of glyceraldehyde 3-phosphate to an acyl phosphate, which, after transfer of the phospho group to ADP, becomes a carboxylate group (Fig. 15-6). The Gibbs energy of oxidation of the aldehyde to the carboxylate group provides the energy for the synthesis of ATP. However, this reaction differs from mitochondrial electron transport in that *the product, 3-phosphoglycerate, is not reconverted to glyceraldehyde 3-phosphate*. Electron carriers of the respiratory chain must be regenerated in some cyclic process. Because of this, it was difficult to imagine practical mechanisms for oxidative phosphorylation that could be related to those of substrate level phosphorylation. Nevertheless, many efforts were made over a period of several decades to find such high-energy intermediates.

Search for chemical intermediates. An early hypothetical model, proposed by Lipmann,[166] is shown in Fig. 18-12. Here A, B, and C are three electron carriers in the electron transport chain. Carrier C is a better oxidizing agent than B or A. Carrier B has some special chemistry that permits it, in the reduced state, to react with group Y of a protein (step *b*) to form Y–BH_2. The latter, an unidentified adduct, is converted by oxidation with carrier C (step *c*) to a "high energy" oxidized form indicated as Y ~ B. Once the possibility of generating such an intermediate is conceded, it is easy to imagine plausible ways in which the energy of this intermediate could be transferred into forms with which we are already familiar. For example, another protein X could react (step *d*) to form X ~ Y in which the X ~ Y linkage could be a thioester, an acyl phosphate, or other high-energy form. Furthermore, it might not be necessary to have two proteins; X and Y could be different functional groups of the same protein. They might be nonprotein components, e.g., Y might be a phospholipid.

Generation of ATP by the remaining reactions (steps *e* and *f* of Fig. 18-12) is straightforward. For example, if X ~ Y were a thioester the reactions would be the reverse of Eq. 12-48. These reaction steps would also be responsible for observed exchange reactions, for example, the mitochondrially catalyzed exchange of inorganic phosphate ($H^{32}PO_4^{2-}$) into the terminal position of ATP. Mitochondria and submitochondrial particles also contain ATP-hydrolyzing **(ATPase)** activity, which is thought to depend upon the same machinery that synthesizes ATP in tightly coupled mitochondria. In the scheme of Fig. 18-12, ATPase

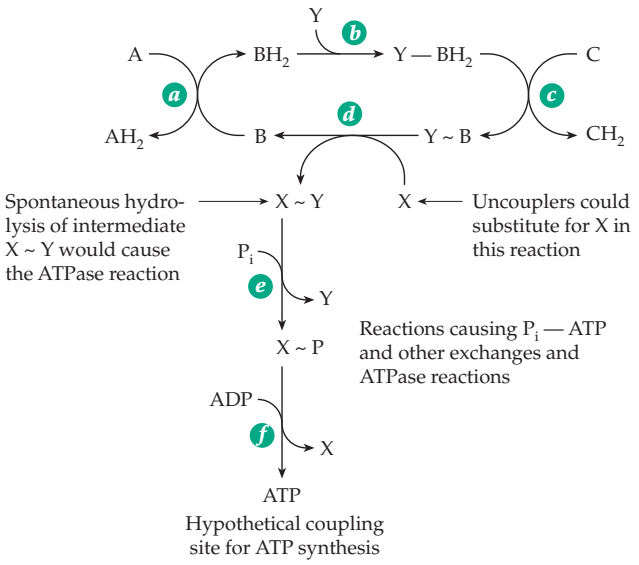


Figure 18-12 An early proposal for formation of ATP via "high-energy" chemical intermediates.

activity would be observed if hydrolysis of X ~ Y were to occur. Partial disruption of the system would lead to increased ATPase reactivity, as is observed. Uncouplers such as the dinitrophenolate ion or arsenate ion, acting as nucleophilic displacing groups, could substitute for a group such as X. Spontaneous breakdown of labile intermediates would permit oxidation to proceed unimpaired. Since there are three different sites of phosphorylation, we might expect to have three different enzymes of the type Y in the scheme of Fig. 18-12, but it would be necessary to have only one X.

In Lipmann's original scheme group Y was visualized as adding to a carbon–carbon double bond to initiate the sequence. Isotopic exchange reactions ruled out the possibility that either ADP or P_i might serve as Y, but it was attractive to think that a bound phosphate ion, e.g., in a phospholipid or coenzyme, could be involved. Y ~ B of Fig. 18-12 would be similar in reactivity to an acyl phosphate or thioester. However, whatever the nature of Y ~ B, part of group Y would be left attached to B after the transfer of Y to X. For example, if Y were Y'OH compound X ~ OY' would be formed, and the carrier would be left in step *d* in the form of B–OH. Elimination of a hydroxyl group would be required to regenerate B. Perhaps nature has shunned this mechanism because there is no easy way to accomplish such an elimination. Many variations on the scheme of Fig. 18-12 were proposed,[166] and some were discussed in the first edition of this textbook.[167] However, as attractive as these ideas may have seemed, *all attempts to identify discrete intermediates that might represent X ~ Y failed. Furthermore, most claims to have seen Y ~ B by any means have been disproved.*

Peter Mitchell's chemiosmotic theory. To account for the inability to identify high energy intermediates as well as the apparent necessity for an intact membrane, Peter Mitchell, in 1961, offered his **chemiosmotic theory** of oxidative phosphorylation.[168–175a] aThis theory also accounts for the existence of **energy-linked processes** such as the accumulation of cations by mitochondria. The principal features of the Mitchell theory are illustrated in Fig. 18-13. Mitchell proposed

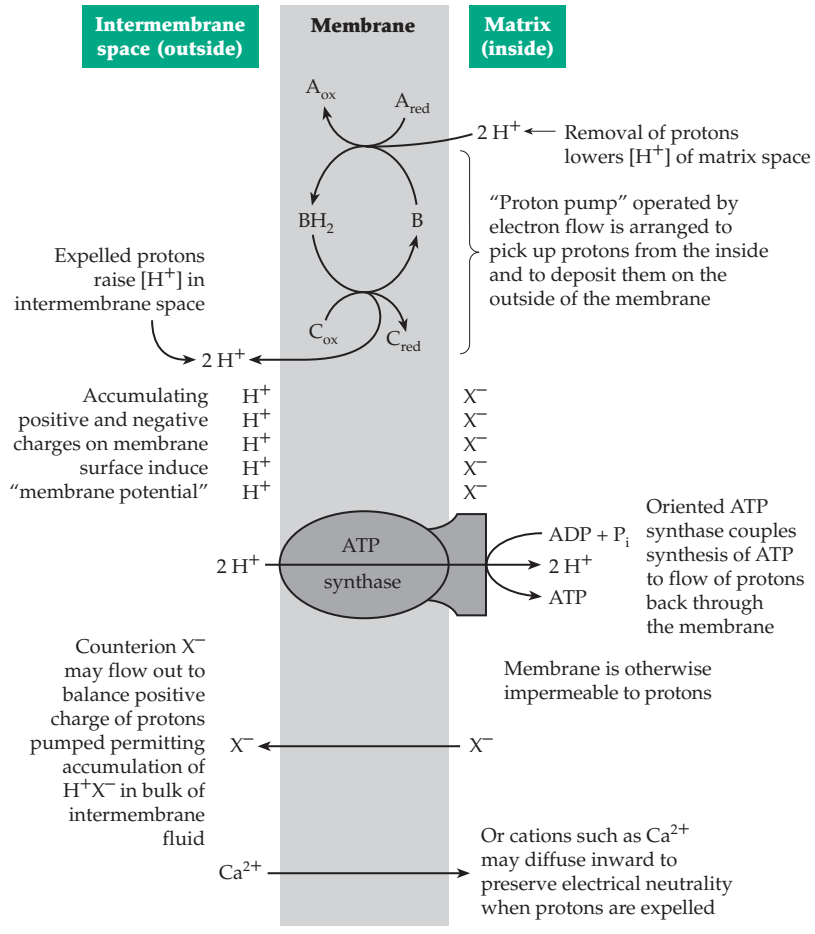


Figure 18-13 Principal features of Mitchell's chemiosmotic theory of oxidative phosphorylation.

that the inner membrane of the mitochondrion is a closed, proton-impermeable **coupling membrane**, which contains **proton pumps** operated by electron flow and which cause protons to be expelled through the membrane from the matrix space. As indicated in Fig. 18-13, an oxidized carrier B, upon reduction to BH_2, acquires two protons. These protons do not necessarily come from reduced carrier AH_2, and Mitchell proposed that they are picked up from the solvent on the matrix side of the membrane. Then, when BH_2 is reoxidized by carrier C, protons are released on the outside of the membrane. On the basis of existing data, *Mitchell assumed a stoichiometry of two protons expelled for each ATP synthesized*. It followed that there should be three different proton pumps in the electron transport chain corresponding to the three phosphorylation sites.

The postulated proton pumps would lead either to bulk accumulation of protons in the intermembrane space and cytoplasm, with a corresponding drop in pH, *or to an accumulation of protons along the membrane itself*. The latter would be expected if counterions X^- do not pass through the membrane with the protons. The result in such a case would be the development of a **membrane potential**, a phenomenon already well documented for nerve membranes (Chapter 8).

A fundamental postulate of the chemiosmotic theory is the presence of an oriented ATP synthase that utilizes the Gibbs energy difference of the proton gradient to drive the synthesis of ATP (Fig. 18-9). Since $\Delta G'$ (pH 7) for ATP synthesis is +34.5 kJ mol^{-1} and, if as was assumed by Mitchell, the passage of two protons through the ATP synthase is required to form one ATP, the necessary pH gradient (given by Eq. 6-25 or Eq. 18-9 with $E_m = 0$) would be 34.5/(2 x 5.708) = 3.0 pH units at 25°C. On the other hand, if the phosphorylation state ratio is ~10^4 M^{-1}, the pH difference would have to be 5 units. Most investigators now think that 4 H^+ per ATP are needed by the synthase. If so, a pH difference of 2.5 units would be adequate. Various experiments have shown that passage of electrons does induce a pH difference, and that an artifically induced pH difference across mitochondrial membranes leads to ATP synthesis. However, pH gradients of the required size have not been observed. Nevertheless, if the membrane were charged as indicated in Fig. 18-13, without accumulation of protons in the bulk medium, a membrane potential would be developed, and this could drive the ATP synthase, just as would a proton gradient.

The mitochondrial membrane potential E_m (or $\Delta\psi$) is the potential difference measured across a membrane relative to a reference electrode present in the surrounding solution.[176] For both mitochondria and bacteria E_m normally has a negative value. The Gibbs energy change $\Delta\psi_H+$ for transfer of one mole of H^+ from the inside of the mitochondrion to the outside, against the concentration and potential gradients, is given by Eq. 18-8. This equation follows directly from Eqs. 6-25

$$\begin{aligned}\Delta G_H + &= 2.303\, RT\, \Delta\text{pH} - E_m F \\ &= 5.708\, \Delta\text{pH} - 96.5\, E_m \text{ kJ/mol at 25°C}\end{aligned}$$
$$\text{where } \Delta\text{pH} = \text{pH (inside)} - \text{pH (outside)} \qquad (18\text{-}8)$$

and 6-63 with n = 1. The same information is conveyed in Eq. 18-9, which was proposed by Mitchell for what he calls the **total protonic potential difference Δp**.

$$\begin{aligned}\Delta p \text{ (volts)} &= E_m\text{(volts)} - 2.303\,\frac{RT}{F}\,\Delta\text{pH} \\ \Delta p \text{ (mV)} &= E_m\text{(mV)} - 59.2\,\Delta\text{pH at 25°C} \\ E_m &= \Delta\psi \qquad (18\text{-}9)\end{aligned}$$

Mitchell was struck by the parallel between the force and flow of electrons, which we call electricity, and the force and flow of protons, which he named **proticity**.[174] This led one headline writer in *Nature*[177] to describe Mitchell as "a man driven by proticity," but if Mitchell is right, as seems to be the case, we are all driven by proticity! Mitchell also talked about **protonmotive** processes and referred to Δp as the **protonmotive force**. Although it is a potential rather than a force, this latter name is a popular designation for Δp.

The reader should be aware that considerable confusion exists with respect to names and definitions.[176] For example, the ΔG_H+ of Eq. 18-8 can also be called the **proton electrochemical potential** $\Delta\mu_H+$, which is analogous to the chemical potential μ of an ion (Eq. 6-24) and has units of kJ/mol (Eq. 18-10).

$$\begin{aligned}-\Delta G_H+ &= \Delta\mu_H+ = F\,\Delta p \\ &= 96.5\,\Delta p \text{ kJ/mol at 25°C}\end{aligned} \qquad (18\text{-}10)$$

However, many authors use $\Delta\mu_H+$ as identical to the protonmotive force Δp.

From Eq. 18-9 or Eq. 18-10 it can be seen that a membrane potential E_m of −296 mV at 25°C would be equivalent to a 5.0 unit change in pH and would be sufficient, if coupled to ATP synthesis via 2 H^+, to raise R_p to 10^4 M^{-1}. Any combination of ΔpH and E_m providing Δp of −296 mV would also suffice. If the ratio H^+/ATP = 4, Δp of −148 mV would suffice.

The chemiosmotic hypothesis had the great virtue of predicting the following consequences which could be tested: (1) electron-transport driven proton pumps with defined stoichiometries and (2) a separate ATP synthase, which could be driven by a pH gradient or membrane potential. Mitchell's hypothesis was initially greeted with skepticism but it encouraged many people, including Mitchell and his associate Jennifer Moyle, to test these predictions, which were soon found to be correct.[178]

Observed values of E_m and pH. One of the problems[179] in testing Mitchell's ideas has been the difficulty of reliably measuring Δp. To evaluate the pH term in Eq. 18-10 measurements have been made with microelectrodes and indicator dyes. However, the most reliable approach has been to observe the distribution of weak acids and bases across the mitochondrial membrane.[180] This is usually done with a suspension of freshly isolated active mitochondria. The method has been applied widely using, for example, methylamine. A newer method employs an isotope exchange procedure to measure the pH-sensitive carbonic anhydrase activity naturally present in mitochondria.[181]

The measurement of E_m ($\Delta\psi$) is also difficult.[179] Three methods have been used: (1) measurement with microelectrodes; (2) observation of fluorescent probes; (3) distribution of permeant ions. Microelectrodes inserted into mitochondria[182] have failed to detect a significant value for E_m. Fluorescent probes are not very reliable,[179,183] leaving the distribution of permeant ions the method of choice. In this method a mitochondrial suspension is exposed to an ion that can cross the membrane but which is not pumped or subject to other influences that would affect its distribution. Under such conditions the ion will be distributed according to Eq. 18-11. The most commonly used ions are K^+, the same ion that is thought to reflect the membrane potential of nerve axons (Chapter 30), or Rb^+. To make the inner mitochondrial membrane permeable to K^+, valinomycin (Fig. 8-22) is added. The membrane potential, with n = 1 in Eq. 9-1, becomes:

$$E_m = -59.2\ ([K^+]_{inside} / [K^+]_{outside})\ \text{volts} \qquad (18\text{-}11)$$

In these experiments respiring mitochondria are observed to take up the K^+ or Rb^+ to give a high ratio of K^+ inside to that outside and consequently a negative E_m. There are problems inherent in the method. The introduction of a high concentration of ion perturbs the membrane potential, and there are uncertainties concerning the contribution of the Donnan equilibrium (Eq. 8-5) to the observed ion distribution.[184]

In most instances, either for mitochondrial suspensions or whole bacteria, ΔpH is less negative than −0.5 unit making a contribution of, at most, −30 mV to Δp. The exception is found in the thylakoid membranes of chloroplasts (Chapter 23) in which protons are pumped into the thylakoid vesicles and in which the internal pH falls dramatically upon illumination of the chloroplasts.[185] The ΔpH reaches a value of −3.0 or more units and Δp is ~180 mV, while E_m remains ~0. Reported values of E_m for mitochondria and bacteria range from −100 to −168 mV and Δp from −140 to −230 mV.[172,179] Wilson concluded that E_m for actively respiring mitochondria, using malate or glutamate as substrates, attains maximum (negative) values of E_m = −130 mV and Δp = −160 mV.[179] However, Tedeschi and associates[183,184] argued that E_m is nearly zero for liver mitochondria and seldom becomes more negative than −60 mV for any mitochondria.

A crucially important finding is that submitochondrial particles or vesicles from broken chloroplasts will synthesize ATP from ADP and P_i, when an artificial pH gradient is imposed.[172,186] Isolated purified F_1F_0 ATPase from a thermophilic *Bacillus* has been coreconstituted into liposomes with the light-driven proton pump **bacteriorhodopsin** (Chapter 23). Illumination induced ATP synthesis.[187] These observations support Mitchell's proposal that the ATP synthase is both spatially separate from the electron carriers in the membrane and utilizes the protonmotive force to make ATP. Thus, the passage of protons from the outside of the mitochondria back in through the ATP synthase induces the formation of ATP. What is the stoichiometry of this process?

It is very difficult to measure the flux of protons across the membrane either out of the mitochondria into the cytoplasm or from the cytoplasm through the ATP synthase into the mitochondria. Therefore, estimates of the stoichiometry have often been indirect. One argument is based on thermodynamics. If Δp attains values no more negative than −160 mV and R_p within mitochondria reaches at least $10^4\ M^{-1}$, we must couple ΔG_H of −15.4 kJ/mol to ΔG of formation of ATP of +57.3 kJ/mol. To do this four H^+ must be translocated per ATP formed. Recent experimental measurements with chloroplast ATP synthase[188] also favor four H^+. It is often proposed that one of these protons is used to pump ADP into the mitochondria via the ATP-ADP exchange carrier (Section D). Furthermore, if R_p reaches $10^6\ M^{-1}$ in the cytoplasm, it must exceed $10^4\ M^{-1}$ in the mitochondrial matrix.

Proton pumps driven by electron transport. What is the nature of the proton-translocating pumps that link Δp with electron transport? In his earliest proposals Mitchell suggested that electron carriers, such as flavins and ubiquinones, each of which accepts two protons as well as two electrons upon reduction, could serve as the proton carriers. Each pump would consist of a pair of oxidoreductases. One, on the inside (matrix side) of the coupling membrane, would deliver two electrons (but no protons) to the carrier (B in Fig. 18-13). The two protons needed for the reduction would be taken from the solvent in the matrix. The second oxidoreductase would be located on the outside of the membrane and would accept two electrons from the reduced carrier (BH_2 in Fig. 18-13) leaving the two released protons on the outside of the membrane. To complete a "loop" that would allow the next carrier to be reduced, electrons would have to be transferred through fixed electron carriers embedded in the

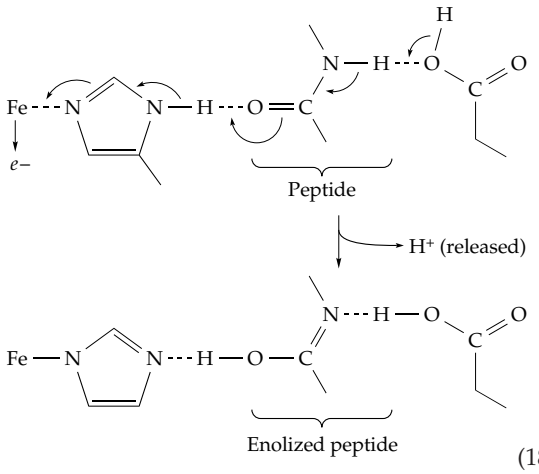
membrane from the reduced electron acceptor (C_{red} in Fig. 18-13) to the oxidized form of the oxidoreductase to be used as reductant for the next loop. These loops, located in complexes I and III of Fig. 18-5, would pump three protons per electron or six H^+/O. With a P/O ratio of three this would provide two H^+ per ATP formed. Mitchell regarded this stoichiometry as appropriate.

The flavin of NAD dehydrogenase was an obvious candidate for a carrier, as was ubiquinone. However, the third loop presented a problem. Mitchell's solution was the previously discussed **Q cycle**, which is shown in Fig. 18-9. This accomplishes the pumping in complex III of 2 H^+/e^-, the equivalent of two loops.[111] However, as we have seen, the magnitude of Δp suggests that 4 H^+, rather than 2 H^+, may be coupled to synthesis of one ATP. If this is true, mitochondria must pump 12 H^+/O rather than six when dehydrogenating NADH, or eight H^+/O when dehydrogenating succinate.

The stoichiometry of proton pumping was measured by Lehninger and associates using a fast-responding O_2 electrode and a glass pH electrode.[189,190] They observed an export of eight H^+/O for oxidation of succinate rat liver mitochondria in the presence of a permeant cation that would prevent the buildup of E_m, and four H^+/O (2 H^+/e^-) for the cytochrome oxidase system. These are equivalent to two H^+/e^- at each of sites II and III as is indicated in Fig. 18-4. Some others have found lower H^+/e^- ratios.

If two H^+/e^- are pumped out of mitochondria, where do we find the pumping sites? One possibility is that protons are pumped through the membrane by a **membrane Bohr effect**, so named for its similarity to the Bohr effect observed upon oxygenation of hemoglobin. In the latter case (Chapter 7), the pK_a values of certain imidazole and terminal amino groups are decreased when O_2 binds. This may result, in part, from an electrostatic effect of O_2 in inducing a partial positive charge in the heme. This partial charge may then cause a decrease in the pK_a values of nearby groups. Similarly, complete loss of an electronic charge from a heme group or an iron–sulfur protein in the electron transport chain would leave a positive charge, an electron "hole," which could induce a large change in the pK_a of a neighboring group. One manifestation of this phenomenon may be a strong pH dependence of the reduction potential (Eq. 16-19).

Protons that could logically be involved in a membrane Bohr effect are those present on imidazole rings coordinated to Fe or Cu in redox proteins. Removal of an electron from the metal ion could be accompanied by displacement of electrons within the imidazole, within a peptide group that is hydrogen-bonded to an imidazole, or within some other acidic group. A hypothetical example is illustrated in Eq. 18-12 in which a carboxyl group loses a proton when "handed" a second. If the transiently enolized peptide linkage formed in



(18-12)

this process is tautomerized back to its original state before the iron is reduced again, the proton originally present on the carboxyl group will be released. It is easy to imagine that a proton could then be "ferried" in (as in Eq. 9-96) from the opposite side of the membrane to reprotonate the imidazole group and complete the pumping process.

In view of the large number of metal-containing electron carriers in the mitochondrial chain, there are many possible locations for proton pumps. However, the presence of the three isopotential groups of Table 18-7 suggests that the pumps are clustered in complexes I-III as pictured in Fig. 18-5. One site of pumping is known to be in the cytochrome *c* oxidase complex. When reconstituted into phospholipid, the purified complex does pump protons in response to electron transport, H^+/e^- ratios of ~1 being observed.[136,137,147,191] As mentioned in Section B,3 a large amount of experimental effort has been devoted to identifying proton transport pathways in cytochrome *c* oxidase and also in the cytochrome bc_1 (complex II).[192] Proton pumping appears to be coupled to chemical changes occurring between intermediates P and F of Fig. 18-11, between F and O,[136,193] and possibly between O and R.[137,138] Mechanisms involving direct coupling of chemical changes at the A_3Cu_B center and at the Cu_A dimetal center have been proposed.[147,194]

How do protons move from the pumping sites to ATP synthase molecules? Since protons, as H_3O^+, are sufficiently mobile, ordinary diffusion may suffice. Because of the membrane potential they will tend to stay close to the membrane surface, perhaps being transported on phosphatidylethanolamine head groups (see Chapter 8). According to the view of R. J. P. Williams protons are not translocated across the entire membrane by the proton pumps, but flow through the proteins of the membrane to the ATP synthase.[195] There the protons induce the necessary conformational changes to cause ATP synthesis. A related idea is that transient high-energy intermediates

generated by electron transport within membranes are proton-carrying conformational isomers. When an electron is removed from an electron-transporting metalloprotein, the resulting positively charged "hole" could be stable for some short time, while the protein diffused within the membrane until it encountered an F_0 protein of an ATP synthase. Then it might undergo an induced conformational change at the same time that it "handed" the Bohr effect proton of Eq. 18-14 to the F_0 protein and simultaneously induced a conformational change in that protein. The coupling of proton transport to conformational changes seems plausible, when we recall that the induction of conformational changes within proteins almost certainly involves rearrangement of hydrogen bonds.

A consequence of the chemiosmotic theory is that there is no need for an integral stoichiometry between protons pumped and ATP formed or for an integral P/O ratio. There are bound to be inefficiencies in coupling, and Δp is also used in ways other than synthesis of ATP.

4. ATP Synthase

In 1960, Racker and associates[196,197] discovered that the "knobs" or "little mushrooms" visible in negatively stained mitochondrial fragments or fragments of bacterial membranes possess ATP-hydrolyzing (**ATPase)** activity. Earlier the knob protein had been recognized as one of several **coupling factors** required for reconstitution of oxidative phosphorylation by submitochondrial particles.[197] Electron micrographs showed that the submitochondrial particles consist of closed vesicles derived from the mitochondrial cristae, and that the knobs (Fig. 18-14A) are on the *outside* of the vesicles. They can be shaken loose by ultrasonic oscillation with loss of phosphorylation and can be added back with restoration of phosphorylation. The knob protein became known as **coupling factor F_1**. Similar knobs present on the outside of the thylakoids became **CF_1**and those inside thermophilic bacteria **TF_1**. The ATPase activity of F_1 was a clue that *the knobs were really ATP synthase*. It also became clear that a portion of the ATP synthase is firmly embedded in the membranes. This part became known as **F_0**. Both the names F_1F_0 ATP synthase and F_1F_0 ATPase are applied to the complex, the two names describing different catalytic activities. The ATPase activity is usually not coupled to proton pumping but is a readily measurable property of the F_1 portion. In a well-coupled submitochondrial particle the ATPase activity will be coupled to proton transport and will represent a reversal of the ATP synthase activity.

The synthase structure. The F_1 complex has been isolated from *E. coli*,[204,205] other bacteria,[206,207] yeast,[208a,b] animal tissues,[199,209–211] and chloroplasts.[212–214] In every case it consists of five kinds of subunits with the stoichiometry $\alpha_3\beta_3\gamma\delta\varepsilon$.[214a,b] The F_0 complex of *E. coli* contains three subunits designated a,b, and c. All of these proteins are encoded in one gene cluster, the *unc* operon (named for uncoupled mutants), with the following order:

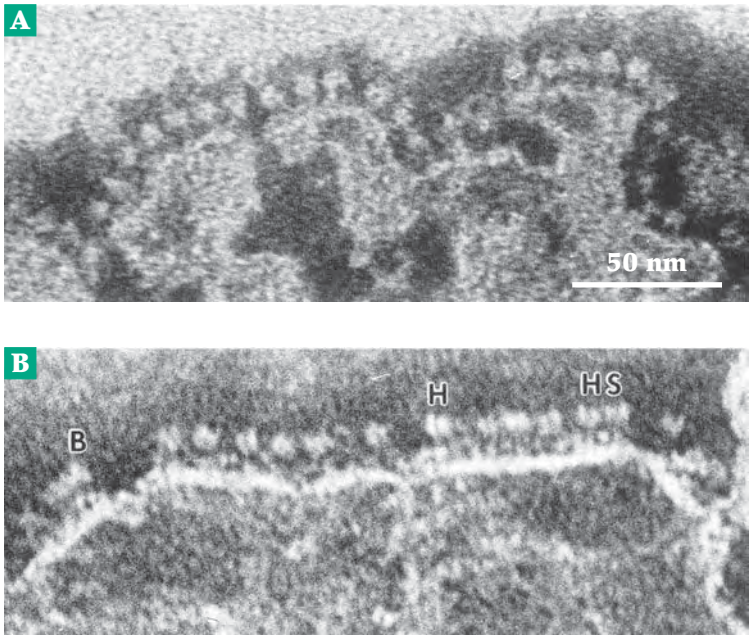
I	*B*	*E*	*F*	*H*	*A*	*G*	*D*	*C*	– – – Gene symbol
i	a	c	b	δ	α	γ	β	ε	– – – Subunit symbol

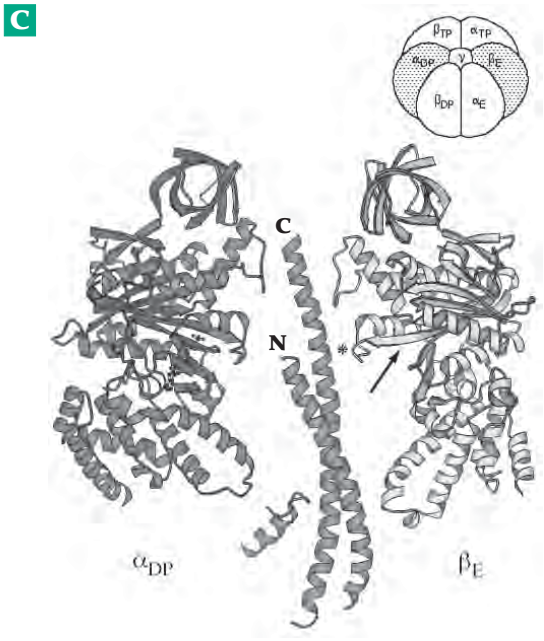
Here I is the regulatory gene (as in Fig. 28-1). The *E. coli* F_0 appears to have approximately the unusual stoichiometry $ab_2c_{9\text{-}11}$. This suggested the possibility that 12 c subunits form a ring with D_6 or D_{12} symmetry, the latter being illustrated in the structural proposal shown in Fig. 18-14E. However, crystallographic evidence suggests that there may be 10, not 12 subunits.[214c]

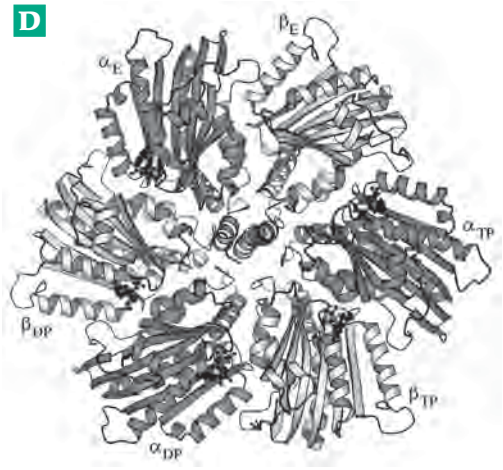
Mitochondrial ATP synthase of yeast contains at least 13 different kinds of subunits[208] and that of animals[215] 16, twice as many as in *E. coli*. Subunits α, β, γ, a, b, and c of the mitochondrial synthase correspond to those of *E. coli*. However, the mitochondrial homolog of *E. coli* δ is called the **oligomycin-sensitivity-conferring protein** (OSCP).[216–218] It makes the ATPase activity sensitive to oligomycin. The mitochondrial δ subunit corresponds to ε of *E. coli* or of chloroplasts.[217,219] Mitochondrial ε has no counterpart in bacteria.[209,220] In addition,[209,215] mitochondria contain subunits called d, e, f, g, A6L, F6, and IF_1, the last being an 84-residue inhibitor, a regulatory subunit.[221] The subunits of yeast ATP synthase correspond to those of the animal mitochondrial synthase but include one additional protein (**h**).[208a]

	F_1							F_0							
E. coli	α	β	γ		δ		ε	a	b	c					
Mitochondria	α	β	γ	OSCP		δ	ε	IF_1	a	b	c	d	e	f	g	A6L	F6

Six of the relatively large (50–57 kDa) α and β subunits associate to an $\alpha_3\beta_3$ complex that constitutes the knobs.[202,210] Chemical crosslinking, directed mutation, electron cryomicroscopy,[222,222a] and high-resolution X-ray diffraction measurements[199,207,211,223,224] have established that the α and β subunits alternate in a quasisymmetric cyclic head that contains active sites for ATP formation in the three β subunits (Fig. 18-14C–E). The α subunits also contain ATP-binding sites, but they are catalytically inactive, and their bound MgATP does not exchange readily with external ATP and can be replaced by the nonhydrolyzable AMP-PNP (Fig. 12-31) with retention of activity. The $\alpha_3\beta_3$ complex is associated with the F_0 part by a slender **central stalk**







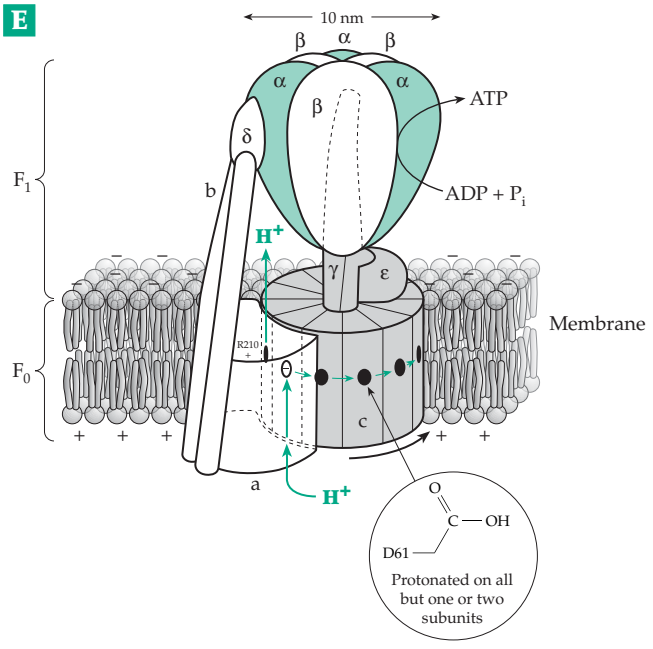


Figure 18-14 ATP synthase and vacuolar ATPase. (A) "Knobs" of ATP synthase on mitochondrial membranes negatively stained with phosphotungstate. (B) Vacuolar proton-pumping ATPase from an intact vacuolar membrane stained in the same way. Some images have been marked to indicate well-resolved head groups (H), stalks (S), and basal components (B). (A) and (B) are from Dschida and Bowman.[198] Courtesy of Barry J. Bowman. (C) Ribbon model of the atomic structure of the F_1 part of bovine heart mitochondrial ATP synthase. This section through the knob, which is drawn schematially at the upper right, shows one α subunit, containing bound ADP, α_{DP}, to the left and one empty β subunit, β_E, to the right. In the center are the N and C termini of subunit γ. The arrow points to a disruption in the β sheet of the subunit structure in the β_E conformation. The asterisk marks a loop that would collide with the C-terminal part of subunit γ if the latter were rotated. (D) View of the F_1 ATP synthase from the membrane side. The section shown contains the nucleotide binding sites. Subunits with empty sites are labeled α_E and β_E. Those with bound ADP are labeled α_{DP} and β_{DP}, and those with bound ATP α_{TP} and β_{TP}. Deviation from perfect threefold symmetry can be seen in this view. (C) and (D) are from Abrahams *et al.*[199] Courtesy of John E. Walker. (E) A recent model of the *E. coli* ATP synthase. The $\alpha_3\beta_3$ head contains the ATP-synthesizing sites in the β subunits. The $\alpha_3\beta_3$ complex and also subunits a, b, and δ remain stationary and form the stator for a protic engine, whose rotor consists of 12 c subunits and attached γ and ε subunits. Rotation is induced by the membrane potential or difference in H^+ activity on the two sides of the membrane. The carboxylate group of Asp 61 in each c subunit must be protonated to –COOH in order for it to move away from the entrance channel in subunit a. The presence of the positively charged Arg 210 near the exit channel in subunit a induces release of the proton when the c subunit has rotated almost 360°. According to this picture, 12 protons are required for one rotation with synthesis of three molecules of ATP. After Elston *et al.*[200] and Zhou *et al.*[201] See also Junge *et al.*[202] and Engelbrecht and Junge.[203]

or **shaft**. Much effort has gone into establishing the subunit composition of the shaft and the F_0 parts of the structure. As is indicated in Fig. 18-14E, subunits γ and ε of the *E. coli* enzyme are both part of the central shaft.[219,225,226] The same is true for the mitochondrial complex, in which the δ subunit corresponds to bacterial ε.[227] The role of this subunit is uncertain. It is part of the shaft but is able to undergo conformational alterations that can permit its C-terminal portion to interact either with F_0 or with the $\alpha_3\beta_3$ head.[227,227a] The unique ε subunit of mitochondrial ATPase appears also to be part of the shaft.[220]

The most prominent component of the central shaft is the 270-residue subunit γ, which associates loosely with the $\alpha_3\beta_3$ head complex but more tightly with F_0. About 40 residues at the N terminus and 60 at the C terminus form an α-helical coiled coil, which is visible in Fig. 18-14E[199,211] and which protrudes into the central cavity of the $\alpha_3\beta_3$ complex. Because it is asymmetric, the γ subunit apparently acts as a rotating camshaft to physically alter the α and β subunits in a cyclic manner. Asymmetries are visible in Fig. 18-14D.[211] The central part of subunit γ forms a more globular structure, which bonds with the c subunits of F_0.[205] Exact structures are not yet clear.

The δ subunit of *E. coli* ATP synthase (OSCP of mitochondria) was long regarded as part of the central stalk. However, more recent results indicate that it is found in a **second stalk**, which joins the $\alpha_3\beta_3$ complex to F_0. The central stalk rotates, relative to the second stalk. The second stalk may be regarded as stationary and part of a **stator** for a protic engine.[228,229] This stalk has been identified[230] in electron micrographs of chloroplast F_1F_0 and by crosslinking studies. As is depicted in Fig. 18-14E, a major portion of the second stalk is formed by two molecules of subunit b. Recent results indicate that bacterial subunit δ (mitochondrial OSCP) extends further up than is shown in Fig. 18-14, and together with subunit F6 may form a cap at the top of the $\alpha_3\beta_3$ head.[230a, 230b]

The F_0 portion of bacterial ATP synthase, which is embedded in the membrane, consists of one 271-residue subunit a, an integral membrane protein probably with five transmembrane helices,[231,232] two 156-residue b subunits, and ~twelve 79-residue c subunits. The c subunit is a proteolipid, insoluble in water but soluble in some organic solvents. The structure of monomeric c in chloroform:methanol:H_2O (4:4:1) solution has been determined by NMR spectroscopy. It is a hairpin consisting of two antiparallel α helices.[233] Twelve of the c subunits are thought to assemble into a ring with both the N and C termini of the subunit chains in the periplasmic (or intermembrane) face of the membrane.[234,235] The ratio of c to a subunits has been difficult to measure but has been estimated as 9–12. The fact that both genetically fused c_2 dimers and c_3 trimers form function F_0 suggested that they assemble to a c_{12} ring as shown in Fig. 18-14E.[236] However, the recent crystallographic results that revealed a C_{10} ring[214c] raise questions about stoichiometry.

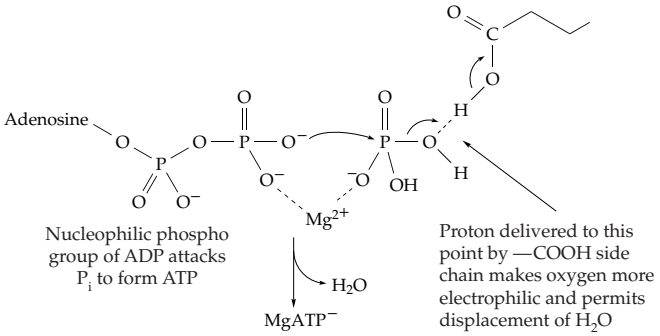
Since ATP synthesis takes place in F_1, it has long been thought that the F_0 part of the ATP synthase contains a "proton channel," which leads from the inside of the mitochondria to the F_1 assemblage.[146] Such a channel would probably not be an open pore but a chain of hydrogen-bonded groups, perhaps leading through the interior of the protein and able to transfer protons via icelike conduction. One residue in the c subunit, Asp 61, which lies in the center of the second of the predicted transmembrane helices, is critical for proton transport.[236a] Natural or artificial mutants at this position (e.g., D61G or D61N) do not transport protons. This carboxyl group also has an unusually high reactivity and specificity toward the protein-modifying reagent dicyclohexylcarbodiimide (DCCD; see Eq. 3-10).[146,237] Modification of a single c subunit with DCCD blocks the proton conductance.

An interesting mutation is replacement of alanine 62 of the c subunit with serine. This mutant will support ATP synthase using Li^+ instead of H^+.[237] Certain alkylophilic bacteria, such as *Propionigenium modestum*, have an ATP synthase that utilizes the membrane potential and a flow of Na^+ ions rather than protons through the c subunits.[238–240c] The sodium transport requires glutamate 65, which fulfills the same role as D61 in *E. coli*, and also Q32 and S66. Study of mutants revealed that the polar side chains of all three of these residues bind Na^+, that E65 and S66 are needed to bind Li^+, and that only E65 is needed for function with H^+.

The a subunit is also essential for proton translocation.[231,232,241] Structural work on this extremely hydrophobic protein has been difficult, but many mutant forms have been studied. Arginine 210 is essential as are E219 and H245. However, if Q252 is mutated to glutamate, E219 is no longer essential.[241] One of the OXPHOS diseases (NARP; Box 18-B) is a result of a leucine-to-arginine mutation in human subunit a.[241a] The b subunit is an elongated dimer, largely of α-helical structure.[242,243] Its hydrophobic N terminus is embedded in the membrane,[229,244] while the hydrophilic C-terminal region interacts with subunit σ of F_1, in the stator structure (Fig. 18-14E). Some of the F_0 subunits (d, e, f, g, A6L) may form a collar around the lower end of the central stalk.[210a,b]

How is ATP made? No covalent intermediates have been identified, and isotopic exchange studies indicate a direct dehydration of ADP and P_i to form bound ATP.[245] For the nucleophilic terminal phospho group of ADP to generate a high-energy linkage directly by attack on the phosphorus atom of P_i an OH^- ion must be eliminated (Eq. 18-13). This is not a probable reaction at pH 7, but it would be reasonable at low pH. Thus, one function of the oriented ATP

synthase might be to deliver one or more protons flowing in from F_0 specifically to the oxygen that is to be eliminated (Eq. 18-13). As we have seen (Section 2), on thermodynamic grounds 3–4 protons would probably be needed. Perhaps they could be positioned nearby to exert a large electrostatic effect, or they could assist in releasing the ATP formed from the synthase by inducing a conformational change. However, it isn't clear how protons could be directed to the proper spots.



(18-13)

Paul Boyer's binding change mechanism. Boyer and associates suggested that ATP synthesis occurs rapidly and reversibly in a closed active site of the ATP synthase in an environment that is essentially anhydrous. ATP would then be released by an energy-dependent conformational change in the protein.[245–249] Oxygen isotope exchange studies verified that a rapid interconversion of bound ADP, P_i, and ATP does occur. Studies of soluble ATP synthase, which is necessarily uncoupled from electron transport or proton flow, shows that ATP is exceedingly tightly bound to F_1 as expected by Boyer's mechanism.[248] According to his **conformational coupling** idea, protons flowing across the membrane into the ATP synthase would in some way induce the conformational change necessary for release of ATP.

The idea of conformational coupling of ATP synthesis and electron transport is especially attractive when we recall that ATP is used in muscle to carry out mechanical work. Here we have the hydrolysis of ATP coupled to motion in the protein components of the muscle. It seems reasonable that ATP should be formed as a result of motion induced in the protein components of the ATPase. Support for this analogy has come from close structural similarities of the F_1 ATPase β subunits and of the active site of ATP cleavage in the muscle protein myosin (Chapter 19).

A simple version of Boyer's binding change mechanism is shown in Figure 18-15. The three F_1 β subunits are depicted in three different conformations. In O the active site is open, in T it is closed, and if ATP is present in the active site it is tightly bound. In the low affinity L conformation ligands are bound weakly. In step *a* MgADP and P_i enter the L site while MgATP is still present in the T site. In step *b* a protonic-energy-dependent step causes synchronous conformational changes in all of the subunits. The tight site opens and MgATP is free to leave. At the same time MgADP and P_i in the T site are converted spontaneously to tightly bound ATP. The MgATP is in reversible equilibrium with MgADP + P_i, which must be bound less tightly than is MgATP. That is, the high positive value of $\Delta G'$ for formation of ATP must be balanced by a corresponding negative $\Delta G'$ for a conformational or electronic reorganization of the protein in the T conformation. Opening of the active site in step *b* of Fig. 18-15 will have a high positive $\Delta G'$ unless it is coupled to proton flow throught F_0. Of three sites in the subunits, one binds MgATP very tightly (K_d ~ 0.1 μM) while the other sites bind less tightly (K_d ~ 20 μM).[250,251] However, it has been very difficult to establish binding constants or K_m values for the ATPase reaction.[248] Each of the three β sites probably, in turn, becomes the high-affinity site, consistent with an ATP synthase mechanism involving protein conformational changes.

Rotational catalysis. Boyer suggested that there is a cyclic rotation in the conformations of the three β subunits of the ATP synthase, and that this might involve rotation about the stalk. By 1984, it had been shown that bacterial flagella are rotated by a protonic motor (Chapter 19), and a protic rotor for ATP synthase had been proposed by Cox *et al.*[252] and others.[245] However, the b subunits were thought to be in the central stalk.[222] More recently chemical crosslinking experiments,[201,253] as well as electron microscopy, confirmed the conclusion that an intact stator structure must also be present as in Fig. 18-14E.[202] The necessary second stalk is visible in CF_1F_0 ATPase of chloroplasts[230] and also in the related vacuolar ATPase, a proton or Na^+ pump from a clostridium.[254] See also Section 5. Another technique, **polarized absorption recovery after photobleaching**, was applied after labeling of Cys 322, the penultimate residue at the C terminus of the γ subunit with the dye eosin. After photobleaching with a laser beam the polarization of the light absorption by the dye molecule relaxed because of rotation. Relaxation was observed when ATP was added but not with ADPPNP.[202,255,256]

The most compelling experiments were performed by Noji *et al.*[202,257–260] They prepared the $\alpha_3\beta_3\gamma$ subcomplex of ATPase from a thermophilic bacterium. The complex was produced in *E. coli* cells from the cloned genes allowing for some "engineering" of the proteins. A ten-histidine "tag" was added at the N termini of the β subunits so that the complex could be "glued" to a microscope coverslip coated with a nickel complex with a high affinity for the His tags. The γ subunit shafts protrude upward as shown in Fig. 18-16. The γ subunit was mutated to replace its

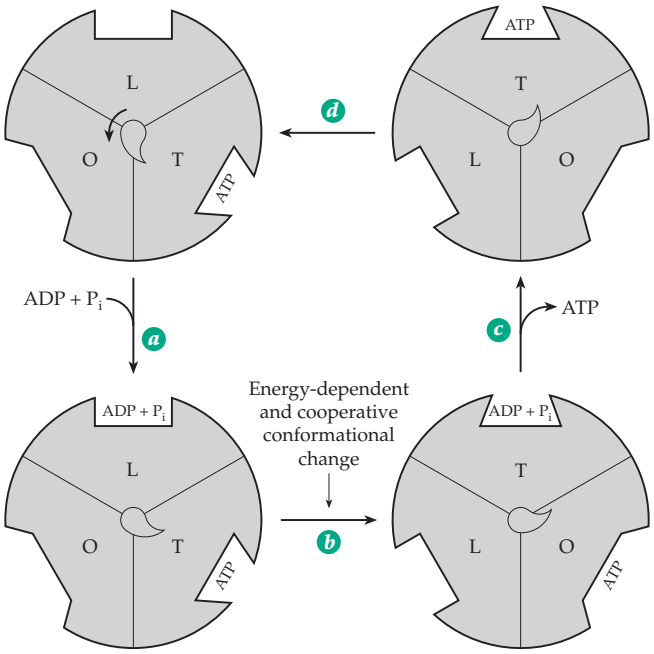
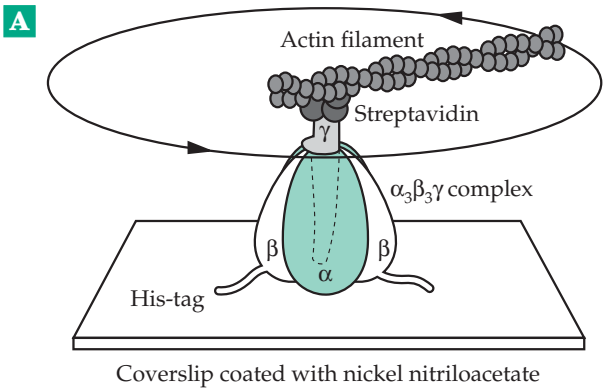


Figure 18-15 Boyer's binding change mechanism for ATP synthase in a simple form. After Boyer[245] but modified to include a central camshaft which may drive a cyclic alteration in conformations of the subunits. The small "pointer" on this shaft is not to be imagined as real but is only an indicator of rotation with induced conformational changes. The rotation could occur in 120° steps rather than the smaller steps suggested here.

only cysteine by serine and to introduce a cysteine in place of Ser 107 of the stalk region of γ. The new cysteine was biotinylated and attached to streptavidin (see Box 14-B) which was also attached to a fluorescently labeled actin filament (Fig. 7-10) ~1–3 μm in length as shown in Fig. 18-16. The actin fiber rotated in a counterclockwise direction when ATP was added but did not rotate with AMPPNP. At low ATP concentrations the rotation could be seen to occur in discrete 120° steps.[258,261,262] Each 120° step seems to consist of ~90° and ~30° substeps, each requiring a fraction of a millisecond.[262a] The ATPase appears to be acting as a **stepper motor**, hydrolysis of a single ATP turning the shaft 120°. Rotation at a rate of ~14 revolutions per second would require the hydrolysis of ~42 ATP per second. If the motor were attached to the F_0 part it would presumably pump four (or perhaps three) H^+ across a membrane for each ATP hydrolyzed. Acting in reverse, it would make ATP. A modification of the experiment of Fig. 18-16 was used to demonstrate that the c subunits also rotate with respect to the $\alpha_3\beta_3$ head.[262b] Other experiments support rotation of the c ring relative to subunit a.[262c,d]

Still to be answered are important questions. How does ATP hydrolysis turn the shaft? Are four H^+ pumped for each step, or are there smaller single proton substeps? Is the simple picture in Fig. 18-15 correct or, as proposed by some investigators,[263–265] must all three β subunits be occupied for maximum catalytic activity?[266] How is the coupling of H^+ transport to mechanical motion accomplished?[267,267a–d]



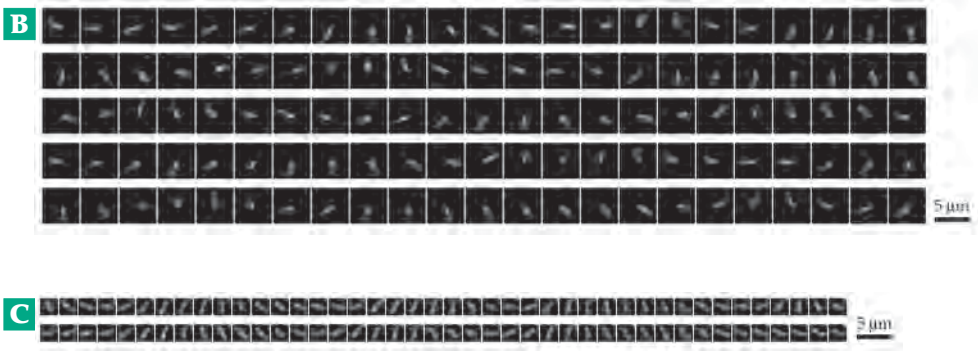


Figure 18-16 (A) The system used for observation of the rotation of the γ subunit in the $\alpha_3\beta_3\gamma$ subcomplex. The observed direction of the rotation of the γ subunit is indicated by the arrows. (B) Sequential images of a rotating actin filament attached as in (A). (C) Similar images obtained with the axis of rotation near the middle of the filament. The images correspond to the view from the top in (A). Total length of the filament, 2.4 μm; rotary rate, 1.3 revolutions per second; time interval between images, 33 ms. From Noji *et al.*[258]

5. ATP-driven Proton Pumps

Not all proton pumps are driven by electron transport. ATP synthase is reversible, and if Δp is low, hydrolysis of ATP can pump protons out of mitochondria or across bacterial plasma membranes.[268] Cells of *Streptococcus faecalis*, which have no respiratory chain

and form ATP by glycolysis, use an F_1F_0 ATP synthase complex to pump protons out to help regulate cytoplasmic pH.[268] Similar **vacuolar (V-type) H^+-ATPases** or V_1V_0 ATPases pump protons into vacuoles, Golgi and secretory vesicles, coated vesicles, and lysosomes[198,269-270] in every known type of eukaryotic cell.[271,272] These proton pumps are similar in appearance (Fig. 18-14,B) and in structure to F_1F_0 ATPases.[272a–c] The 65- to 77-kDa A subunits and 55- to 60-kDa B subunits are larger than the corresponding F_1F_0 α and β subunits. Accessory 40-, 39-, and 33-kDa subunits are also present in V_1. The V_0 portion appears to contain a hexamer of a 16-kDa proteolipid together with 110- and 21-kDa subunits.[271] V-type ATPases are also found in archaebacteria[271,273] and also in some clostridia[254] and other eubacteria.[273a] A type of proton pump, the **V-PPase**, uses hydrolysis of inorganic pyrophosphate as a source of energy.[274] It has been found in plants, in some phototrophic bacteria, and in acidic calcium storage vesicles (acidocalcisomes) of trypanosomes.[274a]

Other ATP-dependent proton pumps are present in the plasma membranes of yeast and other fungi[274b] and also in the acid-secreting parietal cells of the stomach (Fig. 18-17). The H^+-ATPase of *Neurospora* pumps H^+ from the cytoplasm without a counterion. It is electrogenic.[275,275a] However, the gastric H^+,K^+-ATPase exchanges H_3O^+ for K^+ and cleaves ATP with formation of a phosphoenzyme.[276] It belongs to the family of P-type ion pumps that includes the mammalian Na^+,K^+-ATPase (Fig. 8-25) and Ca^{2+}-ATPase (Fig. 8-26). These are discussed in Chapter 8. The H^+,K^+-ATPases, which are widely distributed within eukaryotes, are also similar, both in sequence and in the fact that a phospho group is transferred from ATP onto a carbox-ylate group of an aspartic acid residue in the protein. All of them, including a Mg-ATPase of *Salmonella*, are two-subunit proteins. A large catalytic α subunit contains the site of phosphorylation as well as the ATP- and ion-binding sites. It associates noncovalently with the smaller heavily glycosylated β subunit.[276–278] For example, the rabbit H^+,K^+-ATPase consists of a 1035-residue α chain which has ten transmembrane segments and a 290-residue β chain with a single transmembrane helix and seven N-linked glycosylation sites.[278]

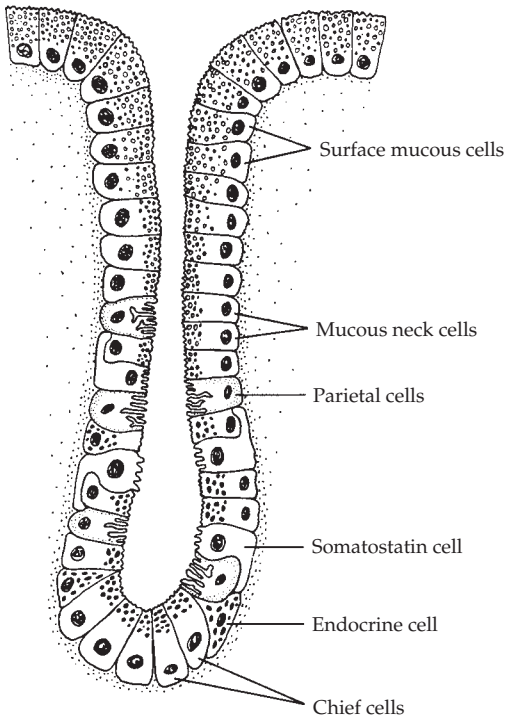
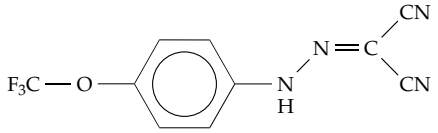


Figure 18-17 Schematic diagram of an acid-producing oxyntic gland of the stomach. The normal human stomach contains about 10^9 parietal (oxyntic) cells located in the walls of these glands. From Wolfe and Soll.[279] Modified from Ito. These glands also produce mucus, whose role in protecting the stomach lining from the high acidity is uncertain.[280]

6. Uncouplers and Energy-linked Processes in Mitochondria

Many compounds that uncouple electron transport from phosphorylation, like 2,4-dinitrophenol, are weak acids. Their anions are nucleophiles. According to the scheme of Fig. 18-12, they could degrade a high energy intermediate, such as Y ~ B, by a nucleophilic attack on Y to give an inactive but rapidly hydrolyzed derivative of Y. On the other hand, according to Mitchell's hypothesis uncouplers facilitate the transport of protons back into the mitochondria thereby destroying Δp. The fact that the anions of the uncouplers are large, often aromatic, and therefore soluble in the lipid bilayer supports this interpretation; the protonated uncouplers can diffuse into the mitochondria and the anion can diffuse back out. Mitochondria can also be uncoupled by a combination of ionophores, e.g., a mixture of valinomycin (Fig. 8-22), which carries K^+ into the mitochondria, plus nigericin, which catalyzes an exchange of K^+ (out) for H^+ (in).[172]

The uncoupler carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP) and related compounds are widely used in biochemical studies. Their action can be explained only partially by increased proton conduction.

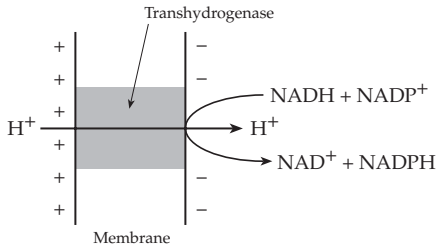


Carbonyl cyanide *p*-(trifluoromethoxy)phenylhydrazone (FCCP)

Uncoupling is sometimes important to an organism. The generation of heat by uncoupling is discussed in Box 18-C. The fungus *Bipolaris maydis* caused a crisis in maize production when it induced pore formation in mitochondrial membranes of a special strain used in production of hybrid seeds.[281,282]

Synthesis of ATP by mitochondria is inhibited by oligomycin, which binds to the OSCP subunit of ATP synthase. On the other hand, there are processes that require energy from electron transport and that are not inhibited by oligomycin. These **energy-linked processes** include the transport of many ions across the mitochondrial membrane (Section E) and reverse electron flow from succinate to NAD^+ (Section C,2). Dinitrophenol and many other uncouplers block the reactions, but oligomycin has no effect. This fact can be rationalized by the Mitchell hypothesis if we assume that Δp can drive these processes.

Another energy-linked process is the **transhydrogenase** reaction by which NADH reduces $NADP^+$ to form NADPH. In the cytoplasm various other reactions are used to generate NADPH (Chapter 17, Section I), but within mitochondria a membrane-bound transhydrogenase has this function.[283–286a] It couples the transhydrogenation reaction to the transport of one (or possibly more than one) proton back into the mitochondria (Eq. 18-14). A value of Δp of −180 mV could increase the ratio of [NADPH] / [$NADP^+$] within mitochondria by a factor of as much as 1000.



(18-14)

Transhydrogenases function in a similar way within bacteria. Whether from *E. coli*, photosynthetic bacteria, or bovine mitochondria, transhydrogenases have similar structures.[285] Two 510-residue α subunits associate with two 462-residue β subunits to form an $\alpha_2\beta_2$ tetramer with 10–14 predicted transmembrane helices. The α subunits contain separate NAD(H) and NADP(H) binding sites. A conformational change appears to be associated with the binding or release of the $NADP^+$ or NADPH.[287]

D. Transport and Exchange across Mitochondrial Membranes

Like the external plasma membrane of cells, the inner mitochondrial membrane is selective. Some nonionized materials pass through readily but the transport of ionic substances, including the anions of the dicarboxylic and tricarboxylic acids, is restricted. In some cases energy-dependent active transport is involved but in others one anion passes inward in exchange for another anion passing outward. In either case specific translocating carrier proteins are needed.

Solutes enter mitochondria through pores in thousands of molecules of the **voltage-gated anion-selective channel VDAC**, also known as mitochondrial porin.[15,16,288,289] In the absence of a membrane potential these pores allow free diffusion to molecules up to ~1.2 kDa in mass and may selectively permit passage of anions of 3- to 5-kDa mass. However, a membrane potential greater than ~20 mV causes the pores to close. NADH also decreases permeability. In the closed state the outer membrane becomes almost impermeant to ATP.[289,290]

An example of energy-dependent transport is the uptake of Ca^{2+} by mitochondria. As indicated in the lower part of Fig. 18-13, there are two possibilities for preservation of electrical neutrality according to the chemiosmotic theory. Counterions X^- may flow out to balance the protons discharged on the outside. On the other hand, if a cation such as Ca^{2+} flows inward to balance the two protons flowing outward, neutrality will be preserved and the mitochondrion will accumulate calcium ions. Experimentally such accumulations via a **calcium uniporter**[4] are observed to accompany electron transport. In the presence of a suitable ionophore energy-dependent accumulation of potassium ions also takes place.[291] In contrast, an **electroneutral exchange** of one Ca^{2+} for two Na^+ is mediated by a Na^+–Ca^{2+} exchanger.[292,293] It permits Ca^{2+} to leave mitochondria. A controversial role of mitochondria in accumulating Ca^{2+} postulates a special **rapid uptake mode** of exchange (see p. 1049).[294]

It is thought that glutamate enters mitochondria as the monoanion Glu^- in exchange for the dianion of aspartate Asp^{2-}. Like the uptake of Ca^{2+} this exchange is driven by Δp. Since a membrane potential can be created by this exchange in the absence of Δp, the process is electrogenic.[4] In contrast, an **electroneutral** exchange of malate^{2-} and 2-oxoglutarate^{2-} occurs by means of carriers that are not energy-linked.[295,296] This dicarboxylate transporter is only one of 35 structurally related mitochondrial carriers identified in the complete genome of yeast.[296,297] Another is the **tricarboxylic transporter** (citrate transport protein) which exchanges the dianionic form of citrate for malate, succinate, isocitrate, phosphoenolpyruvate, etc.[298,298a,b]

The important **adenine nucleotide carrier** takes ADP into the mitochondrial matrix for phosphorylation in a 1:1 ratio with ATP that is exported into the cytoplasm.[299-300b] This is one of the major rate-determining processes in respiration. It has been widely accepted that the carrier is electrogenic,[300]

BOX 18-C USING METABOLISM TO GENERATE HEAT: THERMOGENIC TISSUES

A secondary but important role of metabolism in warm-blooded animals is to generate heat. The heat evolved from ordinary metabolism is often sufficient, and an animal can control its temperature by regulating the heat exchange with the environment. Shivering also generates heat and is used from birth by pigs.[a] However, this is insufficient for many newborn animals, for most small mammals of all ages, and for animals warming up after hibernation. The need for additional heat appears to be met by **brown fat**, a tissue which contrasts strikingly with the more abundant white adipose tissue. Brown fat contains an unusually high concentration of blood vessels, many mitochondria with densely packed cristae, and a high ratio of cytochrome *c* oxidase to ATP synthase. Also present are a large number of sympathetic nerve connections, which are also related to efficient generation of heat. Newborn humans have a small amount of brown fat, and in newborn rabbits it accounts for 5–6% of the body weight.[b–d] It is especially abundant in species born without fur and in hibernating animals. Swordfish also have a large mass of brown adipose tissue that protects their brains from rapid cooling when traveling into cold water.[a]

The properties of brown fat pose an interesting biochemical question. Is the energy available from electron transport in the mitochondria dissipated as heat because ATP synthesis is uncoupled from electron transport? Or does ATP synthesis take place but the resulting ATP is hydrolyzed wastefully through the action of ATPases? Part of the answer came from the discovery that mitochondria of brown fat cells synthesize a 32-kDa **uncoupling protein** (UCP1 or thermogenin). It is incorporated into the inner mitochondrial membranes where it may account for 10–15% of total protein.[d–f] This protein, which is a member of a family of mitochondrial membrane metabolic carriers (Table 18-8), provides a "short-circuit" that allows the protonmotive force to be dissipated rapidly, perhaps by a flow of protons out through the uncoupling protein.[g–i] Synthesis of the uncoupling protein is induced by exposure to cold, but when an animal is warm the uncoupling action is inhibited.

The uncoupling protein resembles the ATP/ADP and phosphate *anion* carriers (Table 18-8),[g,i] which all have similar sizes and function as homodimers. Each monomeric subunit has a triply repeated ~100-residue sequence, each repeat forming two transmembrane helices. Most mitochondrial transporters carry anions, and UCP1 will transport Cl^-.[h,i] However, the relationship of chloride transport to its real function is unclear. Does the protein transport H^+ into, or does it carry HO^- out from, the mitochondrial matrix?[g,h] Another possibility is that a fatty acid anion binds H^+ on the intermembrane surface and carries it across into the matrix as an unionized fatty acid. The fatty acid anion could then pass back out using the anion transporter function and assisted by the membrane potential.[h,i]

The uncoupling protein is affected by several control mechanisms. It is inhibited by nucleotides such as GDP, GTP, ADP, and ATP which may bind at a site corresponding to that occupied by ATP or ADP in the ADP/ATP carrier.[i] Uncoupling is stimulated by noradrenaline,[f] which causes a rapid increase in heat production by brown fat tissues, apparently via activation of adenylate cyclase. Uncoupling is also stimulated by fatty acids.[j] Recently UCP1 and related uncoupling proteins have been found to require both fatty acids and **ubiquinone** for activity.[jj,jk]

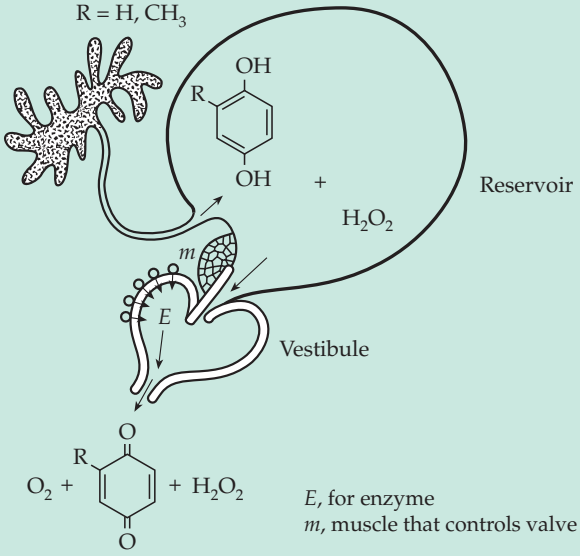
It has been suggested that brown adipose tissue may also function to convert excess dietary fat into heat and thereby to resist obesity.[k–m] Mice lacking the gene for the mitochondrial uncoupling protein are cold-sensitive but not obese. However, other proteins, homologous to UCP1, have been discovered. They may partially compensate for the loss.[m,h]

The bombardier beetle generates a hot, quinone-containing defensive discharge, which is sprayed in a pulsed jet from a special reaction chamber at a temperature of 100°C.[n–p] The reaction mixture of 25% hydrogen peroxide and 10% hydroquinone plus methylhydroquinone is stored in a reservoir as shown in the accompanying figure and reacts with explosive force when it comes into contact with catalase and peroxidases in the reaction chamber. The synthesis and storage of 25% H_2O_2 poses interesting biochemical questions!

Some plant tissues are thermogenic. For example, the spadix (or inflorescence, a sheathed floral spike) of the skunk cabbage *Symplocarpus foetidus* can maintain a 15–35°C higher temperature than that of the surrounding air.[q] The voodoo lily in a single day heats the upper end of its long spadex to a temperature 22°C above ambient, volatilizing a foul smelling mixture of indoles and amines.[r,s] This is accomplished using the alternative oxidase system[s] (Box D in Fig. 18-6). The lotus flower maintains a temperature of 30–35°C, while the ambient temperature may vary from 10–30°C.[t] While the volatilization of insect attractants may be the primary role for thermogenesis in plants, the warm flowers may also offer an important reward to insect pollinators. Beetles and bees require thoracic temperatures above 30°C to initiate flight and, therefore,

BOX 18-C (continued)

benefit from the warm flowers.[t] While in flight bees vary their metabolic heat production by altering their rate of flight, hovering, and other changes in physical activity.[u]



Reservoir and reaction vessel of the bombardier beetle. From D. J. Aneshansley, *et al*.[n]

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bringing in ADP^{3-} and exporting ATP^{4-} in an exchange driven by Δp. However, an electroneutral exchange, e.g., of ADP^{3-} for ATP^{3-}, may also be possible. The carrier is an ~300-residue 32-kDa protein, which is specifically inhibited by the plant glycoside **atractyloside** or the fungal antibiotic **bongkrekate**. The carrier is associated with bound cardiolipin.[301] This one transporter accounts for ~10% of all of the mitochondrial protein.[302,303]

A separate dimeric carrier allows P_i to enter, probably as $H_2PO_4^-$.[304-305a] This ion enters mitochondria in an electroneutral fashion, either in exchange for OH^- or by cotransport with H^+. A less important carrier[306] exchanges HPO_4^{2-} for $malate^{2-}$. Several other transporters help to exchange organic and inorganic ions. One of them allows pyruvate to enter mitochondria in exchange for OH^- or by cotransport with H^+. Some of the identified carriers are listed in Table 18-8. As discussed in Chapter 8, exchange carriers are also important in plasma membranes of organisms from bacteria to human beings. For example, many metabolites enter cells by cotransport with Na^+ using the energy of the Na^+ gradient set up across the membrane by the Na^+,K^+ pump.

Under some circumstances the inner membrane develops one or more types of large-permeability pore. An increase in Ca^{2+} may induce opening of an unselective pore which allows rapid uptake of Ca^{2+}.[294,307,307a] A general anion-specific channel may be involved in volume homeostasis of mitochondria.[308]

Mitochondria are not permeable to NADH. However, reactions of glycolysis and other dehydrogenations in the cytoplasm quickly reduce available NAD^+ to NADH. For aerobic metabolism to occur, the "reducing equivalents" from the NADH must be transferred into the mitochondria. Fungi and green plants have solved

the problem by providing *two* NADH dehydrogenases embedded in the inner mitochondrial membranes (Fig. 18-6). One faces the matrix space and oxidizes the NADH produced in the matrix while the second faces outward to the intermembrane space and is able to oxidize the NADH formed in the cytoplasm. In animals the reducing equivalents from NADH enter the mitochondria indirectly. There are several mechanisms, and more than one may function simultaneously in a tissue.

In insect flight muscle, as well as in many mammalian tissues, NADH reduces dihydroxyacetone phosphate. The resulting *sn*-3-glycerol *P* passes through the permeable outer membrane of the mitochondria, where it is reoxidized to dihydroxyacetone phosphate by the FAD-containing glycerol-phosphate dehydrogenase embedded in the outer surface of the inner membrane (Figs. 18-5, 18-6). The dihydroxyacetone can then be returned to the cytoplasm. The overall effect of this **glycerol-phosphate shuttle** (Fig. 18-18A) is to provide for mitochondrial oxidation of NADH produced in the cytoplasm. In heart and liver the same function is served by a more complex **malate–aspartate shuttle** (Fig. 18-18B).[309] Reduction of oxaloacetate to malate by NADH, transfer of malate into mitochondria, and reoxidation with NAD^+ accomplishes the transfer of reduction equivalents into the mitochondria. Mitochondrial membranes are not very permeable to oxaloacetate. It returns to the cytoplasm mainly via transamination to aspartate, which leaves the mitochondria together with 2-oxoglutarate. At the same time glutamate enters the mitochondria in exchange for aspartate. The 2-oxoglutarate presumably exchanges with the entering malate as is indicated in Fig. 18-18B. The export of aspartate may be energy-linked as a result of the use of an electrogenic carrier that exchanges glutamate$^-$ + H^+ entering mitochondria for aspartate$^-$ leaving the mitochondria. Thus, Δp may help to expel aspartate from mitochondria and to drive the shuttle.

The glycerol-phosphate shuttle, because it depends upon a mitochondrial flavoprotein, provides ~ 2 ATP per electron pair (P / O = 2), whereas the malate–aspartate shuttle may provide a higher yield of ATP. The glycerol-phosphate shuttle is essentially irreversible, but the reactions of the malate–aspartate shuttle can be reversed and utilized in gluconeogenesis (Chapter 17).

TABLE 18-8
Some Mitochondrial Membrane Transporters[a]

Ion Diffusing In	Ion Diffusing Out	Comment[b]
ADP^{3-}	ATP^{3-}	
or ADP^{3-}	ATP^{4-}	Electrogenic symport
$H_2PO_4^- + H^+$		Electroneutral symport
or $H_2PO_4^-$	OH^-	
HPO_4^{2-}	$Malate^{2-}$	
$Malate^{2-}$	$2\text{-Oxoglutarate}^{2-}$	
$Glutamate^{2-} + H^+$	$Aspartate^{2-}$	
$Glutamate^-$	OH^-	
$Pyruvate^-$	OH^-	
or $Pyruvate^- + H^+$		Electroneutral symport
$Citrate^{3-} + H^+$	$Malate^{2-}$	
$Ornithine^+$	H^+	
Acylcarnitine	Carnitine	
$2\ Na^+$	Ca^{2+}	
H^+	K^+	
H^+	Na^+	
General transporters		
VDAC (porin)		Outer membrane
Large anion pores		Inner membrane

[a] From Nicholls and Ferguson[172] and Tyler[4].
[b] Unless indicated otherwise the transporters are *antiporters* that catalyze an electroneutral ion exchange.

E. Energy from Inorganic Reactions

Some bacteria obtain all of their energy from inorganic reactions. These **chemolithotrophs** usually have a metabolism that is similar to that of heterotrophic organisms, but they also have the capacity to obtain all of their energy from an inorganic reaction. In order to synthesize carbon compounds they must be able to fix CO_2 either via the reductive pentose phosphate cycle or in some other way. The chloroplasts of green plants, using energy from sunlight, supply the organism with both ATP and the reducing agent NADPH (Chapter 17). In a similar way the lithotrophic bacteria obtain both energy and reducing materials from inorganic reactions.

Chemolithotrophic organisms often grow slowly, making study of their metabolism difficult.[310] Nevertheless, these bacteria usually use electron transport chains similar to those of mitochondria. ATP is formed by oxidative phosphorylation, the amount formed per electron pair depending upon the number of proton-pumping sites in the chain. This, in turn, depends upon the electrode potentials of the reactions involved. For example, H_2, when oxidized by O_2, leads to passage of electrons through the entire electron transport chain with synthesis of ~3 molecules of ATP per electron pair. On the other hand, oxidation by O_2 of nitrite, for which $E^{\circ\prime}$ (pH 7) = +0.42 V, can make use only of the site III part of the chain. Not only is the yield of ATP less than in the oxidation of H_2 but also there is another problem. Whereas reduced pyridine

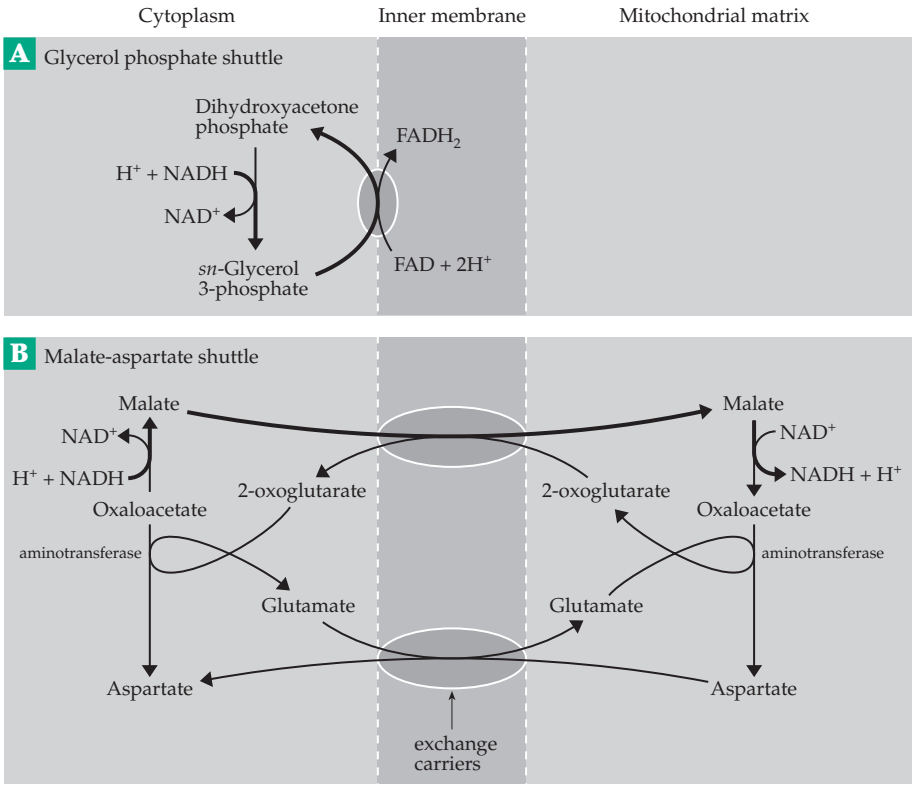


Figure 18-18 (A) The glycerol-phosphate shuttle and (B) the malate–aspartate shuttle for transport from cytoplasmic NADH into mitochondria. The heavy arrows trace the pathway of the electrons (as 2H) transported.

nucleotides needed for biosynthesis can be generated readily from H_2, nitrite is not a strong enough reducing agent to reduce NAD^+ to NADH. The only way that reducing agents can be formed in cells utilizing oxidation of nitrite as an energy source is via *reverse electron flow* driven by hydrolysis of ATP or by Δp. Such reverse electron flow is a common process for many chemolithotrophic organisms.

Let us consider the inorganic reactions in two groups: (1) oxidation of reduced inorganic compounds by O_2 and (2) oxidation reactions in which an inorganic oxidant, such as nitrate or sulfate, substitutes for O_2. The latter reactions are often referred to as **anaerobic respiration**.

1. Reduced Inorganic Compounds as Substrates for Respiration

The hydrogen-oxidizing bacteria. Species from several genera including *Hydrogenomonas*, *Pseudomonas*, and *Alcaligenes* oxidize H_2 with oxygen:

$$H_2 + 1/2\ O_2 \rightarrow H_2O$$
$$\Delta G^\circ = -237.2 \text{ kJ mol}^{-1} \qquad (18\text{-}15)$$

Some can also oxidize carbon monoxide:

$$CO + 1/2\ O_2 \rightarrow CO_2$$
$$\Delta G^\circ = -257.1 \text{ kJ mol}^{-1} \qquad (18\text{-}16)$$

The hydrogen bacteria can also oxidize organic compounds using straightforward metabolic pathways. The key enzyme is a membrane-bound nickel-containing hydrogenase (Fig. 16-26), which delivers electrons from H_2 into the electron transport chain.[310a] A second soluble hydrogenase (sometimes called **hydrogen dehydrogenase**) transfers electrons to $NADP^+$ to form NADPH for use in the reductive pentose phosphate cycle and for other biosynthetic purposes.

Nitrifying bacteria. Two genera of soil bacteria oxidize ammonium ion to nitrite and nitrate (Eqs. 18-17 and 18-18).[311]

$$NH_4^+ + 3/2\ O_2 \rightarrow NO_2^- + 2\ H^+ + H_2O \text{ (}\textit{Nitrosomonas}\text{)}$$
$$\Delta G' \text{ (pH 7)} = -272 \text{ kJ mol}^{-1} \qquad (18\text{-}17)$$

$$NO_2^- + 1/2\ O_2 \rightarrow NO_3^- \text{ (}\textit{Nitrobacter}\text{)}$$
$$\Delta G' \text{ (pH 7)} = -76 \text{ kJ mol}^{-1} \qquad (18\text{-}18)$$

The importance of these reactions to the energy metabolism of the bacteria was recognized in 1895 by Winogradsky, who first proposed the concept of chemiautotrophy. Because the nitrifying bacteria grow

slowly (generation time ~10–12 h) it has been hard to get enough cells for biochemical studies and progress has been slow. The reaction catalyzed by *Nitrosomonas* (Eq. 18-17) is the more complex; it occurs in two or more stages and is catalyzed by two enzymes as illustrated in Fig. 18-19. The presence of hydrazine blocks oxidation of hydroxylamine (NH_2OH) in step *b* and permits that intermediate to accumulate. The oxidation of ammonium ion by O_2 to hydroxylamine (step *a*) is endergonic with $\Delta G'$ (pH 7) = 16 kJ mol^{-1} and is incapable of providing energy to the cell. It occurs by a hydroxylation mechanism (see Section G). On the other hand, the oxidation of hydroxylamine to nitrite by O_2 in step *b* is highly exergonic with $\Delta G'$ (pH 7) = −228 kJ mol^{-1}. The hydroxylamine oxidoreductase that catalyzes this reaction is a trimer of 63-kDa subunits, each containing seven *c*-type hemes and an unusual heme P450, which is critical to the enzyme's function[312–314a] and which is covalently linked to a tyrosine as well as to two cysteines.

The electrode potentials for the two- and four-electron oxidation steps are indicated in Fig. 18-19. It is apparent that step *b* can feed four electrons into the electron transport system at about the potential of ubiquinone. Two electrons are needed to provide a cosubstrate (Section G) for the ammonia monooxygenase and two could be passed on to the terminal cytochrome aa_3 oxidase. The stoichiometry of proton pumping in complexes III and IV is uncertain, but if it is assumed to be as shown in Fig. 18-19 and similar to that in Fig. 18-5, there will be ~13 protons available to be passed through ATP synthase to generate ~3 ATP per NH_3 oxidized. However, to generate NADH for reductive biosynthesis *Nitrosomonas* must send some electrons to NADH dehydrogenase (complex I) using reverse electron transport, a process that depends upon Δp to drive the reaction via a flow of protons through the NADH dehydrogenase from the periplasm back into the bacterial cytoplasm (Fig. 18-19).

Nitrobacter depends upon a simpler energy-yielding reaction (Eq. 18-18) with a relatively small Gibbs energy decrease. The two-electron oxidation delivers electrons to the electron transport chain at $E^{\circ\prime}$ = +0.42 V. The third oxygen in NO_3^- originates from H_2O, rather than from O_2 as might be suggested by Eq. 18-18.[316,317] It is reasonable to anticipate that a single molecule of ATP should be formed for each pair of electrons reacting with O_2. However, *Nitrobacter* contains a confusing array of different cytochromes in its membranes.[311] Some of the ATP generated by passage of electrons from nitrite to oxygen must be used to drive a reverse flow of electrons through both a bc_1-type complex and NADH dehydrogenases. This generates reduced pyridine nucleotides required for biosynthetic reactions (Fig. 18-20).

An interesting feature of the structure of *Nitrobacter* is the presence of several double-layered membranes which completely envelop the interior of the cell. Nitrite entering the cell is oxidized on these membranes and cannot penetrate to the interior, where it might have toxic effects.

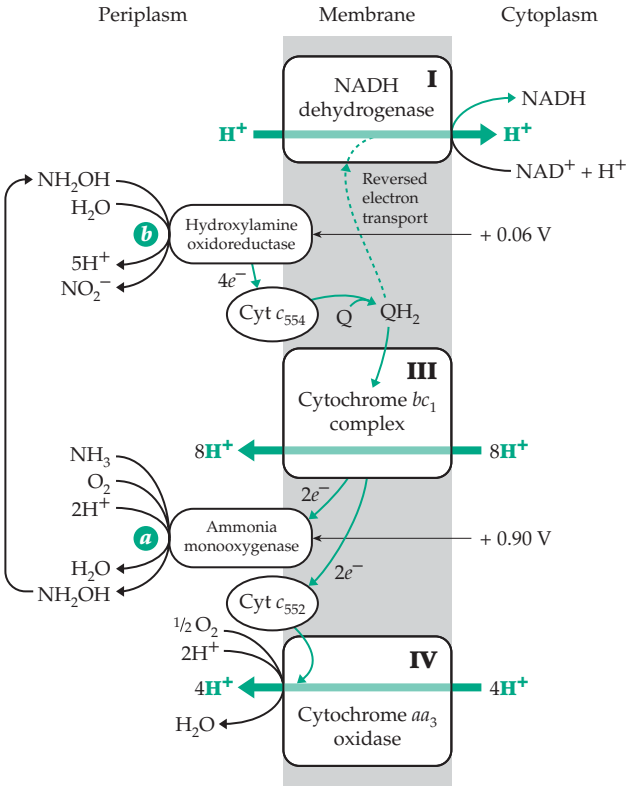


Figure 18-19 The ammonia oxidation system of the bacterium *Nitrosomonas*. Oxidation of ammonium ion (as free NH_3) according to Eq. 18-17 is catalyzed by two enzymes. The location of ammonia monooxygenase (step *a*) is uncertain but hydroxylamine oxidoreductase (step *b*) is periplasmic. The membrane components resemble complexes I, III, and IV of the mitochondrial respiratory chain (Fig. 18-5) and are assumed to have similar proton pumps. Solid green lines trace the flow of electrons in the energy-producing reactions. This includes flow of electrons to the ammonia monooxygenase. Complexes III and IV pump protons out but complex I catalyzes reverse electron transport for a fraction of the electrons from hydroxylamine oxidoreductase to NAD^+. Modified from Blaut and Gottschalk.[315]

The sulfur-oxidizing bacteria. Anaerobic conditions prevail in marine sediments, in poorly stirred swamps, and around hydrothermal vents at the bottom of the sea. Sulfate-reducing bacteria form high concentrations (up to mM) of H_2S (in equilibrium with HS^- and S^{2-})[318–320] This provides the substrate for bacteria of the genus *Thiobacillus*, which are able to oxidize sulfide, elemental sulfur, thiosulfate, and sulfite to sulfate and live where the aerobic and anaerobic regions meet.[311,321–323] Most of these small gram-negative

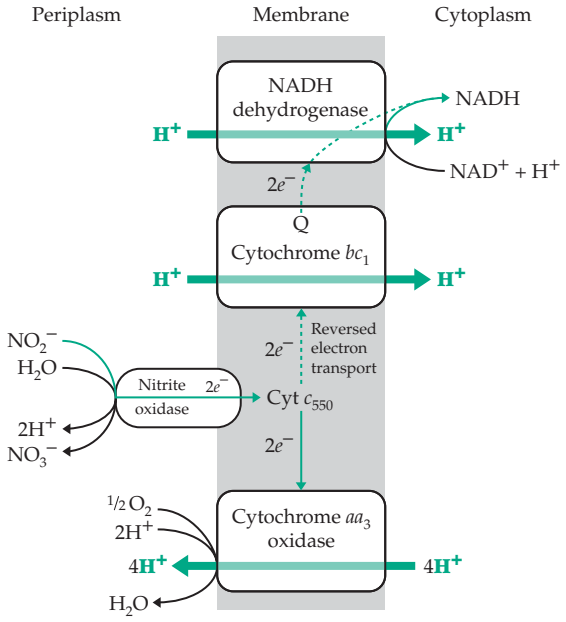
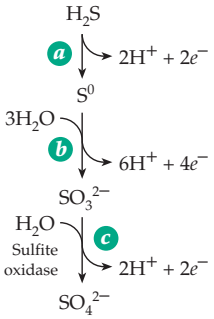


Figure 18-20 Electron transport system for oxidation of the nitrite ion to the nitrate ion by *Nitrobacter*. Only one site of proton pumping for oxidative phosphorylation is available. Generation of NADH for biosynthesis requires two stages of reverse electron transport.

organisms, found in water and soil, are able to grow in a simple salt medium containing an oxidizable sulfur compound and CO_2. One complexity in understanding their energy-yielding reactions is the tendency of sulfur to form chain molecules. Thus, when sulfide is oxidized, it is not clear that it is necessarily converted to monoatomic elemental sulfur as indicated in Eq. 18-19. Elemental sulfur (S_8^0) often precipitates. In *Beggiotoa*, another sulfide-oxidizing bacterium, sulfur is often seen as small globules within the cells. Fibrous sulfur precipitates are often abundant in the sulfide-rich layers of ponds, lakes, and oceans.[318]



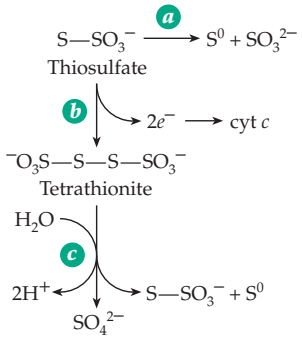
(18-19)

The reactions of Eq. 18-19 occur in the periplasmic space of some species.[315,323a,324] Steps *a* and *b* of Eq. 18-19 are catalyzed by a 67-kDa sulfide dehydrogenase in the periplasm of a purple photosynthetic bacterium.[324] The enzyme consists of a 21-kDa subunit containing two cytochrome *c*-like hemes, presumably the site of binding of S^{2-}, and a larger 46-kDa FAD-containing flavoprotein resembling glutathione reductase.[324] The molybdenum-containing sulfite oxidase (Fig. 16-32), which is found in the intermembrane space of mitochondria, may be present in the periplasmic space of these bacteria. However, there is also an intracellular pathway for sulfite oxidation (see Eq. 18-22).

The sulfide-rich layers inhabited by the sulfur oxidizers also contain thiosulfate, $S_2O_3^{2-}$. It may arise, in part, from reaction of glutathione with elemental sulfur:

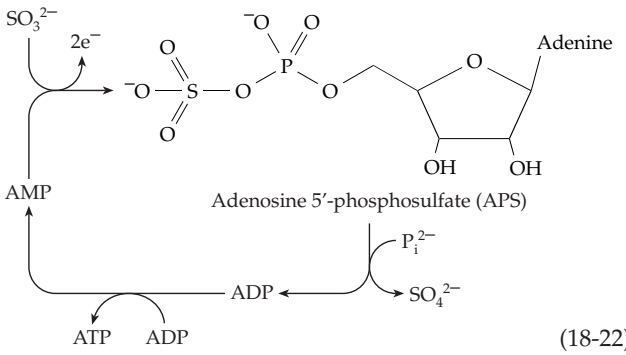
$$\text{GSH} + S_8^0 \rightarrow \text{G—S—}S_8\text{—H} \qquad (18\text{-}20)$$

The linear polysulfide obtained by this reaction may be oxidized, the sulfur atoms being removed from the chain either one at a time to form sulfite or two at a time to form thiosulfate.[322,322a] Thiosulfate is oxidized by all species, the major pathway beginning with cleavage to S^0 and SO_3^{2-} (Eq. 18-21, step *a*). At high thiosulfate concentrations some may be oxidized to tetrathionate (step *b*), which is hydrolyzed to sulfate (step *c*).



(18-21)

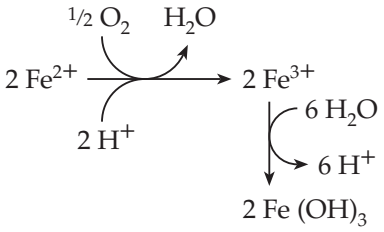
Oxidation of sulfite to sulfate within cells occurs by a pathway through **adenosine 5'-phosphosulfate** (**APS**, adenylyl sulfate). Oxidation via APS (Eq. 18-22) provides a means of substrate-level phosphorylation,



(18-22)

the only one known among chemolithotrophic bacteria. No matter which of the two pathways of sulfite oxidation is used, thiobacilli also obtain energy via electron transport. With a value of $E°'$ (pH 7) of −0.454 V [$E°'$ (pH 2) = −0.158 V] for the sulfate–sulfite couple an abundance of energy may be obtained. Oxidation of sulfite to sulfate produces hydrogen ions. Indeed, pH 2 is optimal for the growth of *Thiobacillus thiooxidans*, and the bacterium withstands 5% sulfuric acid.[322]

The "iron bacterium" *Thiobacillus ferrooxidans* obtains energy from the oxidation of Fe^{2+} to Fe^{3+} with subsequent precipitation of ferric hydroxide (Eq. 18-23). However, it has been recognized recently that a previously unknown species of Archaea is much more important than *T. ferrooxydans* in catalysis of this reaction.[324a]



(18-23)

Since the reduction potential for the Fe(II) / Fe(III) couple is + 0.77 V at pH 7, the energy obtainable in this reaction is small. These bacteria always oxidize reduced sulfur compounds, too. Especially interesting is their oxidation of **pyrite**, ferrous sulfide (Eq. 18-24). The Gibbs energy change was calculated from published data[325] using a value of $G_f°$ for Fe $(OH)_3$ of

$$2\ FeS + 4\tfrac{1}{2}\ O_2 + 5\ H_2O \rightarrow 2\ Fe(OH)_3 + 2\ SO_4^{2-} + 4\ H^+$$
$$\Delta G'\ (pH\ 2) = -1340\ kJ \qquad (18\text{-}24)$$

−688 kJ mol^{-1} estimated from its solubility product. Because sulfuric acid is generated in this reaction, a serious water pollution problem is created by the bacteria living in abandoned mines. Water running out of the mines often has a pH of 2.3 or less.[326]

Various invertebrates live in S^{2-}-containing waters. Among these is a clam that has symbiotic sulfur-oxidizing bacteria living in its gills. The clam tissues apparently carry out the first step in oxidation of the sulfide.[327] Among the animals living near sulfide-rich thermal vents in the ocean floor are giant 1-meter-long tube worms. Both a protective outer tube and symbiotic sulfide-oxidizing bacteria protect them from toxic sulfides.[319,320]

2. Anaerobic Respiration

Nitrate as an electron acceptor. The use of nitrate as an alternative oxidant to O_2 is widespread among bacteria. For example, *E. coli* can subsist anaerobically by reducing nitrate to nitrite (Eq. 18-25).[311,328] The respiratory (dissimilatory) nitrate reductase that

$$NO_3^- + 2\ H^+ + 2e^- \rightarrow NO_2^- + H_2O$$
$$E°'\ (pH7) = +0.421\ V \qquad (18\text{-}25)$$

catalyzes the reaction is a large three-subunit molybdenum-containing protein. The enzyme is present in the plasma membrane, and electrons flow from ubiquinone through as many as six heme and Fe–S centers to the molybdenum atom.[328-329] A second molybdoenzyme, formate dehydrogenase (discussed in Chapter 16), appears to be closely associated with nitrate reductase. Formate is about as strong a reducing agent as NADH (Table 6-8) and is a preferred electron donor for the reduction of NO_3^- (Eq. 18-26).[329a,b] Since cytochrome *c* oxidase of the electron transport chain is bypassed, one less ATP is formed than when O_2 is the oxidant. Nitrate is the oxidant preferred by bacteria grown under anaerobic conditions. The

$$HCOO^- + NO_3^- + H^+ \rightarrow NO_2^- + CO_2 + H_2O$$
$$\Delta G'\ (pH\ 7) = 165\ kJ\ mol^{-1} \qquad (18\text{-}26)$$

presence of NO_3^- induces the synthesis of nitrate reductase and represses the synthesis of alternative enzymes such as **fumarate reductase**,[330,331] which reduces fumarate to succinate (see also p. 1027). On the other hand, if NO_3^- is absent and fumarate, which can be formed from pyruvate, is present, synthesis of fumarate reductase is induced. Although it is a much weaker oxidant than is nitrate ($E^{o'}$ = 0.031 V), fumarate is still able to oxidize H_2 or NADH with oxidative phosphorylation. Like the related succinate dehydrogenase, fumarate reductase of *E. coli* is a flavoprotein with associated Fe / S centers. It contains covalently linked FAD and Fe_2S_2, Fe_4S_4, and three-Fe iron–sulfur centers.[332] In some bacteria a soluble periplasmic cytochrome c_3 carried out the fumarate reduction step.[332a] Trimethylamine *N*-oxide[330,333] or dimethylsulfoxide (DMSO; Eq. 16-62)[334,335] can also serve as alternative oxidants for anaerobic respiration using appropriate molybdemum-containing reductases (Chapter 16).

Reduction of nitrite: denitrification. The nitrite formed in Eq. 18-25 is usually reduced further to ammonium ions (Eq. 18-27). The reaction may not be important to the energy metabolism of the bacteria, but it provides NH_4^+ for biosynthesis. This six-electron reduction is catalyzed by a hexaheme protein containing six *c*-type hemes bound to a single 63-kDa polypeptide chain.[336,337]

$$NO_2^- + 8\ H^+ + 6\ e^- \rightarrow 2\ H_2O + NH_4^+ \qquad (18\text{-}27)$$

Several types of **denitrifying bacteria**[315,338–340]

use either nitrate or nitrite ions as oxidants and reduce nitrite to N_2. A typical reaction for *Micrococcus denitrificans* is oxidation of H_2 by nitrate (Eq. 18-28). *Thiobacillus denitrificans*, like other thiobacilli, can oxidize

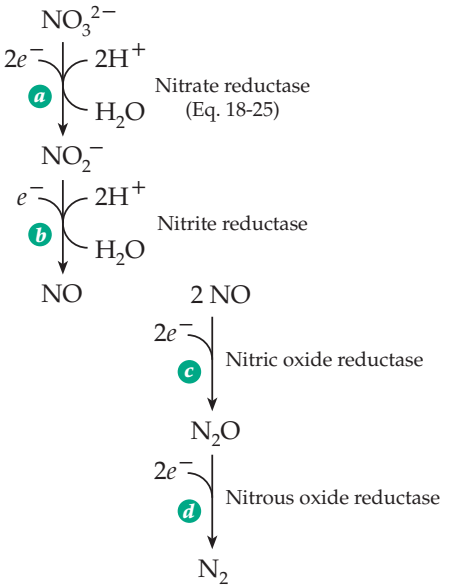
$$5\ H_2 + 2\ NO_3^- + 2\ H^+ \rightarrow N_2 + 6\ H_2O$$
$$\Delta G'\ (\text{pH } 7) = -561\ \text{kJ mol}^{-1}\ \text{of nitrate reduced or}$$
$$-224\ \text{kJ mol}^{-1}\ \text{of } H_2\ \text{oxidized} \qquad (18\text{-}28)$$

sulfur as well as H_2S or thiosulfate using nitrate as the oxidant (Eq. 18-29):

$$5\ S + 6\ NO_3^- + 2\ H_2O \rightarrow 5\ SO_4^{2-} + 3\ N_2 + 4\ H^+$$
$$\Delta G'\ (\text{pH } 7) = -455\ \text{kJ mol}^{-1}\ \text{of nitrate reduced or}$$
$$-546\ \text{kJ mol}^{-1}\ \text{of S oxidized} \qquad (18\text{-}29)$$

The reactions begin with reduction of nitrate to nitrite (Eq. 18-25) and continue with further reduction of nitrite to nitric oxide, **NO**; nitrous oxide, $\mathbf{N_2O}$; and dinitrogen, $\mathbf{N_2}$. A probable arrangement of the four enzymes needed for the reactions of Eq. 18-30 in *Paracoccus denitrificans* is shown in Fig. 18-21. See also pp. 884, 885.

Two types of dissimilatory nitrite reductases catalyze step *b* of Eq. 18-30. Some bacteria use a copper-containing enzyme, which contains a type 1 (blue) copper bound to a β barrel domain of one subunit and a type 2 copper at the catalytic center. The type 1 copper is thought to receive electrons from the small copper-containing carrier pseudoazurin (Chapter 16).[341–342b]



(18-30)

More prevalent is **cytochrome cd_1** nitrite reductase.[340,343–346] The water-soluble periplasmic enzyme is a homodimer of ~60-kDa subunits, each containing a *c*-type heme in a small N-terminal domain and **heme d_1**, a ferric dioxoisobacteriochlorin (Fig. 16-6). The

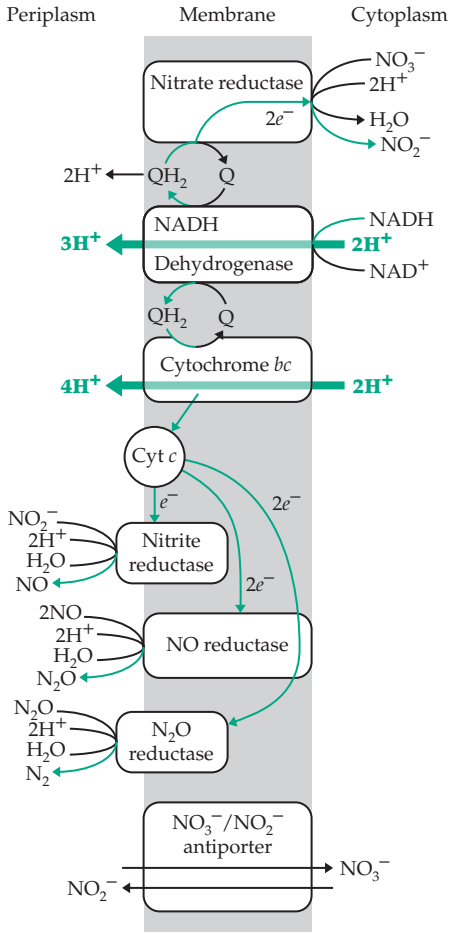


Figure 18-21 Organization of the nitrate reduction system in the outer membrane of the bacterium *Paracoccus dinitrificans* as outlined by Blaut and Gottschalk.[315] The equations are not balanced as shown but will be balanced if two NO_3^- ions are reduced to N_2 by five molecules of NADH (see also Eq. 18-28). Although this will also require seven protons, about 20 additional H^+ will be pumped to provide for ATP synthesis.

latter is present in the central channel of an eight-bladed β-sheet propeller[345-346g] similar to that in Fig. 15-23A. The heme d_1 is unusual in having its Fe atom ligated by a tyrosine hydroxyl oxygen, which may be displaced to allow binding of NO_3^-. The electron required for the reduction is presumably transferred from the electron transfer chain in the membrane to the heme *d*, via the heme *c* group.[347] Cytochrome cd_1 nitrite reductases have an unexpected second enzymatic activity. They catalyze the four-electron reduction of O_2 to H_2O, as does cytochrome *c* oxidase. However, the rate is much slower than that of nitrite reduction.[340,348]

The enzyme catalyzing the third step of Eq. 18-30 (step *c*), **nitric oxide reductase**, is an unstable membrane-bound protein cytochrome *bc* complex.[349,350]

It has been isolated as a two-subunit protein, but genetic evidence suggests the presence of additional subunits.[350] The small subunit is a cytochrome *c*, while the larger subunit is predicted to bind two protohemes as well as a nonheme iron center. This protein also shows sequence homology with cytochrome *c* oxidase. It contains no copper, but it has been suggested that a heme *b*–nonheme Fe center similar to the heme *a*–Cu_B center of cytochrome *c* oxidase may be present. It may be the site at which the nitrogen atoms of two molecules of NO are joined to form N_2O.[350,351] A different kind of NO reductase is utilized by the denitrifying fungus *Fusarium oxysporum*. It is a cytochrome P450 but with an unusually low redox potential (−0.307 V). This **cytochrome P450$_{nor}$** does not react with O_2 (as in Eq. 18-57) but binds NO to its heme Fe^{3+}, reduces the complex with two electrons from NADH, then reacts with a second molecule of NO to give N_2O and H_2O.[352]

Reduction of N_2O to N_2 by bacteria (Eq. 18-30, step *d*) is catalyzed by the copper-containing nitrous oxide reductase. The purple enzyme is a dimer of 66-kDa subunits, each containing four atoms of Cu.[353] It has spectroscopic properties similar to those of cytochome *c* oxidase and a dinuclear copper–thiolate center similar to that of Cu_A in cytochrome *c* oxidase (p. 1030). The nature of the active site is uncertain.[354]

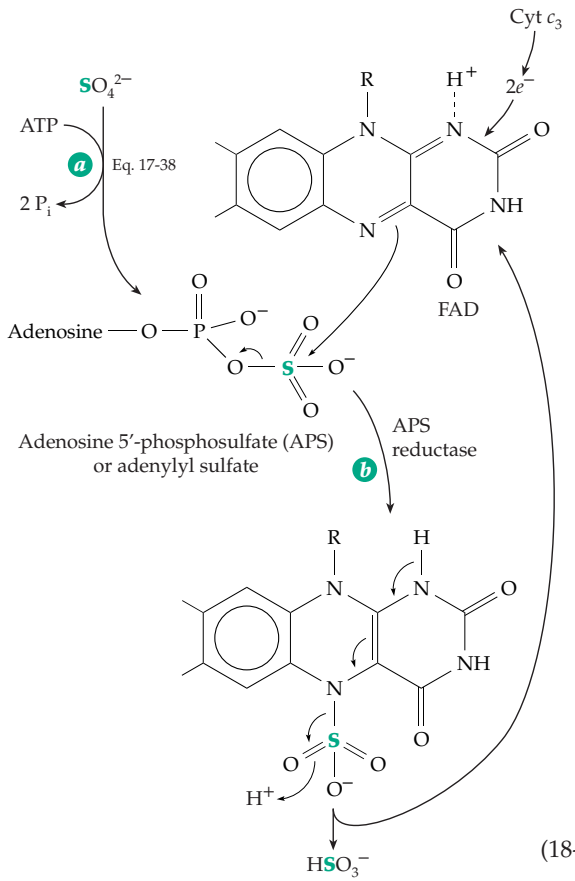
Sulfate-reducing and sulfur-reducing bacteria. A few obligate anaerobes obtain energy by using sulfate ion as an oxidant.[355–356a] For example, *Desulfovibrio desulfuricans* catalyzes a rapid oxidation of H_2 with reduction of sulfate to H_2S (Eq. 18-31).

$$4\,H_2 + SO_4^{2-} + 2\,H^+ \rightarrow H_2S + 4\,H_2O$$
$$\Delta G'\ (\text{pH } 7) = -154\ \text{kJ mol}^{-1}\ \text{of sulfate reduced} \qquad (18\text{-}31)$$

While this may seem like an esoteric biological process, the reaction is quantitatively significant. For example, it has been estimated that within the Great Salt Lake basin bacteria release sulfur as H_2S in an amount of 10^4 metric tons (10^7 kg) per year.[357]

The reduction potential for sulfate is extremely low ($E°'$, pH 7 = −0.454 V), and organisms are not known to reduce it directly to sulfite. Rather, a molecule of ATP is utilized to form adenosine 5'-phosphosulfate (APS) through the action of **ATP sulfurylase** (ATP:sulfate adenylyltransferase, Eq. 17-38).[358,359] APS is then reduced by cytochrome c_3 (Eq. 18-32, step *b*). The 13-kDa low-potential ($E°'$, pH 7 = 0.21 V) cytochrome c_3 contains four heme groups (Figure 16-8C) and is found in high concentration in sulfate-reducing bacteria.[360,361] Some of these bacteria have larger polyheme cytochromes *c*.[361a] For example, *Desulfovibrio vulgaris* forms a 514-residue protein carrying 16 hemes organized as four cytochrome c_3-like domains.[362] Each heme in cytochrome c_3 has a distinct redox potential within the range −0.20 to −0.40 V.[361–363]

APS is reduced (Eq. 18-32, step *b*) by **APS reductase**, a 220-kDa iron–sulfur protein containing FAD and several Fe−S clusters. An intermediate in the reaction may be the adduct of sulfite with FAD, which may be formed as in Eq. 18-32. The initial step in this hypothetical mechanism is displacement on sulfur by a strong nucleophile generated by transfer of electrons from reduced ferredoxin to cytochrome c_3 to the flavin.[364]



(18-32)

Bisulfite produced according to Eq. 18-32 is reduced further by a **sulfite reductase,** which is thought to receive electrons from flavodoxin, cyt c_3, and a hydrogenase. ATP synthesis is coupled to the reduction. Sulfite reductases generally contain both siroheme and Fe_4S_4 clusters (Fig. 16-19). They appear to be able to carry out the 6-electron reduction to sulfide without accumulation of intermediates.[365,366] However, in contrast to the assimilatory sulfite reductases present in many organisms, the dissimilatory nitrite reductases of sulfur-reducing bacteria may also release some thiosulfate $S_2O_3^{2-}$.[367] A possible role of menaquinone (vitamin K_2), present in large amounts in *Desulfovibrio*, has been suggested.[311] Although *Desulfovibrio* can obtain their energy from Eq. 18-31, they are not true autotrophs and must utilize compounds such as acetate together with CO_2 as a carbon source.

Some thermophilic archaeobacteria are able to live with CO_2 as their sole source of carbon and reduction of elemental sulfur with H_2 (Eq. 18-33) as their sole source of energy.[368,369]

$$H_2 + S^0 \rightarrow H_2S$$
$$\Delta G^\circ = -27.4 \text{ kJ mol}^{-1} \qquad (18\text{-}33)$$

This is remarkable in view of the small standard Gibbs energy decrease. Some species of the archaeobacterium *Sulfolobus* are able either to live aerobically oxidizing sulfide to sulfate with O_2 (Eq. 18-22) or to live anaerobically using reduction of sulfur by Eq. 18-33 as their source of energy.[369]

The sulfate-reducing bacterium *Desulfovibrio sulfodismutans* carries out what can be described as "inorganic fermentations" which combine the oxidation of compounds such as sulfite or thiosulfate (as observed for sulfur-oxidizing bacteria; Eq. 18-22), with reduction of the same compounds (Eq. 18-34).[370,370a] Dismutation of $S_2O_3^{2-}$ plus H_2O to form SO_4^{2-} and H_2S also occurs but with a less negative Gibbs energy change.

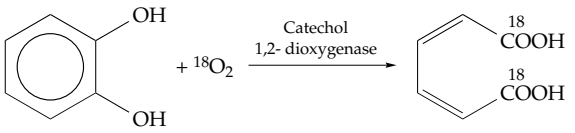
$$4\ SO_3^{2-} + H^+ \rightarrow 3\ SO_4^{2-} + HS^-$$
$$\Delta G' \text{ (pH 7)} = -185 \text{ kJ/mol} \qquad (18\text{-}34)$$

A strain of *Pseudomonas* obtains all of its energy by reducing sulfate using phosphite, which is oxidized to phosphate.[370b]

Methane bacteria. The methane-producing bacteria (Chapter 15) are also classified as chemiautotrophic organisms. While they can utilize substances such as methanol and acetic acid, they can also reduce CO_2 to methane and water using H_2 (Fig. 15-22). The electron transport is from hydrogenase, perhaps through ferredoxin to formate dehydrogenase and via the deazaflavin F_{420} and $NADP^+$ to the methanopterin-dependent dehydrogenases that carry out the stepwise reduction of formate to methyl groups (Fig. 16-28). Generation of ATP probably involves proton pumps, perhaps in internal coupling membranes .[315,371]

F. Oxygenases and Hydroxylases

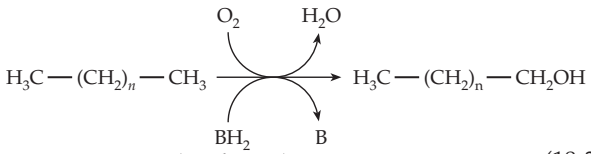
For many years the idea of dehydrogenation dominated thinking about biological oxidation. Many scientists assumed that the oxygen found in organic substances always came from water, e.g., by addition of water to a double bond followed by dehydrogenation of the resulting alcohol. Nevertheless, it was observed that small amounts of O_2 were essential, even to anaerobically growing cells.[372] In 1955, Hayaishi and Mason independently demonstrated that ^{18}O was sometimes incorporated into organic compounds directly from $^{18}O_2$ as in Eq. 18-35. Today a bewildering variety of **oxygenases** are



Catechol *Cis,cis*-muconic acid (18-35)

known to function in forming such essential metabolites as sterols, prostaglandins, and active derivatives of vitamin D. Oxygenases are also needed in the catabolism of many substances, often acting on nonpolar groups that cannot be attacked readily by other types of enzyme.[372]

Oxygenases are classified either as **dioxygenases** or as **monooxygenases**. The monooxygenases are also called mixed function oxidases or **hydroxylases**. Dioxygenases catalyze incorporation of two atoms of oxygen as in Eq. 18-35, but monooxygenases incorporate only one atom. The other oxygen atom from the O_2 is converted to water. A typical monooxygenase-catalyzed reaction is the hydroxylation of an alkane to an alcohol (Eq. 18-36).



(18-36)

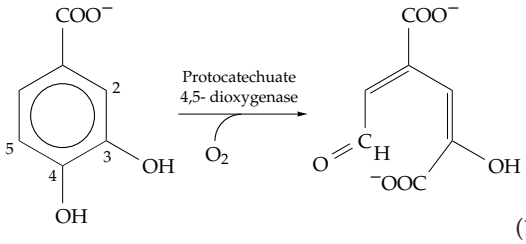
A characteristic of the monooxygenases is that an additional reduced substrate, a **cosubstrate** (BH_2 in Eq. 18-36), is usually required to reduce the second atom of the O_2 molecule to H_2O.

Since O_2 exists in a "triplet" state with two unpaired electrons, it reacts rapidly only with transition metal ions or with organic radicals (Chapter 16). For this reason, most oxygenases contain a transition metal ion, usually of iron or copper, or contain a cofactor, such as FAD, that can easily form a radical or act on a cosubstrate or substrate to form a free radical.

1. Dioxygenases

Among the best known of the oxygenases that incorporate both atoms of O_2 into the product are those that participate in the biological degradation of aromatic compounds by cleaving double bonds at positions between two OH groups as in Eq. 18-35 or adjacent to one OH group of an *ortho* or *para* hydroxyl pair.[373] A much studied example is **protocatechuate 3,4-dioxygenase**,[373–375] which cleaves its substrate

between the two OH groups (*intradiol cleavage*) as in Eq. 18-35. A different enzyme, **protocatechuate 4,5-dioxygenase**,[357] cleaves the same substrate next to just one of the two OH groups (*extradiol cleavage*; Eq. 18-37) to form the aldehyde α-hydroxy-δ-carboxymuconic semialdehyde. Another extradiol cleaving enzyme, **protocatechuate 2,3-dioxygenase**, acts on the same substrate. Many other dioxygenases attack related substrates.[376–380] Intradiol-cleaving enzymes are usually iron-tyrosinate proteins (Chapter 16) in which the



(18-37)

iron is present in the Fe(III) oxidation state and remains in this state throughout the catalytic cycle.[375] The enzymes usually have two subunits and no organic prosthetic groups. For example, a protocatechuate 3,4-dioxygenase from *Pseudomonas* has the composition $(\alpha\beta Fe)_{12}$ with subunit masses of 23 (α) and 26.5 (β) kDa. The iron is held in the active site cleft between the α and β subunits by Tyr 408, Tyr 447, His 460, and His 462 of the β subunit and a water molecule.[375] These enzymes and many other oxygenases probably assist the substrate in forming radicals that can react with O_2 to form organic peroxides. Some plausible intermediate species are pictured in Fig. 18-22. The reactions are depicted as occurring in two-electron steps. However, O_2 is a diradical, and it is likely that the Fe^{3+}, which is initially coordinated to both phenolate groups of the ionized substrate, assists in forming an organic free radical that reacts with O_2.

Extradiol dioxygenases have single Fe^{2+} ions in their active sites. The O_2 probably binds to the Fe^{2+} and may be converted transiently to an Fe^{3+}-superoxide complex which adds to the substrate. Some extradiol dioxygenases require an Fe_2S_2 ferredoxin to reduce any Fe^{3+}-enzyme that is formed as a side reaction back to the Fe^{2+} state.[381] Possible intermediates are given in Fig. 18-22 (left side) with two-electron steps used to save space and to avoid giving uncertain details about free radical intermediates. Formation of the organic radical is facilitated by the iron atom, which may be coordinated initially to both phenolate groups of the ionized substrate. The peroxide intermediates, for both types of dioxygenases, may react and be converted to various final products by several mechanisms.[382]

Tryptophan dioxygenase (indoleamine 2,3-dioxygenase)[383] is a heme protein which catalyzes the reaction of Eq. 18-38. The oxygen atoms designated by

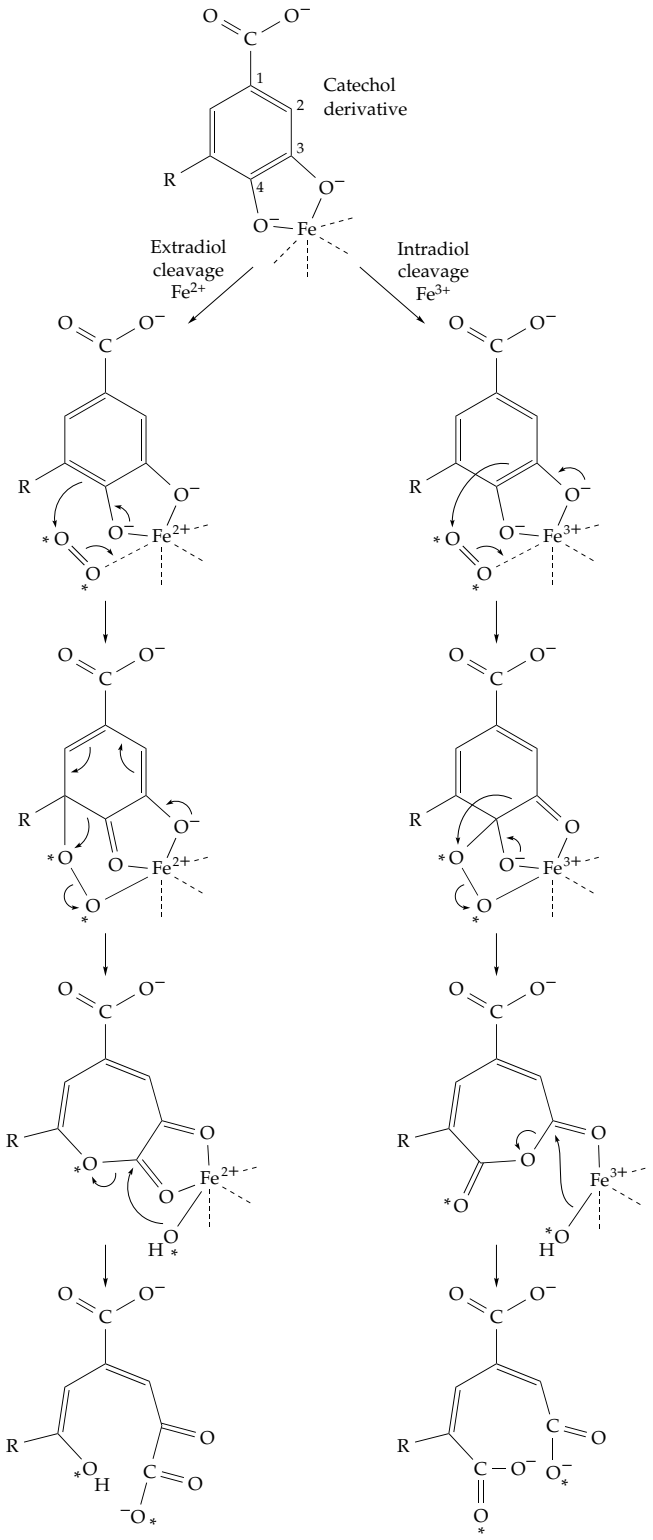
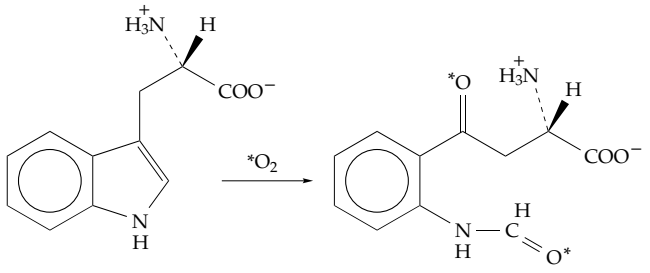


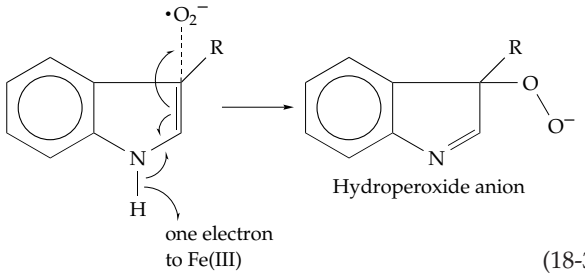
Figure 18-22 Some possible intermediates in the action of extradiol (left) and intradiol (right) aromatic dioxygenases. Although the steps depict the flow of pairs of electrons during the formation and reaction of peroxide intermediates, the mechanisms probably involve free radicals whose formation is initiated by O_2. The asterisks show how two atoms of labeled oxygen can be incorporated into final products. After Ohlendorf *et al.*[374]

the asterisks are derived from O_2. Again, the first step is probably the formation of a complex between Fe(II) and O_2, but tryptophan must also be present before this can occur. At 5°C the enzyme, tryptophan, and O_2 combine to give an altered spectrum reminiscent of that of compound III of peroxidase (Fig. 16-14). This oxygenated complex may, perhaps, then be converted to a complex of Fe(II) and superoxide ion.



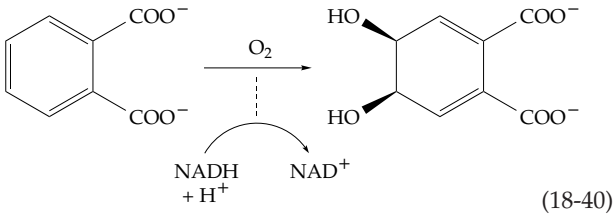
(18-38)

There is much evidence, including inhibition by superoxide dismutase and stimulation by added potassium superoxide,[384] that the superoxide anion radical is the species that attacks the substrate (Eq. 18-39). In this reaction one electron is returned to the Fe(III) form of the enzyme to regenerate the original Fe(II) form. Subsequent reaction of the hydroperoxide anion would give the observed products.



(18-39)

Some dioxygenases require a cosubstrate. For example, **phthalate dioxygenase**[385] converts phthalate to a *cis*-dihydroxy derivative with NADH as the cosubstrate (Eq. 18-40). Similar double hydroxylation reactions catalyzed by soil bacteria are known for benzene, benzoate,[386] toluene, naphthalene, and several other aromatic compounds.[386a] The formation of the *cis*-glycols is usually followed by dehydrogenation or oxidative decarboxylation by NAD^+ to give a catechol, whose ring is then opened by another dioxygenase reaction (Chapter 25). An elimination of Cl^- follows dioxygenase action on *p*-chlorophenylacetate and produces 3,4-dihydroxyphenylacetate as a product. Pthalate dioxygenases consist of two subunits. The 50-kDa dioxygenase subunits receive electrons from reductase subunits that contain a Rieske-type Fe–S



(18-40)

centers and bound FMN.[387] The dioxygenase also contains an Fe_2S_2 center, and electrons flow from NADH to FMN and through the two Fe–S centers to the Fe^{2+} of the active site.[387–388]

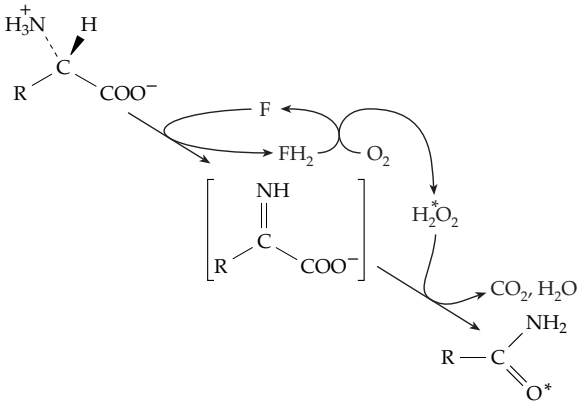
Lipoxygenases catalyze oxidation of polyunsaturated fatty acids in plant lipids. Within animal tissues the lipoxygenase-catalyzed reaction of arachidonic acid with O_2 is the first step in formation of **leukotrienes** and other mediators of inflammation. These reactions are discussed in Chapter 21.

2. Monooxygenases

Two classes of monooxygenases are known. Those requiring a cosubstrate (BH_2 of Eq. 18-36) in addition to the substrate to be hydroxylated are known as **external monooxygenases**. In the other group, the **internal monooxygenases**, some portion of the substrate being hydroxylated also serves as the cosubstrate. Many internal monooxygenases contain flavin cofactors and are devoid of metal ions.

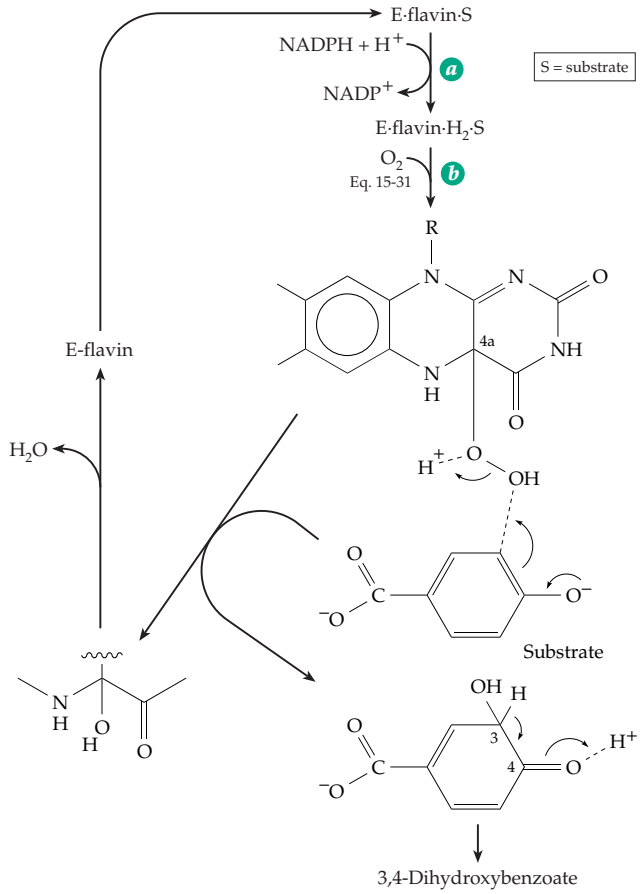
Flavin-containing monooxygenases. One group of flavin-dependent monooxygenases form H_2O_2 by reaction of O_2 with the reduced flavin and use the H_2O_2 to hydroxylate 2-oxoacids. An example is **lactate monooxygenase,** which apparently dehydrogenates lactate to pyruvate and then oxidatively decarboxylates the pyruvate to acetate with H_2O_2 (Eq. 15-36). One atom of oxygen from O_2 is incorporated into the acetate formed.[389,390] In a similar manner, the FAD-containing bacterial **lysine monooxygenase** probably catalyzes the sequence of reactions shown in Eq. 18-41.[391] When native lysine monooxygenase was treated with sulfhydryl-blocking reagents the resulting modified enzyme produced a 2-oxoacid, ammonia, and H_2O_2, just the products predicted from the hydrolytic decomposition of the bracketed intermediate of Eq. 18-41. Similar bacterial enzymes act on tryptophan and phenylalanine.[392]

NADPH can serve as a cosubstrate of flavoprotein monooxygenase by first reducing the flavin, after which the reduced flavin can react with O_2 to generate the hydroxylating reagent.[393] An example is the bacterial **4-hydroxybenzoate hydroxylase** which forms 3,4-dihydroxybenzoate.[394] The 43-kDa protein consists of three domains, the large FAD-binding domain being folded in nearly the same way as that of glutathione reductase (Fig. 15-10). The 4-hydroxybenzoate binds



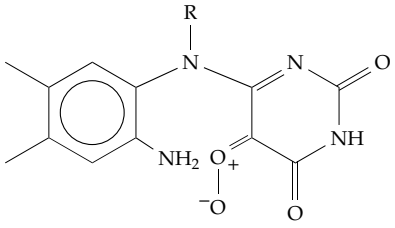
(18-41)

first into a deep cleft below the N-5 edge of the isoalloxazine ring of the FAD; then the NADH binds. Spectroscopic studies have shown the existence of at least three intermediates. The first of these has been identified as the 4a-peroxide whose formation (Eq. 15-31) is discussed in Chapter 15. The third intermediate is the corresponding 4a-hydroxyl compound. The substrate hydroxylation must occur in a reaction with the flavin peroxide, presumably with the phenolate anion form of the substrate (Eq. 18-42).[395] The initial hydroxylation product is tautomerized to form the product 3,4-dihydroxybenzoate.

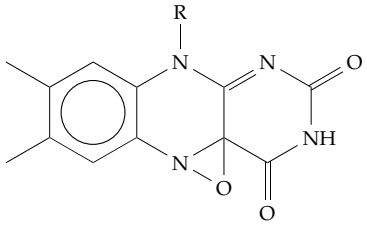


(18-42)

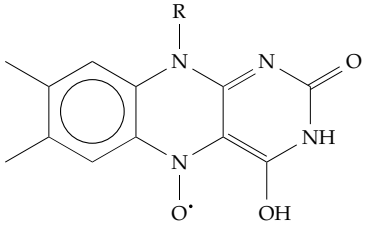
According to this mechanism, one of the two oxygen atoms in the hydroperoxide reacts with the aromatic substrate, perhaps as OH^+ or as a superoxide radical. A variety of mechanisms for activating the flavin peroxide to give a more potent hydroxylating reagent have been proposed. These include opening of the central ring of the flavin to give a carbonyl oxide intermediate which could transfer an oxygen atom to the substrate,[396] elimination of H_2O to form an **oxaziridine,**[397] or rearrangment to a **nitroxyl radical**.[398] Any of these might be an active electrophilic hydroxylating reagent. However, X-ray structural studies suggest that conformational changes isolate the substrate–FAD-enzyme complex from the medium stabilizing the 4a peroxide via hydrogen bonding[399–400] in close proximity to the substrate. Reaction could occur by the simple mechanism of Eq. 18-42, a mechanism also supported by ^{19}F NMR studies with fluorinated substrate analogs[401] and other investigations.[401a,b]



Carbonyl oxide



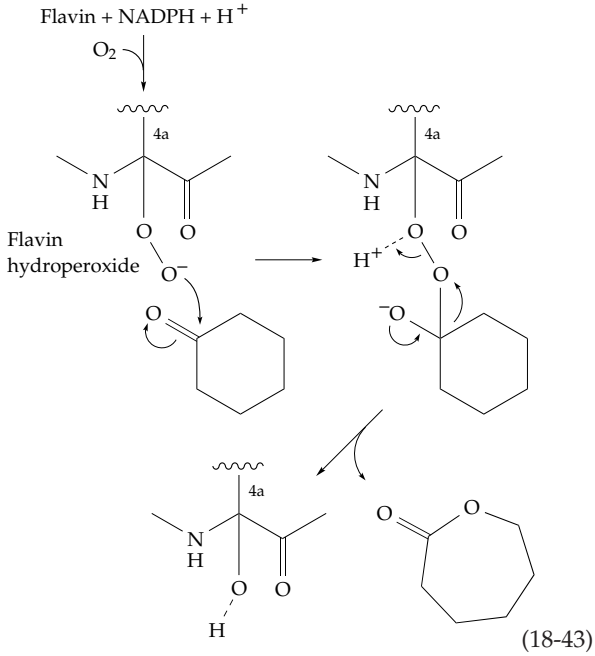
An oxaziridine



A nitroxyl radical

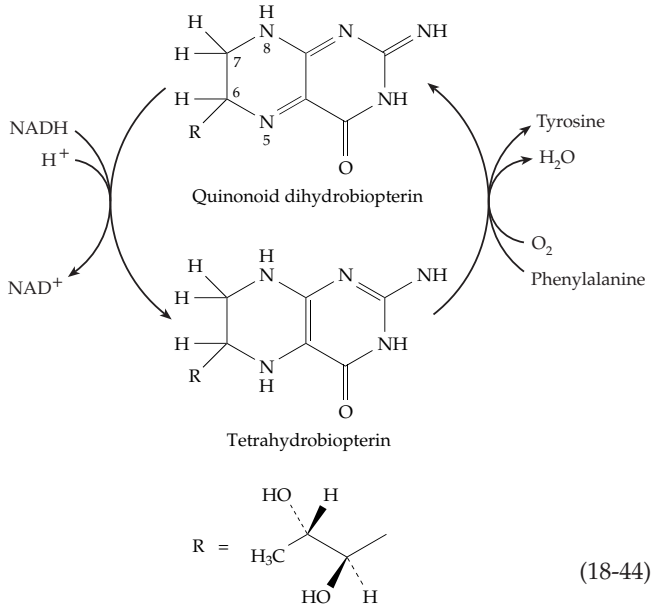
Related flavin hydroxylases act at nucleophilic positions on a variety of molecules[393,402] including phenol,[403] salicylate,[404] anthranilate,[405] *p*-cresol,[406] 4-hydroxyphenylacetate,[407,408] and 4-aminobenzoate.[409] Various microsomal flavin hydroxylases are also known.[410] Flavin peroxide intermediates are also able to hydroxylate some electrophiles.[411] For example, the bacterial **cyclohexanone oxygenase** catalyzes

the ketone to lactone conversion of Eq. 18-43.[411a] The mechanism presumably involves the nucleophilic attack of the flavin hydroperoxide on the carbonyl group of the substrate followed by rearrangement. This parallels the Baeyer–Villiger rearrangement that results from treatment of ketones with peracids.[393] Cyclohexanone oxygenase also catalyzes a variety of other reactions,[412] including conversion of sulfides to sulfoxides.



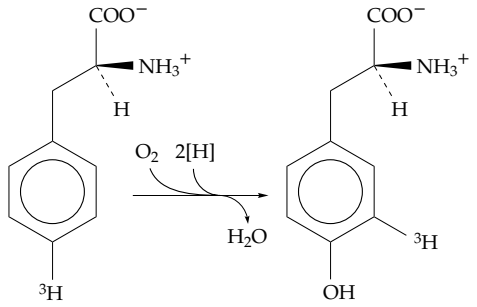
(18-43)

Reduced pteridines as cosubstrates. A dihydro form of biopterin (Fig. 15-17) serves as a cosubstrate, that is reduced by NADPH (Eq. 18-44) in hydroxylases that act on phenylalanine, tyrosine, and tryptophan.



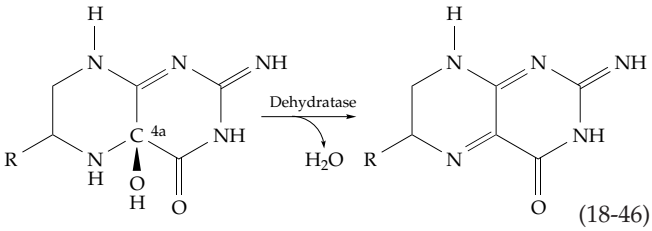
(18-44)

The tetrahydrobiopterin formed in this reaction is similar in structure to a reduced flavin. The mechanism of its interaction with O_2 could reasonably be the same as that of 4-hydroxybenzoate hydroxylase. However, **phenylalanine hydroxylase**, which catalyzes the formation of tyrosine (Eq. 18-45), a dimer of 451-residue subunits, contains one Fe per subunit,[413–415a] whereas flavin monooxygenases are devoid of iron. **Tyrosine hydroxylase**[416–419a] and **tryptophan hydroxylase**[420] have very similar properties. All three enzymes contain regulatory, catalytic, and tetramerization domains as well as a common Fe-binding motif in their active sites.[413,421,421a]



(18-45)

The role of the iron atom in these enzymes must be to accept an oxygen atom from the flavin peroxide, perhaps forming a reactive ferryl ion and transferring the oxygen atom to the substrate, e.g., as do cytochromes P450 (see Eq. 18-57). The 4*a*-hydroxytetrahydrobiopterin, expected as an intermediate if the mechanism parallels that of Eq. 18-42, has been identified by its ultraviolet absorption spectrum.[422] A ring-opened intermediate has also been ruled out for phenylalanine hydroxylase.[423] However, the 4*a* –OH adduct has been observed by ^{13}C-NMR spectroscopy. Its absolute configuration is 4*a*(S) and the observation of an ^{18}O-induced shift in the ^{13}C resonance of the 4*a*-carbon atom[424] confirms the origin of this oxygen from $^{18}O_2$ (see Eq. 18-42). A "stimulator protein" needed for rapid reaction of phenylalanine hydroxylase has been identified as a **4*a*-carbinolamine dehydratase** (Eq. 18-46).[425–426] This protein also has an unexpected function as part of a complex with transcription factor HNF1 which is found in nuclei of liver cells.[425a,426]



(18-46)

Dihydrobiopterin can exist as a number of isomers. The quinonoid form shown in Eqs. 18-44 and 18-46 is

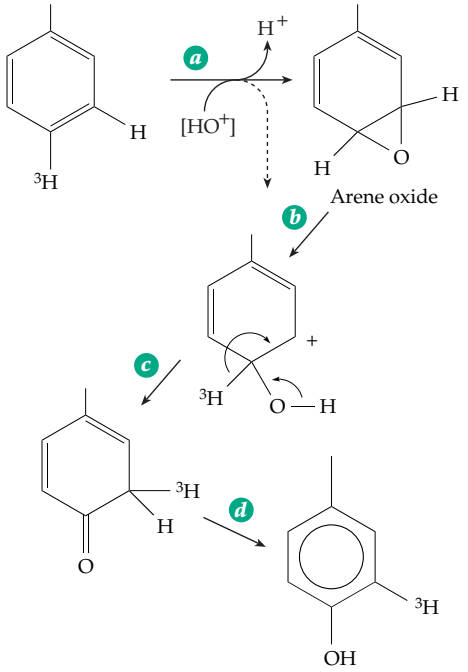
a tautomer of 7,8-dihydrobiopterin, the form generated by dihydrofolate reductase (Chapter 15). A pyridine nucleotide-dependent **dihydropteridine reductase**[427–429] catalyzes the left-hand reaction of Eq. 18-44.

The hereditary absence of phenylalanine hydroxylase, which is found principally in the liver, is the cause of the biochemical defect **phenylketonuria** (Chapter 25, Section B).[430,430a] Especially important in the metabolism of the brain are tyrosine hydroxylase, which converts tyrosine to 3,4-dihydroxyphenylalanine, the rate-limiting step in biosynthesis of the catecholamines (Chapter 25), and tryptophan hydroxylase, which catalyzes formation of 5-hydroxytryptophan, the first step in synthesis of the neurotransmitter 5-hydroxytryptamine (Chapter 25). All three of the pterin-dependent hydroxylases are under complex regulatory control.[431,432] For example, tyrosine hydroxylase is acted on by at least four kinases with phosphorylation occurring at several sites.[431,433,433a] The kinases are responsive to nerve growth factor and epidermal growth factor,[434] cAMP,[435] Ca^{2+} + calmodulin, and Ca^{2+} + phospholipid (protein kinase C).[436] The hydroxylase is inhibited by its endproducts, the catecholamines,[435] and its activity is also affected by the availability of tetrahydrobiopterin.[436]

Hydroxylation-induced migration. A general result of enzymatic hydroxylation of aromatic compounds is the intramolecular migration of a hydrogen atom or of a substituent atom or group as is shown for the ^3H atom in Eq. 18-45.[437] Dubbed the NIH shift (because the workers discovering it were in a National Institutes of Health laboratory), the migration tells us something about possible mechanisms of hydroxylation. In Eq. 18-45 a tritium atom has shifted in response to the entering of the hydroxyl group. The migration can be visualized as resulting from electrophilic attack on the aromatic system, e.g., by an oxygen atom from Fe(N)=O or by OH^+ (Eq. 18-47).

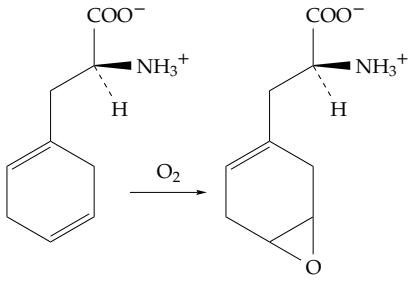
Such an attack could lead in step *a* either to an **epoxide** (**arene oxide**) or directly to a carbocation as shown in Eq. 18-47. Arene oxides can be converted, via the carbocation step *b*, to end products in which the NIH shift has occurred.[438] The fact that phenylalanine hydroxylase also catalyzes the conversion of the special substrate shown in Eq. 18-48 to a stable epoxide, which cannot readily undergo ring opening, also supports this mechanism.

Operation of the NIH shift can cause migration of a large substituent as is illustrated by the hydroxylation of 4-hydroxyphenylpyruvate (Eq. 18-49), a key step in the catabolism of tyrosine (Chapter 25). Human 4-hydroxyphenylpyruvate dioxygenase is a dimer of 43-kDa subunits.[439] A similar enzyme from *Pseudomonas* is a 150-kDa tetrameric iron-tyrosinate protein, which must be maintained in the reduced Fe(II) state for catalytic activity.[440] Although this enzyme is a



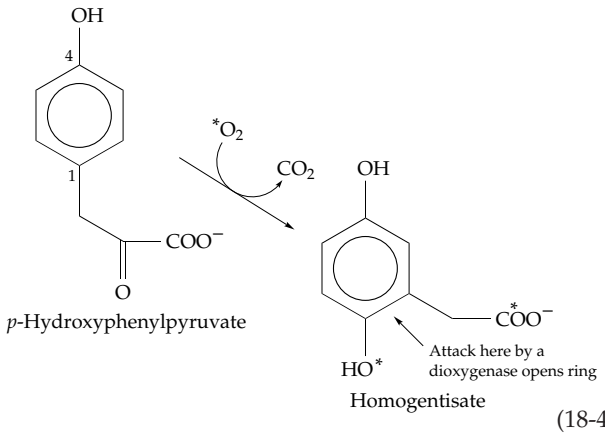
(18-47)

dioxygenase, it is probably related in its mechanism of action to the 2-oxoglutarate-dependent monooxygenases discussed in the next section (Eqs. 18-51, 18-52). It probably uses the oxoacid side chain of the substrate



(18-48)

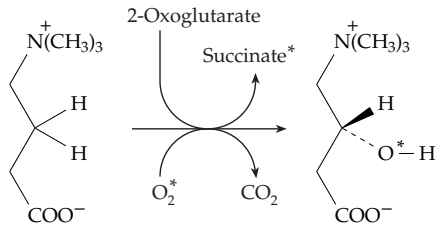
to generate a reactive oxygen intermediate such as Fe(IV)=O by the decarboxylative mechanism of Eqs. 18-50 and 18-51. The iron-bound oxygen attacks C1 of



(18-49)

the aromatic ring, the electron-donating *p*-hydroxyl group assisting. This generates a hydroxylated carbocation of the type shown in Eq. 18-47 in which the whole two-carbon side chain undergoes the NIH shift.

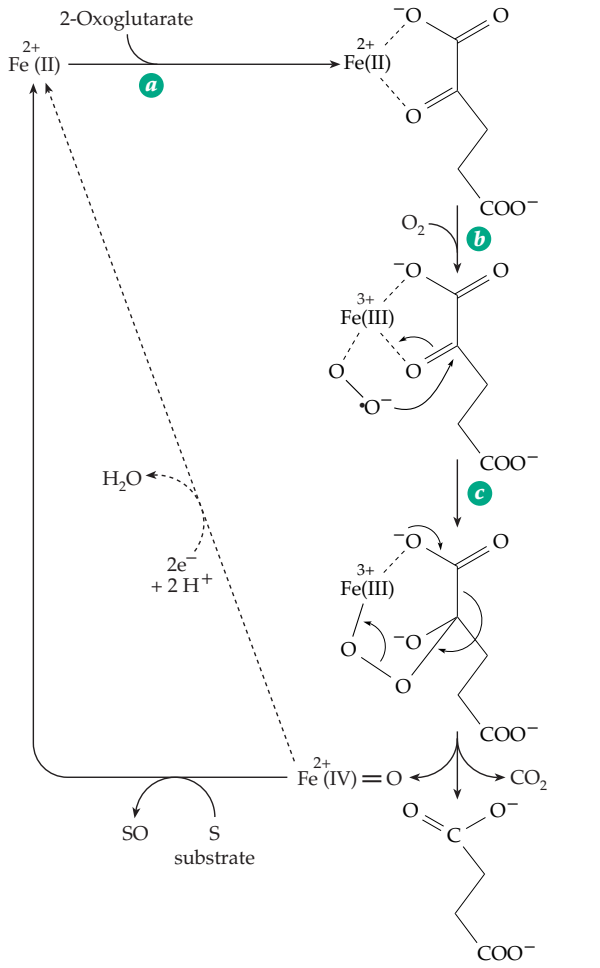
2-Oxoglutarate as a decarboxylating cosubstrate. Several oxygenases accept hydrogen atoms from 2-oxoglutarate, which is decarboxylated in the process to form succinate. Among these are enzymes catalyzing hydroxylation of residues of proline in both the 3- and 4-positions (Eq. 8-6)[441–444] and of lysine in the 5-position (Eq. 8-7)[445,446] in the collagen precursor **procollagen**. The hydroxylation of prolyl residues also takes place within the cell walls of plants.[447] Similar enzymes hydroxylate the β-carbon of aspartyl or asparaginyl side chains in EGF domains (Table 7-3) of proteins.[441] Thymine[448] and taurine[449,449a] are acted on by related dioxygenases. A bacterial oxygenase initiates the degradation of the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) using another 2-oxoglutarate-dependent hydroxylase.[450,451] In the human body a similar enzyme hydroxylates γ-butyrobetaine to form carnitine (Eq. 18-50).[452] All of



γ-Butyrobetaine ʟ-Carnitine (18-50)

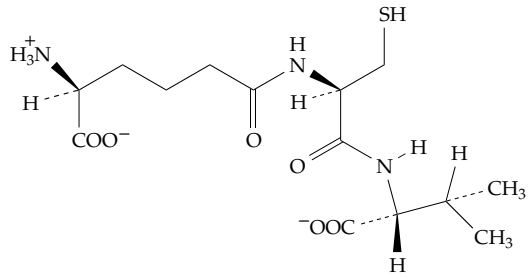
these enzymes contain iron and require ascorbate, whose function is apparently to prevent the oxidation of the iron to the Fe(III) state.

When $^{18}O_2$ is used for the hydroxylation of γ-butyrobetaine (Eq. 18-51), one atom of ^{18}O is found in the carnitine and one in succinate. The reaction is stereospecific and occurs with retention of configuration at C-3, the *pro*-R hydrogen being replaced by OH while the *pro*-S hydrogen stays.[453] Under some conditions these enzymes decarboxylate 2-oxoglutarate in the absence of a hydroxylatable substrate, the iron being oxidized to Fe^{3+} and ascorbate being consumed stoichiometrically.[454] A plausible mechanism (Eq. 18-51) involves formation of an Fe(II)–O_2 complex, conversion to Fe(III)$^+$ $^\bullet O_2^-$, and addition of the superoxide ion to 2-oxoglutarate to form an adduct.[451,455] Decarboxylation of this adduct could generate the oxidizing reagent, perhaps Fe(IV)=O. In the absence of substrate S the ferryl iron could be reconverted to Fe(II) by a suitable reductant such as ascorbate. In the absence of ascorbate the Fe(IV) might be reduced to a catalytically inert Fe(III) form.

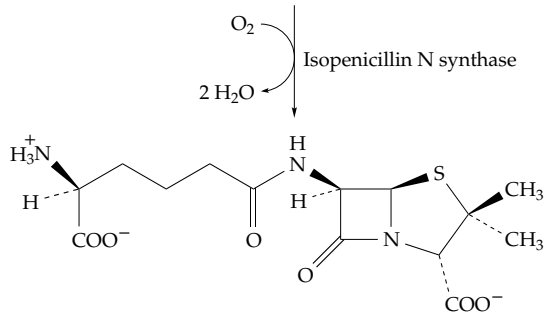


(18-51)

An unusual oxygenase with a single Fe^{2+} ion in its active site closes the four-membered ring in the biosynthesis of penicillins (Eq. 18-52). It transfers four



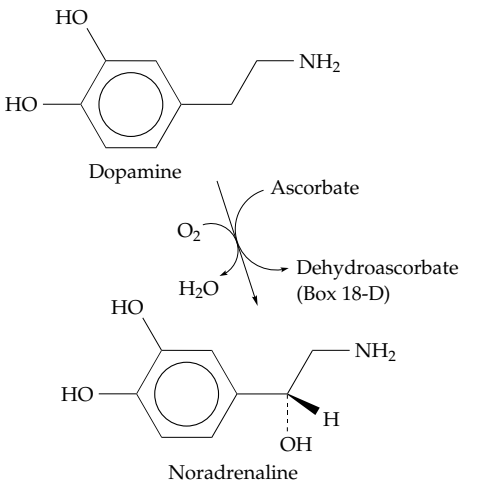
ʟ-δ-(α-Aminoadipoyl)-ʟ-cysteinyl-ᴅ-valine



Isopenicillin N (18-52)

hydrogen atoms from its dipeptide substrate to form two molecules of water and the product isopenicillin N.[456,457] Sequence comparison revealed several regions including the Fe-binding sites that are homologous with the oxoacid-dependent oxygenases. A postulated mechanism for **isopenicillin N synthase** involves formation of an Fe^{3+} superoxide anion complex as in Eq. 18-51. However, instead of attack on an oxoacid as in Eq. 18-51, it removes a hydrogen from the substrate to initiate the reaction sequence.[457] Other related oxygenases include **aminocyclopropane-1-carboxylate oxidase** (Eq. 24-35); **deacetoxycephalosporin C synthase**,[457a] an enzyme that converts penicillins to cephalosporins (Box 20-G); and **clavaminate synthase**,[458,459] an enzyme needed for synthesis of the β-lactamase inhibitor clavulanic acid, and **clavaminate synthase**.[458,459] This 2-oxoglutarate-dependent oxygenase catalyzes three separate reactions in the synthesis of the clinically important β-lactamase inhibitor clavulanic acid. The first step is similar to that in Eq. 18-50. The second is an oxidative cyclization and the third a desaturation reaction.

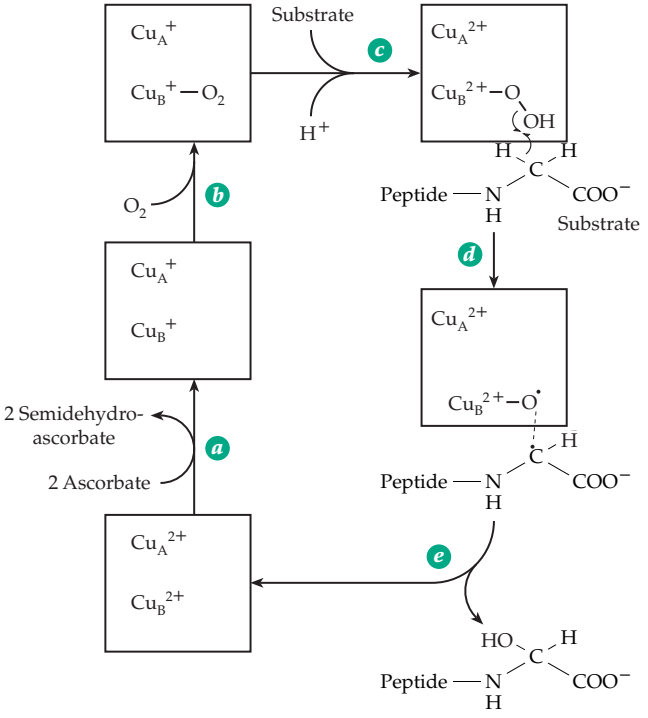
Copper-containing hydroxylases. Many Fe(II)-containing hydroxylases require a reducing agent to maintain the iron in the reduced state, and ascorbate is often especially effective. In addition, ascorbate is apparently a true cosubstrate for the copper-containing **dopamine β-hydroxylase**, an enzyme required in the synthesis of noradrenaline according to Eq. 18-53. This reaction takes place in neurons of the brain and in the adrenal gland, a tissue long known as especially rich in ascorbic acid. The reaction requires two molecules of ascorbate, which are converted in two one-electron steps to **semidehydroascorbate**.[460] Both the structure of this free radical and that of the fully oxidized form of vitamin C, **dehydroascorbic acid**, are shown in Box 18-D. Dopamine β-hydroxylase is a 290-kDa tetramer, consisting of a pair of identical disulfide-crosslinked homodimers, which contains two Cu ions per subunit.[461]



(18-53)

A similar copper-dependent hydroxylase constitutes the N-terminal domain of the **peptidylglycine α-amidating enzyme** (Eq. 10-11). This bifunctional enzyme hydroxylates C-terminal glycines in a group of neuropeptide hormones and other secreted peptides. The second functional domain of the enzyme cleaves the hydroxylated glycine to form a C-terminal amide group and glyoxylate.[462–464b] The three-dimensional structure of a 314-residue catalytic core of the hydroxylase domain is known.[463] Because of similar sequences and other properties, the structures of this enzyme and of dopamine β-hydroxylase are thought to be similar. The hydroxylase domain of the α-amidating enzyme is folded into two eight-stranded antiparallel jelly-roll motifs, each of which binds one of the two copper ions. Both coppers can exist in a Cu(II) state and be reduced by ascorbate to Cu(I). One Cu (Cu_A) is held by three imidazole groups and is thought to be the site of interaction with ascorbate. The other copper, Cu_B, which is 1.1 nm away from Cu_A, is held by two imidazoles. The substrate binds adjacent to Cu_B.[463]

The reaction cycle of these enzymes begins with reduction of both coppers from Cu(II) to Cu(I) (Eq. 18-54, step *a*). Both O_2 and substrate bind (steps *b* and *c*, but not necessarily in this order). The O_2 bound to Cu_B is reduced to a peroxide anion that remains bound to Cu_B. Both Cu_A and Cu_B donate one electron, both being oxidized to Cu(II). These changes are also included in step *c* of Eq. 18-54. One proposal is that the resulting peroxide is cleaved homolytically while removing the *pro-S* hydrogen of the glycyl residue.



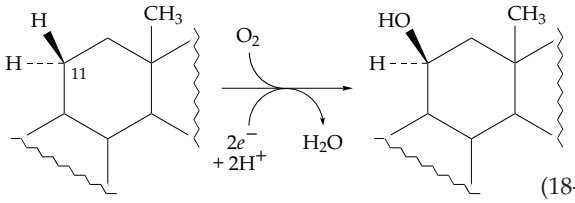
(18-54)

The resulting glycyl radical couples with the oxygen radical that is bound to Cu_B (step *e*).

A variety of other copper hydroxylases are known. For example, **tyrosinase**, which contains a binuclear copper center, catalyzes both hydroxylation of phenols and aromatic amines and dehydrogenation of the resulting catechols or *o*-aminophenols (Eq. 16-57). As in hemocyanin, the O_2 is thought to be reduced to a peroxide which bridges between the two copper atoms. Methane-oxidizing bacteria, such as *Methylococcus capsulatus*, oxidize methane to methanol to initiate its metabolism. They do this with a copper-containing membrane-embedded monooxygenase whose active site is thought to contain a trinuclear copper center. Again a bridging peroxide may be formed and may insert an oxygen atom into the substrate.[465,466] The same bacteria produce a soluble methane monooxygenase containing a binuclear iron center.

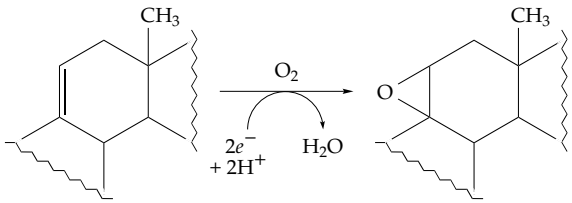
Hydroxylation with cytochrome P450. An important family of heme-containing hydroxylases, found in most organisms from bacteria to human beings, are the cytochromes P450. The name comes from the fact that in their reduced forms these enzymes form a complex with CO that absorbs at 450 nm. In soil bacteria cytochromes P450 attack compounds of almost any structure. In the adrenal gland they participate in steroid metabolism,[467,468] and in the liver microsomal cytochromes P450 attack drugs, carcinogens, and other xenobiotics (foreign compounds).[469–471] They convert cholesterol to bile acids[472] and convert vitamin D,[473] prostaglandins, and many other metabolites to more soluble and often biologically more active forms. In plants cytochromes P450 participate in hydroxylation of fatty acids at many positions.[474] They play a major role in the biosynthetic phenylpropanoid pathway (Fig. 25-8) and in lignin synthesis.[475] More than 700 distinct isoenzyme forms have been described.[476,476a]

Cytochromes P450 are monooxygenases whose cosubstrates, often NADH or NADPH, deliver electrons to the active center heme via a separate flavoprotein and often via an iron-sulfur protein as well.[476a,b] A typical reaction (Eq. 18-55) is the 11 β-hydroxylation of a steroid, an essential step in the biosynthesis of steroid hormones (Fig. 22-11). The hydroxyl group is introduced without inversion of configuration. The same enzyme converts unsaturated derivatives to epoxides (Eq. 18-56), while other cytochromes P450



(18-55)

epoxidize olefins.[477] Epoxide hydrolases, which act by a mechanism related to haloalkane dehalogenase (Fig. 12-1), convert the epoxides to diols.[478] Cytochromes P450 are able to catalyze a bewildering array of other



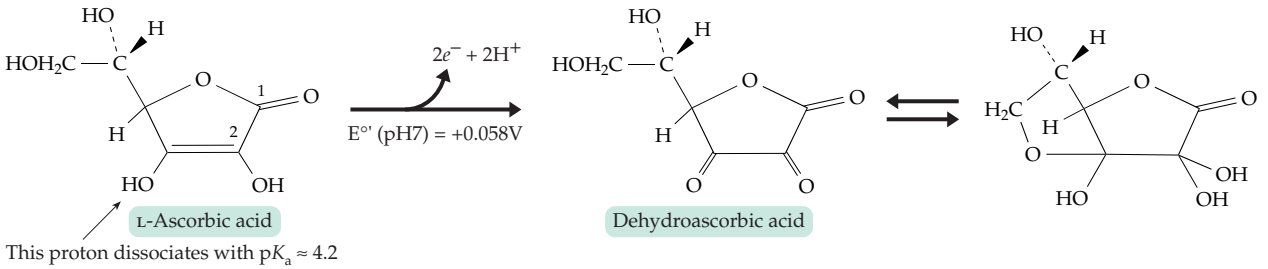
(18-56)

reactions[479–481] as well. Most of these, such as conversion of amines and thioesters to *N*- or *S*-oxides, also involve transfer of an oxygen atom to the substrate. Others, such as the reduction of epoxides, *N*-oxides, or nitro compounds, are electron-transfer reactions.

Several different cytochromes P450 are present in mammalian livers.[470] All are bound to membranes of the endoplasmic reticulum and are difficult to solubilize. Biosynthesis of additional forms is induced by such agents as phenobarbital,[470] 3-methylcholanthrene,[469] dioxin,[482] and ethanol.[483] These substances may cause as much as a 20-fold increase in P450 activity. Another family of cytochrome P450 enzymes is present in mitochondria.[483a] A large number of cytochrome P450 genes have been cloned and sequenced. Although they are closely related, each cytochrome P450 has its own gene. There are at least ten families of known P450 genes and the total number of these enzymes in mammals may be as high as 200. Microorganisms, from bacteria to yeast, produce many other cytochromes P450.

Microsomal cytochromes P450 receive electrons from an **NADPH-cytochrome P450** reductase, a large 77-kDa protein that contains one molecule each of FAD and FMN.[484–485a] It is probably the FAD which accepts electrons from NADPH and the FMN which passes them on to the heme of cytochrome P450. Cytochrome b_5 is also reduced by this enzyme,[486] and some cytochromes P450 may accept one electron directly from the flavin of the reductase and the second electron via cytochrome b_5. However, most bacterial and mitochondrial cytochromes P450 accept electrons only from small iron–sulfur proteins. Those of the adrenal gland receive electrons from the 12-kDa **adrenodoxin**.[487,488] This small protein of the ferredoxin class contains one Fe_2S_2 cluster and is, therefore, able to transfer electrons one at a time from the FAD-containing NADPH-adrenodoxin reductase[489] to the cytochrome P450. The camphor 5-monoxygenase from *Pseudomonas putida* consists of three components: an FAD-containing reductase, the Fe_2S_2 cluster-containin **putidaredoxin**,[489a] and cytochrome $P450_{cam}$.[490] Some other bacterial

BOX 18-D VITAMIN C: ASCORBIC ACID



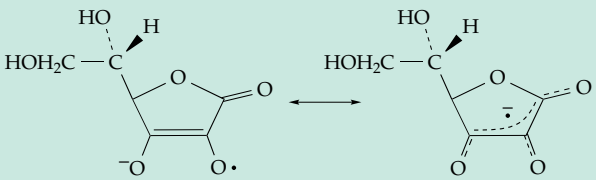
Hemorrhages of skin, gums, and joints were warnings that death was near for ancient sea voyagers stricken with **scurvy**. It was recognized by the year 1700 that the disease could be prevented by eating citrus fruit, but it was 200 years before efforts to isolate vitamin C were made. Ascorbic acid was obtained in crystalline form in 1927,[a–e] and by 1933 the structure had been established. Only a few vertebrates, among them human beings, monkeys, guinea pigs, and some fishes, require ascorbic acid in the diet; most species are able to make it themselves. Compared to that of other vitamins, the nutritional requirement is large.[e] Ten milligrams per day prevents scurvy, but subclinical deficiency, as judged by fragility of small capillaries in the skin, is present at that level of intake. "Official" recommendations for vitamin C intake have ranged from 30 to 70 mg / day. A more recent study[f] suggests 200 mg / day, a recommendation that is controversial.[g]

The biological functions of vitamin C appear to be related principally to its well-established reducing properties and easy one-electron oxidation to a free radical or two-electron reduction to **dehydroascorbic acid**. The latter is in equilibrium with the hydrated hemiacetal shown at the beginning of this box as well as with other chemical species.[h–j] Vitamin C is a weak acid which also has metal complexing properties.

Ascorbate, the anion of ascorbic acid, tends to be concentrated in certain types of animal tissues and may reach 3 mM or more in leukocytes, in tissues of eyes and lungs, in pituitary, adrenal, and parotid glands,[k,l] and in gametes.[m] Uptake into vesicles of the endoplasmic reticulum may occur via glucose transporters.[n] Ascorbate concentrations are even higher in plants and may exceed 10 mM in chloroplasts.[o] In animals the blood plasma ascorbate level of 20–100 μM is tightly controlled.[p,q] Cells take up ascorbate but any excess is excreted rapidly in the urine.[q] Both in plasma and within cells most vitamin C exists as the reduced form, ascorbate. When it is formed, the oxidized dehydroascorbate is reduced back to ascorbate or is degraded. The lactone ring is readily hydrolyzed to 2,3-dioxogulonic acid, which can undergo decarboxylation and oxidative degradation, one product being oxalate (see Fig. 20-2).[r] Tissues may also contain smaller amounts of L-ascorbic acid 2-sulfate, a compound originally discovered in brine shrimp. It is more stable than free ascorbate and may be hydrolyzed to ascorbate in tissues.[s]

In the chromaffin cells of the adrenal glands and in the neurons that synthesize catecholamines as neurotransmitters, ascorbate functions as a cosubstrate for dopamine β-hydroxylase (Eq. 18-53).[t,u] In fibroblasts it is required by the prolyl and lysyl hydroxylases and in hepatocytes by homogentisate dioxygenase (Eq. 18-49). Any effect of ascorbic acid in preventing colds may be a result of increased hydroxylation of procollagen and an associated stimulation of procollagen secretion.[v] High levels of ascorbate in guinea pigs lead to more rapid healing of wounds.[w] An important function of ascorbate in the pituitary and probably in other endocrine glands is in the α-amidation of peptides (Eq. 10-11).[x,y] Together with Fe(II) and O_2 ascorbate is a powerful nonenzymatic hydroxylating reagent for aromatic compounds. Like hydroxylases, the reagent attacks nucleophilic sites, e.g., converting phenylalanine to tyrosine. Oxygen atoms from $^{18}O_2$ are incorporated into the hydroxylated products. While H_2O_2 is formed in the reaction mixture, it cannot replace ascorbate. The relationship of this system to biochemical functions of ascorbate is not clear. An unusual function for vitamin C has been proposed for certain sponges that are able to etch crystalline quartz (SiO_2) particles from sand or rocks.[z]

Ascorbate is a major antioxidant, protecting cells and tissues from damage by free radicals, peroxides, and other metabolites of O_2.[p,r,aa,bb] It is chemically suited to react with many biologically important radicals and is present in high enough concentrations to be effective. It probably functions in cooperation with glutathione (Box 11-B),[cc] α-tocopherol (Fig. 15-24),[dd] and lipoic acid.[ee] Ascorbate can react with radicals in one-election transfer reactions to give the monodehydroascorbate radical[aa]:



BOX 18-D (continued)

Two ascorbate radicals can react with each other in a disproportionation reaction to give ascorbate plus dehydroascorbate. However, most cells can reduce the radicals more directly. In many plants this is accomplished by NADH + H^+ using a flavoprotein **monodehydroascorbate reductase**.[o] Animal cells may also utilize NADH or may reduce dehydroascorbate with reduced glutathione.[cc,ff] Plant cells also contain a very active blue copper ascorbate oxidase (Chapter 16, Section D,5), which catalyzes the opposite reaction, formation of dehydroascorbate.[gg] A heme ascorbate oxidase has been purified from a fungus.[hh] Action of these enzymes initiates an oxidative degradation of ascorbate, perhaps through the pathway of Fig. 20-2.

Ascorbate can also serve as a signal. In cultured cells, which are usually deficient in vitamin C, addition of ascorbate causes an enhanced response to added iron, inducing synthesis of the iron storage protein ferritin.[ii] Ascorbate indirectly stimulates transcription of procollagen genes[v] and decreases secretion of insulin by the pancreas.[jj] However, since its concentration in blood is quite constant this effect is not likely to cause a problem for a person taking an excess of vitamin C.

Should we take extra vitamin C to protect us from oxygen radicals and slow down aging? Linus Pauling, who recommended an intake of 0.25–10 g / day, maintained that ascorbic acid also has a specific beneficial effect in preventing or ameliorating symptoms of the common cold.[j,kk] However, critics point out that unrecognized hazards may exist in high doses of this seemingly innocuous compound. Ascorbic acid has antioxidant properties, but it also promotes the generation of free radicals in the presence of Fe(III) ions, and it is conceivable that too much may be a bad thing.[ll] Catabolism to oxalate may promote formation of calcium oxalate kidney stones. Under some conditions products of dehydroascorbic acid breakdown may accumulate in the lens and contribute to cataract formation.[i,mm,nn] However, dehydroascorbate, or its decomposition products, apparently *protects* low-density lipoproteins against oxidative damage.[bb] Pauling pointed out that nonhuman primates synthesize within their bodies many grams of ascorbic acid daily, and that there is little evidence for toxicity. Pauling's claim that advanced cancer patients are benefited by very high (10 g daily) doses of vitamin C has been controversial, and some studies have failed to substantiate the claim.[oo,pp]

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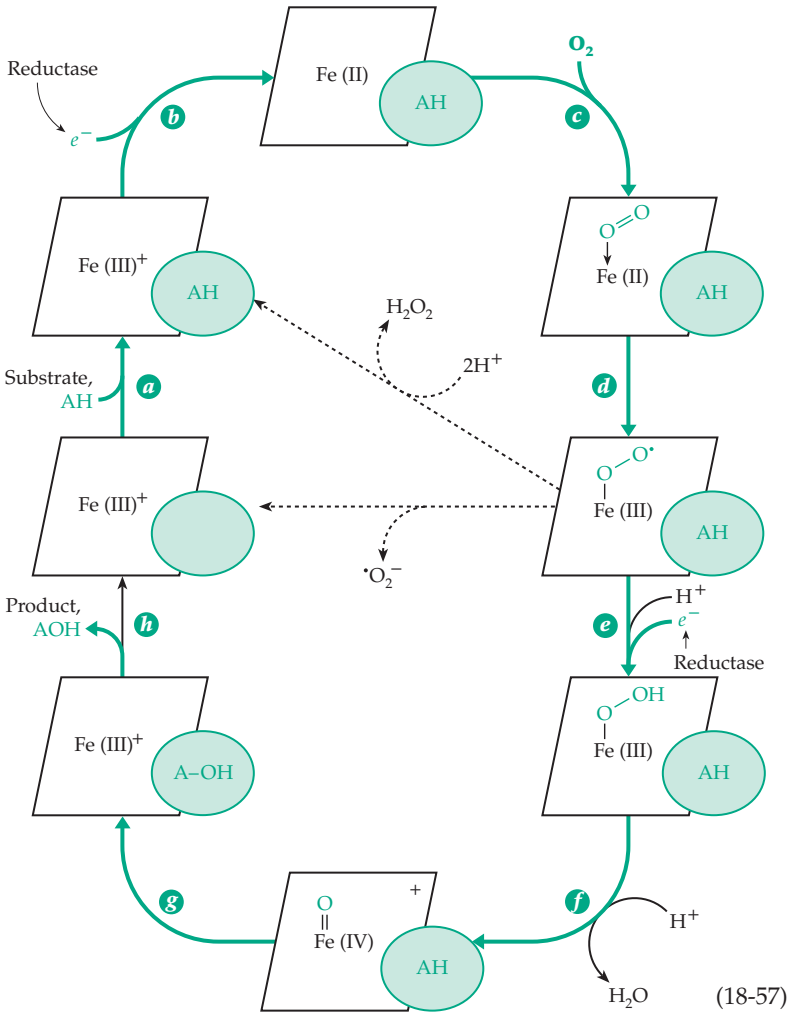
cytochrome P450s, such as a soluble fatty acid hydroxylase from *Bacillus megaterium*, have reductase domains with tightly bound FMN and FAD bound to the same polypeptide chain as is the heme.[491]

All cytochromes P450 appear to have at their active sites a molecule of heme with a thiolate anion as an axial ligand in the fifth position (Fig. 18-23). These relatively large heme proteins of ~ 45- to 55-kDa mass may consist of as many as 490 residues. Only a few three-dimensional structures are known,[490,492–494] and among these there are significant differences. However, on the basis of a large amount of experimental effort[487,495,496] it appears that all cytochromes P450 act by basically similar mechanisms.[474,496a,b,497] As indicated in Eq. 18-57, the substrate AH binds to the protein near the heme, which must be in the Fe(III) form. An electron delivered from the reductase then reduces the iron to the Fe(II) state (Eq. 18-57, step *b*). Then O_2 combines with the iron, the initial oxygenated complex formed in step *c* being converted to an Fe(III)-superoxide complex (Eq. 18-57, step *d*). Subsequent events are less certain.[497a] Most often a second electron is transferred in from the reductase (Eq. 18-57, step *e*) to give a peroxide complex of Fe(III), which is then converted in step *f* to a ferryl iron form, as in the action of peroxidases (Fig. 16-14). This requires transfer of two H^+ into the active site. The ferryl Fe(IV)=O donates its oxygen atom to the substrate regenerating the Fe(III) form of the heme (step *g*) and releasing the product (step *h*).

Microsomal cytochromes P450 often form hydrogen peroxide as a side product. This may arise directly from the Fe–O–O^- intermediate shown in Eq. 18-57. Some cytochromes P450 use this reaction in reverse to carry out hydroxylation utilizing peroxides instead of O_2 (Eq. 18-58).

$$AH + ROOH \rightarrow AOH + ROH \qquad (18\text{-}58)$$

Cytochromes P450 often convert drugs or other foreign compounds to forms that are more readily excreted.[499] However, the result is not always beneficial. For example, 3-methylcholanthrene, a strong inducer of cytochrome P450, is converted to a powerful carcinogen by the hydroxylation reaction.[500] See also Box 18-E.



(18-57)

Other iron-containing oxygenases. Hydroxylases with properties similar to those of cytochrome P450 but containing nonheme iron catalyze ω-oxidation of alkanes and fatty acids in certain bacteria, e.g., *Pseudomonas oleovarans*. A flavoprotein rubredoxin reductase, is also required.[501] The methylotrophs *Methylococcus* and *Methylosinus* hydroxylate methane using as cosubstrate NADH or NADPH (Eq. 18-59). A soluble complex consists of 38-kDa reductase containing FAD and an Fe_2S_2

$$CH_4 + O_2 + NADH + H^+ \rightarrow CH_3OH + H_2O + NAD^+ \qquad (18\text{-}59)$$

center, a small 15-kDa component, and a 245-kDa hydroxylase with an $(\alpha\beta\gamma)_2$ composition and a three-dimensional structure[502–503] similar to that of ribonucleotide reductase (Chapter 16, Section A,9). Each large α subunit contains a diiron center similar to that shown in Fig. 16-20C. It is likely that O_2 binds between the two iron atoms in the Fe(II) oxidation state and, oxidizing both irons to Fe(III), is converted to a bridging peroxide group as shown in Eq. 18-60. In this intermediate, in which the two metals are held rigidly by the surrounding ligands including a bridging carboxylate side chain, the O–O bond may be broken as in Eq. 18-60, steps *a* and *b*, to generate an Fe(IV)–$O^\bullet$ radical that may

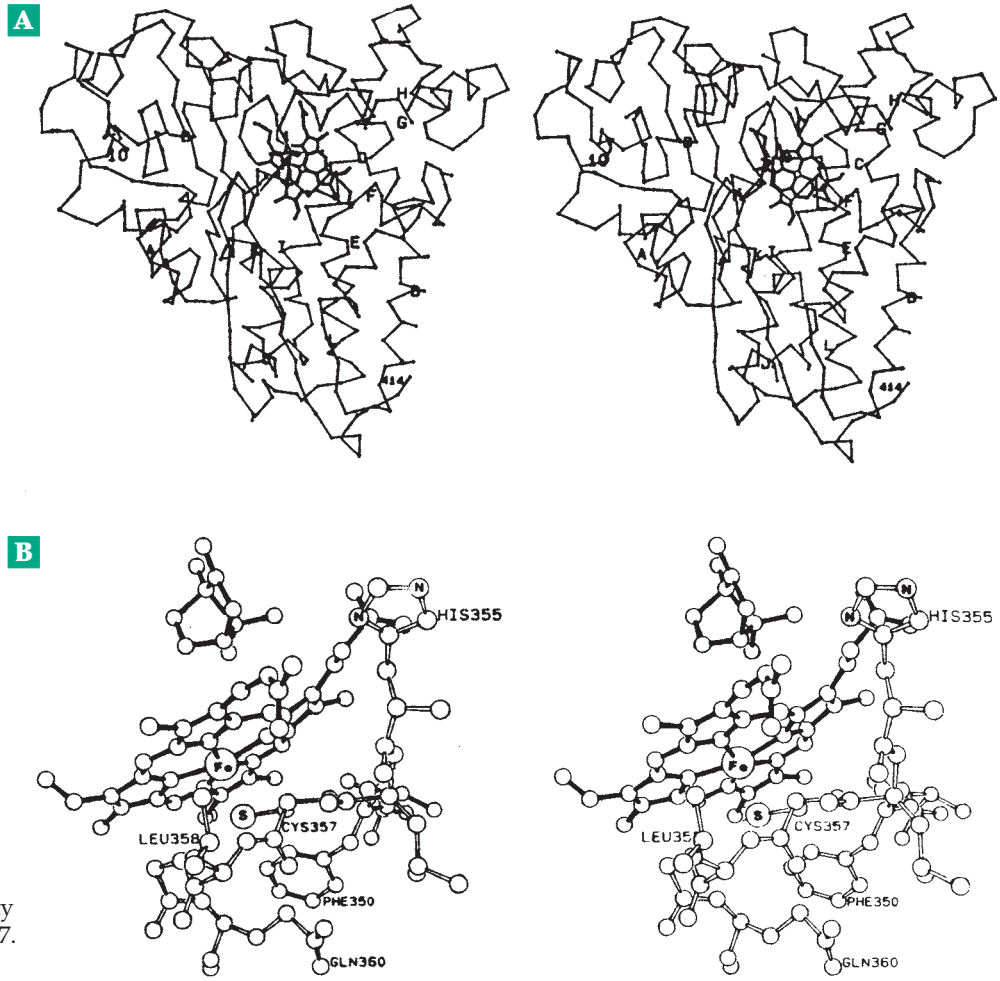
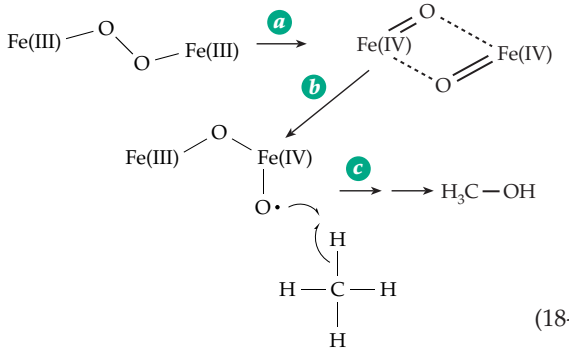


Figure 18-23 (A) Stereoscopic α-carbon backbone model of cytochrome $P450_{cam}$ showing the locations of the heme and of the bound camphor molecule. (B) View in the immediate vicinity of the thiolate ligand from Cys 357. From Poulos *et al.*[498]

remove a hydrogen atom from the substrate (step *c*) and undergo subsequent reaction steps analogous to those in the cytochrome P450 reaction cycle.[504–506]



(18-60)

A group of related bacterial enzymes hydroxylate alkanes,[507] toluene,[508] phenol,[509] and other substrates.[509a] Eukaryotic fatty acid desaturases (Fig. 16-20B) belong to the same family.[508] Some bacteria use cytochrome P450 or other oxygenase to add an oxygen atom to an alkene to form an epoxide. For example, propylene may be converted to either *R* or *S* epoxypropane which may be hydrolyzed, rearranged by a coenzyme M-dependent reaction, and converted to acetoacetate, which can be used as an energy source.[509a,b]

Nitric oxide and NO synthases. Nitric oxide (NO) is a reactive free radical whose formula is often written as •NO to recognize this characteristic. However, NO is not only a toxic and sometimes dangerous metabolite but also an important hormone with functions in the circulatory system, the immune system, and the brain.[510–512] The hormonal effects of NO are discussed in Chapter 30, but it is appropriate here to mention a few reactions. Nitric oxide reacts rapidly with O_2 to form nitrite (Eq. 18-61).

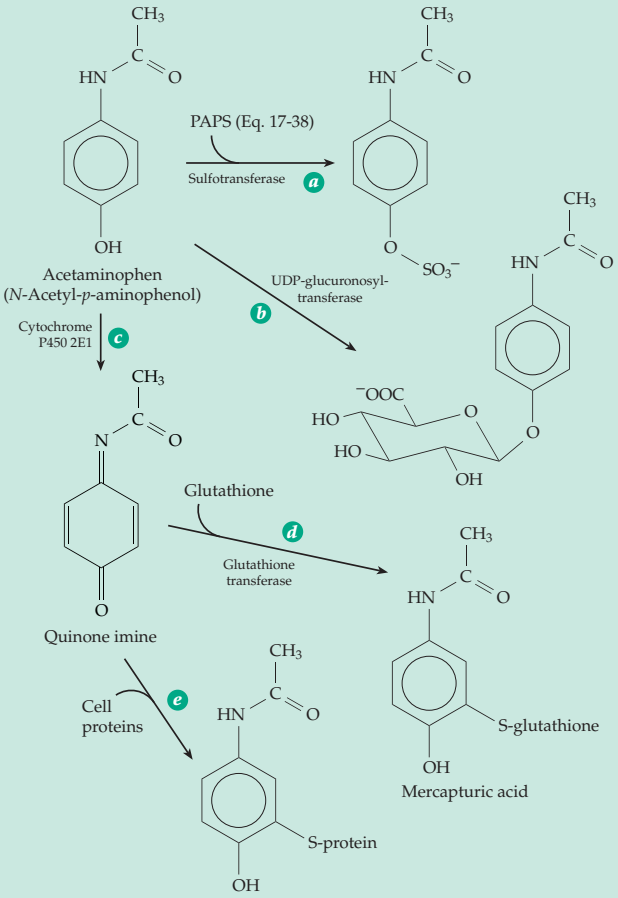
$$4\ NO + O_2 + 2\ H_2O \rightarrow 4\ NO_2^- + 4\ H^+ \qquad (18\text{-}61)$$

It also combines very rapidly with superoxide anion radical to form **peroxynitrite** (Eq. 18-62).[513] This is another reactive oxidant which, because of its relatively high pK_a of 6.8, is partially protonated and able to diffuse through phospholipids within cells.[514,515]

BOX 18-E THE TOXICITY OF ACETAMINOPHEN

Most drugs, as well as toxins and other xenobiotic compounds, enter the body through membranes of the gastrointestinal tract, lungs, or skin. Drugs are frequently toxic if they accumulate in the body. They are often rather hydrophobic and are normally converted to more polar, water-soluble substances before elimination from the body. Two major types of reaction take place, usually in the liver. These are illustrated in the accompanying scheme for acetaminophen (*N*-acetyl-*p*-aminophenol), a widely used analgesic and antipyretic (fever relieving) nonprescription drug sold under a variety of trade names: (1) A large water-soluble group such as sulfate[a] or glucuronate is transferred onto the drug by a nucleophilic displacement reaction (steps *a* and *b* of scheme). (2) Oxidation, demethylation, and other alterations are catalyzed by one or more of the nearly 300 cytochrome P450 monooxygenases present in the liver (step *c*). Oxidation products may be detoxified by glutathione S-transferases, step *d* (see also Box 11-B).[b,c,cc]

These reactions protect the body from the accumulation of many compounds but in some cases can cause serious problems. The best known of these involves acetaminophen. Its oxidation by cytochrome P450 2E1 or by prostaglandin H synthase[d] yields a highly reactive quinone imine which reacts with cell proteins.[e] Since the cytochrome P450 oxidation can occur in two steps, a reactive intermediate radical is also created.[c,f] At least 20 drug-labeled proteins arising in this way have been identified.[c] Both addition of thiol groups of proteins to the quinone imine (step *e* of scheme) and oxidation of protein thiols occur.[g] Mitochondria suffer severe damage,[h] some of which is related to induction of Ca^{2+} release.[i]



Acetaminophen is ordinarily safe at the recommended dosages, but large amounts exhaust the reserve of glutathione and may cause fatal liver damage. By 1989, more than 1000 cases of accidental or intentional (suicide) overdoses had been reported with many deaths. Prompt oral or intravenous administration of *N*-acetylcysteine over a 72-hour period promotes synthesis of glutathione and is an effective antidote.[j]

Similar problems exist for many other drugs. Both acetaminophen and phenacetin, its ethyl ether derivative, may cause kidney damage after many years of use.[k,l] Metabolism of phenacetin and several other drugs varies among individuals. Effective detoxification may not occur in individuals lacking certain isoenzyme forms of cytochrome P450.[m] Use of the anticancer drugs daunomycin (daunorubicin; Figs. 5-22 and 5-23) and adriamycin is limited by severe cardiac toxicity arising from free radicals generated during oxidation of the drugs.[n] These are only a few examples of the problems with drugs, pesticides, plasticizers, etc.

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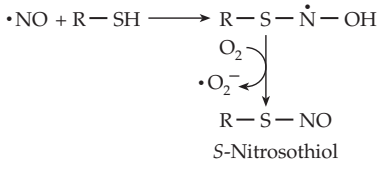
$$^{\bullet}NO + {}^{\bullet}O_2^- \rightarrow O{=}N{-}O{-}O^- \qquad (18\text{-}62)$$

NO binds to the iron atoms in accessible heme groups such as those of hemoglobin[516] and of guanylate cylases,[517,518] and in some Fe – S proteins such as aconitase. Some blood-sucking insects utilize salivary heme proteins called **nitrophorins** to carry NO into host tissues where it activates guanylate cyclase causing vasodilation. Nitrophorins also bind histamine and inhibit blood coagulation, which assists feeding.[518a,b] In the presence of a suitable oxidant such as O_2, nitric oxide reacts with thiol groups of proteins and small molecules to give **S-nitrosothiols** (Eq. 18-63).[511,519-520]



S-Nitrosothiol (18-63)

However, the physiological mechanisms of formation of these *S*-nitroso compounds is not clear.[520–521] One mechanism may involve conversion by O_2 to nitrous anhydride.[511,522]

$$2\ NO + O_2 \rightarrow ON{-}O{-}NO \qquad (18\text{-}64)$$

Nucleophilic attack on this compound by –SH, $-NH_2$, and other nucleophiles would yield *S*-nitroso and *N*-nitroso compounds with release of nitrous oxide N_2O.

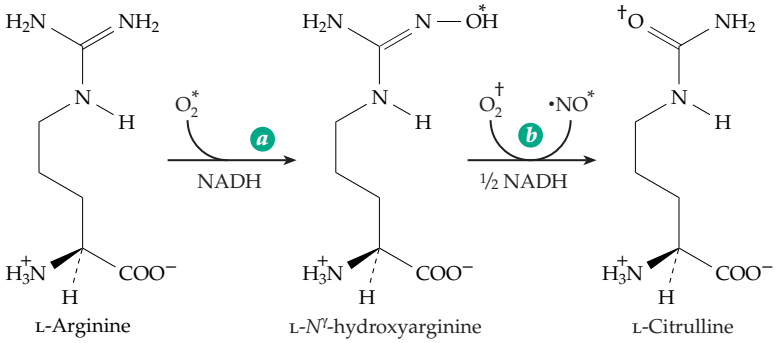
The relatively stable *S*-nitrosothiols derived from glutathione, cysteine, and proteins such as hemoglobin[516] may be important storage and transport forms of NO. If so, mechanisms of release of NO are important. A simple homolytic cleavage of R–S–NO to NO plus a thiyl radical R–S$^{\bullet}$ has often been assumed. However, rapid cleavage requires catalysis by a transition metal ion or reaction with reducing agents such as ascorbate or other thiols.[523,524] *S*-Nitrosothiols may also give rise to nitrosonium (NO^+) or nitroxy (NO^-) ions.[524]

NO synthases are oxygenases that carry out a two-step oxidation of L-arginine to L-citrulline with production of NO. In the first step, a normal monooxygenase reaction, L-N^{γ}-hydroxyarginine is formed (Eq. 18-65, step *a*). In the second step (Eq. 18-65, step *b*) NO is formed in a three-electron oxidation. In this equation the symbols $_*$ and † indicate positions of incorporation of labeled O_2 atoms in the intermediate and final products.

The human body contains three types of nitric oxide synthase known as **neuronal** (nNOS or NOS1), **inducible** (iNOS or NOS2), and **endothelial** (eNOS or NOS3).[511a–f] These enzymes have a broad distribution within various tissues, but NOS1 is especially active in neurons and NOS3 in endothelial cells. The inducible NOS2 originally discovered in macrophages is transcriptionally regulated. When these phagocytic cells are at rest the activity of NOS2 is very low, but it becomes highly active after induction by cytokines or by the lipopolysaccharides of bacterial cell membranes.[525,526] Both NOS1 and NOS2 are constitutively expressed but require calcium ions, which bind to a calmodulin domain of the protein. The inducible NOS2 doesn't require added Ca^{2+} but does contain the calmodulin domain. NOS3 carries an N-terminal myristoyl group as well as cysteines that may be palmitoylated. It is located in caveolae of plasma membranes and in Golgi complexes.[527,528]

Nucleotide sequences revealed a close similarity of NO synthases to cytochrome P450 reductase.[525,529,530] Study of spectra suggested that NO synthases, in which the heme is held by a thiolate sulfur of a cysteine side chain, might be specialized cytochromes P450.[531] However, although the heme in NO synthases is bound by a thiolate group the protein fold is unlike that of cytochromes P450.[532,533] The NO synthases all share a three-component structure.[534] In the NOS2 from macrophages residues 1–489 form the catalytic oxygenase domain, residues 499–530 bind calmodulin, and residues 531–1144 form the reductase domain.[535] The last contains a binding site for NADPH as well as bound FMN and FAD. The reductase domain structure can be modeled after that of cytochrome P450 reductase.[533] Electrons from NADPH are apparently transferred to FAD, then to FMN, and then to the catalytic site.[525]

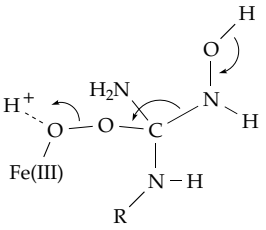
The least understood aspect of NO synthases is the requirement for tetrahydrobiopterin, BH_4, the same coenzyme required by the other pterin-dependent monooxygenases (Eq. 18-44). The presence of this coenzyme in the reduced BH_4 form is essential for step *a* of Eq. 18-65 but not for step *b*. This suggests that in step *a* an organic peroxide might be generated by BH_4 and used to form an oxo-iron hydroxylating reagent.



L-Arginine L-N^{γ}-hydroxyarginine L-Citrulline (18-65)

However, there is no evidence for the expected quinonoid dihydropterin, and the three-dimensional structure suggested that BH_4 plays a structural role in mediating essential conformational changes.[532,535a,b] Nevertheless, newer data indicate a role in electron transfer.[535c]

Step *b* of Eq. 18-65 is an unusual three-electron oxidation, which requires only one electron to be delivered from NADPH by the reductase domain. Hydrogen peroxide can replace O_2 in this step.[536] A good possibility is that a peroxo or superoxide complex of the heme in the Fe(III) state adds to the hydroxyguanidine group. For example, the following structure could arise from addition of Fe(III)–O–O$^-$:

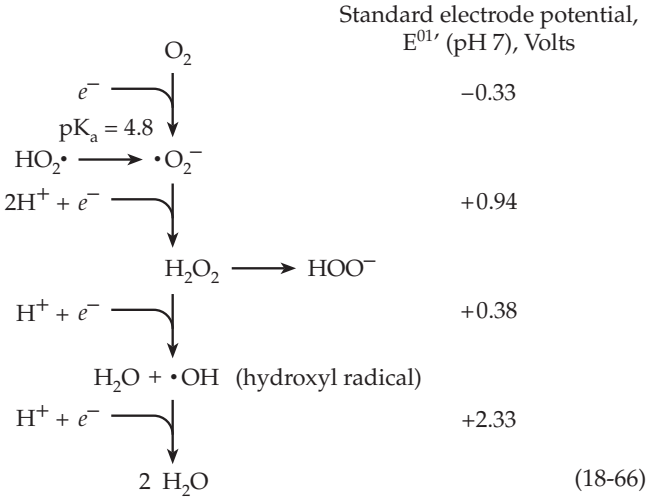


Breakup as indicated by the arrows on this structure would give Fe(III)–OH, citrulline, and O=N–H, **nitroxyl**. This is one electron ($e^- + H^+$) more reduced than •NO. Perhaps the adduct forms from Fe(III)–O–O•. On the other hand, there is evidence that NO synthases may produce nitroxyl or nitroxyl ion NO^- as the initial product.[537–538] NO and other products such as N_2O and NO_2^- may arise rapidly in subsequent reactions. Nitrite is a major oxidation product of NO in tissues.[538a] The chemistry of NO in biological systems is complex and not yet fully understood. See also pp. 1754, 1755.

G. Biological Effects of Reduced Oxygen Compounds

Although molecular oxygen is essential to the aerobic mode of life, it is toxic at high pressures. Oxidative damage from O_2 appears to be an important cause of aging and also contributes to the development of cancer. Reduced forms of oxygen such as superoxide, hydrogen peroxide, and hydroxyl radicals are apparently involved in this toxicity.[539,540] The same agents are deliberately used by phagocytic cells such as the neutrophils (polymorphonuclear leukocytes) to kill invading bacteria or fungi and to destroy malignant cells.[541]

The reactions shown with vertical arrows in Eq. 18-66 can give rise to the reduced oxygen compounds. The corresponding standard redox potential at pH 7 for each is also given.[539,542–544] As indicated by the low value of the redox potential for the O_2 / $O_2^{\bullet -}$ couple, the formation of superoxide by reduction of O_2 is spontaneous only for strongly reducing one-electron donors. Superoxide ion is a strong reductant, but at the same time a powerful one-electron oxidant, as is indicated by the high electrode potential of the $O_2^{\bullet -}$/ H_2O_2 couple.



(18-66)

1. The Respiratory Burst of Neutrophils

Some 25 x 10^9 neutrophils circulate in an individual's blood, and an equal number move along the surfaces of red blood cells. Invading microorganisms are engulfed after they are identified by the immune system as foreign. Phagocytosis is accompanied by a rapid many-fold rise in the rate of oxygen uptake as well as an increased glucose metabolism. One purpose of this **respiratory burst**[545–548] is the production of reduced oxygen compounds that kill the ingested microorganisms. In the very serious **chronic granulomatous disease** the normal respiratory burst does not occur, and bacteria are not killed.[549] The respiratory burst seems to be triggered not by phagocytosis itself, but by stimulation of the neutrophil by chemotactic formylated peptides such as formyl-Met-Leu-Phe[550] and less rapidly by other agonists such as phorbol esters.

The initial product of the respiratory burst appears to be superoxide ion O_2^-. It is formed by an **NADPH oxidase,** which transports electrons from NADPH to O_2 probably via a flavoprotein and cytochrome b_{558}. Either the flavin or the cytochrome b_{558} must donate one electron to O_2 to form the superoxide anion.

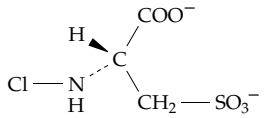
$$\text{NADPH} \rightarrow \text{FAD} \rightarrow \text{cyt } b_{558} \rightarrow O_2 \qquad (18\text{-}67)$$

Flavocytochrome b_{558} (also called b_{-245}) has the unusually low redox potential of −0.245 V. It exists in phagocytic cells as a heterodimer of membrane-associated subunits p22-*phox* and gp91-*phox* where *phox* indicates phagocytic oxidase. The larger 91-kDa

subunit contains two heme groups as well as one FAD and the presumed NADPH binding site.[548,551–552a] The mechanism of interaction with O_2 is unclear. Unlike hemoglobin but like other cytochromes *b*, cytochrome b_{558} does not form a complex with CO.[553] NADPH oxidase also requires two cytosolic components p47-*phox* and p67-*phox*. In resting phagocytes they reside in the cytosol as a 240-kDa complex with a third component, p40-*phox*, which may serve as an inhibitor.[548,554] Upon activation of the phagocyte in response to chemotactic signals the cytosolic components undergo phosphorylation at several sites,[554] and protein p47-*phox* and p67-*phox* move to the membrane and bind to and with the assistance of the small G protein Rac[552a,554a] activate flavocytochrome b_{558}. Phosphorylation of p47-*phox* may be especially important.[555]

In the X-chromosome-linked type of chronic granulomatous disease flavocytochrome b_{558} is absent or deficient, usually because of mutation in gp91-*phox*.[556,557] In an autosomal recessive form the superoxide-forming oxidase system is not activated properly. In some patients protein kinase C fails to phosphorylate p47-*phox*.[556,558] Less severe symptoms arise from deficiences in myeloperoxidase, chloroperoxidase (Chapter 16), glucose 6-phosphate dehydrogenase, glutathione synthetase, and glutathione reductase. The importance of these enzymes can be appreciated by examination of Fig. 18-24, which illustrates the relationship of several enzymatic reactions to the formation of superoxide anion and related compounds. Not only neutrophils but monocytes, macrophages, **natural killer cells** (NK cells), and other phagocytes apparently use similar chemistry in attacking ingested cells (Chapter 31).[559] Superoxide-producing NADH oxidases have also been found in nonphagocytic cells in various tissues.[559a]

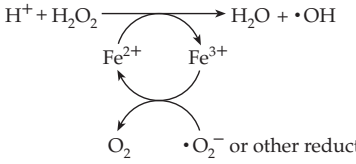
What kills the ingested bacteria and other microorganisms? Although superoxide anion is relatively unreactive, its protonated form $HO_2\bullet$, is very reactive. Since its pK_a is 4.8, there will be small amounts present even at neutral pH. Some of the $^\bullet O_2^-$ may react with NO to form peroxynitrite (Eq. 18-62).[559b] Peroxynitrite, in turn, can react with the ubitquitous CO_2 to give $^\bullet CO_3^-$ and $^\bullet NO_2$ radicals.[559c] Peroxynitrite anion also reacts with metalloenzyme centers[559d] and causes nitration and oxidation of aromatic residues in proteins.[559d,e] However, neutrophils contain active superoxide dismutases, and most of the superoxide that is formed is converted quickly to O_2 and H_2O_2. The latter may diffuse into the phagosomes as well as into the extracellular space. The H_2O_2 itself is toxic, but longer lived, more toxic oxidants are also formed. Reaction of H_2O_2 with **myeloperoxidase** (Chapter 16) produces hypochlorous acid, **(HOCl;** Eqs. 16-12, Fig. 18-24) and **chloramines** such as NH_2Cl, RNHCl, and $RNCl_2$. An important intracellular chloramine may be that of taurine.



Chloramine formed from taurine

Human neutrophils use HOCl formed by myeloperoxidase to oxidize α-amino acids such as tyrosine to reactive aldehydes that form adducts with –SH, $–NH_2$, imidazole, and other nucleophilic groups.[560] They also contain NO synthases, which form NO, peroxynitrite (Fig. 18-24), and nitrite.[561,562]

Hydroxyl radicals •OH, which attack proteins, nucleic acids, and a large variety of other cellular constituents, may also be formed. Although too reactive to diffuse far, they can be generated from H_2O_2 by Eq. 18-68. This reaction involves catalysis by Fe ions as shown.[562a]



(18-68)

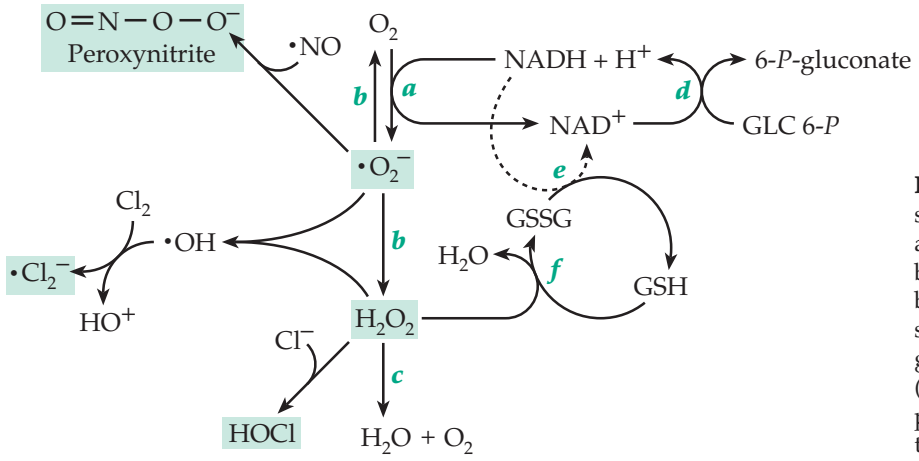


Figure 18-24 Some reactions by which superoxide anions, hydrogen peroxide and related compounds are generated by neutrophils and to a lesser extent by other cells: (*a*) NADPH oxidase, (*b*) superoxide dismutase, (*c*) catalase, (*d*) glucose-6-phosphate dehydrogenase, (*e*) glutathione reductase, (*f*) glutathione peroxidase. Abbrevations: GSH, glutathione; GSSB, oxidized glutathione.

Because Fe^{3+} is present in such low concentrations, there is uncertainty as to the biological significance of this reaction.[563] However, other iron compounds may function in place of Fe^{3+} and Fe^{2+} in Eq. 18-68.[564] A mixture of ferrous salts and H_2O_2 (Fenton reagent) has long been recognized as a powerful oxidizing mixture, which generates •OH or compounds of similar reactivity.[564–567] Ascorbate, and various other compounds, can also serve as the reductant in Eq. 18-68.[568]

Eosinophils, whose presence is stimulated by parasitic infections, have a peroxidase which acts preferentially on Br^- to form HOBr.[569] This compound can react with H_2O_2 more efficiently than does HOCl (Eq. 16-16) to form the very reactive **singlet oxygen.**[570] Singlet oxygen can also be generated from H_2O_2 and $•O_2$ by Eq. 18-69[571] and also photochemically.[572]

$$•O_2^- + H_2O_2 \rightarrow {}^1O_2({}^1\Delta_g) + OH + OH^- \qquad (18\text{-}69)$$

Additional killing mechanisms used by phagocytes include acidification of the phagocytic lysosomes with the aid of a proton pump[573] and formation of toxic peptides. For example, bovine neutrophils produce the bacteriocidal peptide RLCRIVVIRVCR which has a disulfide crosslinkage between the two cysteine residues.[574] Microorganisms have their own defenses against the oxidative attack by phagocytes. Some bacteria have very active superoxide dismutases. The protozoan *Leishmania* produces an acid phosphatase that shuts down the production of superoxide of the host cells in response to activating peptides.[575]

A respiratory burst accompanies fertilization of sea urchin eggs.[576,577] In this case, the burst appears to produce H_2O_2 as the major or sole product and is accompanied by release of **ovoperoxidase** from cortical granules. This enzyme uses H_2O_2 to generate **dityrosine crosslinkages** between tyrosine side chains during formation of the fertilization membrane. Defensive respiratory bursts are also employed by plant cells.[578,579] See also Box 18-B.

2. Oxidative Damage to Tissues

Superoxide anion radicals are formed not only in phagocytes but also as an accidental by-product of the action of many flavoproteins,[580,581] heme enzymes, and other transition metal-containing proteins. An example is xanthine oxidase. It is synthesized as xanthine dehydrogenase which is able to use NAD^+ as an oxidant, but upon aging, some is converted into the $•O_2^-$-utilizing xanthine oxidase (Chapter 16). This occurs extensively during ischemia. When oxygen is readmitted to a tissue in which this conversion of xanthine dehydrogenase to xanthine oxidase has occurred, severe oxidative injury may occur.[582] In animals the intravenous administration of superoxide dismutase or pretreatment with the xanthine oxidase inhibitor **allopurinol** (Chapter 25) prevents much of the damage, suggesting that superoxide is the culprit.

Hydrogen peroxide is also generated within cells[583] by flavoproteins and metalloenzymes and by the action of superoxide dismutase on $•O_2^-$. Since H_2O_2 is a small uncharged molecule, it can diffuse out of cells and into other cells readily. If it reacts with Fe(II), it can be converted within cells to •OH radicals according to Eq. 18-68. Such radicals and others have been detected upon readmission of oxygen to ischemic animal hearts.[584,585] It has also been suggested that NADH may react with Fe(III) compounds in the same way as does O_2^- in Eq. 18-68 to provide a mechanism for producing hydroxyl radicals from H_2O_2.[539] Nitric oxide, formed by the various NO synthases in the cytosol and in mitochondria[586] and by some cytochromes P450,[587] is almost ubiquitous and can also lead to formation of peroxynitrate (Eq. 18-62). Thus, the whole range of reduced oxygen compounds depicted in Eq. 18-24 are present in small amounts throughout cells.

There is little doubt that these compounds cause extensive damage to DNA, proteins, lipids, and other cell constituents.[539,540,563,588] For example, one base in 150,000 in nuclear DNA is apparently converted from guanine to 8-hydroxyguanine presumably as a result of attack by oxygen radicals.[589] In mitochondrial DNA one base in 8000 undergoes this alteration. This may be a result of the high rate of oxygen metabolism in mitochondria and may also reflect the lack of histones and the relatively inefficient repair of DNA within mitochondria. Proteins undergo chain cleavage, crosslinking, and numerous side chain modification reactions.[588] Dissolved O_2 can react directly with exposed glycyl residues in protein backbones to create glycyl radicals which may lead to chain cleavage as in Eq. 15-39.[588a] Iron–sulfur clusters, such as the Fe_4S_4 center of aconitase (Fig. 13-4), are especially sensitive to attack by superoxide anions.[588a–c] "Free iron" released from the Fe–S cluster may catalyze formation of additional damaging radicals.

Antioxidant systems. Cells have numerous defenses against oxidative damage.[563,590,591] Both within cells and in extracellular fluids superoxide dismutase (Eq. 16-27) decomposes superoxide to O_2 and H_2O_2. The H_2O_2 is then broken down by catalase (Eq. 16-8) to O_2 and H_2O. In higher animals the selenoenzyme glutathione peroxidase (Chapter 16) provides another route for decomposition of H_2O_2 and lipid peroxides of membranes. The oxidized glutathione formed is reduced by NADPH. The system has a critical role within erythrocytes (Box 15-H). In chloroplasts an analogous system utilizes ascorbate peroxidase, ascorbate, and glutathione to break down peroxides.[592]

$$NADPH + H^+ \rightarrow \text{glutathione} \rightarrow \text{ascorbate} \rightarrow H_2O_2 \quad (18\text{-}70)$$

Ascorbate,[593–594] glutathione, NADPH,[594a] and tocopherols (Box 15-G)[595] all act as scavengers of free radicals such as O_2^-, $^\bullet OH$ and $ROO^\bullet$, $^\bullet CO_3^-$, and of singlet oxygen. Antioxidant protection is needed in extracellular fluids as well as within cells. In addition to glutathione and ascorbate, bilirubin,[596] uric acid,[597] melatonin,[598,598a] circulating superoxide dismutase, and the copper protein ceruloplasmin (Chapter 16) all act as antioxidants. Methionine residues of proteins may have a similar function.[599] Various proteins and small chelating compounds such as citrate tie up Fe^{3+} preventing it from promoting radical formation. Tocopherols, ubiquinols, and lipoic acid[600,600a–c] protect membranes. Beta carotene (Fig. 22-5), another lipid-soluble antioxidant, is the most effective quencher of singlet O_2 that is known. Even nitric oxide, usually regarded as toxic, sometimes acts as an antioxidant.[601] Trehalose protects plants against oxidative damage.[601a]

An increasing number of proteins are being recognized as protectants against oxidative damage. The exposed –SH and $-SCH_3$ groups of cysteine and methionine residues in proteins may function as appropriately located scavengers which may donate electrons to destroy free radicals or react with superoxide ions to become sulfonated. The thioredoxin (Box 15-C) and glutathione (Eq. 18-70) systems, in turn, reduce the protein radicals formed in this way.[601b–d] Methionine sulfoxide, both free and in polypeptides, is reduced by **methionine sulfoxide reductase** in organisms from bacteria to humans.[601e–g] Biotin, together with biotin sulfoxide reductase,[601h] may provide another antioxidant system. Some bacteria utilize glutathione-independent **alkylperoxide reductases** to scavenge organic peroxides.[601i] while mammals accomplish the same result with **peroxiredoxins** and with thioredoxin.[601j] Many other proteins will doubtless be found to participate in defense against oxidative damage. Oxygen is always present and its reactions in our bodies are essential. Generation of damaging reduced oxygen compounds and radicals is inevitable. Evolution will select in favor of many proteins that have been modified to minimize the damage.

Antioxidant enzymes do not always protect us. There was great excitement when it was found that victims of a hereditary form of the terrible neurological disease **amyotrophic lateral sclerosis** (**ALS**; see also Chapter 30) carry a defective gene for Cu / Zn-superoxide dismutase (SOD; Eq. 16-27).[602–603b] This discovery seemed to support the idea that superoxide anions in the brain were killing neurons. However, it now appears that in some cases of ALS the defective SOD is *too active*, producing an excess of H_2O_2, which damages neurons.

Transcriptional regulation of antioxidant proteins. Certain proteins with easily accessible Fe–S clusters, e.g., aconitase, are readily inactivated by oxidants such as peroxynitrite.[540,604] At least two proteins of this type function as transcription factors in *E. coli*. These are known as **SoxR** and **OxyR**. The SoxR protein is sensitive to superoxide anion, which carries out a one-electron oxidation on its Fe_2S_2 centers.[540,605–607] In its oxidized form SoxR is a transcriptional regulator that controls 30–40 genes, among them several that are directly related to "**oxidative stress.**"[608] These include genes for manganese SOD, glucose-6-phosphate dehydrogenase, a DNA repair nuclease, and aconitase (to replace the inactivated enzyme). The OxyR protein, which responds to elevated $[H_2O_2]$, is activated upon oxidation of a pair of nearby –SH groups to form a disulfide bridge.[607,609] It controls genes for catalase, glutathione reductase, an alkyl hydroperoxide reductase,[610,610a] and many others. Similar transcriptional controls in yeast result in responses to low doses of H_2O_2 by at least 167 different proteins.[608] Animal mitochondria also participate in sensing oxidant levels.[611,612] (See also Chapter 28, Section C,6.)

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Study Questions

1. Reticulocytes (immature red blood cells) contain mitochondria that are capable of both aerobic and anaerobic oxidation of glucose. In an experiment using these cells, incubated in oxygenated Krebs–Ringer solution with 10 mM glucose, the addition of antimycin A produced the following changes in metabolite concentration after 15 min (From Ghosh, A. K. and Sloviter, H. A. (1973) *J. Biol. Chem.* **248**, 3035–3040). Interpret the observed changes in ATP, ADP, and AMP concentrations (see tabulation). Express the concentration of each component after addition of antimycin as a percentage of that before addition. Then plot the resulting figures for each compound in the sequence found in glycolysis, i.e., label the *X* axis as follows:

Metabolite	Abbreviation	Concentrations (mmol/1 of cells) Before addition of antimycin	After addition of antimycin
Glucose 6-phosphate	G6P	460	124
Fructose 6-phosphate	F6P	150	30
Fructose 1,6-bisphosphate	FBP	8	33
Triose phosphates	TP	18	59
3-Phosphoglycerate	3PGA	45	106
2-Phosphoglycerate	2PGA	26	19
Phosphoenolpyruvate	PEP	46	34
Pyruvate	Pyr	126	315
Lactate	Lac	1125	8750
ATP		2500	1720
ADP		280	855
AMP		36	206

2. The following problem can be solved using standard reduction potentials (Table 6-8). Use $E°'$ (pH 7) values for NAD^+, enzyme-bound FAD, and fumarate of −0.32, 0.0, and −0.03 volts, respectively. Values of numerical constants are given in Table 6-1.

   a) Derive an equation relating the equilibrium constant for a reaction, K_{eq}, to differences in E_0'.

   b) Calculate the numerical values of K_{eq} for the reactions

   $$\text{Succinate} + NAD^+ \rightarrow \text{Fumarate} + NADH + H^+$$

   $$\text{Succinate} + FAD \rightarrow \text{Fumarate} + FADH_2$$

   at pH 7 and 25°C. The values should be calculated for succinate and the oxidant in the numerator.

3. Compare the catalytic cycles of the following enzymes:

   Peroxidase
   Cytochrome *c* oxidase
   Cytochrome P450

4. What chemical properties are especially important for the following compounds in the electron transport complexes of mitochondria?

   FAD or FMN
   Ubiquinone (coenzyme Q)
   Cytochrome *c*

5. Describe the operation of the F_1F_0ATP synthase of mitochondrial membranes.

6. In studies of mitochondrial function the following stoichiometric ratios have been measured.

   a) The P/O ratio: number of molecules of ATP formed for each atom of oxygen (as O_2) taken up by isolated mitochondria under specified conditions.
   b) The ratio of H^+ ions translocated across a mitochondrial inner membrane to the molecules of ATP formed.
   c) The ratio of H^+ ions pumped out of a mitochondrion to the number of molecules of ATP formed.

   Discuss the experimental difficulties in such measurements. How do uncertainties affect conclusions about the mechanism of ATP synthase? Are the ratios in (b) and (c) above necessarily equal? Explain.

7. Compare P/O ratios observed for mitochondrial respiration with the following substrates and conditions:

Study Questions

a) Oxidation of NADH by O_2.
b) Oxidation of succinate by O_2.
c) Dehydrogenation of ascorbate by O_2.

How would the ratio of ATP formed to the number of electrons passing from NADH through the respiratory chain differ for these three oxidants: O_2, fumarate, nitrite?

8. What is the mitochondrial glycerol phosphate shuttle? Is it utilized by plant cells? Explain.

9. What chemical reactions are included in these two important components of the nitrogen cycle (see also Fig. 24-1)?

Nitrification
Denitrification

10. What is the difference between a dioxygenase and a monooxygenase? What is meant by a cosubstrate for a monooxygenase?

11. The enzyme *p*-hydroxybenzoate hydroxylase utilizes a cosubstrate together with O_2 to form 3,4-dihydroxybenzoate. Indicate the mechanisms by which the bound FAD cofactor participates in the reaction.

12. What pterin-dependent hydroxylation reactions are important to the human body? Point out similarities and differences between flavin and pterin hydroxylase mechanisms.

13. Describe the basic properties of nitric oxide synthases (NOSs) and their varied functions in the body. What are the three different types of NOS? In what ways do they differ?

14. List several compounds that cause oxidative stress in cells and describe some chemical and physiological characteristics of each.

15. Propylene glycol is metabolized by several aerobic bacteria to acetoacetate, which can be catabolized as an energy source (see references 509a and 509b). The first step is conversion to an epoxide which reacts further in coenzyme M-dependent and CO_2-dependent reactions to form acetoacetate. Can you propose chemical mechanisms?

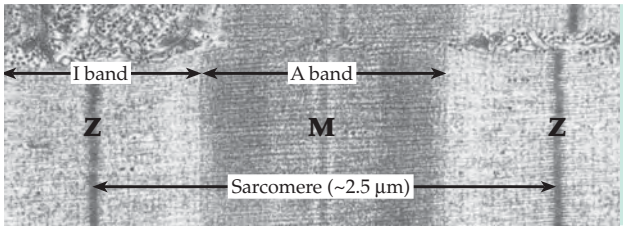
16. A group of slow-growing denitrifying bacteria obtain energy by oxidizing ammonium ions anaerobically with nitrite ions.[613,614]

$$NO_2^- + NH_4^+ \rightarrow N_2 + 2\,H_2O$$

Intermediate metabolites are hydroxylamine (H_2NOH) and hydrazine (N_2H_4). The reaction takes place within internal vesicles known as **anammoxosomes**. Unusual cyclobutane- and cyclohexane-based lipids in their membranes are thought to partially prevent the escape of the toxic intermediates from the anammoxosomes.[614]

Four protons may move from the cytoplasm into the vesicles for each ammonium ion oxidized. Can you write a reaction sequence? What is the Gibbs energy change for the reaction? How is ATP generated? See p. 1052.

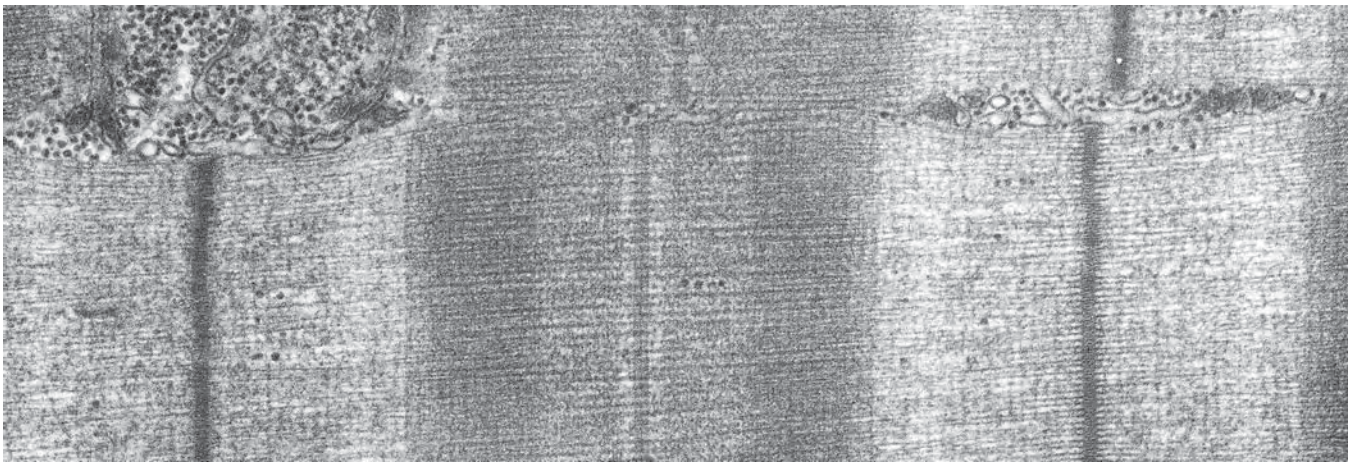
Notes



Electron micrograph of a thin longitudinal section of a myofibril from pig muscle. The basic contractile unit is the **sarcomere**, which extends from one Z line to the next. Thin **actin** filaments are anchored at the M lines and the thick **myosin** filaments at the Z lines. The (anisotropic) A bands are regions of overlap of interdigitated thick and thin filaments, while the I ("isotropic") bands are devoid of thick filaments. The ATP-driven contraction of muscle involves sliding of the interdigitated filaments and shortening of the sarcomere to ~1.8 µm. Micrograph courtesy of Marvin Stromer

Contents

19 The Chemistry of Movement



The swimming of bacteria, the flowing motion of the ameba, the rapid contraction of voluntary muscles, and the slower movements of organelles and cytoplasm within cells all depend upon transduction of chemical energy into mechanical work.

A. Motility of Bacteria

The smallest organs of propulsion are the bacterial flagella (Figs. 1-1, 1-3), and we have been able to unravel some of the mystery of movement by looking at them. When a cell of *E. coli* or *Salmonella* swims smoothly, each flagellum forms a left-handed superhelix with an ~2.3 μm pitch. Rotation of these "propellors" at rates of 100–200 revolutions / s (100–200 Hz) or more[1,2] in a counterclockwise direction, as viewed from the distal end of the flagellum, drives the bacterium forward in a straight line.[3–8] Several flagella rotate side-by-side as a bundle.[4] The observed velocities of 20–60 μm / s are remarkably high in comparison with the dimensions of the bacteria. Also remarkable is the fact that a cell may travel straight for a few seconds, but then tumble aimlessly for about 0.1 s before again moving in a straight line in a different direction. The tumbling occurs when the flagellum reverses its direction of rotation and also changes from a left-handed to a right-handed superhelix, which has just half the previous pitch.

Such behavior raised many questions. What causes reversal of direction of the propellor? Why do the bacteria tumble? How does a bacterium "decide" when to tumble? How is the flagellum changed from a left-handed to a right-handed superhelix? How does this behavior help the bacterium to find food? Most intriguing of all, what kind of motor powers the flagella? The answers are complex, more than 50 genes being needed to specify the proteins required for assembly and operation of the motility system of *E. coli* or *Salmonella typhimurium*.[9]

1. The Structure and Properties of Bacterial Flagella

Twenty or more structural proteins are present from the base to the tip of a complete bacterial flagellum. However, over most of their length the long thin shafts (Figs. 1-1, 19-1) are composed of subunits of single proteins called **flagellins**. Flagellin molecules have a high content of hydrophobic amino acids and, in *Salmonella*, contain one residue of the unusual N^{ε}-methyllysine. The subunits are arranged in a helix of outside diameter ~20 nm in which they also form 11 nearly longitudinal rows or **protofilaments**.[10–12a] Each subunit gives rise to one of the projections seen in the stereoscopic view in Fig. 19-1B. The flagella usually appear under the electron microscope to be supercoiled (Fig 19-1C–E) with a long "wavelength" (pitch) of ~2.5 μm. The supercoiled structure is essential for function, and mutant bacteria with straight flagella are nonmotile. Under some conditions and with some mutant flagellins, straight flagella, of the type shown in Fig. 19-1B, are formed. There is a central hole which is surrounded by what appears to be inner and outer tubes with interconnecting "spokes." However, all of the 494-residue flagellin subunits presumably have identical conformations, and each subunit contributes to both inner and outer tubes as well as to the outer projections. **Basal bodies** (Fig. 19-2) anchor the flagella to the cell wall and plasma membrane and contain the protic motors (Fig. 19-3) that drive the flagella.[14–16]

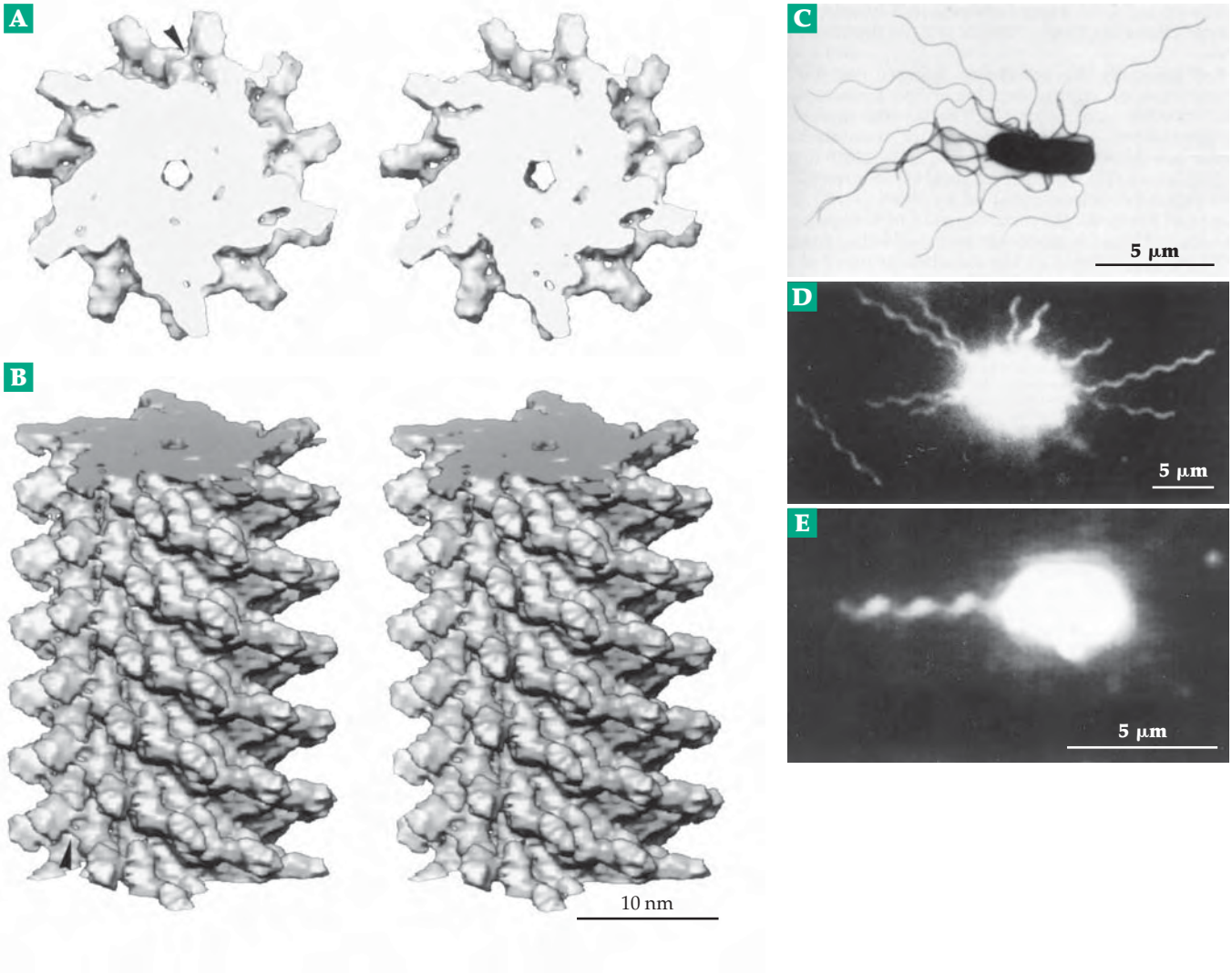


Figure 19-1 (A) Axial view of a 5-nm thick cross-section of the flagellar filament shown in (B). The 11 subunits form two turns of the one-start helix. (B) Stereoscopic oblique view of a 30-nm long section of a flagellum of *Salmonella typhimurium*. This is a straight flagellum from a nonmotile strain of bacteria. The structure was determined to a resolution of 0.9 nm by electron cryomicroscopy. From Mimori *et al.*[11] Courtesy of Keiichi Namba. (C) Electron micrograph of a cell of *S. typhimurium* showing peritrichous (all-around) distribution of flagella. Courtesy of S. Aizawa.[3] (D) Dark-field light micrograph of a flagellated cell of *S. typhimurium* with flagella dispersed during tumbling (see text). Courtesy of R. M. Macnab.[3] (E) Image of a cell of *Vibrio alginolyticus* obtained with dark-field illumination showing the single polar flagellum.[13] Because the illumination was strong, the size of the cell body and the thickness of the flagellum in the image appear large. Courtesy of Michio Homma.

Quasiequivalence. There are two distinct types of straight flagella: one (R) in which the protofilaments have a right-handed twist (as in Fig. 19-1) and the other (L) in which the protofilaments have a left-handed twist. These arise from two different conformations of the subunit proteins. Native supercoiled flagella contain a mixture of flagellins in the R- and L-states with all subunits in a given protofilament being in the same state. The supercoiling of the filament cannot be explained by stacking of identical subunits but is thought to arise because of an asymmetric distribution of protofilaments in a given state around the filament.[17–19a] Here, as with the icosahedral viruses (Chapter 7), quasiequivalence permits formation of a structure that would be impossible with full equivalence of subunits. The corkscrew shape of the flagellum is essential to the conversion of the motor's torque into a forward thrust.[18] Certain mutants of *Salmonella* have "curly" flagella with a superhelix of one-half the normal pitch. The presence of *p*-fluorophenylalanine in the growth medium also produces curly flagella, and normal flagella can be transformed to curly ones by a suitable change of pH. More important for biological function, the transformation from normal to curly also appears to take place during the tumbling of bacteria associated with chemotaxis.[17]

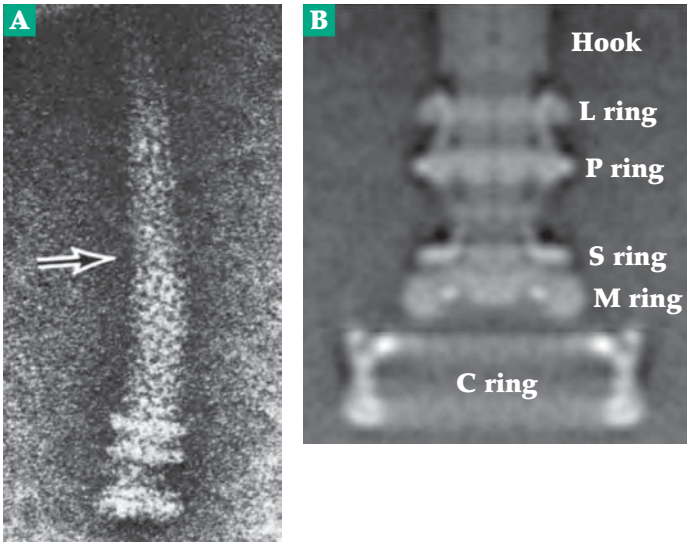


Figure 19-2 (A) Electron micrograph of a flagellum from *E. coli* stained with uranyl acetate. The M-and S-rings are seen at the end. Above them are the P-ring, thought to connect to the peptidoglycan layer, and the L-ring, thought to connect to the outer membrane or lipopolysaccharide layer (see Fig. 8-28). An arrow marks the junction between hook and thinner filament. From DePamphilis and Adler.[14] The hook is often bent to form an elbow. (B) Average of ~100 electron micrographs of frozen-hydrated preparations of basal bodies showing the cytoplasmic C-ring (see Fig. 19-3) extending from the thickened M-ring. From DeRosier.[16]

Growth of flagella. Iino added *p*-fluorophenylalanine to a suspension of bacteria, whose flagella had been broken off at various distances from the body.[20] Curly ends appeared as the flagella grew out. Unlike the growth of hairs on our bodies, the flagella grew from the outer ends. Because no free flagellin was found in the surrounding medium, it was concluded that the flagellin monomers are synthesized within the bacterium, then pass out, perhaps in a partially unfolded form, through the 2- to 3-nm diameter hole[10,12] in the flagella, and bind at the ends.[21] Flagella of *Salmonella* grow at the rate of 1 µm in 2–3 min initially, then more slowly until they attain a length of ~15 µm. More recent studies have provided details. The hook region (Fig. 19-3) grows first to a length of ~55 nm by addition to the basal-body rod of ~140 subunits of protein **FlgE**. During growth a **hook cap** formed from subunits **FlgD** prevents the FlgE subunits from passing out into the medium.[22,23] Hook subunits are added beneath the cap, moving the cap outward. Hook growth is terminated by protein **FlgK** (also called hook-associated protein Hap1). This protein displaces the hook cap and initiates growth of the main filament.[24] The first 10–20 subunits added are those of the FlgK (Fig. 19-3). These are followed by 10–20 subunits of **FlgL** (Hap3), a modified flagellin whose mechanical properties can accomodate the stress induced in the flagella by their rotation.[25] FlgJ is also needed for rod formation.[25a]

Growth of the flagellum to a length of up to 20 µm continues with subunits of **FliC** that are added at the tip, which is now covered by a dodecamer of the **cap protein FliD** (HAP2).[24,26,26a,b] Its 5-fold rotational symmetry means that this "star-cap" does not form a perfect plug for the 11-fold screw-symmetry of the flagellum, a fact that may be important in allowing new flagellin subunits to add at the growing tip. If the cap protein is missing, as in some *FliD* mutants, a large amount of flagellin leaks into the medium.[24]

Still unclear is how the protein synthesis that is taking place on the ribosomes in the bacterial cytoplasm is controlled and linked to "export machinery" at the base of the flagellin. As indicated in Fig. 19-3, the genetically identified proteins FlhA, FliH, and FliI are involved in the process that sends the correct flagellin subunits through the growing flagellum at the appropriate time. FliI contains an ATPase domain.[26c] FliS protein may be an export chaperone.[26d]

2. Rotation of Flagella

A variety of experiments showed that the flagellum is a rigid propellor that is rotated by a "motor" at the base. For example, a bacterium, artificially linked by means of antibodies to a short stub of a flagellum of another bacterium, can be rotated by the second bacterium. Rotation of cells tethered to a cover slip has also been observed. Although it is impossible to see individual flagella on live bacteria directly, bundles of flagella and even single filaments (Fig. 19-1C) can be viewed by dark-field light microscopy.[8,29] Normal flagella appear to have a left-handed helical form, but curly *Salmonella* flagella, which have a superhelix of one-half the normal pitch, form a right-handed helix.[5] Normal bacteria swim in straight lines but periodically "tumble" before swimming in a new random direction. This behavior is part of the system of **chemotaxis** by which the organism moves toward a food supply.[30] Curly mutants tumble continuously. When bacteria tumble the flagella change from normal to curly. The pitch is reversed and shortened. A proposed mechanism for the change of pitch involves propagation of cooperative conformational changes down additional

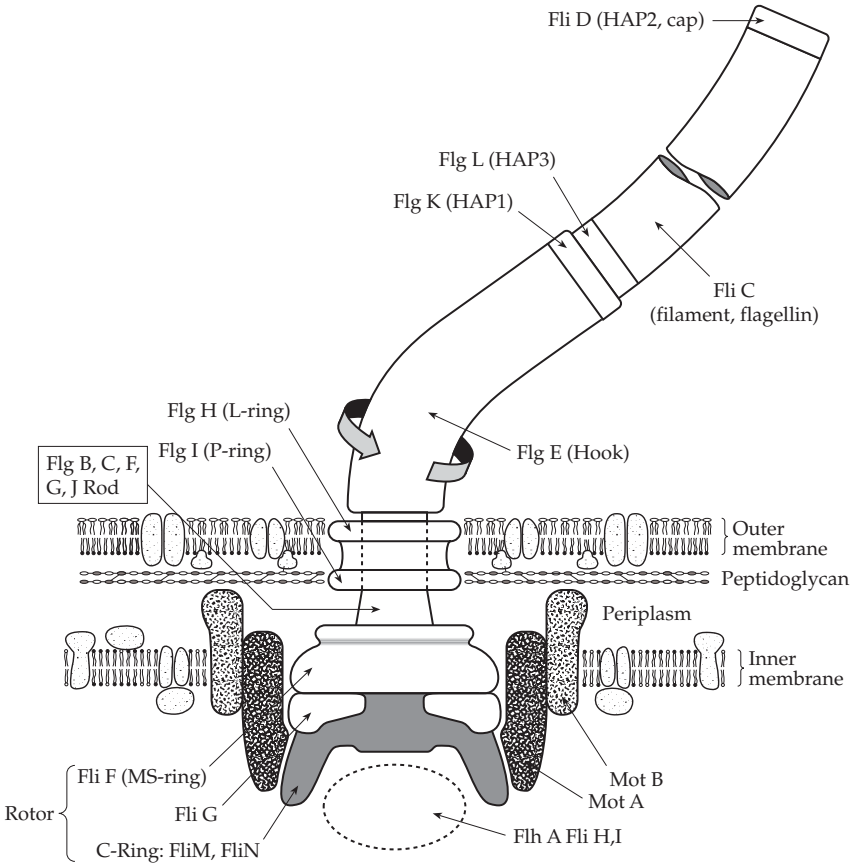


Figure 19-3 Schematic drawing of bacterial flagellar motor. Based on drawings of Berg,[27] Zhou and Blair,[28] and Elston and Oster.[1]

rows of flagellin subunits.[31]

There are no muscle-type proteins in the flagella. By incubating flagellated bacteria with penicillin and then lysing them osmotically, Eisenbach and Adler obtained cell envelopes whose flagella would rotate in a counterclockwise fashion if a suitable artificial electron donor was added.[32] This and other evidence showed that ATP is not needed. Rather, the torque developed is proportional to the **protonmotive force** and, under some circumstances, to ΔpH alone. It is the flow of protons from the external medium into the cytoplasm that drives the flagella.[8] Movement of *E. coli* cells in a capillary tube can also be powered by an external voltage.[33] In alkalophilic strains of *Bacillus* and some *Vibrio* species a sodium ion gradient will substitute.[13] Several hundred protons or Na^+ ions must pass through the motor per revolution.[8] Some estimates, based on energy balance,[29] are over 1000. However, Na^+-dependent rotation at velocities of up to 1700 Hz has been reported for the polar flagellum of *Vibrio alginolyticus*. It is difficult to understand how the bacterium could support the flow of 1000 Na^+ per revolution to drive the flagellum.[2]

What kind of protic motor can be imagined for bacterial flagella? Electron microscopy reveals that the flagellar hook is attached to a rod that passes through the cell wall and is, in turn, attached to a thin disc, the **M-ring** (or MS-ring), which is embedded in the cytoplasmic membrane both for gram-positive and gram-negative bacteria (Fig. 19-3). Two additional rings are present above the M-ring of flagella from gram-negative bacteria. The P-ring interacts with the peptidoglycan layer, and the L-ring contacts the outer membrane (lipopolysaccharide; Fig. 19-3). A logical possibility is that the M-ring, which lies within the plasma membrane, is the rotor, and a ring of surrounding protein subunits is the stator for the motor (Fig. 19-4).[34,35] Glagolev and Skulachev suggested in 1978 that attraction between $-COO^-$ and $-NH_3^+$ groups provides the force for movement.[34] Protons passing down an H^+-conducting pathway from the outer surface could convert $-NH_2$ groups to $-NH_3^+$, which would then be attracted to the $-COO^-$ groups on the stator subunits. When these two oppositely charged groups meet, a proton could be transferred from $-NH_3^+$ to $-COO^-$ destroying the electrostatic attraction. At the same time, movement of the M-ring would bring the next $-NH_2$ group to the H^+-conducting pathway from the outside. The $-COOH$ of the stator would now lose its proton through a conducting pathway to the inside of the bacterium, the proximity of the new $-NH_3^+$ assisting in this proton release. Since that time, other models based on electrostatic interactions have been advanced.[1,29,36]

Approximately 40 genes are required for assembly of the flagella, but mutations in only five motility genes have produced bacteria with intact flagella that do not rotate. Among these genes are *motA*, *mot B*, *FliG*, *FliM*, and *FliN*.[16,29,37,37a] Infection with a lambda transducing bacteriophage carrying functional *motB* genes restores motility to *motB* mutants by inducing synthesis of the motB protein. Block and Berg observed rotation of single bacteria tethered to a coverslip by their flagella. As the synthesis of the motB protein increased,the flagellar rotation rate increased in as many as 16 steps. This suggested that as many as 16 subunits of the *motB* gene product may contribute to the operation of the motor.[38] Later studies suggest eight subunits[39] rather than 16.

Both the M-ring and the thin S-ring, which lies directly above it and is now usually referred to as the MS-ring, are formed from ~20–25 subunits of the 61-kDa **FliF** protein.[39] Both the MotA and MotB proteins are embedded in the inner bacterial membrane and appear to form a circular array of "studs" around the M-ring.[16] MotA has a large cytosolic domain as well as four predicted transmembrane helices[40] while MotB has a large periplasmic domain and probably binds to the peptidoglycan.[37,41,41a] The MotA and MotB proteins, which bind to each other, are thought to form the ~8 functional units in the stator of the motor.[37] Proteins FliG, FliM, and FliN are evidently parts of the rotor assembly. FliM and FliN form an additional ring, the cytoplasmic or **C-ring**, which had been difficult to see in early electron microscopy. As many as 40 of each of these subunits may be present in the ring.[42,43] A ring of FliG subunits joins the C-ring to the MS-ring (Fig. 19-3). FliE is also a part of the basal body.[25a]

From study of mutants it has been concluded that three charged residues of FliG, R279, D286, and D287 are directly involved in generation of torque by the motor.[44] Side chains of these residues may interact with the cytoplasmic domains of MotA and MotB. Residues R90 and E98 of MotA may be involved in controlling proton flow through the motor units.[28,44] The two prolines P173 and P122 are also essential for torque generation.[28]

There are obvious similarities between the flagellar motors and the protic turbines of ATP synthases (Fig. 18-14), but there are also substantial differences. It apparently takes about 12 protons for one revolution of the ATP synthase but about 1000, or ~125 per motor unit, for rotation of a bacterial flagellum. Elston and Oster propose an ion turbine more complex than that of ATP synthase. They suggest that the rotor might contain about 60 slanted rows of positively charged groups spaced as shown in Fig. 19-4. The motor is reversible, i.e., it can rotate in either direction. One possibility is that the subunits alter their conformations cooperatively in such a way that the slant of the rows of charged groups is reversed. Other possibilities for altering the constellation of charges via conformational changes can be imagined.[1] See also Thomas *et al*.[44a]

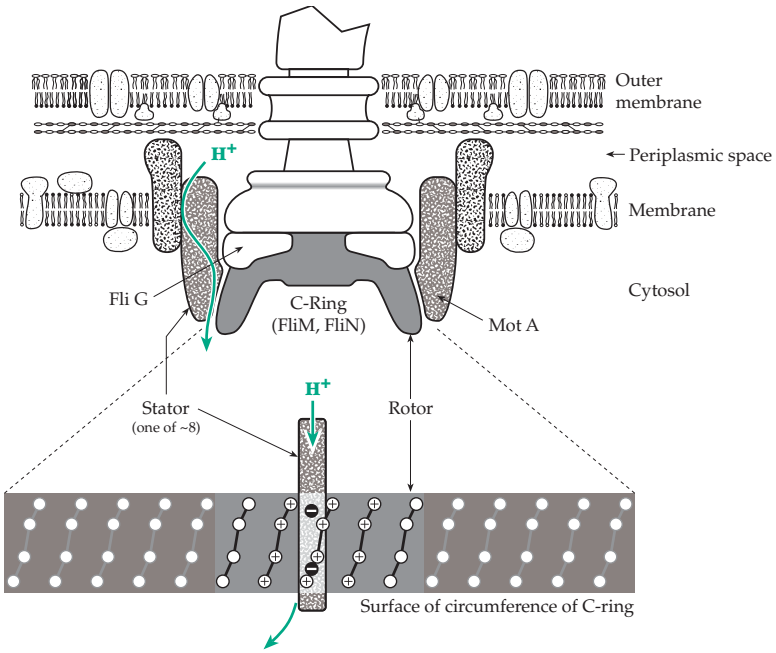


Figure 19-4 Schematic drawing of a hypothetical configuration of rotor and one stator unit in a flagellar motor as proposed by Elston and Oster.[1] The rotor can hold up to 60 positive charges provided by protons flowing from the periplasm through the stator motor units that surround the C-ring and hopping from one site to the next along the slanted lines. The rotor is composed of 15 repeating units, each able to accommodate four protons. Negative charges on the stator units are 0.5 nm from the rotor charges at their closest approach. For details see the original paper.

3. Chemotaxis

The flagellar motor is reversible, and in response to some signal from the bacterium it will turn in the opposite direction. At the same time, the flagellin subunits and those of the hook undergo conformational changes that change the superhelical twist. Perhaps synchronous conformational changes in the M-ring also are associated with the change in direction of rotation and are induced by interaction with a **switch complex** that lies below the M-ring. This consists of proteins FliG, FliM, and FliN.[44b] Mutations in any one of these proteins lead to the following four phenotypes: absence of flagella, paralyzed flagella, or flagella with the switch biased toward clockwise or toward counterclockwise rotation.[45]

What signals a change in direction of rotation? The answer lies in the attraction of bacteria to compounds that they can metabolize. Bacteria will swim toward such compounds but away from repellent substances, a response known as **chemotaxis**. Cells of *E. coli* swim toward higher concentrations of L-serine (but not of D-serine), of L-aspartate, or of D-ribose.

Phenol and Ni^{2+} ions are repellent.[46–48] By what mechanism can a minuscule prokaryotic cell sense a concentration gradient? It is known that the plasma membrane contains receptor proteins, whose response is linked to control of the flagella. Since the dimensions of a bacterium are so small, it would probably be impossible for them to sense the difference in concentration between one end and the other end of the cell. The chemotatic response apparently results from the fact that a bacterium swims for a relatively long time without tumbling when it senses that the concentration of the attractant is increasing with *time*. When it swims in the opposite direction and the concentration of attractant decreases, it tumbles sooner.[49]

Koshland[47] proposed that as the membrane receptors become increasingly occupied with the attractant molecule, the rate of formation v_f of some compound X, within the membrane or within the bacterium, is increased (Eq. 19-1). When [X] rises higher than a threshold level, tumbling is induced. At the same time, X is destroyed at a velocity of v_d.

$$\xrightarrow{v_f} [X] \xrightarrow{v_d} \qquad (19\text{-}1)$$

Subsequently, a readjustment of v_f and v_d occurs such that the concentration of X falls to its normal steady state level. X would act directly on the flagellar motor.

The receptors for L-serine[50–51a] and L-aspartate[52,53] are 60-kDa proteins encoded by genes *tsr* and *tar* in *Salmonella* or *E. coli*.[46,54] These proteins span the inner plasma membrane of the bacteria as shown in Figs. 11-8 and 19-5. The functioning of the receptor has been discussed in some detail in Chapter 11. However, there is still much that is not understood. The symmetric head, whose structure is known (Fig. 11-8), has two binding sites, but the aspartate receptor binds only one aspartate tightly. There is substantial evidence that suggests a piston-type sliding of one helix toward the cytoplasm as part of the signaling mechanism.[54a] While the flagella are distributed around the cell, the receptors appear to be clustered at the cell poles.[55]

Proteins encoded by genes *cheA*, *cheW*, *cheY* and *cheZ, cheB,* and *ChR* are all involved in controlling chemotaxis.[48,56] Their functions are indicated in the scheme of Fig. 19-5. All of the corresponding protein products have been isolated and purified, and the whole chemotaxis system has been reconstituted in phospholipid vesicles.[57] Gene *CheA* encodes a 73-kDa protein kinase, which binds as a dimer to the cytoplasmic domains of the related aspartate, serine, and ribose/galactose receptors with the aid of a coupling protein, cheW (Fig. 19-5). A great deal of effort has been expended in trying to understand how binding of an attractant molecule to the periplasmic domain of the receptor can affect the activity of the CheA kinase, but the explanation is unclear. There is a consensus that a small but distinct conformational alteration is transmitted through the receptor.[58–61a] An apparently α-helical region containing methylation sites (Fig. 19-5) appears to be critically involved in the signaling, responding not only to occupancy of the receptor site but also to intracellular pH and temperature and to methylation. Mutation of the buried Gly 278 found in this region to branched hydrophobic amino acids, such as Val or leucine, locks the receptor in state with a superactivated CheA kinase, while *substitution* of Gly 278 with aspartate leaves the kinase *inactive*.[61] Occupancy of the normal receptor site with ligand (aspartate, serine, etc.,) dramatically decreases the kinase activity.

The CheA protein is an autokinase which, upon activation by the receptor, becomes phosphorylated on N^{ε} of the imidazole ring of His 48. It then transfers this phospho group from His 48 to the carboxylate of Asp 57 of the 654-residue protein **CheY**, which is known as the **response regulator**.[62–65d] The unregulated flagellum rotates counterclockwise (CCW). *Phospho-CheY* (CheY-*P*, which qualifies as X in Eq. 19-1) *carries the message to the flagellar motor to turn clockwise (CW).* This is apparently accomplished through the binding of CheY-*P* to the N-terminal portion of protein FliM. This presumably induces a conformational change, which is propagated to FliG and to all of the proteins of the rotor and flagellar rod, hook, and filament.[45,65,66,66a] The flagella fly apart, and the bacterium tumbles and heads randomly in a new direction.

Tumbling occurs most often when receptors are unoccupied, and the bacteria change directions often, as if lost. However, if a receptor is occupied by an attractant, the activity of CheY is decreased and less CheY-*P* will be made. The carboxyl phosphate linkage in this compound is labile and readily hydrolyzed, a process hastened by the phosphatase **CheZ**.[67–69] Consequently, in the presence of a high enough attractant concentration the tumbling frequency is decreased, CCW flagellar rotation occurs, and the bacterium swims smoothly for a relatively long time.

There are still other important factors. Occupancy of the receptor by a ligand makes the receptor protein itself a substrate for the chemotaxis-specific methyltransferase encoded by the *cheR* gene.[62,70,71] This enzyme transfers methyl groups from *S*-adenosylmethionine to specific glutamate side chains of the receptor to form methyl esters. In the aspartate receptor there are four such glutamate residues in a large cytoplasmic domain that includes the C terminus. Two of these glutamates are initially glutamines and can undergo methylation only if they are deaminated first.[72] An esterase encoded by the *cheB* gene[72] removes the methyl ester groupings as methanol.

The action of the CheR methyltransferase is apparently unregulated, but the esterase activity of CheB is controlled by the phosphorylation state of the

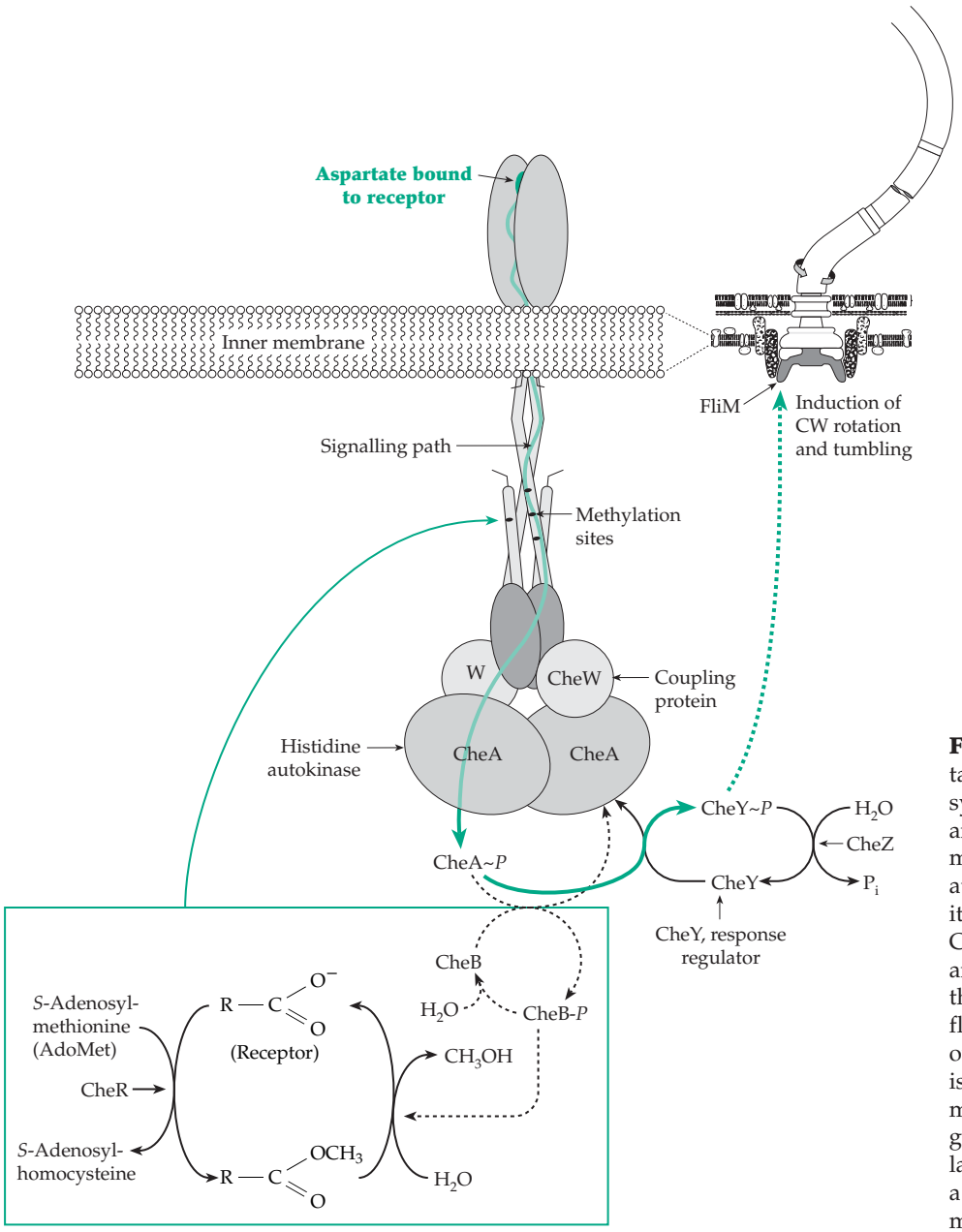


Figure 19-5 Schematic representation of an important chemotactic system of *E. coli*, *S. typhimurium*, and other bacteria. The transmembrane receptor activates the autokinase CheA, which transfers its phospho group to proteins CheY and CheB to form CheY-*P* and CheB-*P*. CheY-*P* regulates the direction of rotation of the flagella, which are distributed over the bacterial surface. CheR is a methyltransferase which methylates glutamate carboxyl groups in the receptor and modulates the CheA activity. CheZ is a phosphatase and CheB-*P* a methylesterase.

autokinase CheA. CheB competes with CheY (Fig. 19-5), and CheB-*P* is the active form of the esterase. After a chemotactic stimulus the level of CheA-*P* falls and so does the activity of the methylesterase. The number of methyl groups per receptor rises making the CheA kinase more active and opposing the decrease in kinase activity caused by receptor occupancy. The system is now less sensitive to the attractant; the bacterium has *adapted* to a higher attractant concentration.[62,73,73a] It tumbles more often unless the attractant concentration rises; if it is headed toward food tumbling is still inhibited. If it is headed away from the attractant the levels of both CheY-*P* and ChB-*P* rise. A high level of fumarate within the cell also acts on the switch–motor complex and favors CW rotation.[74]

For some bacterial attractants such as D-galactose, D-ribose, maltose, and dipeptides[75] the corresponding binding proteins,[38,76] which are required for the sugar uptake (e.g., Fig. 4-18A), are also necessary for chemotaxis. The occupied binding proteins apparently react with membrane-bound receptors to trigger the chemotactic response. The aspartate receptor (*tar* gene product) appears also to be the receptor for the maltose-binding protein complex,[47] and both the aspartate and the serine receptor (*tsr* gene product) also mediate thermotaxis and pH taxis.[77,77a] Clusters of identical receptors may function cooperatively to provide high sensitivity and dynamic range.[77b]

B. Muscle

There is probably no biological phenomenon that has excited more interest among biochemists than the movement caused by the contractile fibers of muscles. Unlike the motion of bacterial flagella, the movement of muscle is directly dependent on the hydrolysis of ATP as its source of energy. Several types of muscle exist within our bodies. **Striated** (striped) **skeletal muscles** act under voluntary control. Closely related are the **involuntary striated heart muscles,** while **smooth involuntary muscles** constitute a third type. Further distinctions are made between fast-twitch and slow-twitch **fibers**. **Fast-twitch fibers** have short isometric contraction times, high maximal velocities for shortening, and high rates of ATP hydrolysis. They occur predominately in white muscle. Because of the absence of the strong oxidative metabolism found in red muscles, fast-twitch fibers fatigue rapidly. Although red muscle sometimes contains fast-twitch fibers, it more often consists of **slow-twitch fibers,** which have a longer contraction time, low shortening velocity, and low ATPase. They are more resistant to fatigue[56] than fast-twitch fibers.[78] Embryonic muscle contains fast-twitch fibers as well as embryonic forms which contract slowly.[79] Some organisms contain specialized types of muscle. For example, the asynchronous flight muscles of certain insects cause the wings to beat at rates of 100–1000 Hz. In these muscles nerve impulses are used only to start and to stop the action; otherwise the cycle of contraction and relaxation continues automatically.[80] The adductor muscles, which close the shells of oysters and clams, can sustain large tensions for long periods of time with little expenditure of energy. This is accomplished by a **catch mechanism**.[81]

1. The Structural Organization of Striated Muscle

Skeletal muscles consist of bundles of long **muscle fibers,** which are *single cells* of diameter 10–100 μm formed by the fusion of many embryonic cells. The lengths are typically 2–3 cm in mammals but may sometimes be as great as 50 cm. Each fiber contains up to 100–200 nuclei. Typical cell organelles are present but are often given special names. Thus, the plasma membrane (plasmalemma) of muscle fibers is called the **sarcolemma**. The cytoplasm is **sarcoplasm,** and mitochondria may be called **sarcosomes**. The major characteristic of muscle is the presence of the contractile **myofibrils**, organized bundles of proteins 1–2 μm in diameter and not separated by membranes from the cytoplasm. Since they occupy most of the cytoplasm, a substantial number of myofibrils are present in each muscle fiber.

In the light microscope cross striations with a repeating distance of ~2.5 μm can be seen in the myofibrils (Figs. 19-6 and 19-7). The space between two of the dense **Z-discs** (Z lines) defines the **sarcomere**, the basic contractile unit. In the center of the sarcomere is a dense **A-band** (anisotropic band). The name refers to the intense birefringence of the band when viewed with plane polarized light. Straddling the Z-discs are less dense **I-bands** (the abbreviation stands for isotropic, a misnomer, for although the bands lack birefringence, they are not isotropic). Weakly staining **M-lines** (usually visible only with an electron microscope) mark the centers of the A-bands and of the sarcomeres.

The fine structure of the sarcomere was a mystery until 1953, when H. E. Huxley, examining thin sections of skeletal muscle with the electron microscope, discovered a remarkably regular array of interdigitated protein filaments.[82,83] **Thick filaments**, 12–16 nm in diameter and ~1.6 μm long, are packed in a hexagonal array on 40- to 50-nm centers throughout the A-bands (Fig. 19-6B). Between these thick filaments are **thin filaments** only 8 nm in diameter and extending from the Z-line for a length of ~1.0 μm. When contracted muscle was examined, it was found that the I-bands had become so thin that they had nearly disappeared and that the amount of overlap between the thick and the thin filaments had increased. This indicated that contraction had consisted of the sliding movement of the thick and thin filaments with respect to each other.[84] In skeletal muscle the sarcomere shortens to a length of ~2 μm, but in insect flight muscle a much smaller shortening occurs repetitively at a very high rate.

2. Muscle Proteins and Their Structures

The myofibrillar proteins make up 50–60% of the total protein of muscle cells. Insoluble at low ionic strengths, these proteins dissolve when the ionic strength exceeds ~0.3 and can be extracted with salt solutions. Analysis of isolated mammalian myofibrils[86] shows that nine proteins account for 96% or more of the protein; **myosin**, which constitutes the bulk of the thick filaments, accounts for 43% and **actin**, the principal component of the thin filaments, 22%.

Actin and the thin filaments. There are at least six forms of actin in adult mammalian tissues: α-cardiac, α-skeletal muscle, α- and γ-smooth muscle, β- and γ-cytoplasmic.[87–89] All of them are closely homologous, e.g., the 42-kDa α-skeletal muscle actin differs in only 4 of 375 residues from the α-cardiac form and only in 6 residues from the γ-smooth form. In almost all organisms actins contain one residue of N^{δ}-methylhistidine at position 73.[87,88,90] Actin is an unusual protein in that

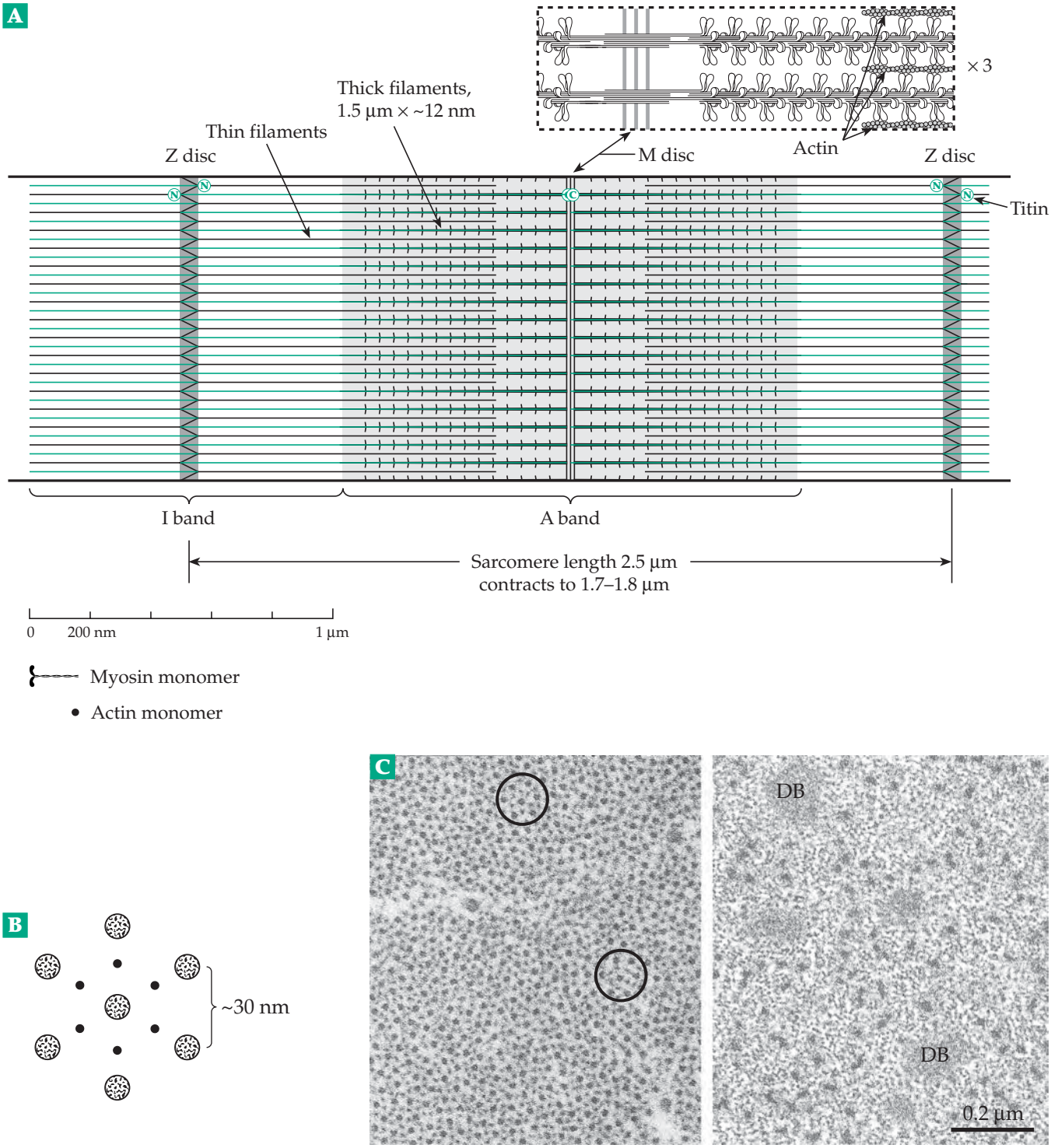


Figure 19-6 (A) The structure of a typical sarcomere of skeletal muscle. The longitudinal section depicted corresponds to that of the electron micrograph, Fig. 19-7A. The titin molecules in their probable positions are colored green. The heads of only a fraction of the myosin molecules are shown protruding toward the thin actin filaments with which they interact. A magnified section at the top is after Spudich.[85] It shows the interactions of the myosin heads with the thin filaments at the right-hand edge. (B) A sketch showing the arrangement of thick and thin filaments as seen in a transverse section of a striated muscle fiber. (C) Left: electron micrograph of a transverse section of a glycerated rabbit psoas muscle. The hexagonal arrangement of six thin filaments around one thick filament can be seen in the center of the circle. Six other thick filaments form a larger concentric circle as in (B). Right: transverse section of a smooth muscle fiber. Notice the irregular arrangement of thick and thin filaments. Filaments of intermediate diameter are also present, as are dense bodies (DB). The latter are characteristically present in smooth muscle.

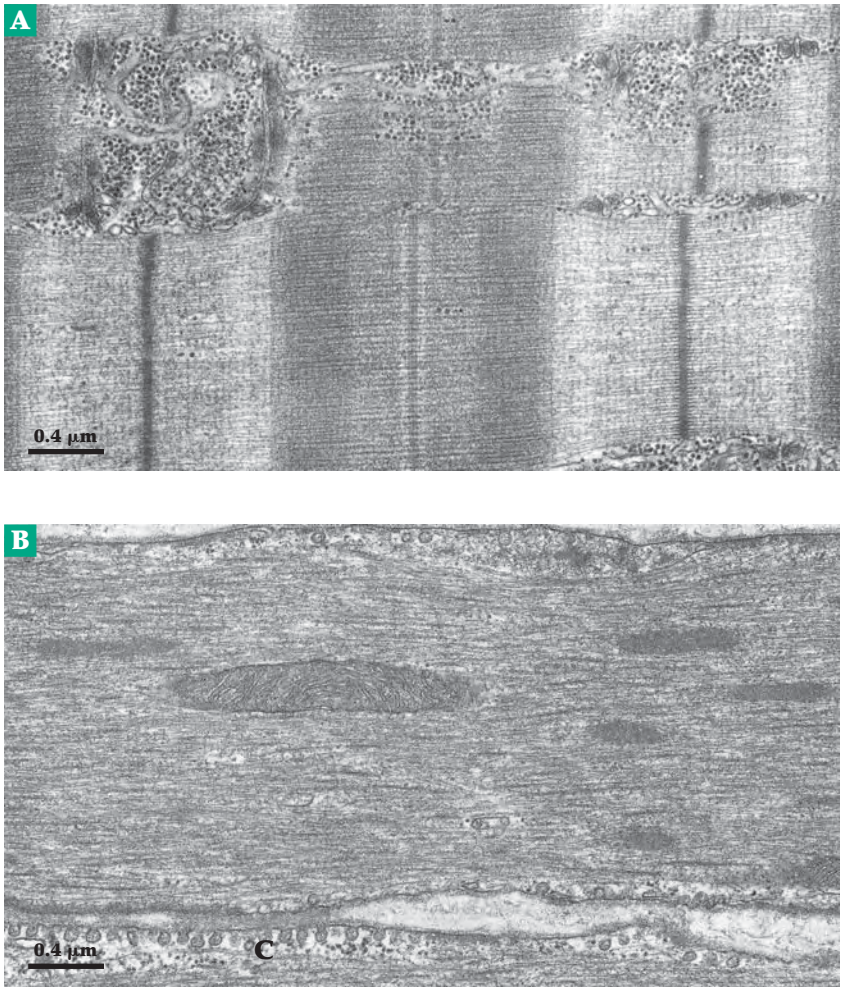


Figure 19-7 (A) Electron micrograph of a longitudinal section of a mammalian skeletal muscle (pig biceps muscle). The tissue was doubly fixed, first with formaldehyde and glutaraldehyde, then with osmium tetroxide. It was then stained with uranyl acetate and lead citrate. The section shows a white muscle fiber containing few mitochondria and narrow Z-lines. The Z-discs (marked Z), M-line, A- and I-bands, and thick and thin filaments can all be seen clearly. The periodicity of ~40 nm along the thin filaments corresponds to the length of the tropomyosin molecules, and the cross striation is thought to represent bound tropomyosin and troponin. The numerous dense particles in the upper part of the micrograph are glycogen granules, while the horizontal membranous structures are longitudinal tubules of the sarcoplasmic reticulum (endoplasmic reticulum). These come into close apposition to the T tubules leading from the surface of the muscle fiber. The T tubules (T) are visible in longitudinal section at the upper left of the micrograph on both sides of the Z-line and in cross-section in the upper right-hand corner. There a T tubule is seen lying between two lateral cisternae of the sarcoplasmic reticulum. (B) Longitudinal section of smooth muscle (chicken gizzard) fixed as in A. Thick filaments (Th), which are considerably thicker than those in striated muscle and less regular, can be seen throughout the section. They are surrounded by many thin filaments, which are often joined to dense bodies (DB). A mitochondrion (Mi) is seen in the center of the micrograph, and at the lower edge is a boundary between two adjacent cells. Notice the caveolae (C), which are present in large numbers in the plasma membrane and which are extremely active in smooth muscle. Micrographs courtesy of Marvin Stromer.

it can exist in both a filamentous and a soluble state. The interconversion between them is of great physiological importance. Actin filaments dissolve in a low ionic strength medium containing ATP to give the soluble, monomeric **G-actin**. Each G-actin monomer usually contains one molecule of bound ATP and a calcium ion.

Because of its tendency to polymerize, G-actin has been difficult to crystallize. However, it forms crystalline complexes with several other proteins, e.g., deoxyribonuclease I,[91] a fragment of gelsolin, and profilin,[92] which block polymerization and it has recently been crystallized as the free ADP complex.[92a] The three-dimensional structure of the actin is nearly the same in all cases. The molecule folds into four domains, the ATP binding site being buried in a deep cleft. The atomic structure (Fig. 7-10) resembles that of hexokinase, of glycerol kinase, and of an ATP-binding domain of a chaperonin of the Hsp 70 family.[90] As with the kinases, actin can exist in a closed and more open conformations, one of which is seen in the profilin complex. Addition of 1 mM Mg^{2+} or 0.1 M KCl to a solution of G-actin leads to spontaneous transformation into filaments of **F-actin** (Figs. 7-10 and 19-9) each containing 340–380 actin monomers and resembling the thin filaments of muscle.[93-94a] The ATP bound in the F-actin filament is hydrolyzed within ~100 s to ADP and P_i. However, the hydrolysis is not as rapid as polymerization so that a "cap" of ATP-containing monomers may be found at each end of the filament.[94,95,95a] There is a striking similarity to the binding of nucleotides to microtubule subunits (Fig. 7-33) and in the contractile tail sheath of bacteriophage (Box 7-C).

The two ends of the F-actin filaments have different surfaces of the monomer exposed and grow at different rates. This has been demonstrated by allowing the myosin fragment called heavy meromyosin (HMM; see Fig. 19-10) to bind to (or "decorate") an actin filament. The

myosin heads bind at an angle, all pointed in one direction. This gives a "pointed" appearance to one end and a "barbed" appearance at the other. When monomeric actin is added to such an HMM-decorated F-actin filament the barbed ends grow much faster than the pointed ends.[94,96] In the intact sarcomere the ends that become pointed when decorated are free, while the opposite barbed ends of the filaments are attached at the Z-line (Fig. 19-6A). The existence in the cytoplasm of proteins that "cap" the fast-growing end of actin filaments thereby preventing further growth[96,97] suggests that cap proteins may be present at the ends of the thin filaments of the myofibrils.

Titin and nebulin. The third most abundant protein (10%), titin (also called **connectin**),[98–100a] is one of the largest of known proteins. Titin cDNA from human cardiac muscle encodes a 26,926-residue chain. Several tissue-specific isoforms of the protein are created by alternative mRNA splicing.[101] A single titin molecule stretches ~1200 nm from the Z-disc, where the N terminus is bound, to the M-line, where the C-terminal domain is attached (Fig. 19-8A). Thoughout much of the A-band titin binds to the thick filament and appears to be part of a scaffold for maintenance of the sarcomere structure. The I-band portion of titin has elastic properties that allow it to lengthen greatly or to shorten as the sarcomere changes length.[98,100,102]

Under the electron microscope titin appears as a flexible beaded string ~4 nm in diameter. Most of the molecule is made up of repetitive domains of two types. In human cardiac titin there are 132 folded domains that resemble type III fibronectin repeats and 112 immunoglobulin-like domains.[98] In a "PEVK region," between residues 163 and 2174, 70% of the residues are Pro, Glu, Val, or Lys. The titin molecule may be organized as polyproline helices in this elastic region.[102a] At the C terminus of titin 800 residues, including a Ser / Thr protein kinase domain, are found within the M-line.

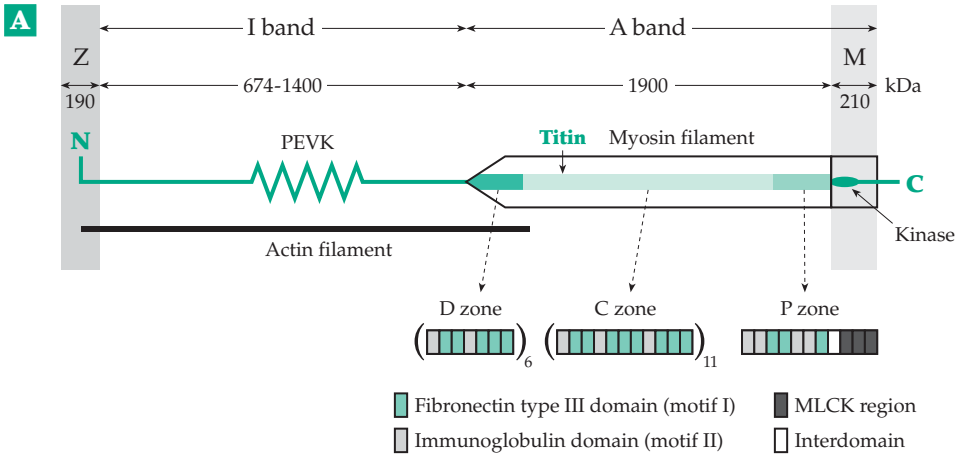
Another very large protein, **nebulin** (3% of the total protein),[103] appears to be stretched alongside the thin filaments. In the electron microscope it appears as a flexible, beaded string ~4 nm in diameter. Ninety-seven percent of the 6669-residue human nebulin is organized as 185 copies of an ~ 35-residue module.[104,105] Nebulin has a proline residue at about every 35th position, possibly corresponding in length to the pitch of the actin helix (Fig. 7-10). At the C terminus is an SH3 domain (see Fig. 11-14), which is preceded by a 120-residue segment rich in potential phosphorylation sites.[106] This part of the peptide chain is anchored in the Z-discs (Fig. 19-8B, C). The three extreme N-terminal modules of nebulin bind to tropomodulin, which caps the pointed ends of thin filments.[106a] Avian cardiac muscle contains a much shorter 100-kDa protein called **nebulette**, which resembles the C-terminal parts of nebulin. Nebulin has been described as encoding a blueprint for thin filament architecture.[99,103]

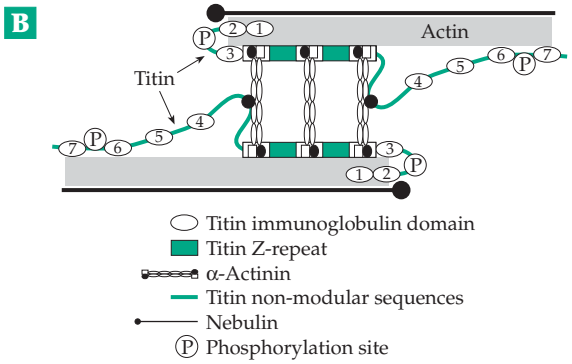
Proteins of the M-line and Z-disc. The M-line region contains the structural protein **myomesin**, which binds to both titin and myosin and holds the two together.[107] Fast skeletal and cardiac fibers also contain another **M-protein**, which may bridge between myosin filaments. Both the C-terminal region of nebulin and the N termini of pairs of titin molecules meet in the Z-disc, where they are bound into a lattice containing **α-actinin**[98,108-109b] and other proteins (Fig. 19-8B). The dimeric α-actinin, a member of the spectrin family, has a subunit mass of ~97 kDa.[109a] Found primarily in the Z-discs, it is also present in nonmuscle cells in stress fibers and at other locations in the cytoskeleton (Chapter 7). It may anchor actin filaments to various structures outside of the sarcomere.[110] In the dense Z-disc of insect flight muscle a regular hexagonal lattice of α-actinin[111] and a large (500–700 kDa) modular protein called **kettin**[112,112a,b] bind the thin filaments of opposite polarity together.

The **C-protein** (thick filaments), myomesin (M-line protein), and α-actinin (Z-line protein)[110,113,114] each provide 2% of the protein in the myofibril. Less than 1% each of 11 or more other proteins may also be present within the sarcomere.[86,115] Several of these, including the cytoskeletal proteins **desmin** and **vimentin**, and **synemin** surround the Z-discs.[116,116a]

The regulatory proteins troponin and tropomyosin. These two proteins are also associated with the filaments, each one contributing ~5% to the total protein of myofibrils. Tropomyosin is an elongated α-helical coiled-coil molecule, each molecule of which associates with seven actin subunits of an actin filament. Troponin consists of three subunits known as troponins C, T, and I. The elongated troponin T binds to tropomyosin. Troponin I is an inhibitor of the interaction of myosin and actin necessary for muscle contraction. Troponin C, a member of the calmodulin family (Fig. 6-8), binds Ca^{2+} and induces conformational changes that relieve the inhibition and allow contraction to occur. Nebulin is also thought to bind to tropomyosin. A possible arrangement of one of the tropomyosin–troponin–nebulin complexes that lie along the length of the thin filaments is shown schematically and as a three-dimensional model in Figs. 19-8C and D. These proteins are discussed further in Section 4. Figure 19-9 shows a model of the thin filaments with tropomyosin coiled-coil molecules on each side. The troponin subunits are not shown.

Myosins. There are 15 distinct families of proteins within the myosin superfamily.[117–120] They vary greatly in size, but all of them bind and hydrolyze ATP, and all bind to actin. Most have C-terminal tails. At their N





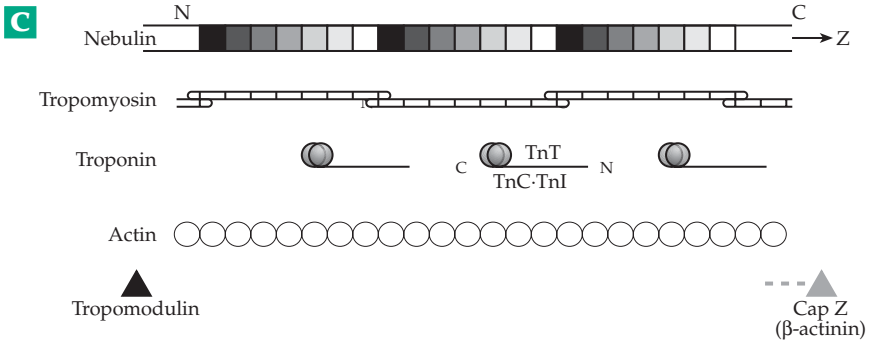


Figure 19-8 (A) Schematic drawing showing one molecule of titin (connectin) in a half sarcomere and its relationship to thick myosin filaments and thin actin filaments. The complex repeat patterns of fibronectin type III, immunoglobulin, in the three zones D, C, and P are also indicated.[98] See Maruyama.[98,98a] (B) Schematic drawing of the molecular structure of the sarcomere Z-disc. Titin, which is thought to parallel the thin filaments through the I-band, consists of various modules that are numbered from the N termini. In the Z-disc titin binds to α-actin, shown here as three vertical rods, and also to actin or actin-binding proteins. The SH3 domain (shown as a sphere) of nebulin and the N terminus of titin may interact. Regulatory phosphorylation sites are marked P. From Young *et al.*[108] Courtesy of Mathias Gautel. (C) Hypothetical model of a composite regulatory complex containing nebulin, tropomyosin, and troponin on the thin filaments of a skeletal muscle sarcomere. Each seven-module nebulin super-repeat (squares with graded shading) binds one tropomyosin, possible through the seven charge clusters along the length of each tropomyosin, and one troponin complex (shaded spheres with a tail). This complex consists of TnT, TnI, and TnC in orientations indicated by the N and C termini. Each nebulin super-repeat binds to seven actin monomers (open circles) along the thin filament. **Tropomodulin** caps the pointed ends of actin filaments and Cap Z, the "barbed ends." From Wang *et al.*[103]

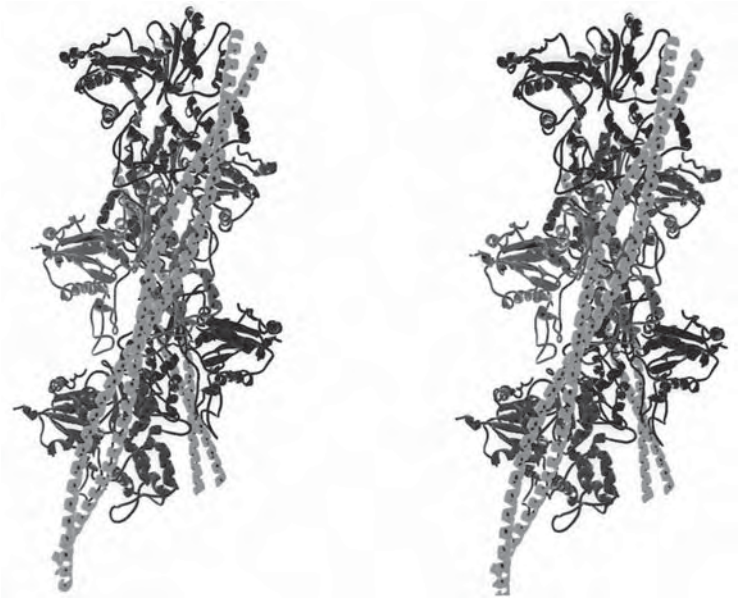


Figure 19-9 Stereoscopic ribbon drawing of the proposed structure of a thin actin filament with tropomyosin coiled-coils bound on opposing sides.[124] Five actin nomomers are assembled in the structure as is also illustrated in Fig. 7-10. From Lorenz *et al*.[125] Courtesy of Michael Lorenz.

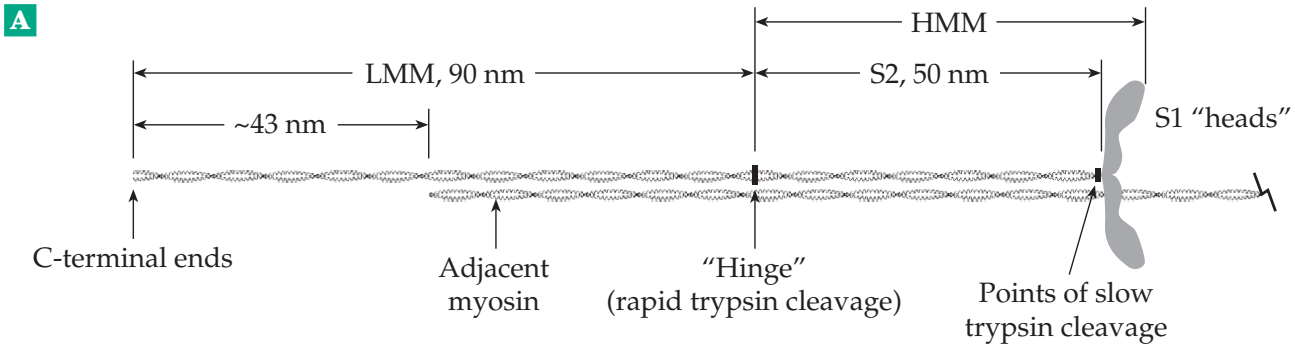
termini are one or two globular heads, which contain the catalytic centers in which ATP hydrolysis occurs. Sizes vary from 93 kDa for a myosin with a very short tail from *Toxoplasma*[118] to over 300 kDa. Myosins I, found in ameboid organisms and also in our own bodies (for example in the microvilli of the brush border of intestinal epithelial cells), are small single-headed molecules.[117,121] Myosins II are the "conventional" myosins of myofibrils and are often referred to simply as myosin. However, each of the three muscle types (skeletal, cardiac, and smooth) has its own kind of myosin II.[121a–c] Likewise, at least six different genes have been identified for the light chains of the myosin heads.[122] Fast and slow muscle as well as embryonic muscle have their own light chains. Each myosin II molecule consists of two identical ~230-kDa **heavy chains,** which are largely α-helical, together with two pairs of smaller 16- to 21-kDa **light chains**. Human skeletal muscle heavy chains contain 1938 residues of which the first ~850 are folded into pear-shaped heads, which contain the catalytic sites involved in harnessing ATP cleavage to movement. Following proline 850 nearly all of the remaining 1088 residues form an α-helical coiled-coil rod of dimensions ~160 x 2 nm (Fig. 19-10) in which the two chains coil around each other. The two heavy chains are parallel, each having its N terminus in one of the two heads and its C terminus bound in the shaft of the thick filament.

Myosins II from other sources have similar structures. For example, analysis of the DNA sequence for a heavy chain gene from the nematode *Caenorhabditis* showed that the protein contains 1966 residues, 1095 of which contain an amino acid sequence appropriate for a 160-nm long coiled coil.[123] There are no prolines within this sequence, which lies between Pro 850 and Pro 1944. Although there are many bands containing positively and negatively charged side chains along the myosin rod, the interactions between the two coiled helices are largely nonpolar. In *Drosophila* 15 different heavy-chain isoforms are created by splicing of a single mRNA.[123a]

While the C-terminal portions of the two parallel myosin heavy chains form a rod, the N-terminal portions fold into two separate heads. Each head also contains two smaller 16- to 21-kDa peptide chains which belong to the calmodulin family. One of these, the **essential light chain**, is tightly bound to the heavy chain. The second, the **regulatory light chain**, is able to bind Ca^{2+} and is less tightly bound to the rest of the head. A short treatment with trypsin or papain cuts the myosin molecule into two pieces. The tail end gives rise to **light meromyosin (LMM)**, a molecule ~90 nm in length. The remainder of the molecule including the heads is designated **heavy meromysin (HMM)**. A longer trypsin treatment leads to cleavage of HMM into one ~62-kDa **S2** fragment 40 nm long, and two ~130-kDa **S1** fragments, each of the latter representing one of the two heads (Fig. 19-10).

The junction of the head and tail portions of myosin appears rigid in Fig. 19-10A. However, there must be considerable conformational flexibility and perhaps some uncoiling of the helices to allow the two heads to interact with a single thin filament as is observed by electron microscopy.[126,128] There also appears to be a hinge between the S2 and LMM segments (Figs. 19-10A and 19-14).

The thick filaments. Dissociated myosin molecules can be induced to aggregate into rods similar to the thick filaments of muscle.[129] Since the filaments



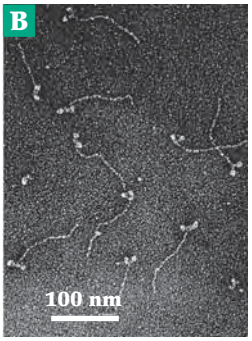


Figure 19-10 (A) An approximate scale drawing of the myosin molecule. The "hinge" is a region that is rapidly attacked by trypsin to yield the light and heavy meromyosins (LMM and HMM). Total length ~160 nm, molecular mass, 470 kDa; two ~200-kDa heavy chains; two pairs of 16- to 21-kDa light chains; heads: ~15 x 4 x 3 nm. (B) Electron micrograph of rabbit myosin monomers that became dissociated from thick filaments in the presence of ATP, fixed and shadowed with platinum.[127] Courtesy of Tsuyoshi Katoh.

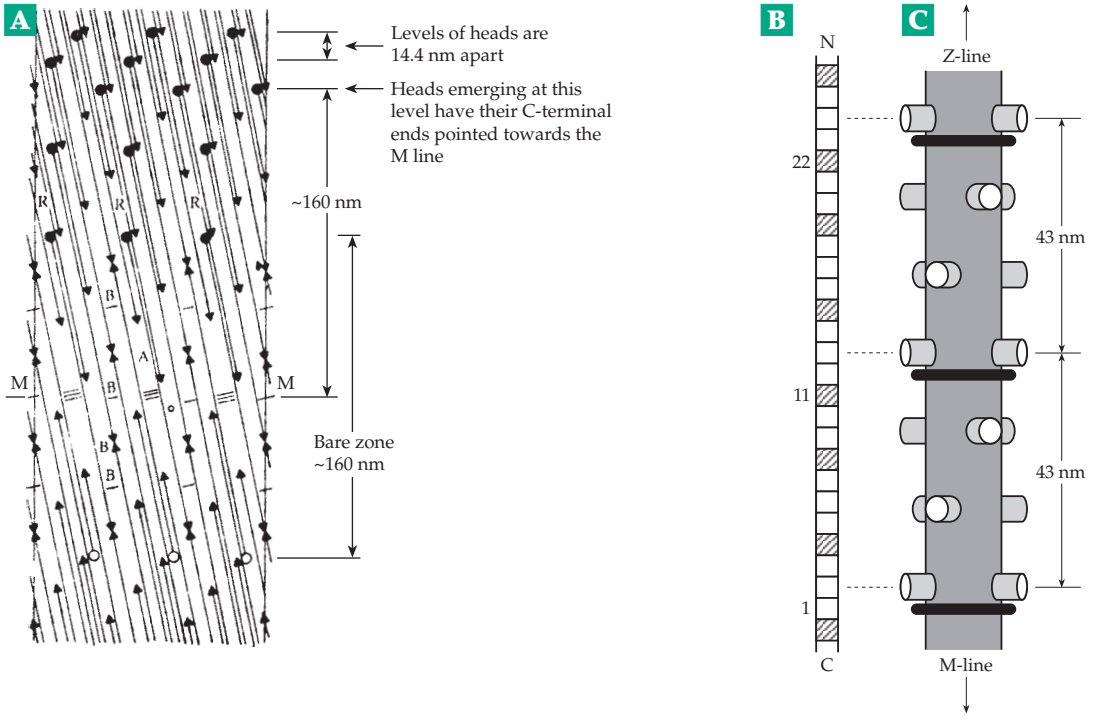


Figure 19-11 (A) Radial projection illustrating packing of myosin rods as suggested by Squire[130] for thick filaments of vertebrate skeletal muscle. The region of the bare zone at the M-line is shown. The filled circles represent the head ends of the myosin molecules and the arrowheads represent the other end of the rod, i.e., the end of the LMM portion. Antiparallel molecules interacting with overlaps of 43 and 130 nm are shown joined by single and triple cross-lines, respectively. Positions where two arrowheads meet are positions of end-to-end butting. O is an "up" molecule (thin lines) and A a "down" molecule (thick lines). The molecules move from the core at the C-terminal end to the filament surface at the head end. The levels marked B may be the levels of attachment of M-bridge material to the myosin filament. The level M-M is the center of the M-line and of the whole filament. The lateral scale is exaggerated more than threefold. (B) A segment of titin showing the 43-nm 11-domain super-repeat. (C) Model of a segment of a thick filament showing the 43-nm repeat, the C-protein, also bound at 43 nm intervals.[99] (B) and (C) Courtesy of John Trinick.

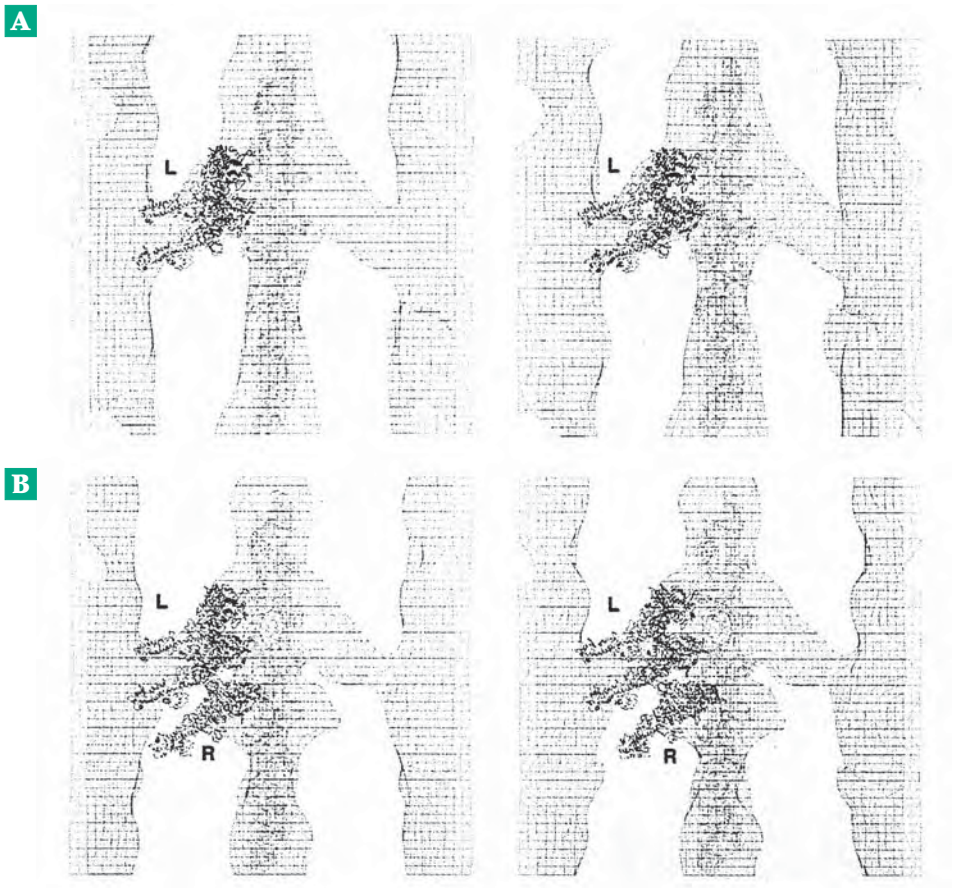


Figure 19-12 (A) Stereoscopic views of computer-assisted reconstructions of images of myosin heads attached to an F-actin filament centered between two thick filaments. Atomic structures of actin (Fig. 7-10) and of myosin heads (Fig. 19-15) have been built into the reconstructed images obtained by electron microscopy. (A) With the nonhydrolyzable ATP analog ATPPNP bound in the active sites. (B) Rigor. Two myosin heads are apparently bound to a single actin filament in (A). If they belong to the same myosin molecule the two C-terminal ends must be pulled together from the location shown here. In (B) a third head is attached, presumably from another myosin rod. This configuration is often seen in rigor. From Winkler *et al.*[134] Courtesy of K. A. Taylor.

have a diameter of ~14 to 20 nm, a large number of the thin 2-nm myosin molecules must be packed together. Electron microscopy reveals the presence of the heads projecting from the thick filaments at intervals of ~43.5 nm. However, there is a bare zone centered on the M-line, a fact that suggests tail-to-tail aggregation of the myosin monomers at the M-line in the centers of the thick filaments (see magnified section of Fig. 19-6, A). A helical packing arrangement involving about 300 myosin molecules (up to 30 rods in a single cross section) in close packing with a small central open core has been proposed for skeletal muscle myosin[130,131] and is illustrated in Fig. 19-11A,B. There are approximately three heads per turn of the helix, each group of three heads spaced 14.3 nm from the preceding one along the thick filament. It is apparently the zones of positive and negative charge, which are especially prevalent in the LMM segment toward the C termini, that lock the successive myosin molecules into this 14.3-nm spacing.[116,132] Titin also binds to the LMM segment of the myosin rod,[99,133] and its 11-domain super repeat of IgG-like and fibronectin-like modules are also 43 nm in length.[98,101] There are typically 47–49 of these super repeats in titin, and if each fits to a turn of the helix, as shown in Fig. 19-11B, there would be 147 myosin molecules in one-half of the thick filament.

Not all muscles have the thick filament structure of Fig. 19-11. In the tarantula muscle, which has a particularly well-ordered structure, there are four myosins per turn.[135,135a] Figure 19-13 shows a reconstruction of scallop myosin which has a 7-fold rotational symmetry. The thick filaments often contain

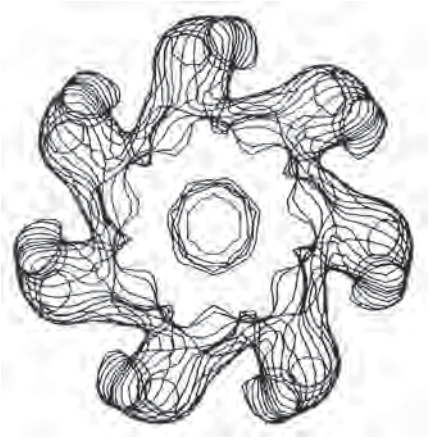


Figure 19-13 Superimposed sections for the 14 nm thickness of a computer-assisted reconstruction of the myosin filaments of the scallop adductor muscle. From Vibert and Craig.[137]

other proteins in addition to myosin. Thus, skeletal muscle contains the C-protein in a series of helical bands along the thick rod.[135b,c] In nematodes, molluscs, and insects the thick filament has a cylindrical core of **paramyosin**, another protein with a structure resembling that of the myosin rod. A minor component of *Drosophila* myosin, the **myosin rod protein**, lacks heads but is transcribed from the myosin heavy chain gene.[136]

3. Actomyosin and Muscle Contraction

That actin and myosin are jointly responsible for contraction was demonstrated long before the fine structure of the myofibril became known. In about 1929, ATP was recognized as the energy source for muscle contraction, but it was not until 10 years later that Engelhardt and Ljubimowa showed that isolated myosin preparations catalyzed the hydrolysis of ATP.[138] Szent-Györgi[139,140] showed that a combination of the two proteins actin (discovered by F. Straub[141]) and myosin was required for Mg^{2+}-stimulated ATP hydrolysis (ATPase activity). He called this combination **actomyosin**.

Under the electron microscope the myosin heads can sometimes be seen to be attached to the nearby thin actin filaments as **crossbridges**. When skeletal muscle is relaxed (not activated by a nerve impulse), the crossbridges are not attached, and the muscle can be stretched readily. The thin filaments are free to move past the thick filaments, and the muscle has some of the properties of a weak rubber band. However, when the muscle is activated and under tension, the crossbridges form more frequently. When ATP is exhausted (e.g., after death) muscle enters the state of **rigor** in which the crossbridges can be seen by electron microscopy to be almost all attached to thin filaments, accounting for the complete immobility of muscle in rigor (Figs. 19-12, 14).[134]

In rigor the crossbridges are almost all firmly attached to the thin actin filaments, making an approximately 45° angle to the actin filaments.[142–144] However, the addition of ATP causes their instantaneous release and the relaxation of the muscle fiber. In contrast, activation by a nerve impulse, with associated release of calcium ions (Section B,4), causes the thin filaments to slide between the thick filaments with shortening of the muscle. An activated muscle shortens if a low tension is applied to the muscle, but at a higher tension it maintains a constant length. Because the maximum tension developed is proportional to the length of overlap between the thick and thin filaments, it was natural to identify the individual crossbridges as the active centers for generation of the force needed for contraction.

The rowing hypothesis. H. E. Huxley[145,146] and A. F. Huxley and R. M. Simmons[147] independently proposed that during contraction the myosin heads attach themslves to the thin actin filaments. The hydrolysis of ATP is then coupled to the generation of a tension that causes the thick and thin filaments to be pulled past each other. The heads then release themselves and become attached at new locations on the actin filament. Repetition of this process leads to the sliding motion of the filaments (Fig. 19-14). The evidence in favor of this "rowing" or "swinging bridge" hypothesis was initially based largely on electron microscopy. For example, contracting muscle was frozen rapidly and fixed for microscopy in the frozen state.[148] Relaxed muscle shows no attached crossbridges, but contracting muscle has many. However,

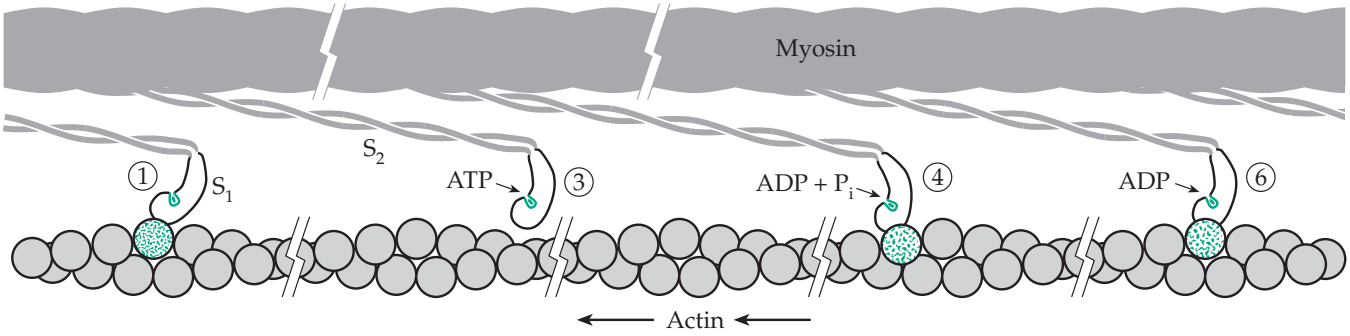


Figure 19-14 A model for the coupling of ATP hydrolysis to force production in muscle based on proposals of H. E. Huxley, and A. F. Huxley and Simmons. The power stroke is depicted here as a rotation of the crossbridge from a 90° to a 45° configuration. Four representative stages are shown: (1) the rigor complex, (3) the dissociated myosin ATP complex, (4) the actomyosin ADP pre-power stroke state in which the actin–myosin band has reformed but with a different actin subunit, which may be distant from that in (1), and (6) the actomyosin ADP post-power stroke state. Force production and contraction result from crossbridges passing cyclically through the steps depicted from left to right. Numbering of the stages corresponds approximately to that in Fig. 19-18. After H. Huxley.[146]

their appearance was distinct from that seen in rigor. The model was also supported by indirect physical methods.

An impressive demonstration that myosin heads do move along the actin filaments was provided by Sheetz and Spudich, who found that myosin-coated fluorescent beads ~0.7 μm in diameter will move along actin filaments from cells of the alga *Nitella* in an ATP-dependent fashion at velocities similar to those required in muscle.[149] The myosin heads literally glide along the thick cables of parallel actin filaments present in these algae.

Why two heads? The actin filament is a two-start helix, and it is natural to ask whether the two myosin heads bind to just one or simultaneously to both of the actin strands. Most evidence supports a 1:1 interaction of a single head with just one strand of actin. However, the other actin strand may associate with heads from a different thick filament. Another question concerns the role of the pairs of myosin heads. Could the two heads bind sequentially to the actin and exert their pull in a fixed sequence? In the reconstruction of the actomyosin complex in rigor (Fig. 19-12B) two different images are seen for the crossbridges. This suggests the existence of two different conformations for the attached myosin heads. Similar images for smooth muscle heavy meromyosin in its inactive (resting) dephosphorylated state (see p. 1116) show the two heads in very different orientations with one binding to the other of the pair and blocking its movement.[121b] Perhaps one head is tightly bound at the end of the power stroke while the other is at a different stage of the catalytic cycle. Nevertheless, single-headed myosin from *Acanthamoeba* will propell organelles along actin filaments,[150] and actin filaments will slide across a surface coated with single-headed myosin formed by controlled proteolysis.[151] The additional interactions seen in rigor may be peculiar to that state.

Structure of the myosin heads. Myosin and myosin fragments can be isolated in large quantities, but they have been difficult to crystallize. However, Rayment and coworkers purified S1 heads cleaved from chicken myosin by papain and subjected them to reductive methylation (using a dimethylamine–borane complex; see also Eq. 3-34). With most of the lysine side chain amino groups converted to dimethylamine groups, high-quality crystals were obtained, and a structure was determined by X-ray diffraction.[152] Since that time various forms of both modified and unmodified myosin heads from several species have been studied by X-ray crystallography.[153–160] Especially clear results were obtained with unmodified myosin from the ameba *Dictyostelium discoideum*. The head structure, shown in Fig. 19-11, includes a 95-kDa piece of the heavy chain and both light chains. A clearer picture of the neck region containing the light chains was provided by the structure of the "regulatory domain" of scallop myosin.[161] Unlike mammalian or avian myosins, molluscan myosins are regulated by binding of Ca^{2+} to a site in the essential light chain, but the structures are similar to those in Figs. 19-10 and 19-15.

Cleavage of the ~850-residue S1 heads with trypsin yields mainly three large fragments that correspond to structural domains of the intact protein as shown in Fig. 19-15. They are known as the 25-kDa (N-terminal), 50-kDa, and 20-kDa fragments, and for myosin from *D. discoideum* correspond to residues 1 to 204, 216 to 626, and 647 to 843, respectively. The ATP-binding site is in a deep cleft between the 20-kDa and 50-kDa

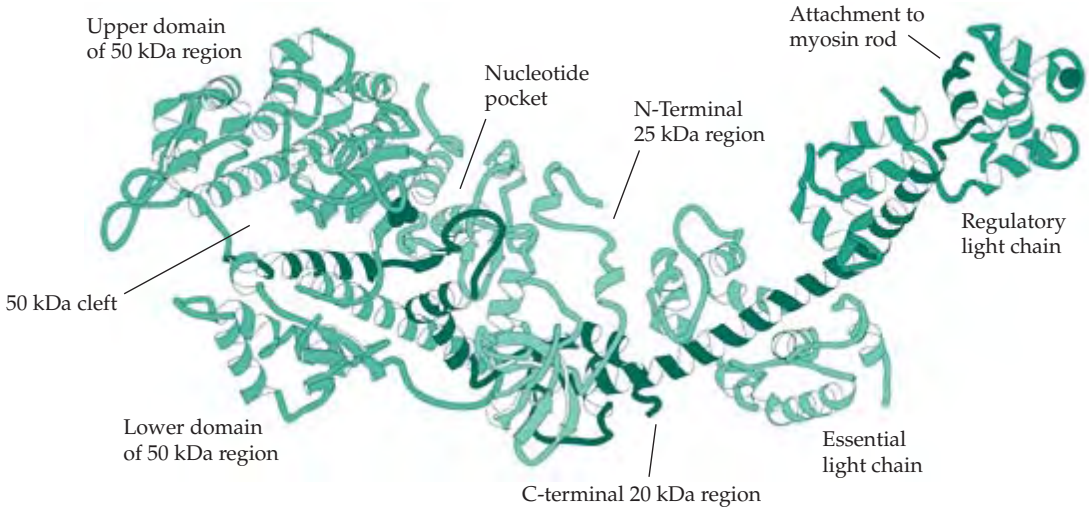


Figure 19-15 Ribbon representation of chicken skeletal myosin subfragment-1 showing the major domains and tryptic fragments. Prepared with the program MolScript. From Rayment.[157]

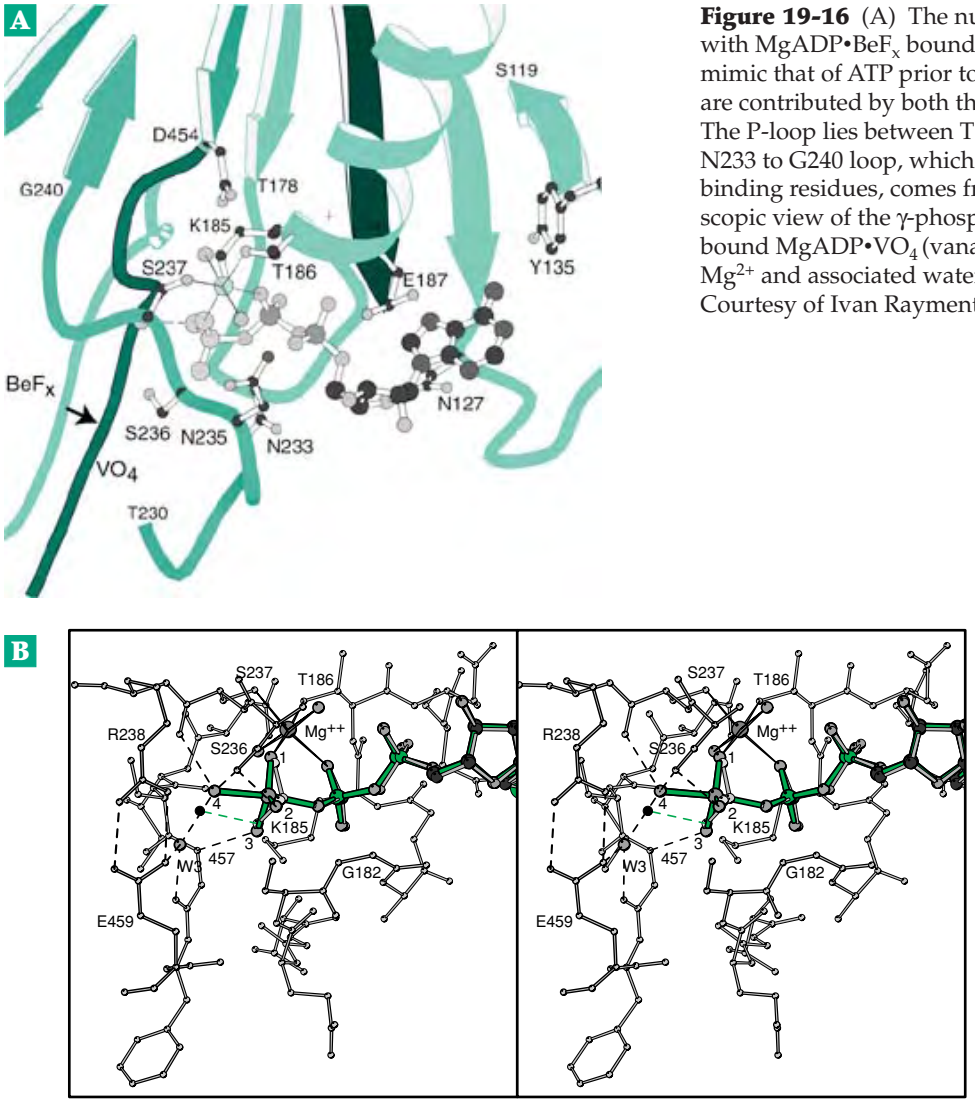


Figure 19-16 (A) The nucleotide binding site of myosin with MgADP•BeF_x bound in a conformation thought to mimic that of ATP prior to hydrolysis. The β-sheet strands are contributed by both the 25-kDa and 50-kDa domains. The P-loop lies between T178 and E187. The conserved N233 to G240 loop, which also contributes important ATP-binding residues, comes from the 50-kDa region. (B) Stereoscopic view of the γ-phospho group binding pocket with the bound MgADP•VO_4 (vanadate) complex. The coordinated Mg^{2+} and associated water molecules are seen clearly. Courtesy of Ivan Rayment.[157]

regions. Figure 19-16 illustrates the binding of an ATP analog, the beryllium fluoride complex of MgADP, in the active site. As can be seen, the ATP binds to loops at the C termini of the β strands of the 8-stranded β sheet from the 25-kDa domain. The conserved P-loop (Chapter 12, E), which lies between T178 and E187, curls around the α and β phospho groups, and has the sequence G(179)ESGAGKT. A second conserved loop N(233)SNSSR-G(240) from the 50-kDa domain contributes to the binding of ATP.

The actin-binding region of the myosin head is formed largely by the 50-kDa segment, which is split by a deep cleft into two separate domains (Fig. 19-15), both of which are thought to participate in binding to actin. A surface loop (loop 1) near the ATP-binding site at the junction of the 25- and 50-kDa regions affects the kinetic properties of myosin, probably by influencing product release. A second loop (loop 2, residues 626–647) at the junction of the 50- and 20-kDa regions interact with actin. Loop 2 contains a GKK sequence whose positive charges may interact with negative charges in the N-terminal part of actin.[162–164]

The C-terminal fragment of myosin contains a globular domain that interacts with both the 20-kDa and 50-kDa regions and contains an α-helical neck that connects to the helix of the coiled-coil myosin rod. This helical region is surrounded by the two myosin light chains (Fig. 19-15).[157] A pair of reactive thiol groups (from C697 and C707) in the globular domain are near the active site. Crosslinking of these cysteines by an –S–S– bridge has been utilized to trap nucleotide analogs in the active site.[165]

How does actin bind? The actin monomer consists of four subdomains, 1, 2, 3, and 4 numbered from the N terminus (Fig. 7-10). The negatively charged N-terminal region of actin contains the sequence

$$\overset{4}{\text{D}}\text{-E-D-}\overset{24}{\text{E}}\text{----D-}\overset{25}{\text{D}}.$$

It may interact with loop 1 of myosin, which contains five lysines. However, to form a strong interaction with the myosin head a conformational change must occur in the myosin. A change may also occur in actin. Modeling suggests that a large nonpolar contact region involves actin residues A144, I341, I345, L349, and F352 and myosin residues P529, M530, I535, M541, F542, and P543. A conformational change in actin, which might involve largely the highly conserved actin subdomain 2, may also be required for tight interaction.[142,166–168]

Kinesins and other molecular motors. Before considering further how the myosin motor may work, we should look briefly at the **kinesins**, a different group of motor molecules,[168a] which transport various cellular materials along microtubule "rails." They also participate in organization of the mitotic spindle and other microtubule-dependent activities.[168a,b,c] See Section C,2 for further discussion. More than 90 members of the family have been identified. Kinesin heads have much shorter necks than do the myosin heads. A myosin head is made up of ~850 residues, but the motor domain of a kinesin contains only ~345. Like myosin, the 950- to 980-residue kinesins have a long coiled-coil C- terminal region that forms a "neck" of ~50 residues, a "stalk" of ~190 and ~330 residue segments with a Pro / Gly-rich hinge between them, and an ~45 residue "tail."[169–171]

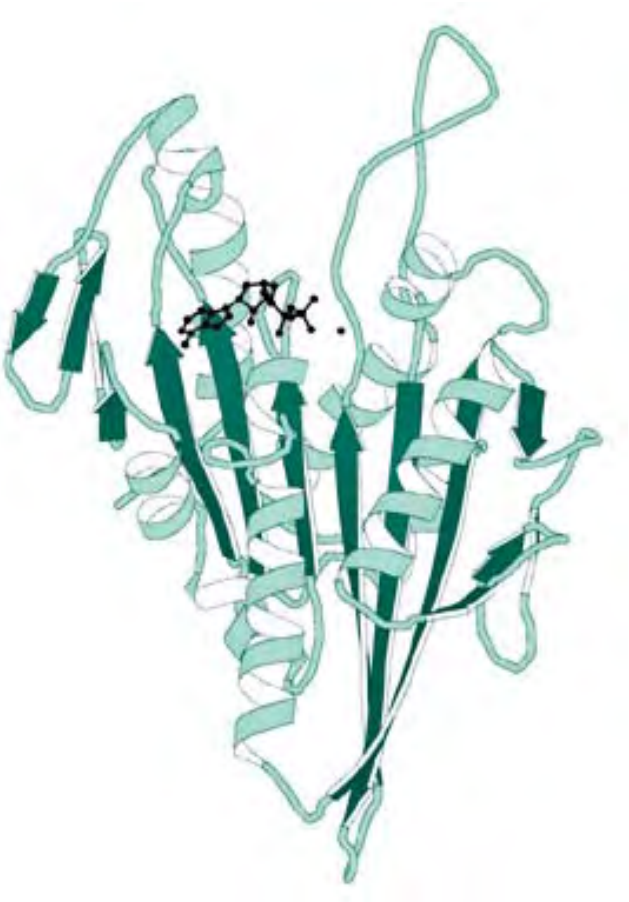


Figure 19-17 Ribbon drawing of human kinesin with bound Mg•ADP. From Gulick *et al.*[174] Courtesy of Ivan Rayment and Andy Gulick.

Crystal structures are known for motor domains of human kinesin[172] and of a kinesin from rat brain.[169,173] The structures of one of six yeast kinesins,[174] a protein called **Kar3**, and also of a *Drosophila* motor molecule designated **Ncd** have also been determined.[175] The last was identified through study of a *Drosophila* mutant called non-claret disjunctional (Ncd). The motor domains of various members of the kinesin family show ~40% sequence identity and very close structural identity (Fig. 19-17).[174] Although the sequences are different from those of the myosin heads or of G proteins, the folding pattern in the core structures is similar in all cases. An 8-stranded β sheet is flanked by three α helices on each side and a P-loop crosses over the ATP-binding site as in Fig. 19-16. Further similarity is found in the active site structures, which, for a monomeric kinesin KIF1A,[174a] have been determined both with bound ADP and with a nonhydrolyzable analog of ATP.[174b,174c] Although there is little similarity in amino acid sequences the structures in the catalytic core are clearly related to each other, to those of dimeric kinesins,[174d] to those of myosins, and to those of the GTP-hydrolyzing G proteins.

A puzzling discovery was that the motor domain of kinesin, which binds primarily to the β subunits of tubulin (Fig. 7-34) and moves toward the fast growing *plus* end of the microtubule,[176] is located at the N terminus of the kinesin molecule, just as is myosin. However, the Ncd and Kar3 motor domains are at the C-terminal ends of their peptide chains and move their "cargos" toward the *minus* ends of microtubules.[174] Nevertheless, the structures of all the kinesin heads are conserved as are the basic chemical mechanisms. The differences in directional preference are determined by a short length of peptide chain between the motor domain and the neck, which allows quite different geometric arrangements when bound to microtubules.[173,177] Like Ncd, myosin VI motor domains also move "backwards" toward the pointed (minus) ends of actin filaments.[178–179a]

Other major differences between kinesins and myosin II heads involve kinetics[180,181] and processivity.[173] Dimeric kinesin is a **processive** molecule. It moves rapidly along microtubules in 8-nm steps but remains attached.[182,182a] Myosins V and VI are also processive[183–183e] but myosin II is not. It binds, pulls on actin, and then releases it. The many myosin heads interacting with each actin filament accomplish muscle contraction with a high velocity in spite of the short time of attachment. Ncd and Kar3 are also nonprocessive and slower than the *plus* end-oriented kinesins.[184]

The ATPase cycles of actomyosin and of the kinesins. The properties of the protein assemblies found in muscle have been described in elegant details, but the most important question has not been fully answered. How can the muscle machinery use the Gibbs energy of hydrolysis of ATP to do mechanical work? Some insight has been obtained by studying the ATPase activity of isolated myosin heads (S1) alone or together with actin. Results of numerous studies of ATP binding, hydrolysis, and release of products using fast reaction techniques[185–191] and cryoenzymology[191a] are summarized in Fig. 19-18. In resting muscle the myosin heads swing freely in the ~20-nm space between the thick and thin filaments. However, in activated muscle some heads are bound tightly to actin as if in rigor (complex A•M in Fig. 19-18). When ATP is added MgATP binds into the active site of the myosin (Fig. 19-18, step *a*) inducing a conformational change to form A•M*•ATP in which the bond between actin and myosin is weakened greatly, while that between myosin and ATP is strengthened. The complex dissociates (step *b*) to give free actin and (M*•ATP), which accumulates at −15°C. However, at higher temperatures the bound ATP is hydrolyzed rapidly (step *c*) to a form M**•ADP•P_i in which the ATP has been cleaved to ADP + P_i but in which the split products remain bound at the active site.[116,192,192a,b] All of these reactions are reversible. That is, the split products can recombine to form ATP. This fact suggests that most of the Gibbs energy of hydrolysis of the ATP must be stored, possibly through a conformational change in the myosin head or through tighter bonding to ATP. As long as calcium ions are absent, there is only a slow release of the bound ADP and P_i and replacement with fresh ATP takes place. Thus, myosin alone shows a very weak ATPase activity.

On the other hand, in activated muscle the head with the split ATP products will bind to actin (step *d*), probably at a new position. The crossbridges that form appear to be attached almost at right angles to the thin filaments. In step *e*, P_i is released following a conformational alteration that is thought to open a "back door" to allow escape of the phosphate ion.[193] In the final two steps (*f* and *g*) the stored energy in the myosin head (or in the actin) is used to bring about another conformational change that alters the angle of attachment of myosin head to the thin filament from ~90° to ~45°. At least some indication of such a change can be observed directly by electron microscopy.[144] Such a change in angle is sufficient to cause the actin filament to move ~10 nm with respect to the thick filaments to complete the movement cycle (Fig. 19-18), if the head is hinged at the correct place. However, the existence of at least four different conformational states suggests a more complex sequence.[193a,193b] Examination of the three-dimensional structures available also suggest a complex sequence of alterations in structure and geometry. X-ray crystallographic structures of myosin heads, in states thought to correspond to states 1 and 3 of Figs. 19-14 and 19-18, are also in agreement on an ~10 (5–12) nm movement of the lever arm.[194,195] Six states of the actomyosin complex are depicted in Fig. 19-18, but a complete kinetic analysis requires at least eight and possibly 12 states.[196,197]

Observing single molecules. A major advance in the study of molecular motors has been the development of ways to observe and study single macromolecules. The methods make use of **optical traps** (optical "tweezers") that can hold a very small (~1 μm diameter) polystyrene or silica bead near the waist of a laser beam focused through a microscope objective.[198–202] In one experimental design an F-actin filament is stretched between two beads, held in a pair of optical traps. The filament is pulled taut and lowered onto a stationary silica bead to which a few myosin HMM fragments have been attached (Fig. 19-19). If ATP is present, short transient movements along the filament are detected by observation of displacements of one of the beads when the actin filament contacts HMM heads. An average lateral displacement of 11 nm was observed. Each HMM head exerted a force of 3–4 pN, a value consistent with expectations for the swinging bridge model.[200] From the duration of a single displacement (≤7 ms) and an estimated k_{cat} for ATP hydrolysis of 10 s^{-1}, the fraction of time that the head was attached during one catalytic cycle of the head was therefore only 0.07. This ratio, which is called the **duty ratio**, is low for actomyosin. However, many myosin heads bind to each actin filament in a muscle. Each head exerts its pull for a short time. but the actin is never totally unattached.[203] Similar measurements with smooth muscle revealed similar displacements but with a 10-fold slower sliding velocity and a 4-fold increase in the duty ratio. This may perhaps account for an observed 3-fold increase in force as compared with skeletal muscle.[204,204a]

Other single molecule techniques involve direct observation of motor molecules or of S1 myosin fragments tagged with highly fluorescent labels.[205,206] All measurements of single molecule movement are subject to many errors. Brownian motion of the beads makes measurements difficult.[207] Not all results are in agreement, and some are difficult to understand.[207a] Most investigators agree that there is a step size of ~4–10 nm. Kitamura *et al.* found 5.3 nm as the average.[206] However, they also reported the puzzling observation that some single S1 molecules moved 11–30 nm in two to five rapid successive steps during the time of hydrolysis of a single molecule at ATP. They suggested that some of the energy of ATP hydrolysis may be stored in S1 or in the actin filament and be released in multiple steps. Veigel *et al.*[208] observed that a brush border myosin I from chicks produced ~ 6 nm

movements, each of which was followed by an additional ~ 5.5 nm step within ~5 ms. They attribute these steps to two stages in the power stroke, e.g., to steps *f* and *g* of Fig. 19-18. A value of ~10nm was reported recently by Piazzesi *et al*.[208a] Myosin V moves along actin filaments with very large 36-nm steps.[208b]

Motion of kinesin heads has been observed by movement of microtubules over biotinylated kinesin fixed to a steptavidin-coated surface,[209] by direct observation of fluorescent kinesin moving along microtubules,[171] and by optical trap interferometry.[210] Kinesin heads move by 8-nm steps, evidently the exact length

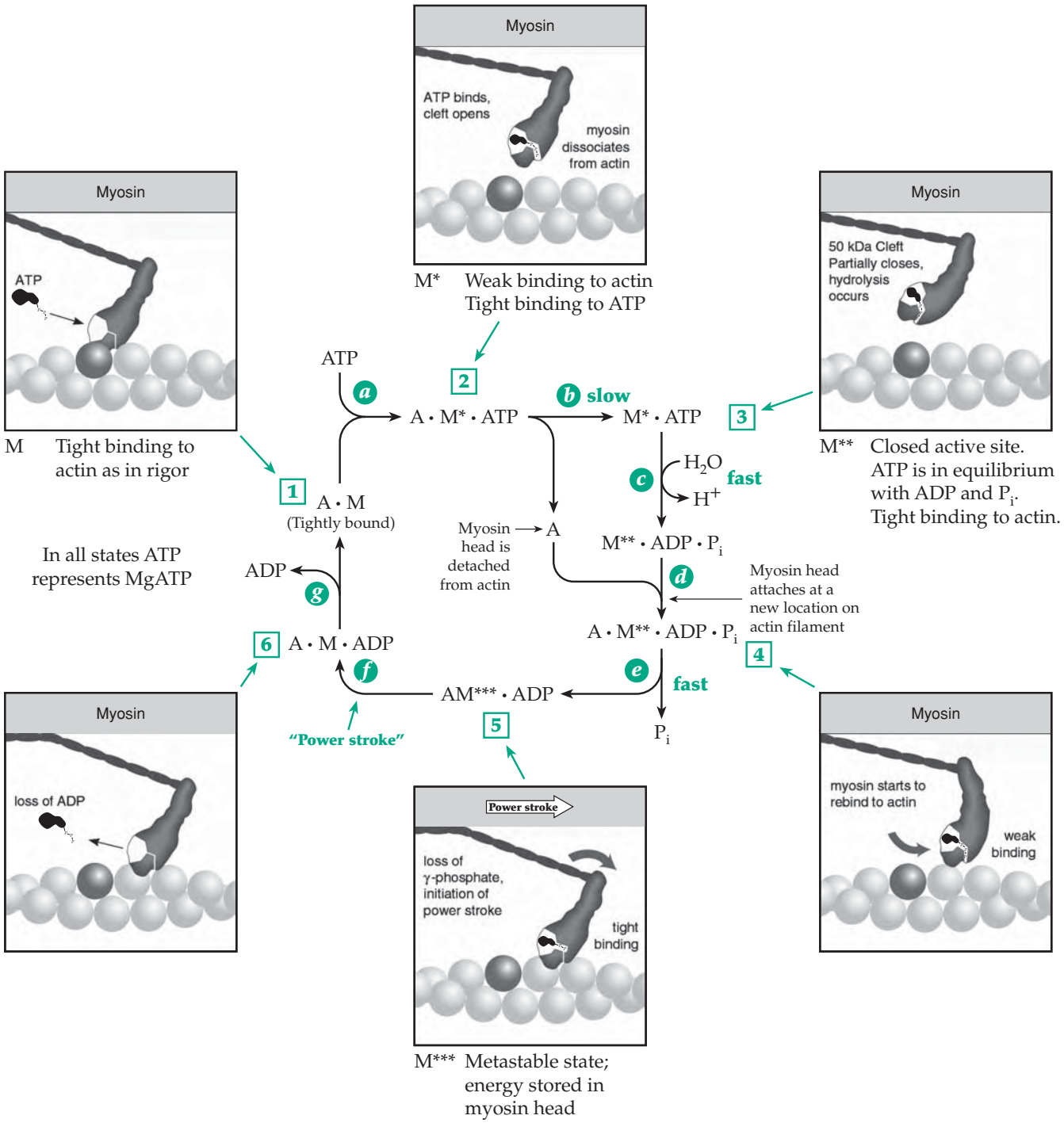


Figure 19-18 Simplified view of the ATP hydrolysis cycle for actomyosin. A similar cycle can be written for kinesins and dyneins. Here A stands for fibrous actin and M, M*, M**, and M*** for four different conformations of the myosin heads. As indicated by the numbers in squares, four of the six states of actomyosin shown here can also be correlated with those in Fig. 19-14.

of an αβ tubulin dimer in the microtubule structure (Fig. 7-33). One ATP is apparently hydrolyzed for each 8-nm step. However, shorter steps of ~5 nm have sometimes been seen.[211,212]

The movement is processive, kinesin motors typically taking 100 steps before dissociating from the microtubule.[201,212a] Kinesin is bound to the microtubule continuously. Its duty ratio is nearly 1.0 (the same is true for the bacterial flagellar motor; Fig. 19-4).[212b] However, single kinesin heads, which lack the coiled-coil neck region, have a duty ratio of <0.45. The movement is nonprocessive.[213] The Ncd motor is also nonprocessive.[214–216] As mentioned previously, the Ncd and kinesin motor domains are at opposite ends of the peptide chain, and the motors move in opposite directions along microtubules.[217,218] The critical difference between the two motor molecules was found in the neck domains, which gave rise to differing symmetrics in the two heads.[219] The latter are shown in Fig. 19-20, in which they have been docked onto the tubulin protofilament structure. One head, both of ncd and of kinesin, occupies a similar position on the microtubule, but the other head points toward the microtubular plus end for kinesin but toward the minus end for Ncd. Cryoelectron microscopy also supports this interpretation.[220]

Still not fully understood is the processive action of kinesin.[221–224] It is often assumed that this protein moves in a hand-over-hand fashion with the two heads binding alternatively to the microtubule. Some substantial reorganization of the peptide chain in the hinge region at the end of the neck is presumably involved.[173] An alternative "inchworm" mechanism has been suggested.[220a]

Thinking about chemical mechanisms. We have now examined the active sites of kinases that cleave ATP (Chapter 12), ATPases that pump ions by cleaving ATP, ATP synthases that form ATP from ADP and P_i (Chapter 18), and GTP hydrolyzing enzymes that cause movement and shape changes that control metabolic processes (Chapter 11). It is striking that the active site regions where the ATP or GTP bind have such a highly conserved structure.[225] This suggests that the secret of movement can be found in the very strong interactions of the nucleotides and their split products with the proteins. In every case there is at least one tight binding or closed conformation in which a large number of hydrogen bonds and ionic interactions bind the nucleotide. This is shown for a kinase in Fig. 12-32 and for myosin in Fig. 19-16. During the actomyosin reaction several conformational changes must occur. Not only does the affinity for the bound nucleotide vary, but also the binding of actin to myosin can be strong, as in the nucleotide-free state or

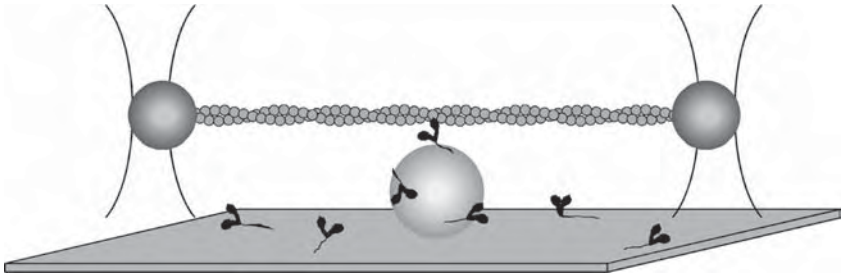


Figure 19-19 Schematic drawing (not to scale) illustrating the use of two optical traps that are focused on beads attached to a single actin filament. The filament is lowered onto a stationary silica bead sparsely coated with HMM fragments of myosin. In the presence of ATP the myosin heads bind transiently for a few milliseconds to the actin, moving it in one direction and displacing the beads from their positions in the optical traps. An image of one of the beads is projected onto photodiode detectors capable of measuring small displacements. The displacing force can also be recorded. For details of the experiments and of the optical traps and measuring devices see Finer *et al.*[200] Courtesy of J. A. Spudich.

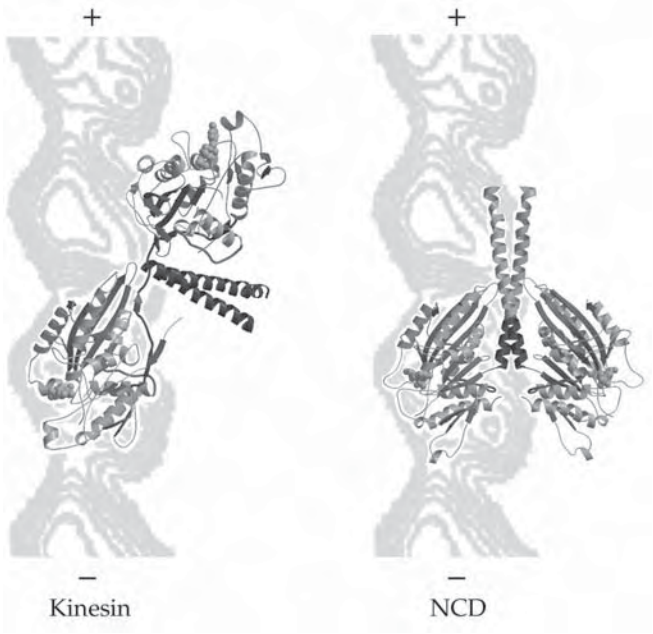
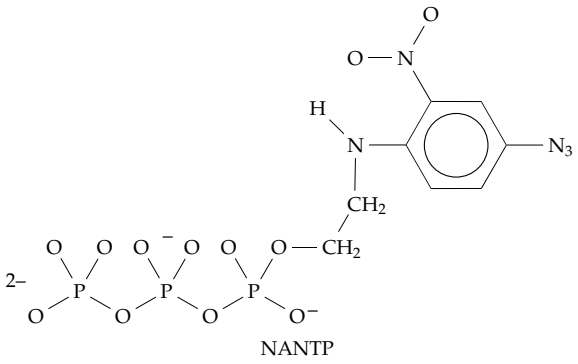


Figure 19-20 Model showing the ncd and kinesin dimer structures docked onto a tubulin protofilament. The bound ncd and kinesin heads are positioned similarly. Because of the distinct architectures of the kinesin and ncd necks, the unbound kinesin head points toward the plus end, whereas the unbound ncd head is tilted toward the minus end of the protofilament. From Sablin *et al.*[219] Courtesy of Ronald Vale.

in the presence of bound ADP. Binding is weak when ATP or the split product ADP + P_i are in the active site.

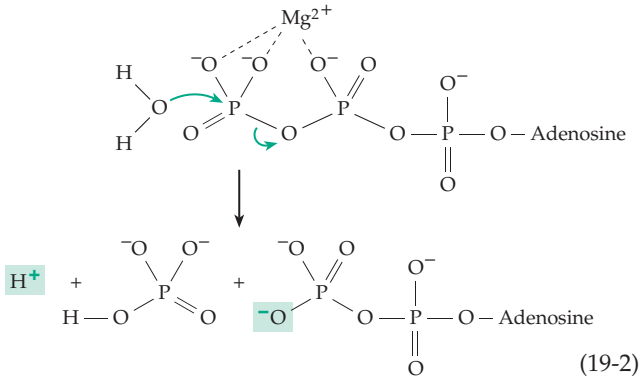
To understand these differences we should look at the structure of ATP itself. The triphosphate group has many negative charges repelling each other. What must happen to allow the binding of ATP to myosin to break the actin-myosin bond? The electrostatic attraction of these phospho groups for active site groups is doubtless one cause of the observed conformational changes. Could it be that electrostatic repulsion, via a proton shuttle mechanism, is also induced at the right point in the actin–myosin interface? Many studies with analogs of ATP have contributed to our understanding. Neither the purine nor the ribose ring of ATP is absolutely essential. The compound 2-[(4-azido-2-nitrophenyl) amino] diphosphate (NANDP) and related nonnucleotide analogs[165,196,226] support muscle contraction and relaxation in the same



way as does ATP. An analog with a rigid five-membered ring, 2',3'-dideoxydidehydro-ATP, is also active.[227] A comparison of kinetic data and X-ray structural data supports the proposal that the ATP must be bound in the conformation shown for MgADP•BeF_x in Fig. 19-16A.[196] When the two SH groups of C697 and C707 of the myosin head are crosslinked by various reagents,[227a] this NANDP analog can be trapped at the active site. Because of the presence of its azide ($-N_3$) group the trapped compound can serve as a photoaffinity label, attaching itself to a tryptophan side chain upon activation with visible light (Eq. 23-27).

How can cleavage of ATP to bound ADP + P_i create a metastable high-energy state of the myosin head ready to hold onto and pull the actin chain? This may be compared with the inverse problem of generating ATP in oxidative phosphorylation, in which ADP and P_i coexist in equilibrium with ATP in a closed active site (Fig. 18-14). Comparison should also be made with the GTP-hydrolyzing G proteins (Fig. 11-7).[227b,c] During hydrolysis of GTP by the Ras protein binding to the protein induces a shift of negative charge from the γ- to β-oxygens of GTP facilitating bond cleavage as in Eq. 19-2. G proteins also couple substrate hydrolysis to mechanical motion. We should also think about the fact that when ATP is cleaved within myosin there will necessarily be a flow of electrical charge from the water to the ADP (Eq. 19-2). This will be followed by some accomodation of the protein to the new charge constellation. As we have seen previously, movement of protons is often the key to conformational changes. In this case, the initial change must be to create a high-energy state of myosin which, following loss of the orthophosphate ion, can cause the major conformational change that swings the lever arm of the myosin. The conformational changes may occur in several steps in which the packing of groups within the myosin head is always tight in some places and rather loose in others. Movement within the head is being observed not only by X-ray crystallography but by **fluorescence resonance energy transfer** (FRET; Chapter 23)[227d] and by the newer **luminescence resonance energy transfer** (LRET). For example, a terbium chelate of azide-ATP was photochemically bound in the active site, and a fluorescent dye was attached to Cys 108 in the regulatory light chain. The terbium ion was irradiated, and fluorescence of the dye was observed. Distance changes, measured in the absence and presence of ATP, were consistent with the swinging arm model.[228] Dyes have been attached to –SH groups engineered into various locations in the myosin molecule to permit other distance measurements.[229,230] In another elegant application of the FRET technique the **green fluorescent protein** of *Aequoria* (Box 23-A) was fused to the C terminus of the motor domain of myosin giving a fluorescent lever arm. Energy transfer to blue fluorescent protein fused to the N terminus of the S1 head was measured. The distance between these was estimated by the FRET technique and was also consistent with expectations for the "rowing model."[231]

The "rowing model" is generally accepted, but other quite different processes have been proposed to account for the elementary cycle of muscle contraction. Muscle contracts nearly *isovolumetrically*; thus, anything that expands the sarcomere will cause a contraction. It has been suggested that the hydrolysis of ATP deposits negatively charged phospho groups on the actin filaments, and that the electrostatic repulsion is responsible for



(19-2)

BOX 19-A HEREDITARY DISEASES OF MUSCLE

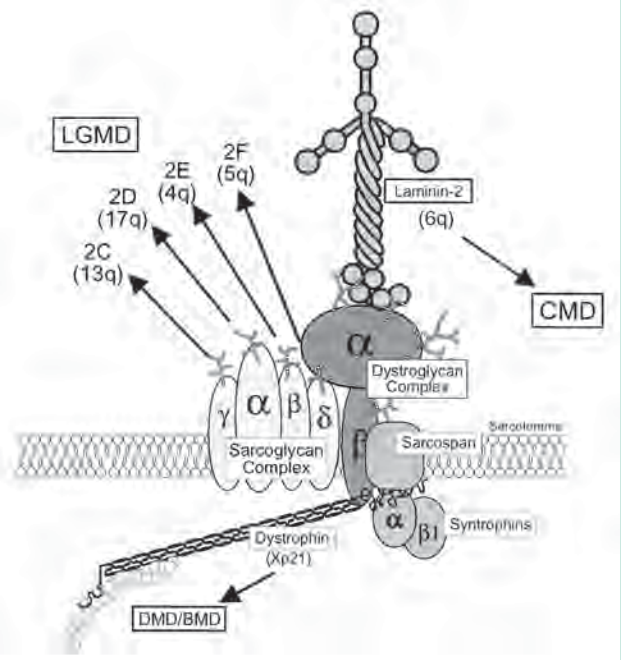
Considering the numerous specialized proteins in muscle it is not surprising that many rare hereditary muscle diseases are known. The most frequent and most studied of these is **Duchenne muscular dystrophy**. An X-linked disease of boys, it may not be recognized until two to three years of age, but victims are usually in a wheelchair by age 12 and die around age 20. Individual muscle fibers disintegrate, die, and are replaced by fibrous or fatty tissue.[a–d] The disease strikes about 1 out of 3500 boys born. The less serious **Becker muscular dystrophy** arises from defects in the same gene but affects only 1 in 30,000 males, some of whom have a normal life span. Because of its frequency and the knowledge that the gene must lie in the X-chromosome, an intensive search for the gene was made. It was found in 1986 after a five-year search.[a,e] This was the first attempt to find a faulty gene whose protein product was totally unknown. The project, which relied upon finding restriction fragment polymorphisms (Chapter 26) that could serve as markers in the genome, made use of the DNA from patients with a range of related diseases. The very rare female patients in whom the faulty gene had been translocated from the X-chromosome to an autosome also provided markers. DNA probes obtained from a young man with a large X-chromosome deletion that included genes related to retinitis pigmentosa and several other diseases provided additional markers. The result was a triumph of "reverse genetics" which has since been applied to the location of many other disease genes.[e]

The muscular dystrophy gene may be the largest human gene. It consists of 2.3 million base pairs, which include 79 exons which encode a huge 427 kDa protein named **dystrophin**. The protein consists of four main domains.[a,f,g] The N-terminal domain binds to actin and is homologous to β-actinin. The central domain is an elongated rod resembling spectrin. It contains repetitive coiled-coil segments and four hinge regions. The third domain is rich in cysteine and binds Ca^{2+}, while the fourth domain has a structure that is shared by several other proteins of the dystrophin family. Dystrophin is quantitatively a minor protein of muscle. It forms part of the cytoskeleton, lying adjacent to the sarcolemma (cell membrane) along with β-spectrin and vinculin (see Fig. 8-16).

While one end of the dystrophin molecule binds to actin filaments, the C-terminal domain associates with several additional proteins to form a **dystrophin–glycoprotein complex** (see figure).[f,h–k] Dystrophin is linked directly to the membrane-spanning protein **β-dystroglycan,** which in the outer membrane surfaces associates with a glycoprotein **α-dystroglycan**. The latter binds to laminin-2 (Fig. 8-33), a protein that binds the cell to the basal lamina. Four other membrane-spanning proteins, α-, β-, γ-, and δ-**sarcoglycans**, are among additional members of the complex.[k–m]

Patients with Duchenne muscular dystrophy are deficient not only in dystrophin but also in the dystroglycan and sarcoglycan proteins.[f,n] Evidently, dystrophin is needed for formation of the complex which plays an essential role in muscle. In both types of X-linked muscular dystrophy there are individuals with a wide range of point mutations, frame-shift mutations, and deletions in the dystrophin gene.[d] The essential function of dystrophin and associated proteins is uncertain but may be related to the linkage from actin filaments through the membrane to laminin. Individuals with Becker muscular dystrophy also have defects in dystrophin, but the protein is partially functional. Some other muscular dystrophies are caused by defects in the autosomal genes of any of the four sarcoglycan subunits.[j,k,o] or in that of laminin α1 chain.[p,q] The arrows in the accompanying drawing indicate chromosome locations of the sarcoglycan subunits, which are sites for mutations causing **limb girdle muscular dystrophy**.[k]

Dystrophin, shorter isoforms, and related proteins are found in many tissues including the brain.[s] One related protein, **utrophin** (dystrophin-related



Schematic model of the dystrophin–glycoprotein complex. Courtesy of Kevin P. Campbell. See Lim and Campbell[r] Abbreviations: LGMD, Limb Girdle muscular dystrophy; CMD, congenital muscular dystrophy; DMD / BMD, Duchenne / Becker muscular dystrophies.

BOX 19-A (continued)

protein 1), is present in the neuromuscular junction of adult skeletal muscle. One approach to therapy of Duchenne muscular dystrophy is to stimulate a higher level of expression of the utrophin gene.[t] Because the dystrophin gene is so large treatment by gene transfer is not practical, but transfer of parts of the gene may be. Myoblast transfer has not been successful, but new approaches will be devised.[d]

Myotonic dystrophy is a generalized adult-onset disorder with muscular spasms, weakness, and many other symptoms.[u–y] It is one of the **triple-repeat diseases** (Table 26-4). The affected gene encodes a protein kinase of unknown function. The corresponding mRNA transcript has ~2400 nucleotides. The gene has a CTG repeat $(CTG)_n$ near the 3'-end with $n < 30$ normally. For persons with the mildest cases of myotonic dystrophy n may be over 50 while in severe cases it may be as high as 2000. As in other triple-repeat diseases the repeat number tends to increase in successive generations of people as does the severity of the disease.[x]

For some individuals, muscular dystrophy causes no obvious damage to skeletal muscle but affects the heart producing a severe **cardiomyopathy,** and persons with Duchenne muscular dystrophy often die from heart failure. Heart failure from other causes, some hereditary, is a major medical problem, especially among older persons. Hereditary forms are often autosomal dominant traits that may cause sudden death in young persons. At least seven genes for cardiac sarcomeric proteins including actin,[z] myosin, both heavy and light chains,[aa–dd] three subunits of troponin,[ee] tropomyosin, and protein C (p. 1104) may all carry mutations that cause cardiomyopathy.

A hereditary disease common in Japan results from a defect in migration of neurons and is associated with brain malformation as well as muscular dystrophy.[ff] In **nemaline myopathy** a defect in nebulin leads to progressive weakness and often to death in infancy. A characteristic is the appearance of "nemaline bodies" or thickened Z-discs containing Z-disc proteins.[gg] Some hereditary diseases involve nonmuscle myosins. Among these is **Usher syndrome**, the commonest cause of deaf-blindness. The disease, which results from a defect in the myosin VIA gene, typically causes impairment of hearing and retinitis pigmentosa (Chapter 23).[hh]

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lateral expansion of the sarcomere.[232] Still other ideas have been advanced.[233,234]

4. Control of Muscle Contraction

Skeletal muscle must be able to rest without excessive cleavage of ATP but able to act rapidly with a high expenditure of energy upon nervous excitation. Even a simple physical activity requires that a person's muscles individually contract and relax in rapid response to nerve impulses from the brain. To allow for this control the endoplasmic reticulum (**sarcoplasmic reticulum, SR**) of striated muscle fibers is organized in a striking regular manner.[235–237] Interconnecting tubules run longitudinally through the fibers among the bundles of contractile elements. At regular intervals they come in close contact with infoldings of the outer cell membrane (the **T system** of membranes, Fig. 19-21; see also Fig. 19-7A). A nerve impulse enters the muscle fiber through the neuromuscular junctions and travels along the sarcolemma and into the T tubules. At the points of close contact the signal is transmitted to the longitudinal tubules of the sarcoplasmic reticulum, which contain a high concentration of calcium ions.

Calcium ions in muscle. A nerve signal arriving at a muscle causes a sudden release of the calcium ions into the cytoplasm from cisternae of the sarcoplasmic reticulum (SR) that are located adjacent to the T-tubules. Diffusion of the Ca^{2+} into the myofibrils initiates contraction. In smooth muscle the signals do not come directly from the nervous system but involve hormonal regulation.[238] Again, calcium ions play a major role, which is also discussed in Chapter 6, Section E, and in Box 6-D. Muscle contains a large store of readily available Ca^{2+} in lateral cisternae of the SR. The free intracellular Ca^{2+} concentration is kept low by a very active ATP-dependent calcium ion pump (Fig. 8-26), which is embedded in the membranes of the SR.[238a] Within the vesicles Ca^{2+} is held loosely by the ~63-kDa protein **calsequestrin,** which binds as many as 50 calcium ions per molecule. When the cytoplasmic concentration of free Ca^{2+} falls below ~10^{-6} M, contraction ceases. In fast-contracting skeletal muscles the Ca^{2+}-binding protein **parvalbumin** (Fig. 6-7) may

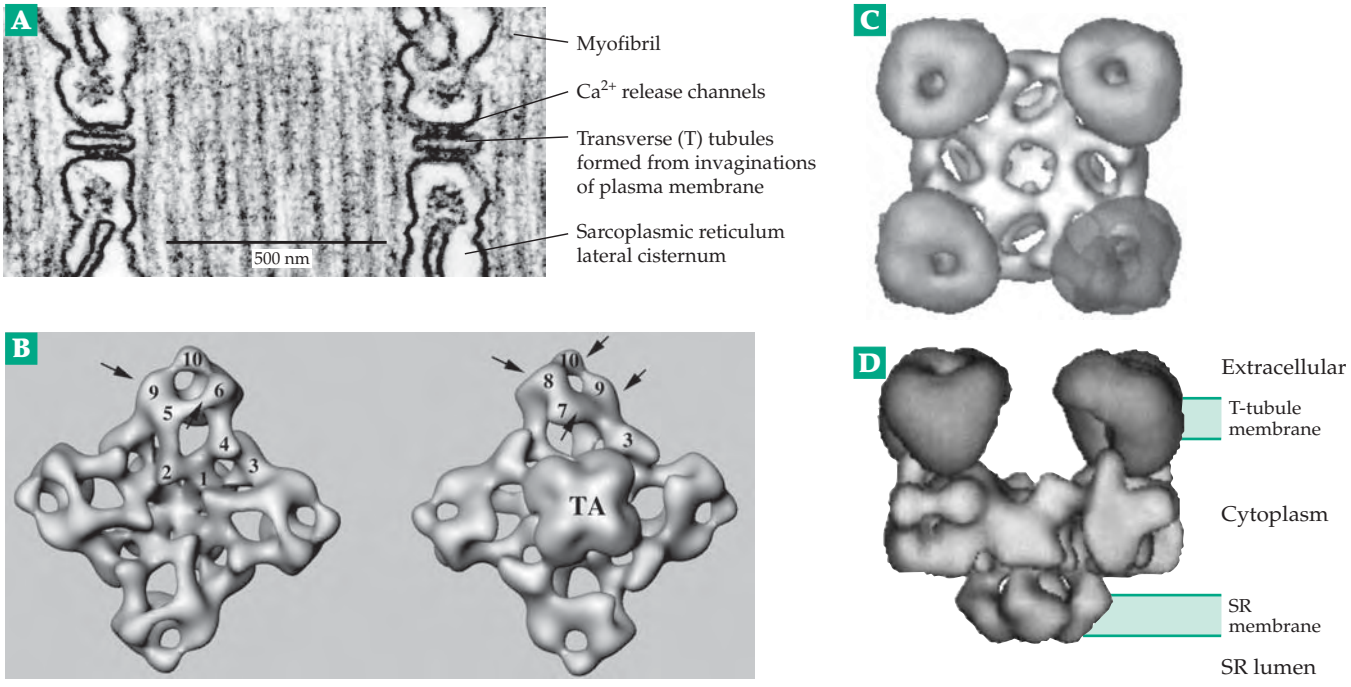
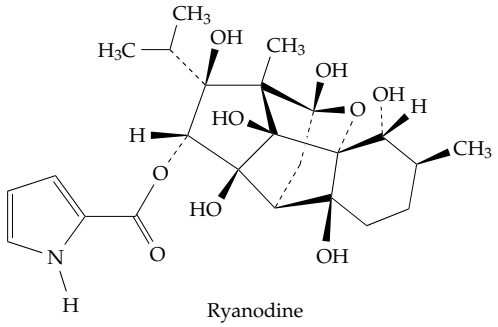


Figure 19-21 (A) Electron micrograph showing two transverse tubules (T-tubules) that are formed by infolding of the plasma membrane. They wrap around a skeletal muscle fiber and carry nerve impulses to all parts of the fiber. From Alberts *et al.*[237] Courtesy of Clara Franzini-Armstrong. (B) Three-dimensional surface representation of the calcium release channels known as ryanodine receptors, type RyR1 based on cryoelectron microscopy and image reconstruction at a resolution of 4.1 nm. The image to the left shows the surface that would face the cytoplasm while that to the right shows the surface that would interact with the sarcoplasmic reticulum, TA representing the transmembrane portion. Notice the fourfold symmetry of the particle, which is composed of four 565-kDa subunits. From Sharma *et al.*[239] Courtesy of Manjuli Rani Sharma. (C), (D) Model showing proposed arrangement of ryanodine receptors and dihydropyridine receptors (round) in the T tubule and SR membranes. From Serysheva *et al.*[245a]

assist in rapid removal of free Ca^{2+} from the cytoplasm. Contraction is activated when Ca^{2+} is released from the SR through the **calcium release channels**,[240–244] which are often called **ryanodine receptors**. The name arises from their sensitivity to the insecticidal plant alkaloid ryanodine, which at low concentration (≤60 μM) causes the channels to open, but a high concentration closes the channels.[243] These calcium release channels consist of tetramers of ~5000-residue proteins. The bulk of the 565-kDa polypeptides are on the cytosolic surface of the SR membranes, where they form a complex "foot" structure (Fig. 19-21B) that spans the ~12 nm gap between the SR vesicles and the T-tubule membrane.[239,240] Ryanodine receptor function is modulated by NO, which apparently binds to –SH groups within the Ca^{2+} channel.[243a,243b] Some ryanodine receptors are activated by cyclic ADP-ribose (cADPR, p. 564).[243c] Some have an oxidoreductase-like structural domain.[243d]

The release channels open in response to an incompletely characterized linkage to the **voltage sensor** that is present in the T-tubule membrane and is known as the **dihydropyridine receptor**.[240,245] This too, is a Ca^{2+} channel, which opens in response to arrival of an **action potential** (nerve impulse; see Chapter 30) that move along the T-tubule membrane. Because the



Ryanodine

action potential arrives almost simultaneously throughout the T-tubules of the muscles, the dihydropyridine receptors all open together. It isn't clear whether the linkage to the calcium channels is via stimulation from released Ca^{2+} passing from the dihydropyridine receptor to the surfaces of the feet of the release channels, or is a result of depolarization of the T-tubule membrane, or involves direct mechanically linked conformational changes.[240,245] The close cooperation of the Ca^{2+} release channel and the voltage sensor is reflected in their close proximity. In the sarcoplasmic reticulum every second release channel is adjacent to a voltage sensor in the opposing T-tubule membrane.[240,245a] The essential nature of the voltage

BOX 19-B MALIGNANT HYPERTHERMIA AND STRESS-PRONE PIGS

Very rarely during surgery the temperature of a patient suddenly starts to rise uncontrollably. Even when heroic measures are taken, sudden death may follow within minutes. This **malignant hyperthermia syndrome** is often associated with administration of halogenated anesthetics such as a widely used mixture of halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) and succinylcholine.[a–d] There is often no warning that the patient is abnormally sensitive to anesthetic. However, development of an antidote together with increased alertness to the problem has greatly decreased the death rate. Nevertheless, severe damage to nerves and kidneys may still occur.[c] Biochemical investigation of the hyperthermia syndrome has been facilitated by the discovery of a similar condition that is prevalent among certain breeds of pigs. Such "stress-prone" pigs are likely to die suddenly of hyperthermia induced by some stress such as shipment to market. The sharp rise in temperature with muscles going into a state of rigor is accompanied by a dramatic lowering of the ATP content of the muscles.

The problem, both in pigs and in humans susceptible to malignant hyperthermia, was found in the Ca^{2+} release channels (ryanodine receptors). Study of inheritance in human families together with genetic studies in pigs led to the finding that the stress-prone pigs have cysteine replacing arginine 615 in the Ca^{2+} channel protein. This modification appears to facilitate opening of the channels but to inhibit their closing.[e] A similar mutation has been found in some human families in which the condition has been recognized. However, there is probably more than one site of mutation in humans.[c,f] Similar mutations in the nematode *C. elegans* are being investigated with the hope of shedding light both on the problem of hyperthermia and on the functioning of the Ca^{2+} release channels.[g]

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sensor is revealed by a lethal mutation (**muscular disgenesis**) in mice. Animals with this autosomal recessive trait generate normal action potentials in the sarcolemma but Ca^{2+} is not released and no muscular contraction occurs. They lack a 170-kDa dihydropyridine-binding subunit of the sensor.[246]

Some aspects of regulation by calcium ions are poorly understood. The frequent observations of oscillations in [Ca^{2+}] in cells is described in Box 6-D. Another phenomenom is the observation of Ca^{2+} "sparks," detected with fluorescent dyes and observation by confocal microscopy.[247] These small puffs of Ca^{2+} have been seen in cardiac muscle[247] and in a somewhat different form in smooth muscle.[248] They may represent the release of Ca^{2+} from a single release channel or a small cluster of channels. When the calcium release channels open, Ca^{2+} ions flow from the cisternae of the SR into the cytoplasm, where they activate both the troponin–tropomyosin system and also the Ca^{2+}-calmodulin-dependent **light chain protein kinase,** which acts on the light chains of the myosin head. These light chains resemble calmodulin in their Ca^{2+}-binding properties. The function of light chain phosphorylation of skeletal muscle myosin is uncertain but it is very important in smooth muscle.[248a]

The regulatory complex of tropomyosin and troponin is attached to the actin filaments as indicated in Fig. 19-8D and also in Fig. 19-9. The latter shows a model at near atomic resolution but without side chains on the tropomyosin and without the troponin components. When the regulatory proteins are completely removed from the fibrils, contraction occurs until the ATP is exhausted. However, in the presence of the regulatory proteins and in the absence of calcium, both contraction and hydrolysis of ATP are blocked. Tropomyosin (Tm) is a helical coiled-coil dimer, a 40-nm rigid rod, in which the two 284-residue 33-kDa monomers have a parallel orientation (Fig. 19-9)[249] as in the myosin tail. However, an 8– to 9– residue overlap at the ends may permit end-to-end association of the Tm molecules bound to the actin filament. As with other muscle proteins there are several isoforms,[250,251] whose distribution differs in skeletal and smooth muscle and in platelets. The elongated Tm rods appear to fit into the grooves between the two strands of actin monomers in the actin filament.[252–254] In resting muscle the Tm is thought to bind to actin near the site at which the S1 portion of the myosin binds. As a consequence, the Tm rod may block the attachment of the myosin heads to actin and prevent actin-stimulated hydrolysis of ATP. The 40-nm Tm rod can contact about seven actin subunits at once (Fig. 19-9). Thus, one Tm–troponin complex controls seven actin subunits synchronously.

Troponin (Tn) consists of three polypeptides (TnC, TnI, TnT) that range in mass from 18 to 37 kDa. The complex binds both to Tm and to actin.[255,256] Peptide TnT binds tightly to Tm and is thought to link the TnI•TnC complex to Tm.[256,257] TnI interacts with actin and inhibits ATPase activity in the absence of Ca^{2+}.[258–261] It may work with the other two peptides to keep the Tm in the proper position to inhibit ATP hydrolysis. TnC binds calcium ions. This ~160-residue protein has a folding pattern almost identical to that of calmodulin (Fig. 6-8) with four Ca^{2+}-binding domains arranged in two pairs at the ends of a long 9-turn helix. When Ca^{2+} binds to TnC, a conformational change occurs[258,259,262-265] (p. 313). This induces changes in the troponin•tropomyosin•actin complex, releases the inhibition of actomyosin ATPase, and allows contraction to occur.[265a] In the heart additional effects are exerted by β-adrenergic stimulation, which induces phosphorylation of two sites on TnI by the action of the cAMP-dependent protein kinase PKA. Dephosphorylation by protein phosphatase 2A completes a regulatory cycle in which the doubly phosphorylated TnI has a decreased sensitivity to calcium ions.[266] Cardiac muscle also contains a specialized protein called **phospholamban**. An oligomer of 52-residue subunits, it controls the calcium ion pump in response to β-adrenergic stimulation. Unphosphorylated phospholamban inhibits the Ca ATPase, keeping [Ca^{2+}] high in the cytoplasm. Phosphorylation of phospholamban by cAMP and/or calmodulin-dependent protein kinase activates the Ca^{2+} pump,[267–268a] removing Ca^{2+} and ending contraction.

X-ray diffraction and electron microscopy in the 1970s suggested that when calcium binds to troponin the tropomyosin moves through an angle of ~ 20° away from S1, uncovering the active site for the myosin–ATP–actin interactions.[252,253] Tropomyosin could be envisioned as rolling along the surface of the actin, uncovering sites on seven actin molecules at once. Side-chain knobs protruding from the tropomyosin like teeth on a submicroscopic gear might engage complementary holes in the actin. At the same time a set of magnesium ion bridges between zones of negative charge on tropomyosin and actin could hold the two proteins together. This proposal has been difficult to test. Although the older image reconstruction is regarded as unreliable, recent work still supports this **steric blocking** model.[255,269-270c] Image reconstruction from electron micrographs of thin filaments shows that, in the presence of Ca^{2+}, the tropomyosin does move 25° away from the position in low [Ca^{2+}]. However, instead of two states of the thin filament, "on" and "off," there may be at least three, which have been called "blocked," "closed," and "open."[255,269,271,271a] The closed state may be attained in rigor.[269] In addition, the possibility that changes in the conformation of actin as well as of myosin occur during the contraction cycle must be considered.[255]

Smooth muscle. The primary regulation of smooth muscle contraction occurs via phosphorylation of the Ser 19 –OH group in the 20-kDa regulatory light chains of each myosin head.[121b,160,272–274] The phosphorylated form is active, participating in the contraction process. Removal of Ca^{2+} by the calcium pump and dephosphorylation of the light chains by a protein phosphatase[275] restores the muscle to a resting state. The N-terminal part of the myosin light chain kinase binds to actin, while the catalytic domain is in the center of the protein. The C-terminal part binds to myosin, and this binding also has an activating effect.[276]

Another protein, **caldesmon**, binds to smooth muscle actin and blocks actomyosin ATPase.[271,277–278a] It is present in smooth muscle in a ratio of actin:tropomyosin:caldesmon of ~14:2:1. Inhibition can be reversed by Ca^{2+}, but there is no agreement on the function of caldesmon.[277] It is an elongated ~756-residue protein with N-terminal domain, which binds to myosin, and a C-terminal domain, which binds to actin, separated by a long helix.[278] Caldesmon may be a substitute for troponin in a tropomyosin-type regulatory system, or it may promote actomyosin assembly. Another possibility is that it functions in a **latch state**, an energy-economic state of smooth muscle at low levels of ATP hydrolysis.[278,279] In molluscan muscles Ca^{2+} binds to a myosin light chain and activates contraction directly. Some molluscan smooth muscles (**catch muscles**) also have a latch state, which enables these animals to maintain muscular tension for long periods of time, e.g., holding their shells tightly closed, with little expenditure of energy.[279a] Catch muscles contain myosin plus a second protein, **catchin**, which is formed as a result of alternative mRNA splicing. Catchin contains an N-terminal sequence that may undergo phosphorylation as part of a regulatory mechanism.[280] However, recent experiments indicate that twitchin (see next paragraph), rather than catchin, is essential to the catch state and is regulated by phosphorylation.[280a] Regulation of the large groups of unconventional myosins is poorly understood. Phosphorylation of groups on the myosin heavy chains is involved in ameba myosins and others.[281]

An unexpected aspect of regulation was discovered from study of the 40 or more genes of *Caenorhabditis elegans* needed for assembly and function of muscle. The mutants designated *unc-22* showed a constant twitch arising from the muscles in the nematode's body. The gene was cloned using transposon tagging (Chapter 27) and was found to encode a mammoth 753-kDa 6839-residue protein which has been named **twitchin**.[282–285] Twitchin resembles titin (Fig. 19-8) and like titin has a protein kinase domain, which is normally inhibited by the end of its peptide chain, which folds over the active site of the kinase. Perhaps the protein kinase activities of twitchin, titin, and related proteins[285] are required in assembly of the sarcomere.

5. The Phosphagens

ATP provides the immediate source of energy for muscles but its concentration is only ~5 mM. As discussed in Chapter 6, Section D, **phosphagens**, such as **creatine phosphate**, are also present and may

$$\text{Creatine-}P + \text{ADP} \longrightarrow \text{Creatine} + \text{ATP}$$

attain a concentration of 20 mM in mammalian muscle. This provides a reserve of high-energy phospho groups and keeps the adenylate system buffered at a high phosphorylation state ratio[286] (see Eq. 6-67).

The concentration of both ATP and creatine phosphate as well as their rates of interconversion can be monitored by ^{31}P NMR within living muscles (Figs. 6-4 and 19-22). Phospho groups were observed to be

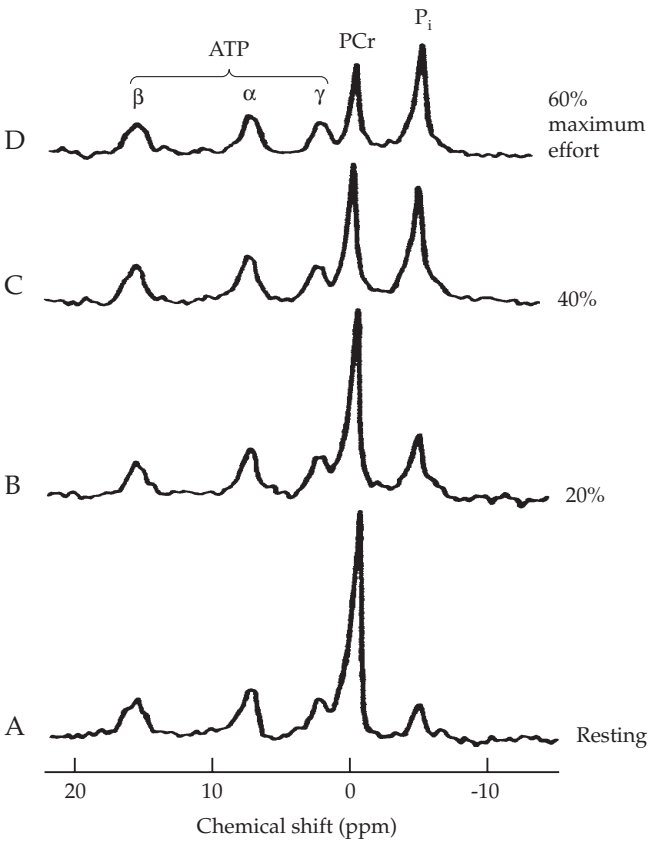

Figure 19-22 Phosphorus-31 magnetic resonance spectra of wrist flexor muscles of the forearm of a trained long-distance runner at rest and during contraction at three different levels of exercise. Ergometer measurements indicating the percent of initial maximum strength (% max) were recorded over each 6-min period. Spectra were obtained during the last 3 min of each period. Times of spectral data collection: A, resting; B, 4–6 min; C, 10–12 min; and D, 16–18 min. The pH ranged from 6.9 to 7.0. From Park *et al.*[288]

transferred from creatine phosphate to ADP to form ATP with a flux of 13 mmol $kg^{-1}s^{-1}$ in rat legs.[287] The reverse reaction must occur at about the same rate because little cleavage of ATP to P_i was observed in the anaesthetized rats. Use of surface coils has permitted direct observation of the operation of this shuttle system in human muscle (Fig. 19-22)[288] as well as in animal hearts (see Chapter 6). Only a fraction of the total creatine present within cells participates in the shuttle, however.[289]

C. Motion in Nonmuscle Cells

At one time actin and myosin were thought to be present only in muscles, but we know now that both actin proteins of the myosin family are present in all eukaryotic cells. Ameboid movement, the motion of cilia and flagella, and movement of materials along microtubules within cells also depend upon proteins of this group.

1. Actin-Based Motility

Ameboid movements of protozoa and of cells from higher organisms, the ruffling movements of cell membranes, phagocystosis, and the cytoplasmic streaming characteristic of many plant cells[289a] have all been traced to actin filaments or actin cables rather

TABLE 19-1
Some Actin-Binding Proteins

Function	Name	Function	Name
Bind and stabilize monomeric actin	Profilin[a,b,c,d,e,f,g,h]	Crosslink actin filaments or monomers	
	ADF/Cofilin[i,j,h,k]	Tight bundles	Villin[c,z,aa]
	Thymosin[f,l]	Loose bundles	α-Actinin[bb]
Cap actin filament ends	CapZ[m,n]		Spectrin[bb]
barbed end	Fragmin[o,p]		Fascin[cc]
pointed end	β-Actin[q]		MARCKS[a]
	Tropomodulin[r]	Network	Filamin[bb,c]
	Arp2/3, a complex of seven polypeptides		Gelactins
Sever or dissociate actin filament	Gelsolin[s,t,u,v,w]	Bind actin filaments to membrane	Talin[dd]
	Depactin		"ERM" proteins[ee,ff]
	Profilin[d,e]		
	ADF/cofilin[h,k,x,y]		

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than to microtubules.[289b] Actin is one of the most abundant proteins in all eukaryotes. Its network of filaments is especially dense in the lamellipodia of cell edges, in microvilli, and in the specialized stereocilia and acrosomal processes (see also pp. 369-370).[289c] Actin filaments and cables are often formed rapidly and dissolve quickly. When actin filaments grow, the monomeric subunits with bound ATP are added most rapidly at the "barbed end" and dissociate from the filament at the "pointed end" (see Section B,2).[94,290] The rate of growth may be ~20–200 nm / s, which requires the addition of 10–100 subunits / s.[291] Various actin-binding proteins control the growth and stability of the filaments. The actin-related proteins **Arp2** and **Arp3**, as a complex Arp2/3, together with recently recognized **formins**.[291a], provide nuclei for rapid growth of new actin filaments as branches near the barbed ends.[290,292–293c]

Growth of the barbed ends of actin filaments is stimulated by phosphoinositides and by members of the Rho family of G proteins (p. 559)[293d] through interaction with proteins of the **WASp** group.[293b,d,e] The name WAS comes from the immune deficiency disorder Wiskott–Aldrich Syndrome. Yet another family, the **Ena/VASP** proteins, are also implicated in actin dynamics. They tend to localize at focal adhesions and edges of lamellipodia.[293e,f] Profilin (Table 19-1) stabilizes a pool of monomeric actin when the barbed ends of actin filaments are capped. However, it catalyzes both the addition of actin monomers to uncapped barbed ends and rapid dissociation of subunits from pointed ends, leading to increased **treadmilling**.[294,295] Actin-severing proteins such as the **actin depolymerizing factor** (**ADF** or **cofilin**, Table 19-1) promote breakdown of the filaments.[296–297a] Treadmilling in the actin filaments of the lamellipods of crawling cells or pseudopods of amebas provides a motive force for many cells[291,298–299] ranging from those of *Dictyostelium* to human leukocytes. A series of proteins known as the **ezrin**, **radixin**, **moesin** (**ERM**) group attach actin to integral membrane proteins (Fig. 8-17)[292,300,301] and may interact directly with membrane lipids.[301a,b] Bound ATP in the actin subunits is essential for polymerization, and excess ATP together with crosslinking proteins stabilize the filaments. However, when the bound ATP near the pointed ends is hydrolyzed to ADP the filaments become unstable and treadmilling is enhanced. Thus, as in skeletal muscle, ATP provides the energy for movement.

Bacteria also contain filamentous proteins that resemble F-actin and which may be utilized for cell-shape determination.[301c] Actin-based motility is used by some bacteria and other pathogens during invasion of host cells (Box 19-C). It is employed by sperm cells of *Ascaris* and of *C. elegans*, which crawl by an ameboid movement that utilizes treadmilling of filaments formed from a motile sperm protein, which does not resemble actin.[302,302a] Cells are propelled on a glass surface at rates up to ~1 μm / s.

Various nonmuscle forms of myosin also interact with actin without formation of the myofibrils of muscle.[299] In most higher organisms nonmuscle myosins often consist of two ~200-kDa subunits plus two pairs of light chains of ~17 and 24 kDa each. These may form bipolar aggregates, which may bind to pairs of actin filaments to cause relative movement of two parts of a cell.[303] Movement depending upon the cytoskeleton is complicated by the presence of a bewildering array of actin-binding proteins, some of which are listed in Table 19-1.

2. Transport along Microtubules by Kinesin and Dynein

Many materials are carried out from the cell bodies of neurons along microtubules in the axons, which in the human body may be as long as 1 m. The rates of this **fast axonal transport** in neurons may be as high as 5 μm / s or 0.43 m / day. The system depends upon ATP and kinesin (Fig. 19-17) and permits small vesicles to be moved along single microtubules.[304–305b] Movement is from the minus end toward the plus end of the microtubule as defined in Figs. 7-33, 7-34. Slower **retrograde axonal transport** carries vesicles from the synapses at the ends of the axons (Fig. 30-8) back toward the cell body. This retrograde transport depends upon the complex motor molecule **cytoplasmic dynein** which moves materials from the plus end of the microtubule toward the minus end.[305,305c] In addition to these movements, as mentioned in Chapter 7, microtubules often grow in length rapidly or dissociate into their tubulin subunits. Growth occurs at one end by addition of tubulin subunits with their bound GTP. The fast growing *plus*-ends of the microtubules are usually oriented toward the cell periphery, while the *minus*-ends are embedded in the **centrosome** or **microtubule-organizing center** (p. 372).[306] Just as with actin, in which bound ATP is hydrolyzed to ADP, the bound GTP in the β-tubulin subunits of microtubules is hydrolyzed to GDP[307–310] decreasing the stability of the microtubules, a phenomenon described as **dynamic instability**. Various **microtubule-associated proteins** (MAPs) have strong effects on this phenomenon.[311] The MAPs are often regulated by phosphorylation–dephosphorylation cycles involving serine / threonine kinases. Microtubules also undergo posttranslational alterations not seen in other proteins. These include addition or removal of tyrosine at the C terminus.[312] Polyglycyl groups containing 3–34 glycine residues may be bound covalently to γ-carboxyl groups of glutamate side chains in both α- and β-tubulins.[312,313] This stabilizes the microtubules and is important to the long-lived microtubules of the axonomes of flagella and

BOX 19-C ACTIN-BASED MOTILITY AND BACTERIAL INVASION

Listeria monocytogenes is a dangerous food-borne bacterium that has become a major problem in the United States. This is one of the best understood *intracellular* pathogens. It is able to enter cells, escape from phagocytic vesicles, spread from cell to cell, and cross intestinal, blood–brain, and placental barriers.[a–c] Within cells these bacteria move using actin-based motility. Actin subunits polymerize at one end of a bacterium leaving a "comet tail" of crosslinked fibrous actin behind (see micrographs). Actin polymerization occurs directly behind the bacteria with subunits of monomeric actin adding to the fast growing "barbed end" (see Section B,2) of the actin strands. Growth has been described as a "Brownian ratchet."[c,d] Continual Brownian movement opens up spaces behind the bacteria, spaces that are immediately filled by new actin subunits. This provides a propulsive force adequate to move the bacteria ahead at velocities of about 0.3 μm / s.

Polymerization of actin is induced by interaction of a dimer of a 610-residue bacterial protein **ActA** with proteins of the host cell.[a,e–h] ActA is a composite protein with an N-terminal region that protrudes from the bacterial cell, a central region of proline-rich repeats that appear to be essential for recognition by host cells, and a C-terminal hydrophobic membrane anchor. There are also regions of close sequence similarity to the human actin-binding proteins vinculin and zyxin. The number of host proteins needed in addition to monomeric actin are:[i,j] the two actin-related proteins, **Arp2** and **Arp3**, which stimulate actin polymerization and branching;[h] **ADF/ cofilin**, which increases the rates, both of growth at the barbed ends and dissociation from the pointed ends of the filaments; and **Cap Z**, which caps barbed ends (Table 19-1). The need for ADF/ cofilin and Cap Z seems paradoxical. Cap Z may cap mostly older and slower growing filaments, restricting rapid filament assembly to the region closest to the bacterium. The need for ADF/ cofilin is unclear.[i] Growth rates are also enhanced by the human protein called **VASP** (vasodilator-stimulated phosphoprotein). The proline-rich region of the bacterial ActA may bind to VASP to initiate polymerization.[g] Both profilin (Table 19-1) and the crosslinking protein α-actinin also stimulate comet tail growth. Myosin does not participate in actin-based motility, but the hydrolysis of ATP drives the process through its link to actin polymerization.[i]

Although *Listeria* has been studied most, actin-based motility is employed by other pathogens as well, e.g., *Shigella flexneri* (the dysentery bacterium),[k] *Rickettsia*,[l] and vaccinia virus.[l] Although enteropathogenic *E. coli* do not use this method of movement, they induce accumulation of actin beneath the bacteria. They also promote formation of actin-rich adherent pseudopods and highly organized cytoskeletal structures that presumably assist the bacteria in entering a cell.[m]

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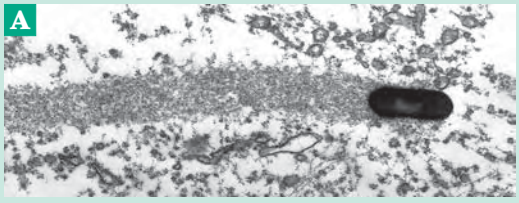

(A) *Listeria* cell with "comet tail" of cross-linked actin filaments. From Kocks *et al*. (1992) *Cell* **68**, 521–531. Courtesy of Pascale Cossart.

(B) Enlarged section of a thin comet tail of high resolution showing the actin filaments. From Sechi *et al*.[b] Courtesy of Antonio Sechi.

cilia. Polyglutamyl groups of 6–7 glutamates are also often added.[314]

Both dynein and several kinesins act as motors for formation of the spindle and for movement of chromosomes toward the minus ends of spindle microtubules during mitosis and meiosis (Fig. 26-11).[314a] In the genome of *Saccharomyces cerevisiae* there is only one dynein gene, but genes for six different kinesin-type motor molecules are present.[315] In higher organisms there may be even more genes for kinesins but there is apparently only one dynein in most species.[316]

Axonemal dyneins drive the motion of eukaryotic flagella and cilia. As with the cytoplasmic dyneins a complete molecule consists of two or three heavy chains with molecular mass ~520 kDa, some localized in the dynein tail, and several lighter chains.[305a,317–321] Like myosin dynein is an ATPase.

3. Eukaryotic Cilia and Flagella

The motion of eukaryotic flagella (Fig. 1-8) involves a sliding of the microtubular filaments somewhat analogous to the sliding of muscle filaments.[305,322–325] Sliding between the outer doublet microtubules (Fig. 19-23) via their inner and outer arms (dynein compounds) is thought to provide the characteristic bending waves.[325a,b] The movement is powered by dynein and ATP hydrolysis. Force and displacement measurements made by optical trapping nanometry suggest that the characteristic rhythmic beating of flagella results from an oscillatory property of the dynein.[326] The extremely complex structure of flagella is illustrated in Fig. 19-23. About 250 individual axonemal proteins have been detected in flagella of the alga *Chlamydomonas* (Fig. 1-11),[327] and a large number of mutants with various defects in their flagella have been isolated. The radial spokes (Fig. 19-23) alone contain 17 different proteins. These spokes protrude at ~29-nm intervals while the dynein molecules lie between pairs of the outer microtubule doublets at ~24-nm intervals. The dynein "arms" protrude from the "A" microtubule of each outer doublet and make contact with the incomplete "B" microtubule of the next doublet (Fig. 19-12). Although the shapes of the molecules are quite different, the basic chemistry of the ATPase activity of the dynein–microtubule system resembles that of actomyosin. However, the complexity of the dynein arms,[328]

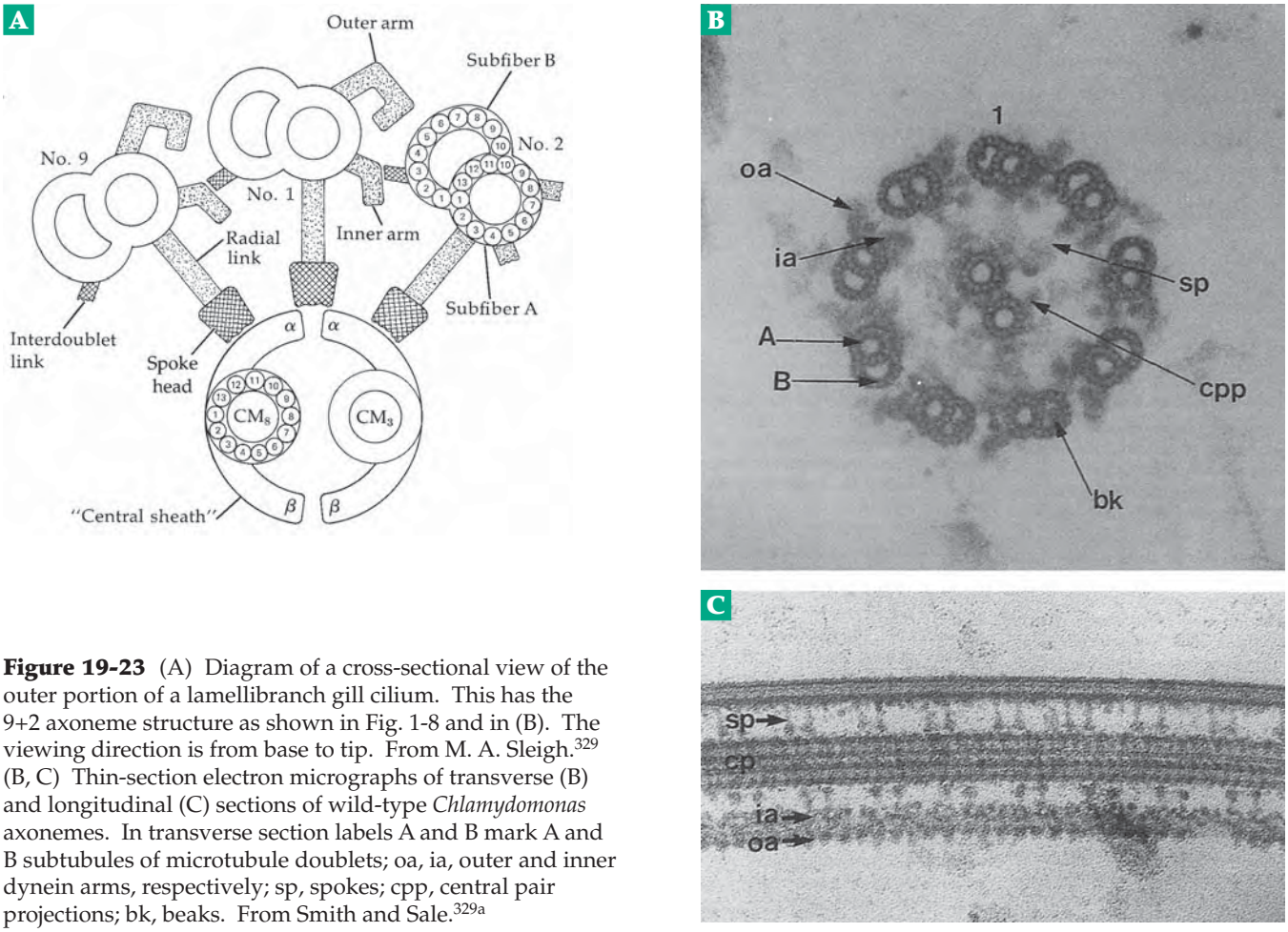

Figure 19-23 (A) Diagram of a cross-sectional view of the outer portion of a lamellibranch gill cilium. This has the 9+2 axoneme structure as shown in Fig. 1-8 and in (B). The viewing direction is from base to tip. From M. A. Sleigh.[329] (B, C) Thin-section electron micrographs of transverse (B) and longitudinal (C) sections of wild-type *Chlamydomonas* axonemes. In transverse section labels A and B mark A and B subtubules of microtubule doublets; oa, ia, outer and inner dynein arms, respectively; sp, spokes; cpp, central pair projections; bk, beaks. From Smith and Sale.[329a]

which exist in two types, inner and outer, suggests a complex contraction cycle.

4. Chemotaxis

As described in Box 11-C, the ameboid cells of the slime mold *Dictyostelium discoideum* are attracted to nutrients such as folic acid during their growth stage. Later, as the cells undergo developmental changes they become attracted by pulses of cyclic AMP.[330] Occupancy of 7-helix receptors for cAMP on the outer plasma membrane appears to induce methylation of both proteins and phospholipids and a rise in cytosolic Ca^{2+} and changes in the cytoskeleton that result in preferential extension of the actin-rich pseudopods toward the chemoattractant.[331]

In a similar manner, human ameboid leukocytes are attracted to sites of inflammation by various **chemotactic factors**.[332] These include the 74-residue cleavage product C5a formed from the fifth component of complement (Chapter 31),[333] various **lymphokines** (Chapter 31) secreted by lymphocytes, and peptides such as VGSE and AGSE, as well as larger peptides released by mast cells, basophils, or stimulated monocytes[334] and oxoicosenoids.[335] Polymorphonuclear leukocytes, upon engulfing sodium urate crystals in gouty joints, release an 8.4-kDa chemotactic protein which may cause a damaging response in this arthritic condition. Leukotriene B is a potent chemotactic agent as are a series of specific bacterial products, formylated peptides such as *N*-formyl-MLF.[332,336]

Neutrophils, monocytes, macrophages, eosinophils, basophils, and polymorphonuclear leukocytes are all affected by several or all of these factors. Binding to specific receptors results in a variety of changes in the cells. These include alterations in membrane potential, cyclic nucleotide levels, and ion fluxes (Na^+, K^+, Ca^{2+}) as well as increased methylation of specific proteins. A reorganization of microtubules and actin fibrils occurs, probably in response to an altered gradient of Ca^{2+}. The morphology of the cells changes, and they begin immediately to crawl toward the chemoattractants. It appears that these ameboid cells detect a gradient of attractant concentration between one end of the cell and the other, even though the anticipated difference may amount to only 0.1% of the total.[337,338]

5. Other Forms of Movement

Movement is characteristic of life and is caused not only by motor proteins but by various springs and ratchets which may be energized in a number of ways.[339] A striking example, which any one with a microscope and some fresh pond water can observe, is contraction of the stalk of protozoa of the genus *Vorticella*. Apparently first reported in 1676 by Leeuwenhoek the organism's 2–3 mm-long stalk contracts into a coiled spring (see p. 1 and also p. 281) when the animal is disturbed. Application of calcium ions causes contraction within a few milliseconds to ~40% of the original length. The process reverses slowly after a few seconds. Contraction is caused by a spring-like organ the **spasmoneme**, which is a bundle of short 2 nm-diameter fibrils inside the stalk. The fibrils are thought to be weakly crosslinked and held in the extended state by electrostatic repulsion between the negatively charged rods. Addition of Ca^{2+} neutralizes the charges permitting an entropy-driven collapse of the fibers.[339]

Another remarkable example is extension of the acrosomal process from a sperm cell of the horseshoe crab *Limulus polyphemus* at fertilization. A bundle of actin filaments in a crystalline state lies coiled around the base of the nucleus. At fertilization the bundle uncoils and slides through a tunnel in the nucleus to form a 60 µm-long acrosomal process within a few seconds. The uncoiled bundle is also crystalline. The coiled bundle is apparently overtwisted and an actin crosslinking protein **scruin** mediates the conformational alteration that takes place.[339] A somewhat related process may be involved in contraction of bacteriophage tails (pp. 363, 364)

Some bacteria glide with a twitching movement induced by rapid retraction of pili.[340] Another type of movement involves the pinching off of vesicles, e.g., of clathrin-coated pits (Fig. 8-27). This is a GTP-driven process that requires a mechanoenzyme called **dynamin**.[341,342]

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Study Questions

1. Describe briefly major aspects of the structure, properties, locations, and functions of each of the following proteins of skeletal muscle.

Actin	Tropomyosin
Myosin	Troponin
Titin	Myomesin
Nebulin	Desmin
α-Actinin	Vimentin
C-protein	

2. Describe the generally accepted sliding filament model of muscle contraction. List some uncertainties in this description.

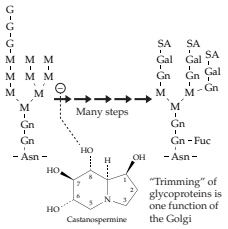
3. Compare mechanisms that regulate contraction in skeletal muscle and in smooth muscle.

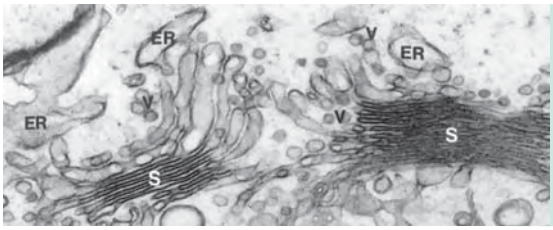
4. Compare myosin with kinesins and dyneins. What features do they have in common? What differences can you describe?

5. Compare the properties of actin in skeletal muscle and in nonmuscle cells. What is meant by "treadmilling?" What is "actin-based motility?"

6. The human genome contains more than 100 genes for proteins of the kinesin superfamily. Why?

7. Describe some of the major diseases that involve muscle proteins.



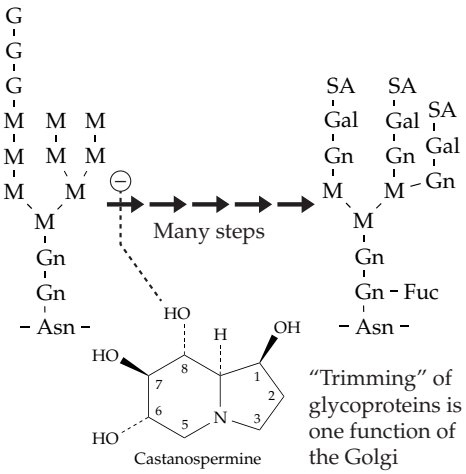


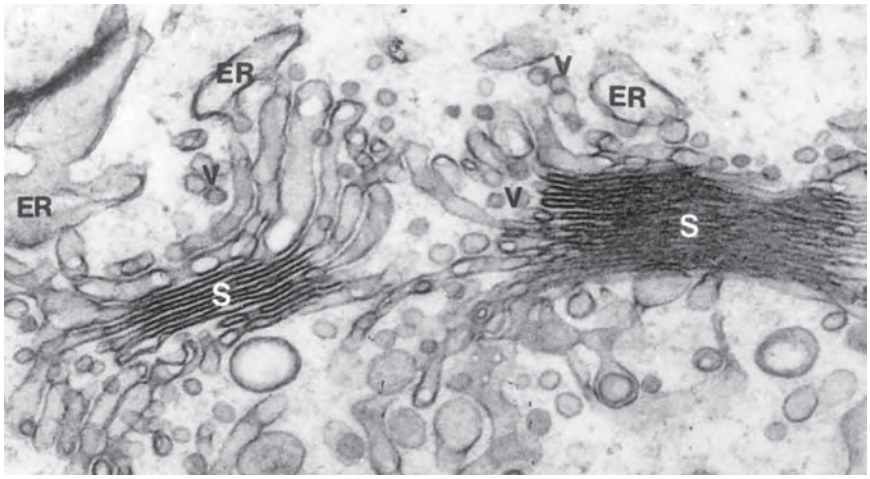
The branched oligosaccharides of glycoprotein surfaces are formed on asparagine side chains of selected cell surface proteins. The oligosaccharide at the left is formed in the ER and is transferred intact (Fig. 20-6) to an acceptor asparagine. It is then trimmed by removal of glucose and mannose units and residues of glucosamine, galactose, and fucose are added as in Fig. 20-7. These reactions begin in the ER and continue in the Golgi apparatus (right). See also Fig. 20-8.

Contents

Some Pathways of Carbohydrate Metabolism

20

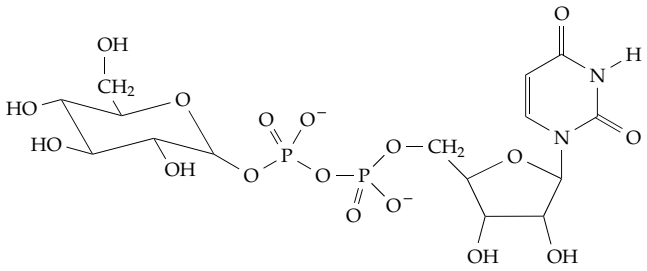




The general principles of biosynthesis, as well as the pathways of formation of major carbohydrate and lipid precursors, are considered in Chapter 17. Also described are the processes of gluconeogenesis, the synthesis of glucose 6-phosphate and fructose 6-phosphate from free glucose, and typical polymerization pathways for formation of polysaccharides. In this chapter, additional aspects of the metabolism of monosaccharides, oligosaccharides, polysaccharides, glycoproteins, and glycolipids are considered. These are metabolic transformations that affect the physical properties of cell surfaces and body fluids. They are essential to signaling between cells, to establishment of the immunological identity of individuals, and to the development of strong cell wall materials. Some of the differences in carbohydrates found in bacteria, fungi, green plants, and mammals are considered.

A. Interconversions of Monosaccharides

Chemical interconversions between compounds are easiest at the level of oxidation of carbohydrates. Consequently, many reactions by which one sugar can be changed into another are known. Most of the transformations take place in the "sugar nucleotide derivatives" (see also Eq. 17-56). The first of this group of compounds to be recognized was **uridine diphosphate glucose** (UDPG), which was discovered around 1950 by L. F. Leloir[1,2] during his investigation of the metabolism of galactose 1-*P*. The fact that interconversions of hexoses take place largely at the sugar nucleotide level was unknown at the time. Leloir's studies led to the characterization of both UDP-glucose and UDP-galactose.



Uridine diphosphate D-glucose

Figure 20-1 summarizes pathways by which glucose 6-phosphate or fructose 6-phosphate can be converted into many of the other sugars found in living things. Galactose and mannose can also be interconverted with the other sugars. A kinase forms **mannose 6-phosphate** which equilibrates with fructose 6-phosphate. Galactokinase converts free galactose to **galactose 1-phosphate**, which can be isomerized to glucose 1-phosphate by the reactions of Eq. 20-1. Fructose, an important human dietary constituent[3] derived largely by hydrolysis of sucrose, can also be formed in tissues via the **sorbitol pathway**[4] (Box 20-A). Fructose can be phosphorylated to fructose 1-phosphate by liver **fructokinase**. We have no mutase able to convert fructose 1-*P* to fructose 6-*P*, but a special aldolase cleaves fructose 1-*P* to dihydroxyacetone phosphate and free glyceraldehyde. Lack of this aldolase leads to occasionally observed cases of fructose intolerance.[5,6] The glyceraldehyde formed from fructose can be metabolized by reduction to glycerol followed by phosphorylation (glycerol kinase) and reoxidation to dihydroxyacetone phosphate. Some phosphorylation of fructose 1-*P* to fructose 1,6-P_2 apparently also occurs.[7] Interconversion of ribose 5-*P* and other sugar phosphates

is a central part of the pentose phosphate pathway (Fig. 17-8). Free ribose can be phosphorylated by a **ribokinase**.[8]

Oxidation of UDP-glucose in two steps[9,9a] by NAD^+ yields **UDP-glucuronic** acid, which can be epimerized to **UDP-galacturonic** acid. Likewise (see bottom of Fig. 20-1), **guanosine disphosphate-mannose** (GDP-mannose) is oxidized to **GDP-mannuronic acid**, which undergoes 4-epimerization to **GDP-guluronic acid**. Looking again at the top of the scheme, notice that UDP-D-glucuronic acid may be epimerized at the 5 position to **UDP-L-iduronic acid**. However, the iduronic acid residues in dermatan sulfate arise by inversion at C-5 of D-glucuronic acid residues in the polymer.[10] The mechanism of these reactions, like that of the decarboxylation of UDP-glucuronic acid to UDP-xylose (near the top of Fig. 20-1), apparently have not been well investigated.

Notice that glucuronic acid is abbreviated GlcA, in accord with IUB recommendations. However, many authors use GlcUA, ManUA, etc., for the uronic acids.

1. The Metabolism of Galactose

The reactions of galactose have attracted biochemists' interest because of the occurrence of the rare (30 cases / million births) hereditary disorder **galactosemia**. When this defect is present, the body cannot transform galactose into glucose metabolites but reduces it to the sugar alcohol **galactitol** or oxidizes it to **galactonate**, both products being excreted in the urine. Unfortunately, severe gastrointestinal troubles often appear within a few days or weeks of birth. Growth is slow and cataracts develop in the eyes, probably as a result of the accumulating galactitol. Death may come quickly from liver damage. Fortunately, galactose-free diets can be prepared for young infants, and if the disease is diagnosed promptly the most serious damage can be avoided. However, it has not been possible to prevent long-term effects that include speech difficulties, learning disabilities, and ovarian dysfunction.[1,11]

In some less seriously affected galactosemic patients **galactokinase** (Eq. 20-1, step *a*) is absent, but it is more often **galactose-1-phosphate uridyltransferase** (Eq. 20-1, step *b*) that is missing or inactive.[12–15a] This enzyme transforms galactose 1-*P* to UDP-galactose by displacing glucose 1-*P* from UDP-glucose. The UDP-galactose is then isomerized by the NAD^+-dependent

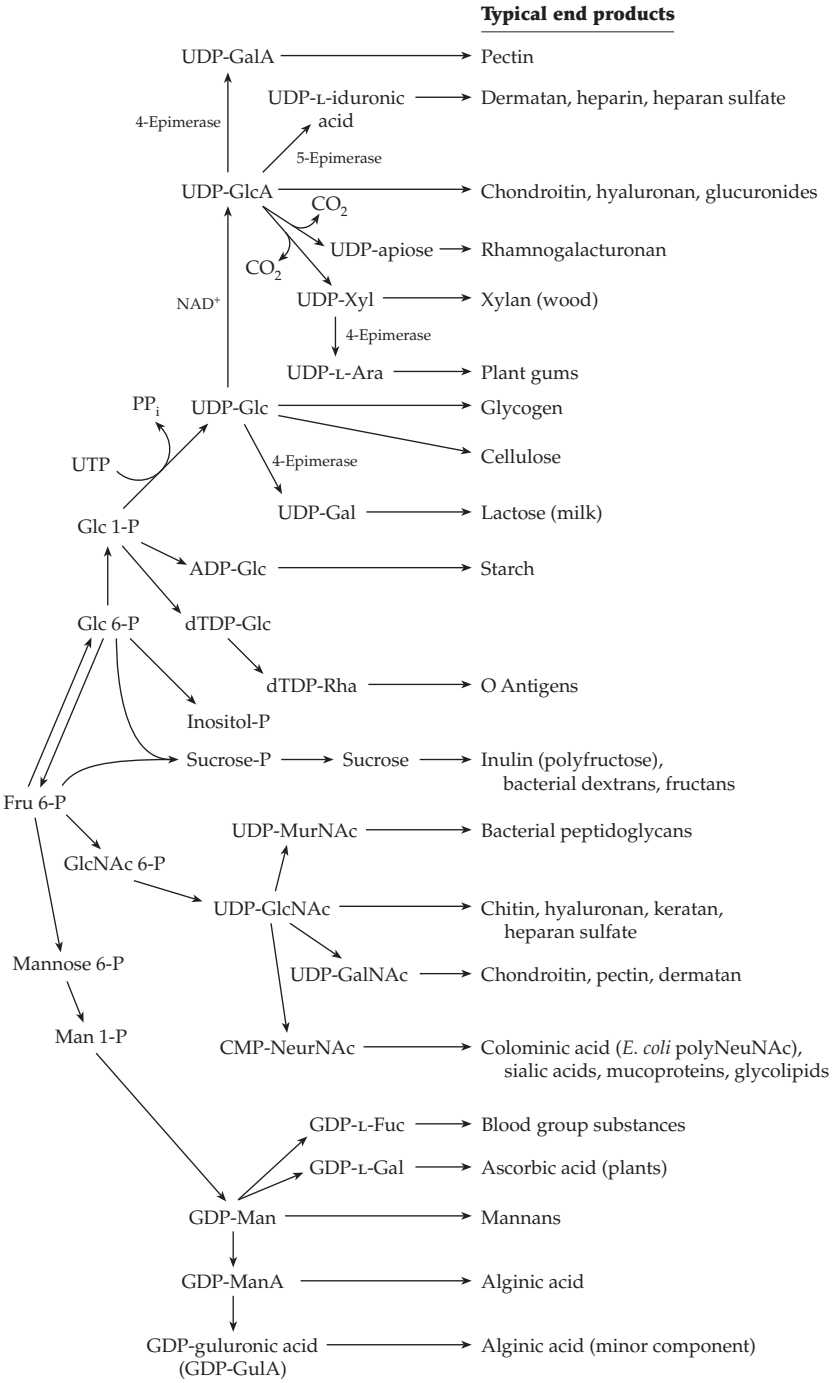
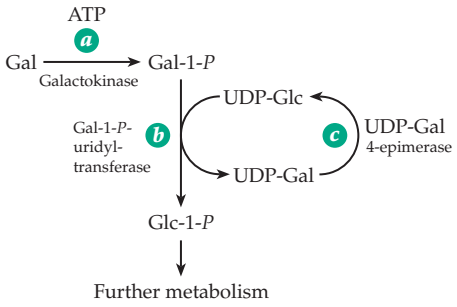


Figure 20-1 Some routes of interconversion of monosaccharides and of polymerization of the activated glycosyl units.



(20-1)

UDP-Gal 4-**epimerase**[16–16b] (Eq. 20-1, step *c*; see also Eq. 15-13 and accompanying discussion). Absence of this enzyme also causes galactosemia.[11] The overall effect of the reactions of Eq. 20-1 is to transform galactose into glucose 1-*P*. At the same time, the 4-epimerase can operate in the reverse direction to convert UDP-glucose to UDP-galactose, when the latter is needed for biosynthesis (Fig. 20-1).

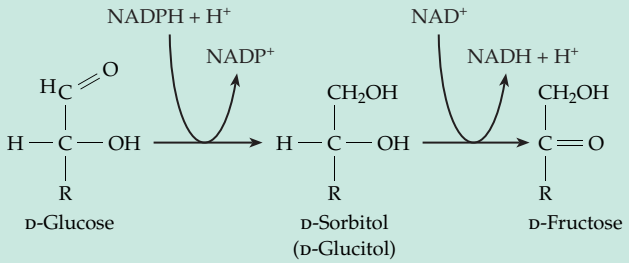
Another enzyme important to galactose metabolism, at least in *E. coli*, is **galactose mutarotase**.[17] Cleavage of lactose by β-galactosidase produces β-D-galactose which must be converted to the α-anomer by the mutarotase before it can be acted upon by galactokinase. Galactose is present in most glycoproteins and glycolipids in the pyranose ring form. However, in bacterial O-antigens, in cell walls of mycobacteria and fungi, and in some protozoa glactose occurs in the furanose form. The precursor is UDP-Gal*f*, which is formed from UDP-Gal*p* by **UDP-Gal*p* mutase**.[17a]

2. Inositol

Related to the monosaccharides is the hexahydroxycyclohexane ***myo*-inositol** (Eq. 20-2). This **cyclitol**, which is apparently present universally within cells (Fig. 11-9), can be formed from glucose-6-*P* according to Eq. 20-2 using a synthase that contains bound

BOX 20-A FRUCTOSE FOR SPERM CELLS VIA THE POLYOL PATHWAY

An interesting example of the way in which the high [NADPH]/[NADP$^+$] and [NAD$^+$]/[NADH] ratios in cells can be used to advantage is found in the metabolism of sperm cells. Whereas D-glucose is the commonest sugar used as an energy source by mammalian cells, spermatozoa use principally D-fructose, a sugar that is not readily metabolized by cells of surrounding tissues.[a–c] Fructose, which is present in human semen at a concentration of 12 mM, is made from glucose by cells of the seminal vesicle by reduction with NADPH to the sugar alcohol D-sorbitol, which in turn is oxidized in the 2 position by NAD$^+$. The combination of high [NADPH]/[NADP$^+$] and high [NAD$^+$]/[NADH] ratio is sufficient to shift the equilibrium far toward fructose formation.[d]



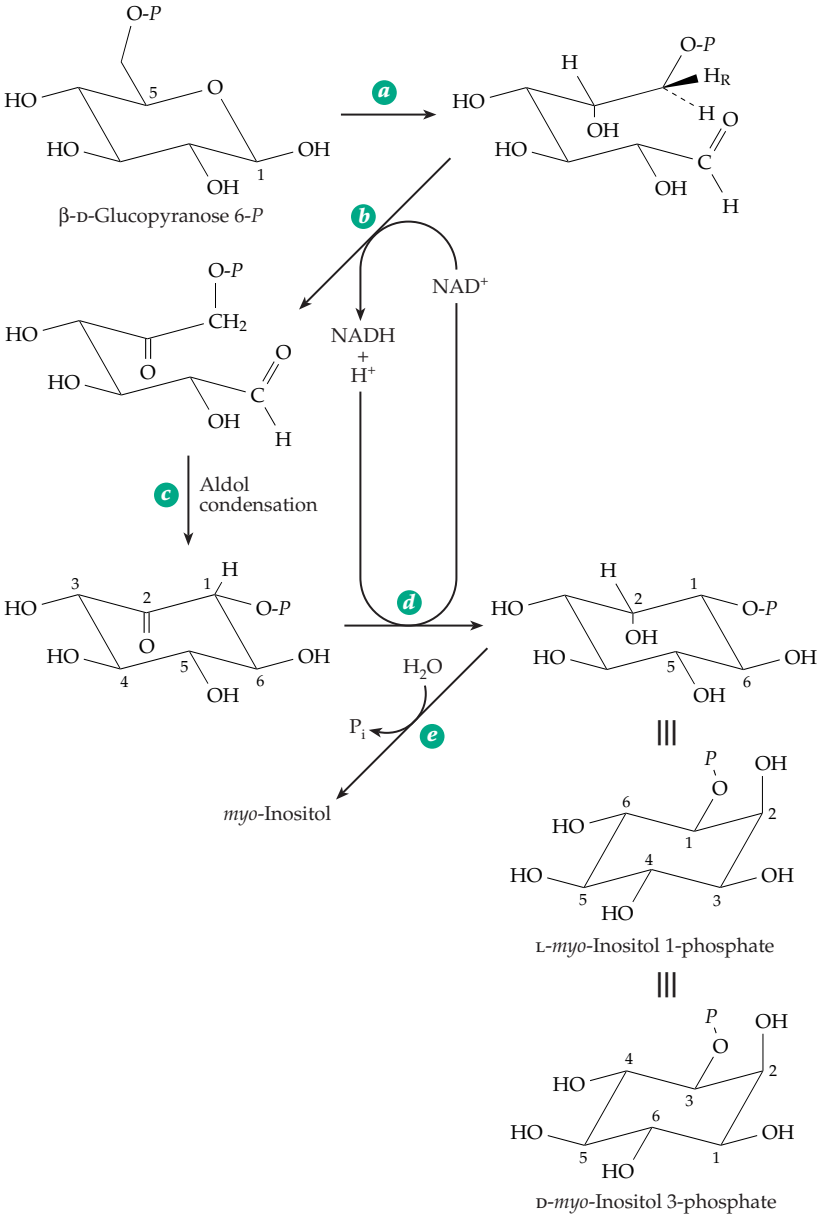
The polyol pathway is an active bypass of the dominant glycolysis pathway in many organisms.[e] Sorbitol and other polyols such as glycerol, erythritol, threitol, and ribitol serve as cryoprotectants in plants, insects, and other organisms.[f] Sorbitol is also an important osmolyte in some organisms (see Box 20-C). On the other hand, accumulation of sorbitol in lenses of diabetic individuals has often been blamed for development of cataract. However, doubts have been raised about this conclusion. The polyol pathway is more active than normal in diabetes, and there is evidence that the increased flow in this pathway may lead to an increase in oxidative damage to the lens. This may result, in part, from the depletion of NADPH needed for reduction of oxidized glutathione in the antioxidant system.[g] Aldose reductase inhibitors, which reduce the rate of sorbitol formation, decrease cataract formation. However, the reason for this is not yet clear.[h]

[a] McGilvery, R. W. (1970) *Biochemistry, A Functional Approach*, Saunders, Philadelphia, Pennsylvania (pp. 631–632)
[b] Hers, H. G. (1960) *Biochim. Biophys. Acta.* **37**, 127–
[c] Gitzelmann, R., Steinmann, B., and Van den Berghe, G. (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. 905–935, McGraw-Hill, New York
[d] Prendergast, F. G., Veneziale, C. M., and Deering, N. G. (1975) *J. Biol. Chem.* **250**, 1282–1289
[e] Luque, T., Hjelmqvist, L., Marfany, G., Danielsson, O., El-Ahmad, M., Persson, B., Jörnvall, H., and Gonzàlez-Duarte, R. (1998) *J. Biol. Chem.* **273**, 34293–34301
[f] Podlasek, C. A., and Serianni, A. S. (1994) *J. Biol. Chem.* **269**, 2521–2528
[g] Lee, A. Y. W., and Chung, S. S. M. (1999) *FASEB J.* **13**, 23–30
[h] Srivastava, S., Watowich, S. J., Petrash, J. M., Srivastava, S. K., and Bhatnagar, A. (1999) *Biochemistry* **38**, 42–54

NAD^+. In addition to the two redox steps (Eq. 20-2, *b* and *d*), this enzyme catalyzes both the conversion of the β anomer of glucose 6-*P* to the open-chain aldehyde form and the internal aldol condensation of Eq. 20-2, step *c*.[18-19b] The pro-*R* hydrogen at C-6 of glucose 1-*P* is lost in step *b* while the pro-*S* hydrogen is retained.[20] The ring numbering system is different for glucose and for the inositols, C-5 of glucose 1-*P* becoming C-2 of L-*myo*-inositol. Since *myo*-inositol contains a plane of symmetry D- and L- forms are identical. However, they are numbered differently (Eq. 20-2). The phosphoinositides and inositol polyphosphates are customarily numbered as derivatives of D-*myo*-inositol.

Synthesis of inositol by animals is limited and *myo*-inositol is sometimes classified as a vitamin. Mice grow poorly and lose some of their hair if deprived of dietary inositol. Various phosphate esters of inositol occur in nature. For example, large amounts of the hexaphosphate (**phytic acid**) are present in grains, usually as the calcium or mixed Ca^{2+}–Mg^{2+} salts known as **phytin**. The two apical cells of the 28-cell larvae of mesozoa (Fig. 1-12A) contain enough magnesium phytate in granular form to account for up to half of the weight of the larvae.[21] Inositol pentaphosphate is an allosteric activator for hemoglobin in birds and turtles (p. 358). Di-*myo*-inositol-1,1'-phosphate is an osmolyte in some hypothermophilic archaea.[19a] Inositol is a component of **galactinol**, the β glycoside of D-galactose with inositol (Eq. 20-15). Galactinol, as well as free inositol, circulates in human blood and in plants and may be a precursor of cell wall polysaccharides. However, in our own bodies the greatest importance for inositol doubtless lies in the inositol-containing phospholipids known as **phosphoinositides** (Figs. 8-2, 11-9, 21-5). Their function in generation of "second messengers" for various hormones is dealt with in Chapters 11 and 21.

A person typically ingests daily about one gram of inositol, some in the free form, some as phosphoinositides, and some as phytin. As much as four grams of inositol per day may be synthesized in the kidneys.[22] Breast milk is rich in inositol and dietary supplementation with inositol has increased survival of premature infants with respiratory distress syndrome.[22] The action of insulin is reported to be improved by administration of D-*chiro*-inositol (p. 998) to women with polycystic ovary syndrome.[22a]



(20-2)

3. D-Glucuronic Acid, Ascorbic Acid, and Xylitol

In bacteria, as well as in animal kidneys,[23] inositol may be converted to D-glucuronic acid (Fig. 20-1) with the aid of an oxygenase. Free glucuronic acid may also be formed by animals from glucose or from UDP-glucose (Fig. 20-2). Within the animal body glucuronic acid can be reduced with NADH (Fig. 20-2, step *a*) to yield **L-gulonic acid**, an aldonic acid that could also be formed by oxidation at the aldehyde end of the sugar **gulose**. Because C-6 of the glucuronic acid has become C-1 of gulonic acid, the latter belongs to the L family of

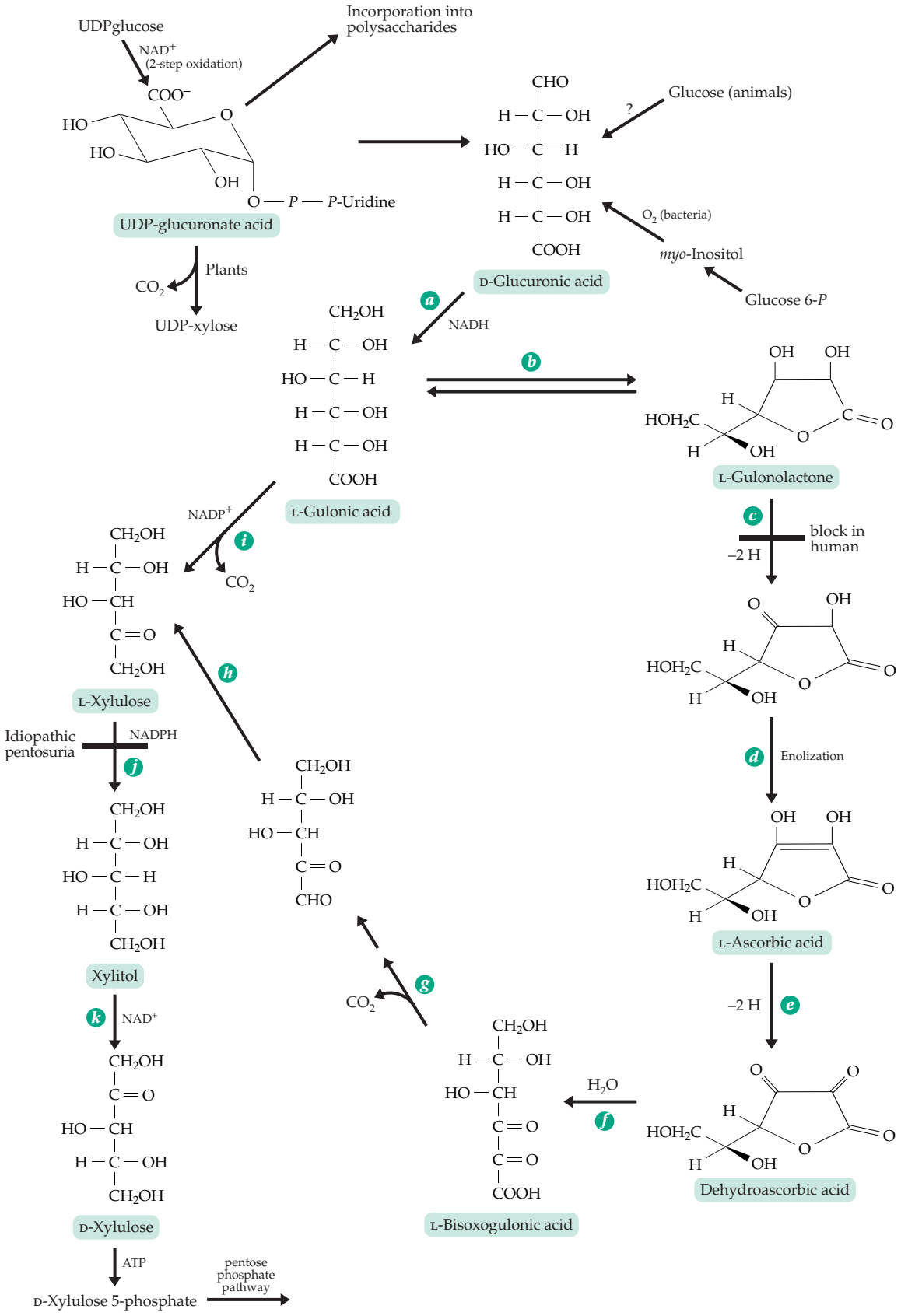
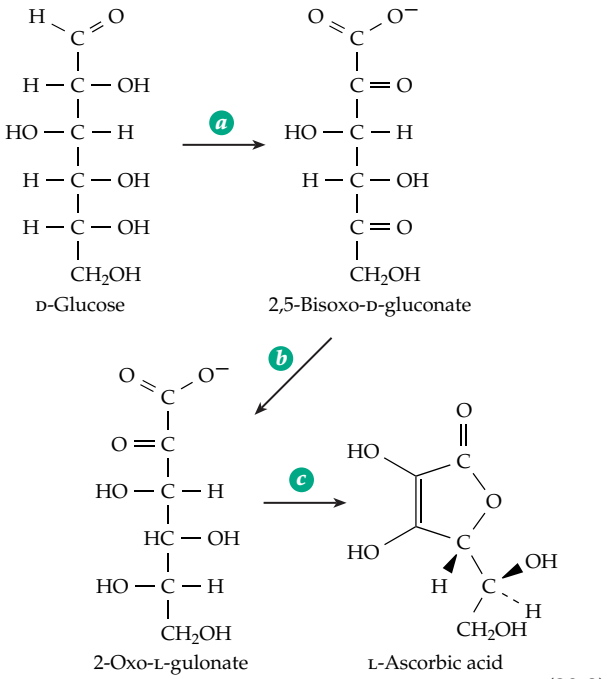


Figure 20-2 Some pathways of metabolism of D-glucuronic acid and of ascorbic acid, vitamin C.

sugars. Gulonic acid can be converted to a cyclic lactone (step *b*) which, in a two-step process involving dehydrogenation and enolization (steps *c* and *d*), is converted to **L-ascorbic acid**. This occurs in most higher animals.[24] However, the dehydrogenation step is lacking in human beings and other primates, in the guinea pig, and in a few other species. One might say that we and the guinea pig have a genetic defect at this point which obliges us to eat relatively large quantities of plant materials to satisfy our bodily needs for ascorbic acid (see Box 18-D). Gulonolactone oxidase is one of the enzymes containing covalently bound 8α-(N^1-histidyl)riboflavin.[25] The defective human gene for this enzyme has been identified, isolated, and sequenced. It is found to have accumulated a large number of mutations, which have rendered it inactive and now only a pseudogene.[26] Mice with an inactivated gulonolactone oxidase have a dietary requirement for vitamin C similar to that of humans. They suffer severe vascular damage on diets marginal in ascorbic acid.[26a] Even in rodents Na^+-dependent ascorbic acid transporters are present in metabolically active tissues to bring the vitamin from the blood into cells.[26b]

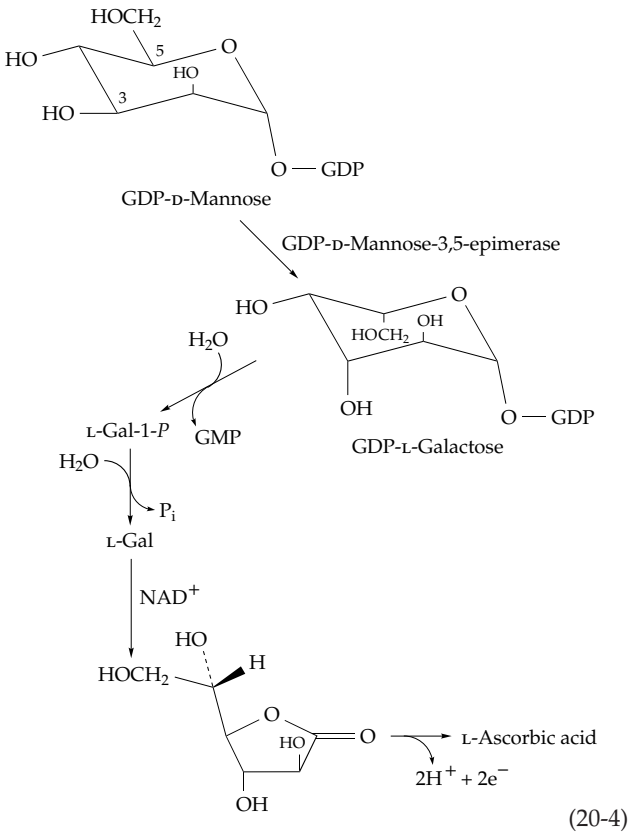
A clever bit of genetic engineering has permitted the conversion of D-glucose to 2-oxo-L-gulonate in the enzymatic sequence of Eq. 20-3, *a*, *b*.



(20-3)

The bacterium *Erwinia herbicola* naturally has the ability to oxidize glucose to 2,5-bisoxo-D-gluconate (Eq. 20-3, step *a*) but cannot carry out the next step, the stereospecific reduction to 2-oxo-L-gulonate. However, a gene encoding a suitable reductase was isolated from a genomic library from *Corynebacterium*. The cloned gene was fused to an *E. coli trp* promoter (see Chapter 28) and was introduced in a multicopy plasmid into *E. herbicola*. The resultant organism can carry out both steps *a* and *b* of Eq. 20-3 leaving only step *c*, a nonenzymatic acid-catalyzed reaction, to complete an efficient synthesis of vitamin C from glucose.[27]

Higher plants make large amounts of L-ascorbate, which in leaves may account for 10% of the soluble carbohydrate content.[28] However, the pathway of synthesis differs from that in Fig. 20-2. Both D-mannose and L-galactose are efficient precursors. The pathway in Eq. 20-4, which starts with GDP-D-mannose and utilizes known enzymatic processes, has been suggested.[28,29] The GDP-D-mannose-3, 5-epimerase is a well documented but poorly understood enzyme. Multistep mechanisms related to that of UDP-glucose 4-epimerase (Eqs. 20-1, 15-14) can be envisioned.

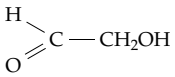


(20-4)

Ascorbic acid is readily oxidized to dehydroascorbic acid (Box 18-D; Fig. 20-2, step *e*), which may be hydrolyzed to L-bisoxogulonic acid (step *f*). The latter, after decarboxylation and reduction, is converted to L-xylulose (steps *g* and *h*), a compound that can also be formed by a standard oxidation and decarboxylation sequence on L-gulonic acid (step *i*). Reduction of xylulose to xylitol and oxidation of the latter with NAD^+ (steps *j* and *k*) produces D-xylulose, which can

be phosphorylated with ATP and enter the pentose phosphate pathways. A metabolic variation produces a condition called **idiopathic pentosuria**. Affected individuals cannot reduce xylulose to xylitol and, hence, excrete large amounts of the pentose into the urine, especially if the diet is rich in glucuronic acid. The "defect" seems to be harmless, but the sugar in the urine can cause the condition to be mistaken for diabetes mellitus.[30]

Xylitol is as sweet as sucrose and has been used as a food additive. Because it does not induce formation of dental plaque, it is used as a replacement for sucrose in chewing gum. It appeared to be an ideal sugar substitute for diabetics. However, despite the fact that it is already naturally present in the body, ingestion of large amounts of xylitol causes bladder tumors as well as oxalate stones in rats and mice. Its use has, therefore, been largely discontinued. A possible source of the problem may lie in the conversion by fructokinase of some of the xylitol to D-xylulose 1-*P*, which can be cleaved by the xylulose 1-*P* aldolase to dihydroxyacetone *P* and glycolaldehyde.

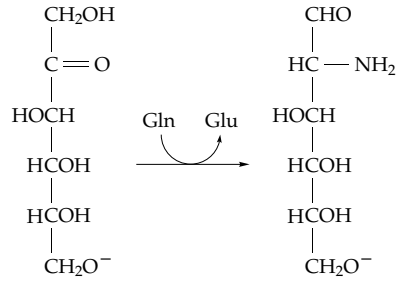


Glycolaldehyde

The latter can be oxidized to oxalate and may also be carcinogenic. As indicated in the upper left corner of Fig. 20-2, UDP-glucuronate can be decarboxylated to UDP-xylose.

4. Transformations of Fructose 6-Phosphate

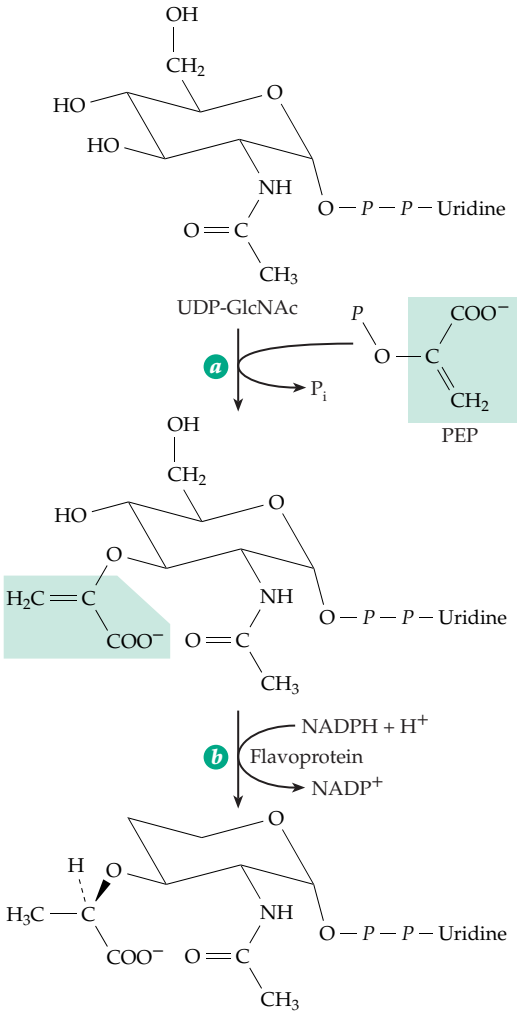
Biosynthesis of **D-glucosamine 6-phosphate** is accomplished by reaction of fructose 6-*P* with glutamine (Eq. 20-5):



Fructose 6-*P* D-Glucosamine 6-*P* (20-5)

Glutamine is one of the principal combined forms of ammonia that is transported throughout the body (Chapter 24). Glucosamine 6-phosphate synthase, which catalyzes the reaction of Eq. 20-5, is an amidotransferase of the N-terminal nucleophile hydrolase superfamily (Chapter 12).[31] It hydrolyzes the amide linkage of glutamine. The released ammonia presumably reacts with the carbonyl group of fructose 6-*P* to form an imine,[32–34a] which then undergoes a reaction analogous to that catalyzed by sugar isomerases.[35] The resulting D-glucosamine 6-*P* is acetylated on its amino group by transfer of an acetyl group,[36] and a mutase moves the phospho group to form *N*-acetylglucosamine 1-*P*. In *E. coli* acetylation occurs on GlcN 1-*P* and is catalyzed by a bifunctional enzyme that also has mutase activity.[37-37b] The resulting *N*-acetylglucosamine 1-*P* is converted to UDP-*N*-acetylglucosamine (UDP-GlcNAc) with cleavage of UTP to inorganic pyrophosphate as in the synthesis of UDP-glucose (Eq. 17-56). Cells of *E. coli* are also able to catabolize glucosamine 6-phosphate. A **deaminase,** with many properties similar to those of GlcN 6-*P* synthase, catalyzes a reaction resembling the reverse of Eq. 20-5 but releasing NH_3.[38,39]

One of the compounds formed from UDP-GlcNAc is **UDP-*N*-acetylmuramic acid**. The initial step in its synthesis is an unusual type of displacement reaction on the α-carbon of PEP by the 3-hydroxyl group of the sugar (Eq. 20-6, step *a*).[40-41c] Inorganic phosphate is



UDP-*N*-acetylmuramic acid (UDP-MurNAc) (20-6)

displaced with formation of an enolpyruvyl derivative of UDP-GlcNAc. This derivative is then reduced by NADPH (Eq. 20-6, step *b*).[42-43a] A second sugar nucleotide formed from UDP-GlcNAc is **UDP-*N*-acetylgalactosamine** (UDP-GalNAc), which may be created by the same 4-epimerase that generates UDP-Gal (Eq. 20-1).[44] Some animal tissues such as kidney and liver also have a **GalNAc kinase** that may salvage, for reuse, GalNAc that arises from the degradation of complex polysaccharides.[44] Bacteria may dehydrogenate UDP-GalNAc to UDP-*N*-acetylgalactosaminuric acid (UDP-GalNAcA).[44a]

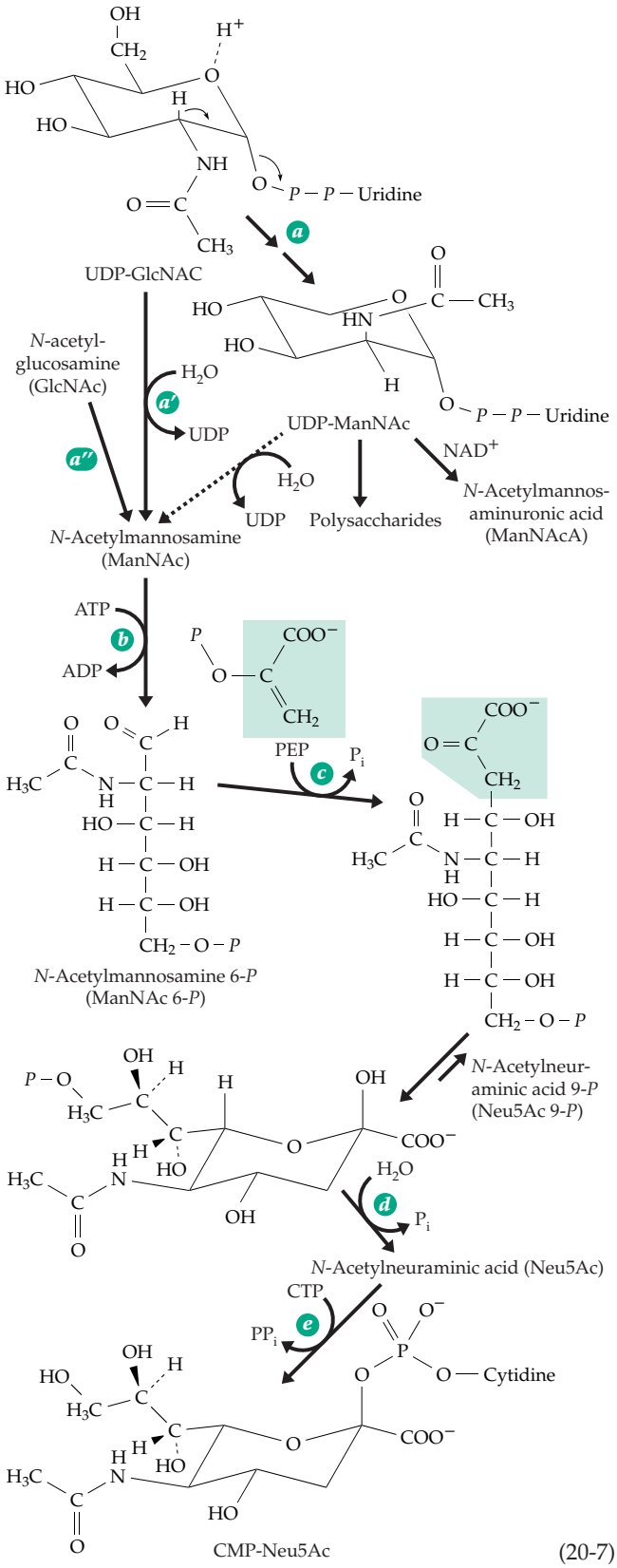
UDP-GlcNAc can be converted to UDP-*N*-acetylmannosamine (UDP-ManNAc) with concurrent elimination of UDP (Eq. 20-7).[45–47b] This unusual epimerization occurs without creation of an adjacent carbonyl group that would activate the 2-H for removal as a proton. As indicated by the small arrows in Eq. 20-7, step *a*′, the UDP is evidently eliminated. In a bacterial enzyme it remains in the E–S complex and is returned after a conformational change involving the acetamido group. This allows the transient C1–C2 double bond to be protonated from the opposite side (Eq. 20-7, step *a*).[47] In bacteria the UDP-ManNAc may be dehydrogenated to UDP-*N*-acetylmannos-aminuronic acid (ManNAcA). Both ManNAc and ManNAcA are components of bacterial capsules.[47]

In mammals the epimerase (Eq. 20-7, step *a*′) probably utilizes a similar chemical mechanism but eliminates UDP and replaces it with HO^- to give free *N*-acetylmannosamine, which is then phosphorylated on the 6-hydroxyl (Eq. 20-7, step *b*). ManNAc may also be formed from free GlcNAc by another 2-epimerase (step a″).[47c,d]

5. Extending a Sugar Chain with Phosphoenolpyruvate (PEP)

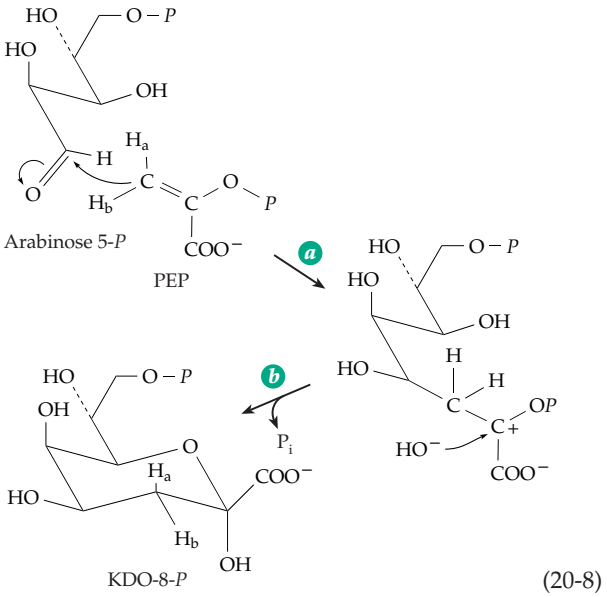
The six-carbon chain of ManNAc 6-*P* can be extended by three carbon atoms using an aldol-type condensation with a three-carbon fragment from PEP (Eq. 20-7, step *c*) to give ***N*-acetylneuraminic acid** (sialic acid).[48] The nine-carbon chain of this molecule can cyclize to form a pair of anomers with 6-membered rings as shown in Eq. 20-7. In a similar manner, arabinose 5-*P* is converted to the 8-carbon **3-deoxy-D-*manno*-octulosonic acid** (**KDO**) (Fig. 4-15), a component of the lipopolysaccharide of gram-negative bacteria (Fig. 8-30), and D-Erythrose 4-*P* is converted to 3-deoxy-D-*arabino*-heptulosonate 7-*P*, the first metabolite in the shikimate pathway of aromatic synthesis (Fig. 25-1).[48a] The arabinose-*P* used for KDO synthesis is formed by isomerization of D-ribulose 5-*P* from the pentose phosphate pathway, and erythrose 4-*P* arises from the same pathway.

The mechanism of the aldol condenstion that



(20-7)

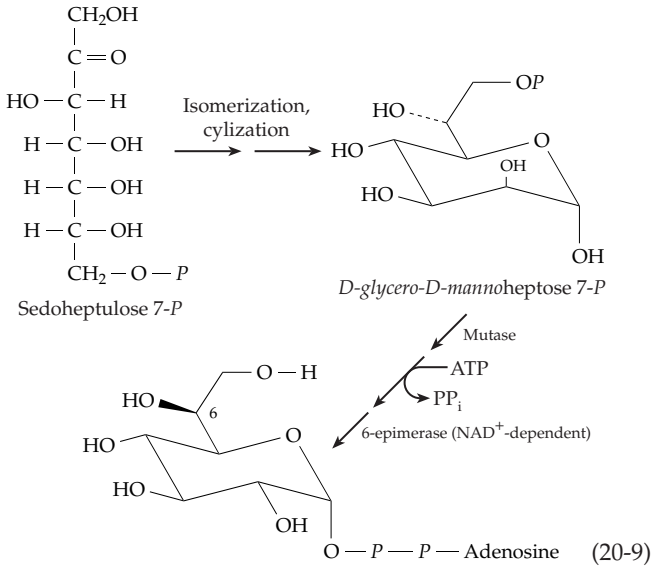
forms these sugars is somewhat unexpected. A reactive enolate anion can be formed from PEP by hydrolytic attack on the phospho group with cleavage of the O–P bond. However, in reactions such as step *a* of



(20-8)

Eq. 20-6, step *c* of Eq. 20-7,and also in EPSP synthase (Eq. 25-4) the initial condensation does not involve O–P cleavage. NMR studies of the action of KDO synthase reveal that the C–O bond of PEP is cleaved as is indicated in Eq. 20-8.[49-52b] The *si* face of PEP faces the *re* face of the carbonyl group of the sugar phosphate. A carbanionic center is generated at C-3 of PEP with possible participation of the phosphate oxygen as well as electrostatic stabilization of the carbocation formed in step *a*. Ring closure (step *b*) occurs with loss of P_i. The immediate product of the aldol condensation, in Eq. 20-7, is *N*-acetylneuraminic acid 9-phosphate, which is cleaved through phosphatase action (step *d*) and is activated to the CMP derivative by reaction with CTP (Eq. 20-7,step *e*).[52c] Further alterations may occur. For example, CMP-Neu5Ac is hydroxylated to form CMP-*N*-glycolylneuraminic acid.[53] Furthermore, an additional type of sialic acid, 2-oxo-3-deoxy-D-*glycero*-D-*galacto*-nononic acid (**KDN**), has been found in human developmentally regulated glycoproteins and also in many other organisms.[54-55a] It has an –OH group in the 5-position rather than the acetamido group of the other sialic acids. Like NeuNAc it is activated by reaction with CTP forming CMP-KDN. These activated monosaccharides differ from most others in being derivatives of a CMP rather than of CDP. More than 40 different naturally occuring variations of sialic acid have been identified.[55b]

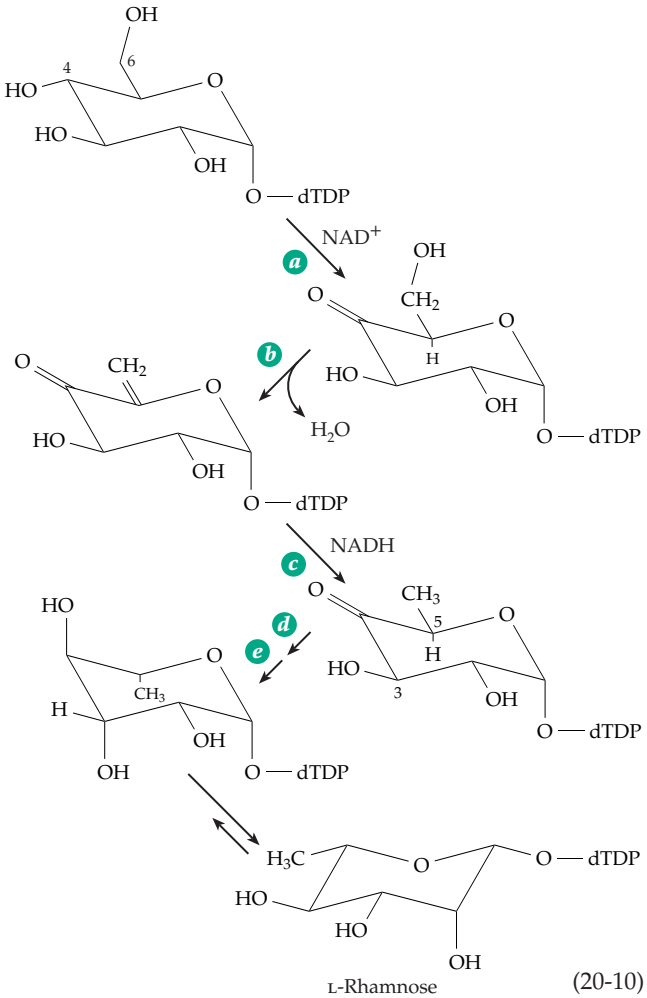
In a similar fashion, KDO is converted to the β-linked **CMP-KDO**,[56-56b] which is incorporated into lipid A as shown in Fig. 20-10. The ADP derivative of the **L-*glycero*-D-*manno*-heptose** (Fig. 4-15), which is also present in the lipopolysaccharide of gram-negative bacteria, is formed from sedoheptulose 7-*P* in a five-step process (Eq. 20-9).[57-58b]



(20-9)

6. Synthesis of Deoxy Sugars

Metabolism of sugars often involves dehydration to α,β-unsaturated carbonyl compounds. An example is the formation of 2-oxo-3-deoxy derivatives of sugar acids (Eq. 14-36). Sometimes a carbonyl group is



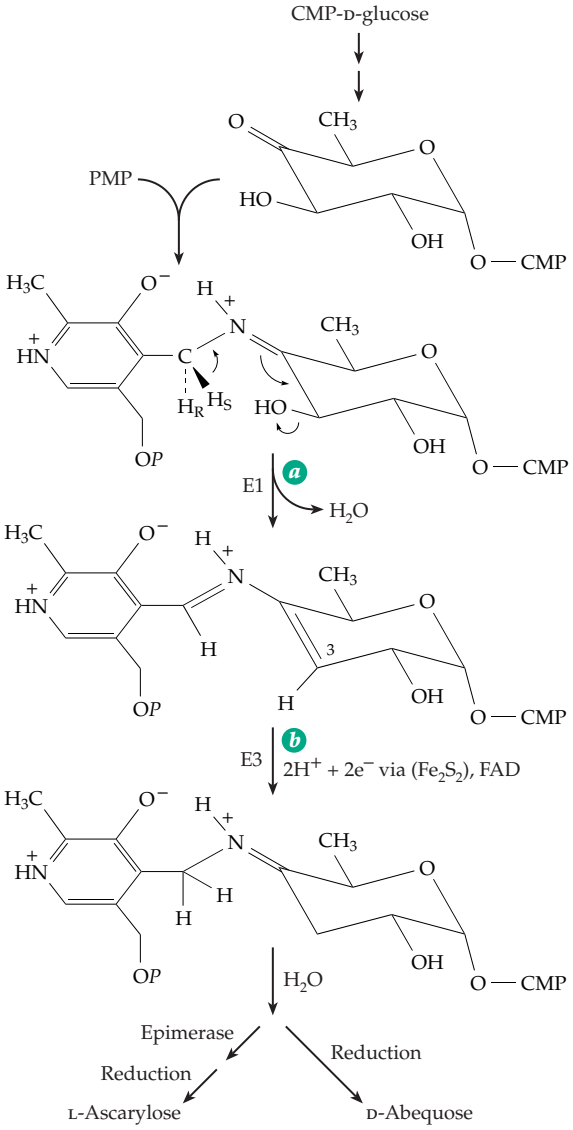
(20-10)

created by oxidation of an –OH group, apparently for the sole purpose of promoting dehydration. For example, the biosynthesis of L-rhamnose from D-glucose is a multistep process (Eq. 20-10) that takes place while the sugars are attached to deoxythymidine diphosphate.[59,59a,b] Introduction of the carbonyl group by dehydrogenation with tightly bound NAD^+ (Eq. 20-10, step *a*) is followed by dehydration (step *b*).[59c,d] To complete the sequence, the double bond formed by dehydration is reduced (step *c*) by the NADH produced in step *a*. A separate enzyme, a 3,5-epimerase catalyzes inversion at both C-3 and C-5 (step *d*).[59e] Finally, a third enzyme is needed for a second reduction (step *e*) using NADPH.[59f] The biosynthesis of **GDP-L-fucose** from GDP-D-mannose occurs by a parallel sequence.[60-61b]

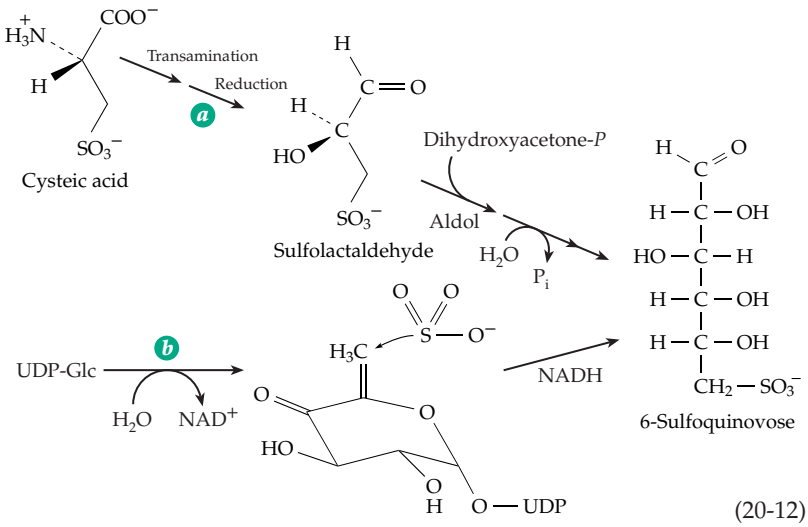
The metabolism of free L-fucose (6-deoxy-L-galactose), which is present in the diet and is also generated by degradation of glycoproteins, resembles the Entner–Doudoroff pathway of glucose metabolism (Eq. 17-18). Similar degradative pathways act on D-arabinose and L-galactose.[60]

Bacterial surface polysaccharides contain a variety of dideoxy sugars. The four 3,6-dideoxy sugars **D-paratose** (3,6-dideoxy-D-glucose), **D-abequose** (3,6-dideoxy-D-galactose), **D-tyvelose** (3,6-dideoxy-D-mannose), and **L-ascarylose** (3,6-dideoxy-L-mannose), whose structures are shown in Fig. 4-15, arise from CDP-glucose.[60a] This substrate is first converted, in reactions parallel to the first three steps of Eq. 20-10, to 4-oxo-6-deoxy-CDP-glucose which reacts in two steps with pyridoxamine 5'-phosphate (PMP) and NADH (Eq. 20-11). This unusual reaction[62–65] is catalyzed by a two-enzyme complex. The first component, E1, catalyzes the formation of a Schiff base of the substrate with PMP and a transamination, which also accomplishes dehydration, to give an unsaturated sugar ring (Eq. 20-11, step *a*). The protein also contains an Fe_2S_2 center suggesting a possible one-electron transfer. The second component, E_3, contains both an Fe_2S_2 plant type ferredoxin center and bound FAD.[65] Observation by EPR spectroscopy revealed accumulation of an organic free radical[64] that may be an intermediate in step *b* of Eq. 20-11. Hydrolysis, epimerization at C-5, and reduction yields L-ascarylose. A similar reaction sequence without the last epimerization would yield D-abequose. CDP-D-tyvelose arises by C-2 epimerization of CDP-D-paratose.[65a] Other unusual sugars[66–68] are formed from intermediates in Eq. 20-11. One is a **3-amino-3,4,6-trideoxyhexose** in which the amino group has been provided by transamination[67] (see also Box 20-B).

The unusual sulfur-containing sugar **6-sulfoquinovose** is present in

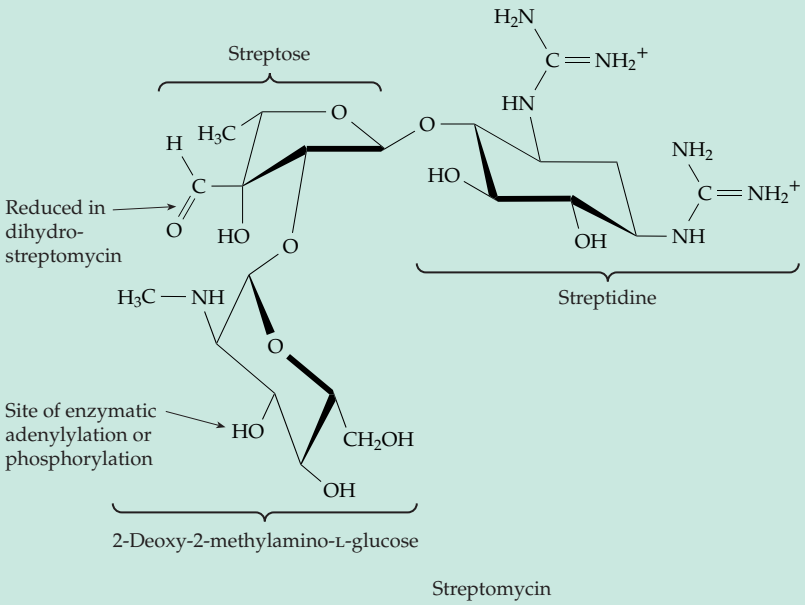


(20-11)

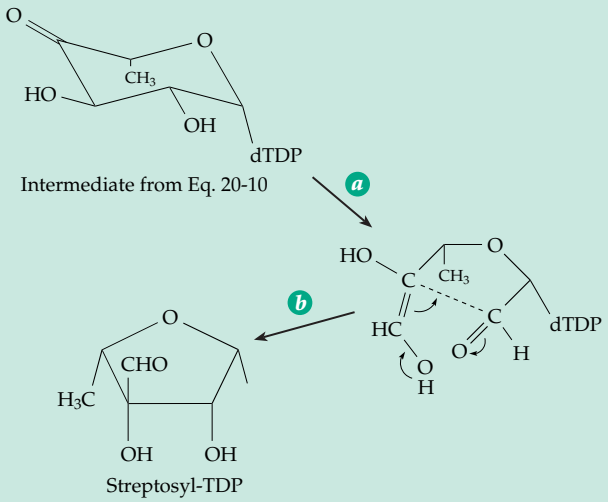


(20-12)

BOX 20-B THE BIOSYNTHESIS OF STREPTOMYCIN

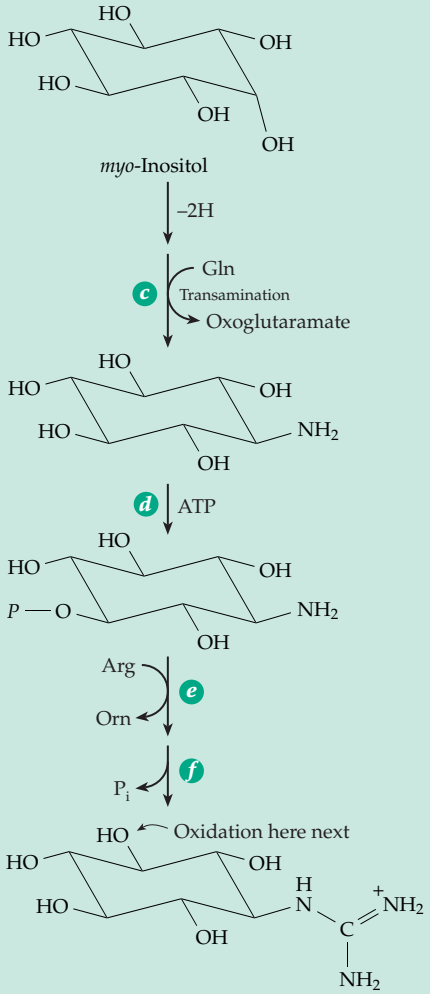


Streptomycin, the kanamycins, neomycins, and gentamycins form a family of medically important **aminoglycoside antibiotics**.[a] They are all water-soluble basic carbohydrates containing three or four unusual sugar rings. D-Glucose is a precursor of streptomycin, all three rings being derived from it. While the route of biosynthesis of 2-deoxy-2-methylamino-L-glucose is not entirely clear, the pathways to L-streptose and streptidine, the other two rings, have been characterized.[b–d] The starting material for streptidine synthesis is a nucleoside diphosphate sugar, which is an intermediate in the synthesis of L-rhamnose (Eq. 20-10). The carbon–carbon chain undergoes an aldol cleavage as shown in step *a* of the following equation:



The ring-open product is written here as an enediol, which is able to recyclize in an aldol condensation (step *b*) to form a five-membered ring with a branch at C-3. The L-streptosyl nucleoside diphosphate formed in this way serves as the donor of streptose to streptomycin.

The basic cyclitol streptidine is derived from *myo*-inositol, which has been formed from glucose 6-*P* (Eq. 20-2). The guanidino groups are introduced by oxidation of the appropriate hydroxyl group to a carbonyl group followed by transamination from a specific amino donor. In the first step, illustrated by the following equation, glutamine is the amino donor for the transamination, the oxoacid product being α-oxoglutaramic acid.



BOX 20-B THE BIOSYNTHESIS OF STREPTOMYCIN (continued)

The amino group on the ring now receives an amidine group, which is transferred from arginine by nucleophilic displacement[e] in a reaction resembling that in the synthesis of urea (see Fig. 24-10, step *h*). However, there is first a phosphorylation at the 2 position. After the amidine transfer has occurred to form the guanidino group, the phospho group is hydrolyzed off by a phosphatase. This is another phosphorylation–dephosphorylation sequence (p. 977) designed to drive the reaction to completion in the desired direction. The second guanidino group is introduced in an analogous way by oxidation at the 3 position followed by transamination, this time with the amino group being donated by alanine. Again, a phosphorylation is followed by transfer of an amidine group from arginine. The final hydrolytic removal of the phospho group (which this time is added at C-6) does not occur until the two other sugar rings have been transferred on from nucleoside diphosphate precursors to form streptomycin phosphate.

As with other antibiotics,[f–i] streptomycin is subject to inactivation by enzymes encoded by genetic resistance factors (Chapter 26). Among these are enzymes that transfer phospho groups or adenylyl groups onto streptomycin at the site indicated by the arrow in the structure.[j,k] Thus, dephosphorylation at one site generates the active antibiotic as the final step in the biosynthesis, while phosphorylation at another site inactives the antibiotic.

[a] Benveniste, R., and Davies, J. (1973) *Ann. Rev. Biochem.* **42**, 471–506
[b] Luckner, M. (1972) *Secondary Metabolism in Plants and Animals*, Academic Press, New York (pp.78–80)
[c] Walker, J. B., and Skorvaga, M. (1973) *J. Biol. Chem.* **248**, 2441–2446
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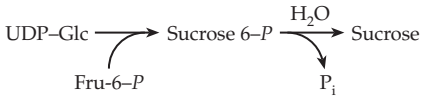
[h] Thompson, P. R., Hughes, D. W., Cianciotto, N. P., and Wright, G. D. (1998) *J. Biol. Chem.* **273**, 14788–14795
[i] Gerratana, G., Cleleand, W. W., and Reinhardt, L. A. (2001) *Biochemistry* **40**, 2964–2971
[j] Roestamadji, J., Grapsas, I., and Mobashery, S. (1995) *J. Am. Chem. Soc.* **117**, 80–84
[k] Thompson, P. R., Hughes, D. W., and Wright, G. D. (1996) *Biochemistry* **35**, 8686–8695

the sulfolipid of chloroplasts (p. 387).[69] A possible biosynthetic sequence begins with transamination of cysteic acid to 3-sulfopyruvate, reduction of the latter to sulfolactaldehyde, and aldol condensation with dihydroxyacetone-*P* as indicated in Eq. 20-12a.[70] See also Eq. 24-47 and Fig. 4-4. However, biosynthesis in chloroplasts appears to start with action of a 4,6-dehydratase on UDP-glucose followed by addition of sulfite and reduction (Eq. 20-12*b*).[70a,b] The sulfite is formed by reduction of sulfate via adenylyl sulfate (Fig. 24-25). However, biosynthesis in chloroplasts appears to start with action of a 4,6-dehydratase on UDP-glucose followed by addition of sulfite and reduction (Eq. 20-12*b*).[70a,b] The sulfite is formed by reduction of sulfate via adenylyl sulfate (Fig. 24-25).

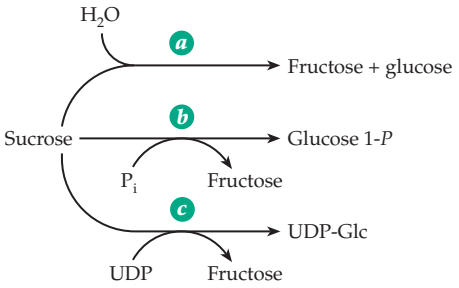
B. Synthesis and Utilization of Oligosaccharides

Our most common food sugar **sucrose** is formed in all green plants and nowhere else. It is made both in the chloroplasts and in the vicinity of other starch deposits. It serves both as a transport sugar and, dissolved within vacuoles, as an energy store. Sucrose is very soluble in water and is chemically inert because the hemiacetal groups of both sugar rings are blocked. However, sucrose is thermodynamically reactive, the glucosyl group having a group transfer potential of 29.3 kJ mol^{-1}. It is extremely sensitive toward hydrolysis catalyzed by acid. Transport of sugar in the form of a disaccharide provides an advantage to plants in that the disaccharide has a lower osmotic pressure than would the same amount of sugar in monosaccharide form.

Biosynthesis of sucrose[71,71a] utilizes both UDP-glucose and fructose 6-*P* (Eq. 20-13). Reaction of UDP-glucose with fructose can also occur to give sucrose directly.[72] Because this reaction is reversible, sucrose serves as a source of UDP-glucose for synthesis of cellulose and other polysaccharides in plants. Metabolism of sucrose in the animal body begins with the action of **sucrase** (invertase), which hydrolyzes the disaccharide to fructose and glucose (Eq. 20-14, step *a*). The same enzyme is also found in higher plants and fungi. Mammalian sucrase is one of several carbohydrases that are anchored to the external surfaces of the microvilli of the small intestines. Sucrose is bound



(20-13)



(20-14)

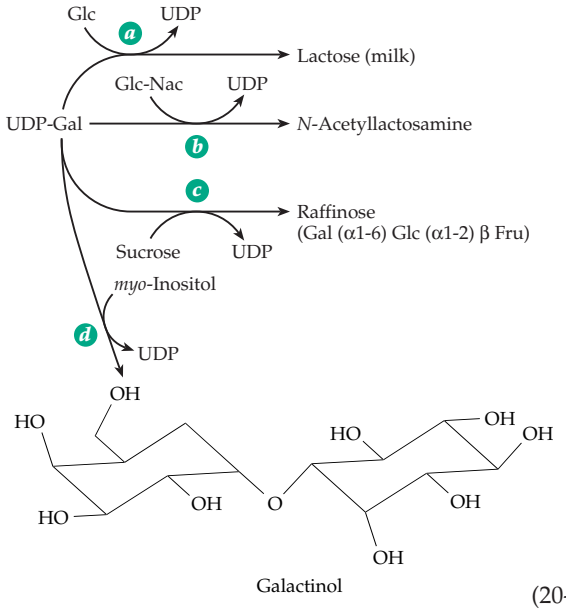
tightly but noncovalently to **isomaltase,** which hydrolyzes the α-1,6-linked isomaltose and related oligosaccharides. A nonpolar N-terminal segment of the isomaltase anchors the pair of enzymes to the microvillus membrane. The two-protein complex arises naturally because the two enzymes are synthesized as a single polypeptide, which is cleaved by intestinal proteases.[73,74]

Because of the relatively high group transfer potential of either the glucosyl or fructosyl parts, sucrose is a substrate for glucosyltransferases such as sucrose phosphorylase (Eq. 20-14, step *b*; see also Eq. 12-7 and associated discussion). In certain bacteria this reaction makes available the activated glucose 1-*P* which may enter catabolic pathways directly. Cleavage of sucrose for biosynthetic purposes can occur by reaction 20-14, step *c*, which yields UDP-glucose in a single step.

A disaccharide with many of the same properties as sucrose is **trehalose,** which consists of two α-glucopyranose units in 1,1 linkage (p. 168). The biosynthetic pathway from UDP-glucose and glucose 6-*P* parallels that for synthesis of sucrose (Eq. 20-13).[75,76] In *E. coli* the genes for the needed glucosyltransferase and phosphatase are part of a single operon. Its transcription is controlled in part by glucose-mediated catabolite repression (Chapter 28) and also by a repressor of the Lac family.[76,76a,77] The repressor is allosterically activated by trehalose 6-*P*, the intermediate in the synthesis. Trehalose formation in bacteria, fungi, plants, and microscopic animals is strongly induced during conditions of high osmolality (see Box 20-C).[77] Both trehalose and maltose can also be taken up via an ABC type transporter (p. 417).[77a,b]

Lactose, the characteristic sugar of milk, is formed by transfer of a galactosyl unit from UDP-galactose directly to glucose (Eq. 20-15, reaction *a*). The similar transfer of a galactosyl unit to *N*-acetylglucosamine to form *N*-acetyllactosamine (Eq. 20-15, reaction *b*) occurs in many animal tissues. An interesting regulatory mechanism is involved. The transferase catalyzing Eq. 20-15, reaction *b*, forms a complex with **α-lactalbumin** to become **lactose synthase**,[78–80b] the enzyme that catalyzes reaction *a*. Lactalbumin was identified as a milk constituent long before its role as a regulatory protein was recognized.

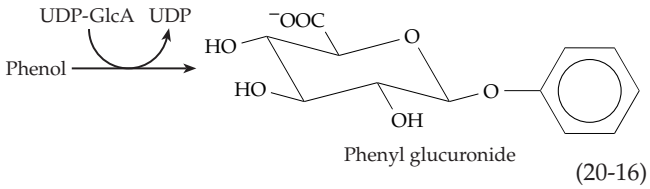
A very common biochemical problem is intolerance to lactose.[81] This results from the inability of the intestinal mucosa to make enough **lactase** to hydrolyze the sugar to its monosaccharide components galactose and glucose. Among most of the peoples of the earth only infants have a high lactase level, and the use of milk as a food for adults often leads to a severe diarrhea. The same is true for most animals. In fact, baby seals and walruses, which drink lactose-free milk, become very ill if fed cow's milk.



(20-15)

The plant trisaccharide **raffinose** arises from UDP-galactose by transfer of a galactosyl unit onto the 6-hydroxyl of the glucose ring of sucrose (Eq. 20-15, reaction *c*). Transfer of a galactosyl unit onto *myo*-inositol (Eq. 20-15, reaction *d*) produces **galactinol**, whose occurrence is widespread within the plant kingdom. Galactinol, in turn, can serve as a donor of activated galactosyl groups. Thus, many plants contain **stachyose** and higher homologs, all of which are formed by transfer of additional α-D-galactosyl units onto the 6-hydroxyl of the galactose unit of raffinose. These sugars appear to serve as antifreeze agents in the plants. The concentration of stachyose in soy beans can be as high as that of sucrose. Some seeds, e.g., those of maize, are coated with a glassy sugar mixture of sucrose and raffinose in a ratio of ~3:1.[81a]

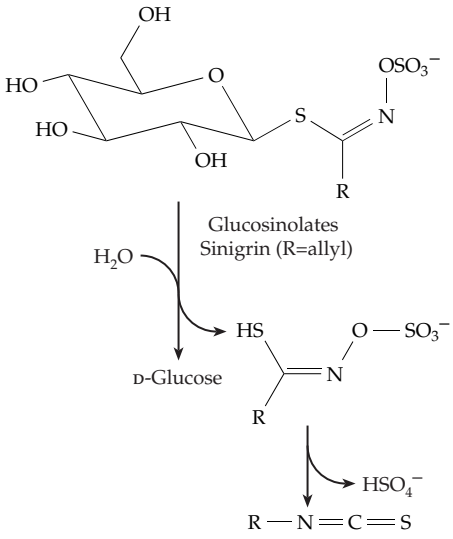
Besides the oligosaccharides, living organisms form a great variety of glycosides that contain nonsugar components. Among these are the **glucuronides** (glucosiduronides), excretion products found in urine and derived by displacement of UDP from UDP-glucuronic acid by such compounds as phenol, benzoic acid, and sterols.[81b,c] Phenol is converted to phenyl glucuronide (Eq. 20-16), while benzoic acid (also excreted in part as hippuric acid, Box 10-A) yields an ester by the same type of displacement reaction. Many other aromatic or aliphatic compounds containing –OH, –SH, $-NH_2$, or $-COO^-$ groups also form glucuronides.[82]



Phenyl glucuronide (20-16)

Among these is bilirubin (Fig. 24-24). UDP-glucuronosyltransferases responsible for their synthesis are present in liver microsomes.

Among the many glycosides and glycosylamines made by plants are the anthocyanin and flavonoid pigments of flowers (Box 21-E), cyanogenic glycosides such as amygdalin (Box 25-B), and antibiotics (e.g., see Box 20-B).[83,84] Some are characteristic of certain families of plants. For example, more than 100 β-thioglucosides known as **glucosinolides** are found in the Cruciferae (cabbages, mustard, rapeseed). The compounds impart the distinctive flavors and aromas of the plants. However, some are toxic and may cause goiter or liver damage. The enzyme **myrosinase** hydrolyzes these compounds releasing isothiocyanates, thiocyanates, and nitriles (Eq. 20-17).[85-86a] L-Ascorbate acts as a cofactor for this enzyme, evidently providing a catalytic base.[86a]



(20-17)

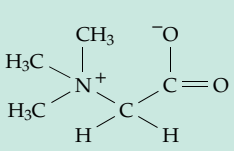
BOX 20-C OSMOTIC ADAPTATION

Bacteria, plants of many kinds, and a variety of other organisms are forced to adapt to conditions of variable osmotic pressure.[a,b] For example, plants must resist drought, and some must adapt to increased salinity. Some organisms live in saturated brine ~6 M in NaCl.[c] The **osmotic pressure** Π in dilute aqueous solutions is proportional to the total molar concentration of solute particles, c_s, as follows.

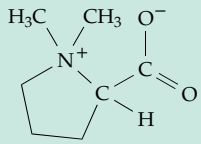
$$\Pi = RTc_s$$ where R is the gas constant and T the Kelvin temperature

At higher solute concentrations c_s must be replaced by the "effective molar concentration," which is called **osmolarity** (OsM) and has units of molarity (see Record *et al.* for discussion).[b] An osmolarity difference across a membrane of 0.04 OsM results in a turgor pressure of ~ 1 atmosphere. To adapt to changes in the environmental osmolarity organisms must alter their internal solute concentrations.

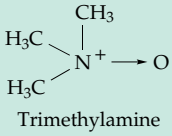
Cells of *E. coli* can adapt to at least 100-fold changes in osmolarity. Because of the porosity of the bacterial outer membrane the osmolarity of the periplasmic space is normally the same as that of the external medium. However, the inner membrane is freely permeable only to water and a few solutes such as glycerol.[b] The bacterial cells avoid loss of water when the external osmolarity is high by accumulating K^+ together with anions such as glutamate⁻ and nonionic **osmoprotectants** such as trehalose, sucrose, and oligosaccharides. *E. coli* cells will also take up other osmoprotectants such as **glycine betaine**, dimethylglycine, choline, proline, and proline betaine. Some methanogens accumulate N^{ε}-acetyl-β-lysine as well as glycine betaine.[d]



Betaine (glycine betaine)



Proline betaine



Trimethylamine *N*-oxide

Functioning in a somewhat different way in *E. coli* are 6- to 12-residue **periplasmic membrane-derived oligosaccharides**. These are β-1,2- and β-1,6-linked glucans covalently linked to *sn*-1-phosphoglycerol, phosphoethanolamine, or succinate (see Fig. 8-28).[b,e,f] They accumulate in the periplasm when cells are placed in a medium of low osmolarity. The resulting increased turgor in the periplasm is thought to buffer the cytoplasm against the loss of external osmolarity and to protect the periplasmic space from being eliminated by expansion of the plasma membrane. Related cyclic glucans, which are attached to *sn*-1-phosphoglycerol or *O*-succinyl ester residues, are accumulated by rhizobia.[g]

C. Synthesis and Degradation of Polysaccharides

Polysaccharides are all formed by transfer of glycosyl groups onto initiating molecules or onto growing polymer chains. The initiating molecule is usually a glycoprotein. However, let us direct our attention first to the growth of polysaccharide chains. The glycosyl are transferred by the action of glycosyltransferases from substrates such as UDP-glucose, other sugar nucleotides, and sometimes sucrose. The glycosyltransferases act by mechanisms discussed in Chapter 12 and are usually specific with respect both to substrate structure and to the type of linkage formed.

1. Glycogen and Starch

The bushlike glycogen molecules grow at their numerous nonreducing ends by the transfer of glucosyl units from UDP-glucose (Eq. 17-56)[87,87a] or in bacteria from ADP-glucose.[88–90] Utilization of glycogen by the cell involves removal of glucose units as glucose 1-*P* by the action of glycogen phosphorylase. The combination of growth and degradation from the same chain ends provides a means of rapidly storing and utilizing glucose units. The synthesis and breakdown of glycogen in mammalian muscle (Fig. 11-4) involves one of the first studied[91] and best known metabolic control systems. Various aspects have been discussed in Chapters 11, 12, and 17. The mechanism[92] and regulatory features[93–96b] have been described. An important recent development is the observation of glycogen concentrations in human muscles *in vivo* with ^{13}C NMR. This can be coupled with observation of glucose 6-*P* by ^{31}P NMR. The concentration of the latter is ~ 1 mM but increases after intense exercise.[94]

Glycogen phosphorylase and glycogen synthase alone are insufficient to synthesize and degrade glycogen. Synthesis also requires the action of the **branching enzyme** amylo-(1,4 → 1,6-transglycosylase,[97]

BOX 20-C (continued)

Fungi, green algae, and higher plants more often accumulate glycerol,[h,i] sorbitol, sucrose,[j] trehalose,[k] or proline.[a,l,m] These compounds are all "compatible solutes" which tend not to disrupt cellular structure.[n] Betaines and proline are especially widely used by a variety or organisms. How is it then that some desert rodents, some fishes, and other creatures accumulate **urea**, a well-known protein denaturant? The answer is that they also accumulate methylamine or trimethylamine *N*-oxide in an approximately 2:1 ratio of urea to amine. The mixture of compounds is compatible, the stabilizing effects of the amines offsetting the destabilizing effect of urea.[c,o]

Adaptation to changes in osmotic pressure involves sensing and signaling pathways that have been partially elucidated for *E. coli*[p] and yeasts.[i,q] Major changes in structure and metabolism may result. For example, in *E. coli* the outer membrane porin OmpF (Fig. 8-20) is replaced by OmpC (osmoporin), which has a smaller pore.[r]

A "resurrection plant" that normally contains an unusual 2-octulose converts this sugar almost entirely into sucrose when desiccated. This is one of a small group of plants that are able to withstand severe desiccation but can, within a few hours, reverse the changes when rehydrated.[j]

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an enzyme with dual specificity. After the chain ends attain a length of about ten glucose units, the branching enzyme attacks a 1,4-glycosidic linkage somewhere in the chain. Acting much as does 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 glycogen molecule. There the enzyme rejoins the bound oligosaccharide chain that it carries to a free 6-hydroxyl group of the glycogen creating a new branch attached in α-1,6-linkage. Degradation of glycogen requires **debranching** after the long nonreducing ends of the polysaccharide have been shortened until only four glycosyl residues remain at each branch point. This is accomplished by **amylo-1,6-glucosidase / 4- α-glucanotransferase**. This 165-kDa bifunctional enzyme transfers a trisaccharide unit from each branch end to the main chain and also removes hydrolytically the last glucosyl residue at each branch point.[98-99a]

How are new glycogen molecules made? There is some evidence that a 37-kDa protein primer **glycogenin** is needed to initiate their formation.[100-101a] Thus, glycogen synthesis may be analogous to that of the glycosaminoglycans considered in Section D,1. Muscle glycogenin is a self-glycosylating protein, which catalyzes attachment of ~7 to 11 glucose units in α–1,4 linkage to the hydroxyl group of Tyr 194. The glucose units are added one at a time and when the chain is long enough it becomes a substrate for glycogen synthase.[100,102] The role of glycogenin in liver has been harder to demonstrate,[103] but a second glycogenin gene, which is expressed in liver, has been identified.[104] Genes for several glycogenins or glycogenin-like proteins have been identified in yeast, *Caenorhabditis elegans*, and *Arabidopsis*.[101a,105]

In contrast to animals, bacteria such as *E. coli* synthesize glycogen via ADP-glucose rather than UDP-glucose.[88] ADP-glucose is also the glucosyl donor for synthesis of starch in plants. The first step in the biosynthesis (Eq. 20-18) is catalyzed by the enzyme ADP-glucose pyrophosphorylase (named for the reverse reaction).

$$\text{Glucose 1-}P + \text{ATP} \rightarrow \text{ADP-glucose} + \text{PP}_i \qquad (20\text{-}18)$$

In bacteria this enzyme is usually inhibited by AMP and ADP and activated by glycolytic intermediates such as fructose 1,6-P_2, fructose 6-P, or pyruvate. In higher plants, green algae, and cyanobacteria the enzyme is usually activated by 3-phosphoglycerate, a product of photosynthetic CO_2 fixation, and is inhibited by inorganic phosphate (P_i).[106–108].

In eukaryotic plants starch is deposited within chloroplasts or in the cytoplasm as granules (Fig. 4-6) in a specifically differentiated and physically fragile plastid, the **amyloplast**.[108–110] Within the granules the starch is deposited in layers ~ 9 nm in thickness. About two-thirds of the thickness consists of nearly crystalline arrays, probably of double helical amylopectin side chains (Figs. 4-7, 4-8, 20-3) with "amorphous" segments between the layers.[111–114] In maize there are at least five starch synthases, one of which forms the straight chain amylose.[115–117] There are also at least three branching enzymes[118] and two or three debranching enzymes.[119,120] As in the synthesis of glycogen the molecules of amylopectin may grow at the many nonreducing ends. A current model, which is related to the broom-like cluster model of French (Fig. 4-7), is shown in Fig. 20-3. The branches are thought to arise, in part, by transglycosylation within the double helical strands. After branching the two chains remain in a double helix but the cut chain can now grow. Only double helical parts of strands pack well in the crystalline layer. A recent suggestion is that debranching enzymes then trim the molecule, removing single-stranded regions.[112]

The location (within the granule) of amylose, which makes up 15–30% by weight of many starches,[121] is uncertain. It may fill in the amorphous layers. It may be cut and provide primer pieces for new amylopectin molecules.[122,122a] Another possibility is that it grows by an insertion mechanism such as that portrayed for cellulose in Fig. 20-5 and is extruded inward from the membrane of the amyloplast. This mechanism might explain a puzzling question about starch. The branched amylopectin presumably grows in much the same way as does glycogen. A branching enzyme transfers part of the growing glycan chain to the $-CH_2-OH$ group of

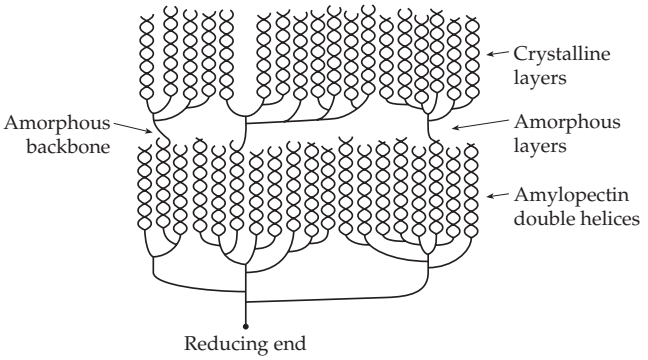

Figure 20-3 Proposed structure of a molecule of amylopectin in a starch granule. The highly branched molecule lies within 9 nm thick layers, about 2 / 3 of which contains parallel double helices of the kind shown in Fig. 4-8 in a semicrystalline array. The branches are concentrated in the amorphous region.[113,114,121] Some starch granules contain no amylose, but it may constitute up to 30% by weight of the starch. It may be found in part in the amorphous bands and in part intertwined with the amylopectin.[122]

BOX 20-D GENETIC DISEASES OF GLYCOGEN METABOLISM

In 1951, B. McArdle described a patient who developed pain and stiffness in muscles after moderate exericse.[a] Surprisingly, this person completely lacked muscle glycogen phosphorylase. Since that time several hundred others have been found with the same defect. Glycogen accumulates in muscle tissue in this disease, one of the several types of **glycogen storage disease**.[b] Severe exercise is damaging, but steady moderate exercise can be tolerated. Until the time of McArdle's discovery, it was assumed that glycogen was synthesized by reversal of the phosphorylase reaction. No hint of the UDP-glucose pathway had appeared, and it was, therefore, not obvious how glycogen could accumulate in the muscles of these patients. Leloir's discovery of UDP-glucose at about the same time provided the answer. Persons with McArdle syndrome are greatly benefitted by a high-protein diet, presumably because amino acids such as alanine and glutamine are converted efficiently to glucose and because branched-chain amino acids may serve as a direct source of muscle energy.[c,d]

Several other rare heritable diseases also lead to accumulation of glycogen because of some block in its breakdown through the glycolysis pathway. The enzyme deficiencies include those of muscle phosphofructokinase,[e] liver phosphorylase kinase, liver phosphorylase, and liver glucose-6-phosphatase. In the last case, glycogen accumulates because the liver stores cannot be released to the blood as free glucose.[b,f,g] This is a dangerous disease because blood glucose concentrations may fall too low at night. The prognosis improved greatly when methods were devised for providing the body with a continuous supply of glucose. The simplest treatment is ingestion of uncooked cornstarch which is digested slowly.[b,h] In one of the storage diseases the branching enzyme of glycogen synthesis is lacking, and glycogen is formed with unusually long outer branches. In another the debranching enzyme is lacking, and only the outer branches of glycogen can be removed readily.[i]

The most serious of the storage diseases involve none of the enzymes mentioned above. Pompe disease is a fatal generalized glycogen storage disease in which a lysosomal α-1,4-glucosidase is lacking. This observation suggested the existence of a new and essential pathway of degradation of glycogen to free glucose in the lysosomes. A few cases of glycogen synthase deficiency have been reported. Little or no glycogen is stored in muscle or liver, and patients must eat at regular intervals to prevent hypoglycemia. Severe diseases in which glycogen synthesis is impaired include deficiencies of the gluconeogenic enzymes pyruvate carboxylase and PEP carboxykinase.

The following tabulation includes deficiencies of glycogen metabolism, glycolysis, and gluconeogenesis.[a] Glycogen storage diseases are often designated as Types I–V and these terms are included.

Deficiency	Organ	Severity
Glycogen phosphorylase (Type V), McArdle disease	Muscle	Moderate, late onset
Glycogen phosphorylase	Liver	Very mild
Phosphorylase kinase	Liver	Very mild
Debranching enzyme (Type III)	Liver	Mild
Lysosomal α-glucosidase (Type II)		Lethal, infant and adult form
Phosphofructokinase	Muscle	Moderate, late onset
Phosphoglycerate mutase[j]	Muscle	Moderate
Pyruvate carboxylase		Lethal
PEP carboxykinase		Lethal
Fructose-1,6-bisphosphatase	Muscle	Severe
Glycogen synthase	Liver	Mild
Branching enzyme (Type IV)		Lethal, liver transplantation
Glucose-6-phosphatase (Type I)		Severe if untreated

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a glucose unit in an adjacent polysaccharide chain that lies parallel to the first, possibly in a double helix. Since amylose and amylopectin are intimately intermixed in the starch granules, it seems strange that the branching enzyme never transfers a branch to molecules of the straight-chain amylose. However, if the linear amylose chains are oriented in the opposite direction from the amylopectin chains, the nonreducing ends of the amylose molecules would be located toward the center of the starch granule. Growth could occur by an insertion mechanism at the reducing ends and the ends could move out continually with the amyloplast membrane as the granule grows.[123] Recent evidence from ^{14}C labeling indicates that both amylose and amylopectin too may grow by insertion at the reducing end of glucose units from ADP-glucose.[123a,b] Branching could occur to give the structure of Fig. 20-3. Starch synthesis in leaves occurs by day but at night the starch is degraded by amylases, α-glucosidases, and starch phosphorylase. Both the starch synthases and catabolic enzymes are present within the amyloplasts where they may be associated with regulatory proteins of the 14-3-3 class.[122a]

Digestion of dietary glycogen and starch in the human body begins with the salivary and pancreatic amylases, which cleave α-1,4 linkages at random. It continues with a **glucoamylase** found in the brush border membranes of the small intestine where it occurs as a complex with **maltase**.[74] Carbohydrases are discussed in Chapter 12, Section B.

2. Cellulose, Chitin, and Related Glycans

Cellulose synthases transfer glucosyl units from UDP-glucose, while chitin synthases utilize UDP-*N*-acetylglucosamine. Not only green plants but some fungi and a few bacteria form cellulose. The ameba *Dictyostelium discoideum* also coats its spores with cellulose.[124] Electron microscopic investigations suggest that both in bacteria[125] and in plants[126] multienzyme aggregates located at the plasma membrane synthesize many polymer chains side by side to generate hydrogen-bonded microfibrils which are extruded through the membrane. Both green plants and fungi also form important β-1,3-linked glycans.

The bacterial cellulose synthase from *Acetobacter xylinum* can be solubilized with detergents, and the resulting enzyme generates characteristic 1.7 nm cellulose fibrils (Fig. 20-4) from UDP-glucose.[125,127–129] These are similar, but not identical, to the fibrils of cellulose I produced by intact bacteria.[125,130] Each native fibril appears as a left-handed helix which may contain about nine parallel chains in a crystalline array. Three of these helices appear to coil together (Fig. 20-4) to form a larger 3.7-nm left-handed helical fibril. Similar fibrils are formed by plants. In both

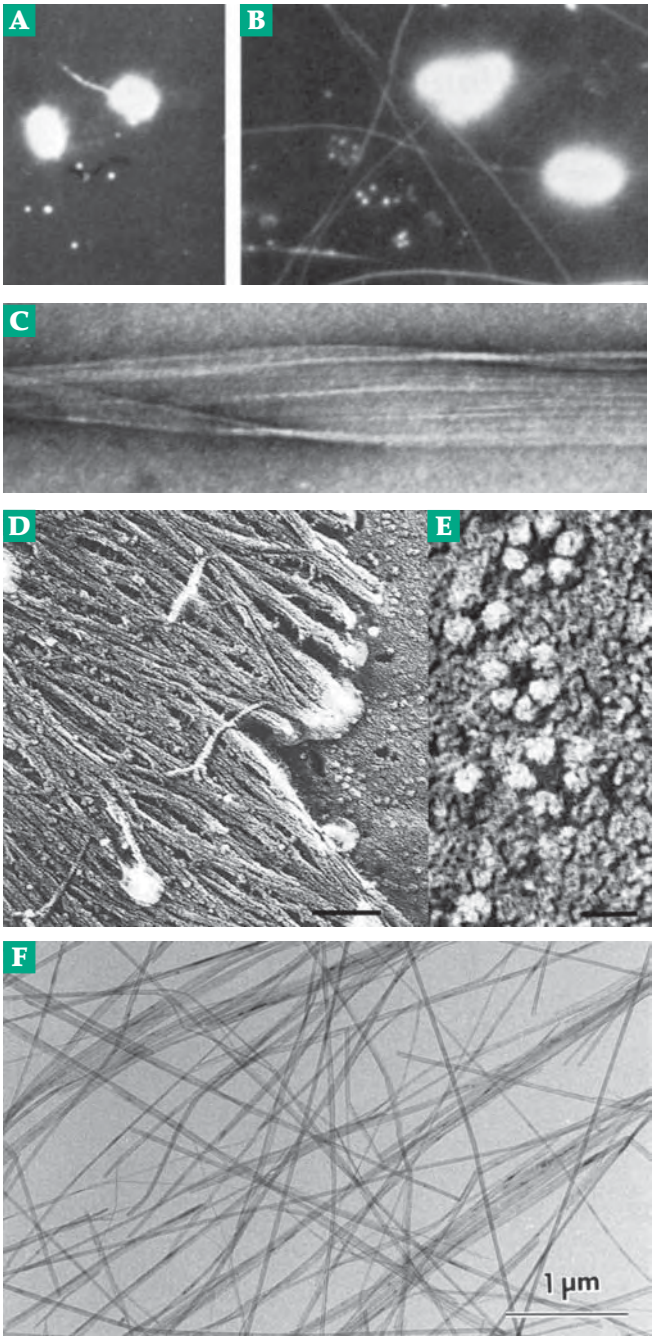


Figure 20-4 Cellulose microfibrils being formed by *Acetobacter xylinum*.[127] (A) Dark-field light micrograph after five minutes of cellulose production (x 1250). (B) After 15 minutes a pellicle of cellulose fibers is forming (x 2000) (C) Negatively stained cellulose ribbon. At the right the subdivision into microfibrils is visible. Courtesy of R. Malcolm Brown, Jr. (D) Cellulose microfibrils overlaying the plasma membrane in the secondary cell wall of a tracheary element of *Zinnia elegans*. Bar = 100 nm. (E) Rosettes in the plasma membrane underlying the cellulose-rich secondary cell wall thickening in *A. elegans*. Bar = 30 nm. (D) and (E) from Haigler and Blanton.[132] Courtesy of Candace H. Haigler. (F) Chitin microfibrils purified from protective tubes of the tube-worm *Lamellibrachia satsuma*.[137] Courtesy of Junji Sugiyama.

bacteria and plants the cellulose I fibrils that are formed are highly crystalline, contain parallel polysaccharide chains (Fig. 4-5), and have the tensile strength of steel. Electron micrographs show that the cell envelope of *A. xylinum* contains 5–80 pores, through which the cellulose is extruded, lying along the long axis of the cell.[129] The biosynthetic enzymes are probably bound to the plasma membrane. Similar, but more labile, cellulose synthases are present in green plants.[131] In *Arabidopsis* there are ten genes. The encoded cellulose synthases appear to be organized as rosettes on some cell surfaces (Fig. 20-4E).[131a-133a] The rosettes may be assembled to provide parallel synthesis of ~36 individual cellulose chains needed to form a fibril.[131a]

Because of the insolubility of cellulose fibrils it has been difficult to determine whether they grow from the reducing ends or the nonreducing ends of the chains. From silver staining of reducing ends and micro electron diffraction of cellulose fibrils attached to bacteria,[134] Koyama *et al.* concluded that the reducing ends are extruded from cells. New glucosyl rings would be added at the *nonreducing ends*, which remain attached noncovalently to the cells.[134] From amino acid sequence similarities it was also concluded that the same is true for *Arabidopsis*.[131,133] A single cellulose chain has a twofold screw axis, each residue being rotated 180° from the preceding residue (Fig. 4-5). It was postulated that two synthases act cooperatively to add cellobiose units. Another suggestion is that sitosterol β-glucoside acts in some fashion as a primer for cellulose synthesis in plants.[133b]

An insertion mechanism for synthesis of cellulose. Using ^{14}C "pulse and chase" labeling Han and Robyt found that new glucosyl units are added at the *reducing ends* of cellulose chains formed by cell membrane preparations from *A. xylinum*.[135] This conclusion is in accord with the generalization that extracellular polysaccharides made by bacteria usually grow from the reducing end by an insertion mechanism that depends upon a polyprenyl alcohol present in the cell membrane.[136] This lipid alcohol, often the C_{55}

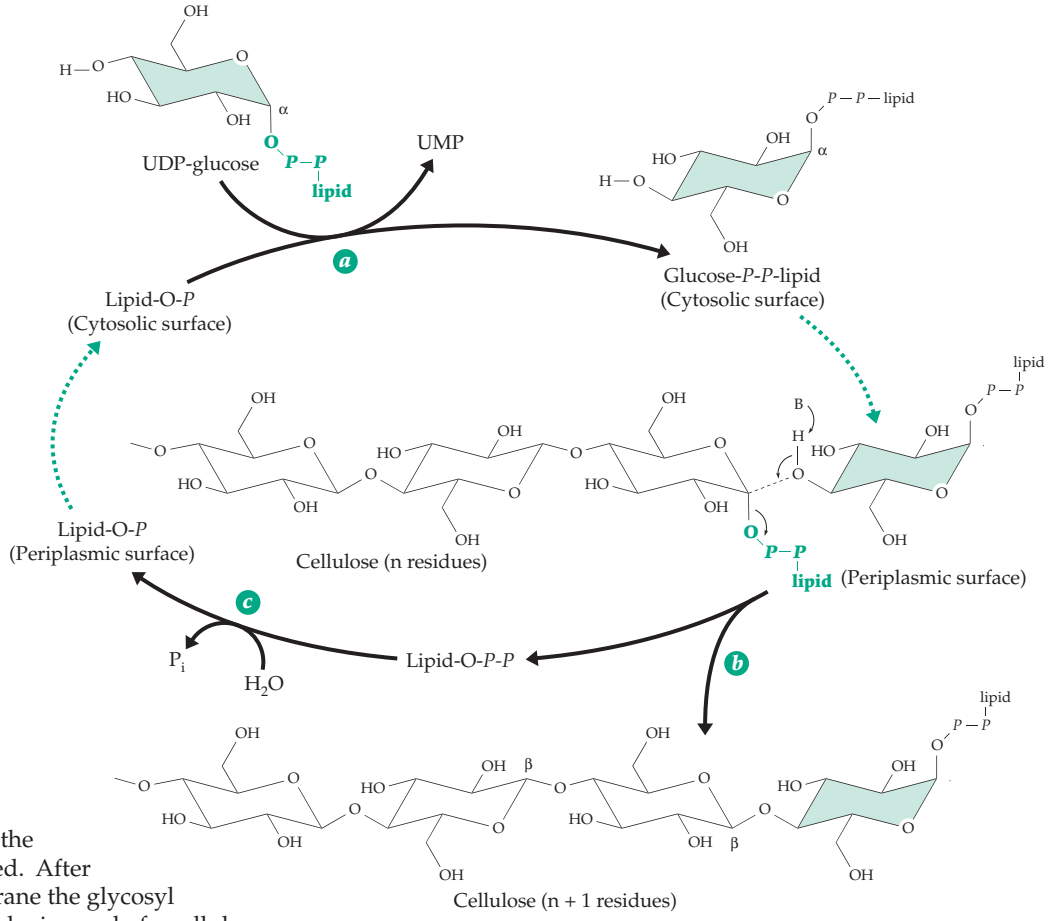
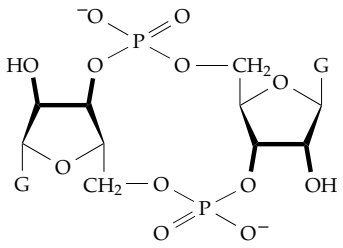


Figure 20-5 Proposed insertion mechanism for biosynthesis of cellulose. Three enzymatic steps are involved: a nucleophilic displacement reaction of a lipid phosphate on UDP-glucose yields a glucosyl diphosphate lipid in which the α-glycosyl linkage is retained. After passage through the membrane the glycosyl group is inserted into the reducing end of a cellulose chain, which is covalently attached by a pyrophosphate linkage to another lipid. The first lipid diphosphate is released and is hydrolyzed (step *c*) to the monophosphate, which crosses the membrane to complete the cycle. After Han and Robyt.[135] As throughout this book *P* represents the phospho group $-PO_3H$. The H may be replaced by groups which may contain oxygen atoms. This explains why an O is included in Lipid-O-*P* but no O is shown between the *P*'s in -O-*P*-*P*.

bactoprenol, reacts with UDP-glucose (or other glycosyl donor) to give a lipopyrophospho-glucose (step *a*, Fig. 20-5). The α linkage of the UDP-glucose is retained in this compound. The growing cellulose chain is attached at the reducing end by a similar linkage to a second lipid molecule. Then, in a displacement on the anomeric carbon of the first glucosyl residue of the cellulose chain, the new glucosyl unit is inserted with inversion of the α linkage to β. In step *c* the pyrophosphate linkage of the lipid diphosphate is hydrolyzed to regenerate the lipid monophosphate and to drive the reaction toward completion. Two of the steps in the cycle involve transport across the bacterial membrane. The first involves the lipid –O-*P*-*P*-glucose and the second the lipid monophosphate. This type of insertion mechanism is a common feature of polyprenol phosphate-dependent synthetic cycles for extracellular polysaccharides (Figs. 20-6, 20-9 and Eq. 20-20). However, further verification is needed for cellulose synthesis.

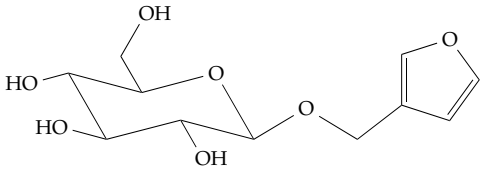
Regulation of cellulose synthesis in bacteria depends on allosteric activator of cellulose synthase, **cyclic diguanylate** (c-di-GMP), and a Ca^{2+}-activated phosphodiesterase that degrades the activator.[129,138–139a] Sucrose is the major transport form of glucose in plants. Synthesis of both cellulose and starch is reduced in mutant forms of maize deficient in sucrose synthase (Eq. 20-13). This synthase, acting in the reverse direction, forms UDP-Glc from sucrose.[140,141] The enzymatic degradation of cellulose is an important biological reaction, which is limited to certain bacteria, to fungi, and to organisms such as termites that obtain cellulases from symbiotic bacteria or by ingesting fungi.[142] These enzymes are discussed in Chapter 12, Section B,6. Genetic engineering methods now offer the prospect of designing efficient cellulose-digesting yeasts[143] that may be used to produce useful fermentation products from cellulose wastes.



Cyclic diguanylate

Callose and other β-1,3-linked glycans.

Attempts to produce cellulose from UDP-Glc using enzymes of isolated plasma membranes from higher plants have usually yielded the β-1,3-linked glucan (callose) instead. This is a characteristic polysaccharide of plant wounds which, as healing occurs, is degraded and replaced by cellulose.[140,144] Callose formation is induced by a specific activator β-furfuryl-β-glucoside, and callose synthase is virtually inactive unless both the activator and Ca^{2+} are present.[144]



β-Furfuryl-β-glucoside

Beta-1,3-linked glycans are major components of the complex layered cell wall of yeasts and other fungi. In the fission yeast *Saccharomyces pombe* ~55% of the cell wall carbohydrate consists of β-1,3-linked glucan with some β-1,4-linked branches, ~ 28% is α-1,3-linked glucan, ~6% is α-1,6-linked glucan, and ~0.5% is chitin. There are two carbohydrate layers, the outer one appearing amorphous. The inner layer contains interwoven fibrils of both α-1,3-linked and α-1,4-linked glucans and holds the shape of the cell. The β-1,3 glucan synthase is localized on the inner side of the cell membrane and is activated by GTP and a small subunit of the Rho family of G proteins.[145]

Plants synthesize 1,3-β-glucanases that hydrolyze the glycans of fungal cell walls. Synthesis is induced by wounding as a defense reaction (see Box 20-E). These glycanases also function in the removal of callose.[146]

Chitin. Like cellulose synthase, fungal chitin synthases are present in the plasma membrane and extrude microfibrils of chitin to the outside.[147–150] In the fungus *Mucor* the majority of the chitin synthesized later has its *N*-acetyl groups removed hydrolytically to form the deacetylated polymer **chitosan**.[151,152] Chitin is also a major component of insect exoskeletons. For this reason, chitin synthase is an appropriate target enzyme for design of synthetic insecticides.[153]

Chitin hydrolyzing enzymes are formed by fungi and in marine bacteria.[154] Chitinases are also present in plant vacuoles, where they participate in defense against fungi and other pathogens[155] (Box 20-E). More recently a chitinase has been identified in human activated macrophages.[156] Another unanticipated discovery was that a developmental gene designated *DG*42, from *Xenopus*, has a sequence similar to that of the *NodC* gene. The latter encodes a synthase for chitin oligosaccharides (Nod factors) that serve as nodulation factors in *Rhizobia* (Chapter 24). The enzyme is synthesized for only a short time during early embryonic development.[157] The significance of this discovery is not yet clear. Synthesis of both the bacterial Nod factors and chitin oligosaccharides in zebrafish embryos occurs by transfer of GlcNAc residues from UDP-GlcNAc at the *nonreducing ends* of the

chains.[158] Whether the same is true of chitin in fungi or arthropods remains uncertain.

Cell walls of plants. The thick walls of higher plant cells (Figs. 1-7, 4-14, and 20-4D) provide strength and rigidity to plants and, at the same time, allow rapid elongation during periods of growth.[159–163a] Northcote[164] likened the wall structure to glass fiber-reinforced plastic (fiber glass). Thus, the cell wall contains microfibrils of cellulose and other polysaccharides embedded in a matrix, also largely polysaccharide. The **primary cell wall** laid down in green plants during early stages of growth contains loosely interwoven cellulose fibrils ~10 nm in diameter and with an ~4 nm crystalline center. The cellulose in these fibrils has a degree of polymerization of 8000–12,000 glucose units. As the plant cell matures, a secondary cell wall is laid down on the inside of the primary wall. This contains many layers of closely packed microfibrils, alternate layers often being laid down at different angles to one another (Fig. 20-4D). The microfibrils in green plants are most often cellulose but may contain other polysaccharides as well. Some algae are rich in fibrils of xylan and mannan.

The materials present in the matrix phase vary with the growth period of the plant. During initial phases **pectin** (polygalacturonic acid derivatives) predominate but later xylans and a variety of other polysaccharides known as **crosslinking glycans** (or hemicelluloses) appear. Primary cell wall constituents of dicotyledons include **xyloglucans** (linear glucan chains with xylose, galactose, and fucose units in

BOX 20-E OLIGOSACCHARIDES IN DEFENSIVE AND OTHER RESPONSES OF PLANTS

Plants that are attacked by bacteria, fungi, or arthropods respond by synthesizing broad-spectrum antibiotics called **phytoalexins**,[a,b] by strengthening their cell walls with lignin and hydroxyproline-rich proteins called **extensins**,[c] and by making **protease inhibitors** and other proteins that help to block the chemical attack.[d] These plant responses seem to be initiated by the release from an invading organism of **elicitors**, which are often small oligosaccharide fragments, sometimes called **oligosaccharins**.[e] These include β-1,6-linked glucans that carry β-1,3-linked branches as well as chitin and chitosan oligomers, derived from fungal cell walls.[f] Other elicitors include galacturonic acid oligomers released from damaged plant cell walls,[g] metabolites such as arachidonic acid and glutathione,[h] and bacterial toxins.[i] Any of these may serve as signals to plants to take defensive measures.

Phytoalexins are often isoflavonoid derivatives (Box 21-E). Their synthesis, like that of lignin, occur via 4-coumarate (4-hydroxycinnamate, Fig. 25-8). The ligase which forms the thioester of 4-coumarate with coenzyme A is one of the **pathogenesis-related proteins** whose synthesis is induced.[j] A second induced enzyme is chalcone synthase, which condenses three acetyl units onto 4-coumaroyl-CoA as shown in Box 21-E. Its induction by elicitors acting on bean cells requires only five minutes.[h] Another rapidly induced gene is that of cinnamoyl alcohol dehydrogenase,[k] essential to lignin synthesis. Other proteins formed in response to infections include **chitinases** that are able to attack invading fungi[l,m] as well as the protease inhibitors. Their synthesis is induced via derivatives of **jasmonate**, a product of the octadecenoic acid pathway (Eq. 21-18).[a] As yet, little is known about the mechanism by which elicitors induce the defensive responses, but the presence of receptors, of phosphorylation, and of release of second messengers have been suggested.[d]

Lipooligosaccharides known as Nod factors (p. 1365) are another group of signaling molecules. These chitin-related *N*-acylated oligomers of *N*-acetylglucosamine (GlcNAc) do not defend against infection but invite infection of roots of legumes by appropriate species of *Rhizobia*[n–p] leading to formation of nitrogen-fixing root nodules.

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branches),[164a] other crosslinking glycans, and galacturonic acid-rich pectic materials.[163a] The xyloglycans, which comprise 20% of the cell wall in some plants, have a backbone of α-1,4-linked glucose units with numerous α-1,6-linked xylose rings, some of which carry attached L-arabinose, galactose, or fucose. The structures, which vary from species to species, are organized as repeating blocks with a continuous glucan backbone. Another crosslinking glycan is **glucuronoarabinoxylan**. The backbone is β-1,4-linked xylose. Less abundant glucomannans, galactomannans, and galactoglucomannans, with β-1,4-linked mannan backbone structures, are also present in most angiosperms.[163a]

Pectins form a porous gel on the inside surface of plant cell walls.[163a,164a] A major component is a **homogalacturonan**, which consists of α-1,4-linked galacturonic acid (GalA). A second is rhamnogalacturonan I, an alternating polymer of (2-L-Rhaα1 → 4GalAα →) units. The most interesting pectin component is **rhamnogalacturonan II**, one of the less abundant constuents of pectin. It is obtained by hydrolytic cleavage of pectin by a polygalacturonidase. Before such release it forms parts (hairy regions) of pectin molecules that are largely homogalacturonans (in smooth regions). A rhamnogalacturonan II segment consists of 11 different monomer units.[164b–f] Attached to the polygalacturonic acid backbone are four oligosaccharides, consisting of rhamnose, galactose and fucose as well as some unusual sugars (see structure in Box 20-E). This polysaccharide is apparently present in all higher plants and is unusually stable, accumulating, for example, in red wine.[164e] It contains two residues of the branched chain sugar **apiose**, one of which is a site of crosslinking by boron (Box 20-F). A borate diol ester linkage binds two molecules of the pectin together as a dimer, perhaps controlling the porosity of the pectin gel. All of the complex cell wall polysaccharides bind, probably through multiple hydrogen bonds, to the cellulose microfibrils (Fig. 4-14). The resulting structures are illustrated in drawings of Carpita and McCann,[163a] which are more current than is Fig. 4-14. The cellulose plus crosslinking glycans form one network in the cell wall. The pectic substances form a second independent network. Some covalent crosslinking occurs, but most interactions are noncovalent.[163a] The site of biosynthesis of pectins and hemicelluloses is probably Golgi vesicles which pass to the outside via exocytosis. However, the cellulose fibrils as well as the chitin in fungi are apparently extruded from the plasma membrane.

Although the principal cell wall components of plants are carbohydrates, proteins account for 5–10% of the mass.[165] Predominant among these are glycoprotein **extensins**. Like collagen, they are rich in 4-hydroxyproline which is glycosylated with arabinose oligosaccharides and galactose (p. 181). Other hydroxyproline-containing proteins with the characteristic sequence $(\text{hydroxyproline})_4$-Ser are also found, e.g., in soybean cell walls.[166] Some plant cell walls contain glycine-rich structural proteins. One in the petunia consists of 67% glycine residues.[167] During advanced stages of formation, as the walls harden into wood, large amounts of **lignins** are laid down in some plant cells. These chemically resistant phenylpropanoid polymers contain many crosslinked aromatic rings (Fig. 25-9).[163a]

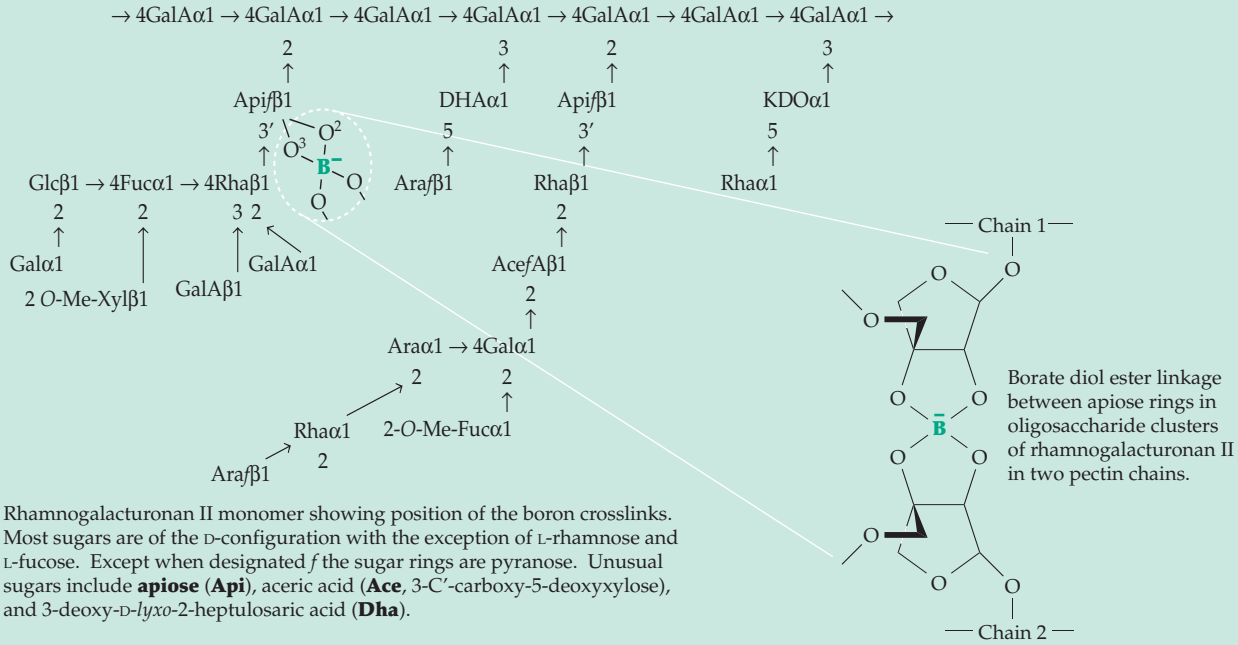
A remarkable aspect of primary plant cell walls is their ability to be elongated extremely rapidly during growth. While the driving force for cell expansion is thought to be the development of pressure within the cell, the manner in which the wall expands is closely regulated. After a certain point in development, elongation occurs in one direction only and under the influence of plant hormones. Most striking is the effect of the **gibberellins** (Eq. 22-5), which cause very rapid elongation. Elongation of plant cell walls may depend to some extent upon chemical cleavage and reforming of crosslinking polysaccharides. However, the cellulose fibrils probably remain intact and slide past each other.[161] A curious effect, which is mediated by proteins called **expansins**,[133a,168] is the ability of plant tissues to extend rapidly when incubated in a mildly acidic buffer of pH <5.5. Expansins are also involved in ripening of fruit. They may disrupt noncovalent bonding between cellulose fibrils and the hemicelluloses.[169,170] The β-expansins of grasses are allergens found in grass pollens.[133a,168] The borate diol ester linkages in the pectin may also facilitate expansion.

3. Patterns in Polysaccharide Structures

How can the many complex polysaccharides found in nature be synthesized? Are there genetically determined patterns? How are these controlled? The answer can be found in the *specificities* of the hundreds of known *glycosyltransferases*[171,172] and in the *patterns of expression of the genes* for transferases and other proteins. As a consequence, a great variety of structurally varied polysaccharide structures arise, especially on cell surfaces. The structures are not random but depend upon the assortment of glycosyltransferases available at the particular stage of development in a tissue. The numerous possibilities can account for much of the variation observed between species, between tissues, and also among individuals.

The simplest pattern is the growth of straight-chain homopolysaccharides such as amylose, cellulose, and chitin. The glycosyltransferases must recognize both the glycosyl donor, e.g., ADP-glucose, UDP-glucose, and also the correct end of the growing polymer, always adding the same monomer unit.

BOX 20-F WHAT DOES BORON DO?



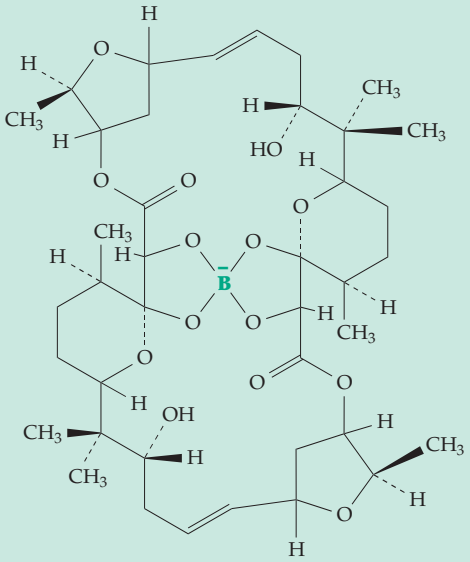
Rhamnogalacturonan II monomer showing position of the boron crosslinks. Most sugars are of the D-configuration with the exception of L-rhamnose and L-fucose. Except when designated *f* the sugar rings are pyranose. Unusual sugars include **apiose** (**Api**), aceric acid (**Ace**, 3-C′-carboxy-5-deoxyxylose), and 3-deoxy-D-*lyxo*-2-heptulosaric acid (**Dha**).

Borate diol ester linkage between apiose rings in oligosaccharide clusters of rhamnogalacturonan II in two pectin chains.

For 75 years or more it has been known that boron is essential for growth of green plants.[a,b] In its absence root tips fail to elongate normally, and synthesis of DNA and RNA is inhibited. Boron in the form of boric acid, $B(OH)_3$, is absorbed from soil. Although deficiency is rare it causes disintegration of tissues in such diseases as "heart rot" of beets and "drought spot" of apples. The biochemical role has been obscure, but is usually thought to involve formation of borate esters with sugar rings or other molecules with adjacent pairs of –OH groups (as in the accompanying structures). A regulatory role involving the plant hormones auxin, gibberelic acid, and cytokinin has also been suggested.

Diatoms also require boron, which is incorporated into the silicon-rich cell walls.[a] Some strains of *Streptomyces griseus* produce boron-containing macrolide antibiotics such as **aplasmomycin** (right).[c] Recently a function in plant cell walls has been identified (see also main text) as crosslinking of rhamnogalacturonan portions of pectin chains by borate diol ester linkages as illustrated.

It was long thought that boron was not required by human beings, but more recent studies suggest that we may need ~30 µg / day.[d] The possible functions are uncertain. Animals deprived of boron show effects on bone, kidney, and brain as well as a relationship to the metabolism of calcium, copper, and nitrogen. Nielson proposed a signaling function, perhaps via phosphoinositides, in animals.[b]



Aplasmomycin, a boron-containing antibiotic

[a] Salisbury, F. B., and Ross, C. W. (1992) *Plant Physiology*, 4th ed., Wadsworth, Belmont, California

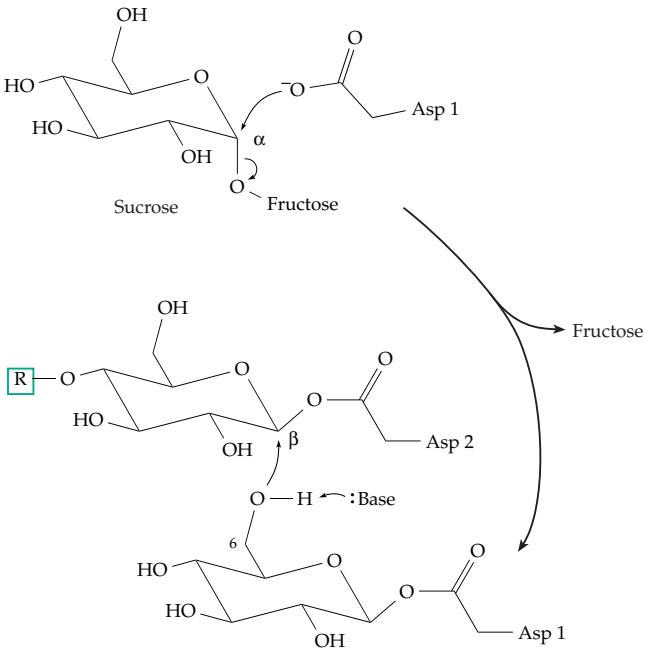
[b] Nielsen, F. H. (1991) *FASEB J.* **5**, 2661–2667

[c] Lee, J. J., Dewick, P. M., Gorst-Allman, C. P., Spreafico, F., Kowal, C., Chang, C.-J., McInnes, A. G., Walter, J. A., Keller, P. J., and Floss, H. G. (1987) *J. Am. Chem. Soc.* **109**, 5426–5432

[d] Nielsen, F. H. (1999) in *Modern Nutrition in Health and Disease*, 9th ed. (Shils, M. E., Olson, J. A., Shike, M., and Ross, A. C., eds), pp. 283–303, Williams & Wilkins, Baltimore, Maryland

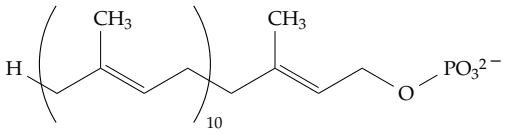
In contrast, hyaluronan and the polysaccharide chains of **glycosaminoglycans** (Fig. 4-11) have an alternating pattern. For a hyaluronan chain growing at the reducing end, one active site of hyaluronan synthase must be specific for UDP-GlcNAc and transfer the sugar unit only to the end of a glucuronic acid ring. A second active site must be specific for UDP-glucuronic acid but attach it only to the end of an acetylglucosamine unit.[172a,172b] There is still uncertainty about the direction of growth of hyaluronan.[173–175] Some hyaluronan synthases are lipid-dependent and their mechanism may resemble that proposed for cellulose synthesis (Fig. 20-5).

Dextrans. Some polysaccharides, such as the bacterial dextrans, are synthesized outside of cells by the action of secreted enzymes. An enzyme of this type, **dextran sucrase** of *Leuconostoc* and *Streptococcus*, adds glucosyl units at the *reducing* ends of the dextran chains (p. 174). Sucrose is the direct donor of the glucosyl groups, which are added by an insertion mechanism.[121,176–178] However, it is not dependent upon a membrane lipid as is that of Fig. 20-5. The glucosyl groups are transferred from sucrose to one of a pair of carboxylate groups of aspartate side chains in the active site.[179,180] If both carboxylates are glucosylated, a dextran chain can be initiated by insertion of one glucosyl group into the second (Eq. 20-19). The dextran grows alternating binding sites between the two carboxylates. Chain growth can be terminated by reaction with a sugar or oligosaccharide that fits into the active site and acts in place of the glucosyl group attached to Asp 1 as pictured in Eq. 20-19. The α-1,3-linked branches can be formed when a 3-OH group of a second dextran chain enters the catalytic site, serving as the glycosyl acceptor. See Robyt for a detailed discussion of synthesis of dextrans and related polysaccharides such as **alternan** and the α-1,3-linked **mutan** (p. 175).[121,176] Some bacteria form β-2,6-linked **fructans** by a similar mechanism, with glucose being released by displacement on C2 of sucrose.[181] Fructans are also formed in green plants, apparently from reaction of two molecules of sucrose with release of glucose to form the trisaccharide Fru*f*β2 → 1Fru*f*β2–1α-Glc*p*, which then transfers a fructosyl group to the growing chain.



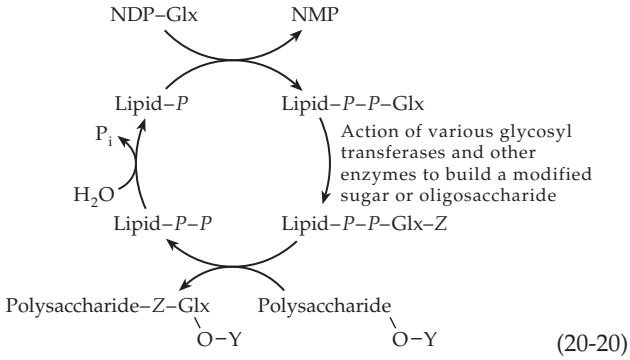
(20-19)

Lipid-dependent synthesis of polysaccharides. Insertion of monomer units at the base of a chain is a major mechanism of polymerization that is utilized for synthesis not only of polysaccharides but also of proteins (Chapter 29). For most carbohydrates the synthesis is dependent upon a polyprenyl lipid alcohol. In bacteria this is often the 55-carbon **undecaprenol** or **bactoprenol**,[136] which functions as a phosphate ester:



Undecaprenol phosphate

It serves as a membrane anchor for the growing polysaccharide. We have already discussed one example in the hypothetical cellulose synthase mechanism of Fig. 20-5. For some polysaccharides the mechanism is better established. The synthetic cycles all resemble that of Fig. 20-5 and can be generalized as in Eq. 20-20. Here NDP-Glx is a suitable nucleotide disphosphate derivative of sugar Glx, and Z-Glx is the repeating unit of the polysaccharide formed by the action of glycosyltransferases and other enzymes.



(20-20)

For example, the biosynthesis of alginate involves GDP-mannuronic acid (GDP-ManA) as NDP-Glx, bactoprenol as the lipid, and a glycosyltransferase that inserts a second mannuronate residue (as Z).

An additional transferase that uses acetyl-CoA as a substrate sometimes acetylates one mannuronate unit. The disaccharide units are then inserted into the growing chain. An additional modification, which occurs after polymerization, is random C5 epimerization of unacetylated D-mannuronate residues to L-guluronate.[136,182] Formation of alginate is of medical interest because infections by alginate-forming bacteria are a major cause of respiratory problems in cystic fibrosis.[182]

Sometimes an oligosaccharide assembled on the polyprenol phosphate represents a substantial block in assembly of a repeating polymer. For example, the xanthan gum (p. 179) produced by the bacterium *Xanthomonas campestris* is formed by several successive glycosyl transfers to bactoprenol-*P*-*P*-Glc. A second glucose is transferred onto the first from UDP-Glc, forming a pair of glucosyl groups in β-1,4 linkage. Mannose is then transferred from GDP-Man and joined in an α-1,3 linkage to the first GDP-Man to form a branch point. A glucuronate residue is then transferred from UDP-GlcA and another mannose from GDP-Man. The last mannose is modified by reaction with PEP to form a ketal (Eq. 4-9). The product of this assembly is the following lipid-bound oligosaccharide block.

$$\begin{array}{lll} & \text{Acetyl} & \text{Glc}\beta 1 \\ \text{Pyruvate} & \downarrow & \downarrow \\ \| & 6 & 4 \end{array}$$
$$\text{Man}\beta 1 \rightarrow 4\,\text{GlcA}\beta 1 \rightarrow 2\,\text{Man}\alpha 1 \rightarrow 3\,\text{Glc}\beta 1 \rightarrow \text{O-}P\text{-}P\text{-lipid}$$

This is inserted into the growing polysaccharide using the free 4-OH on the second glucose to link the units in a cellulose type chain. The twelve separate genes needed for synthesis of xanthan gum are contained in a 16-kb segment of the *X. campestris* genome.[136] Lipid-bound intermediates are also involved in synthesis of peptidoglycans (Fig. 20-9) and in the assembly of bacterial O-antigens (Fig. 8-30). Both of these also yield "block polymers."

D. Proteoglycans and Glycoproteins

The glycoproteins contain oligosaccharides attached to the protein either through *O*-glycosidic linkages with hydroxyl groups of side chains of serine, threonine, hydroxyproline, or hydroxylysine (*O*-linked) or via glycosylaminyl linkages to asparagine side chains (*N*-linked). The "core proteins" of the proteoglycans carry long polysaccharide chains, which are usually *O*-linked and are usually described as glycosaminoglycans.

1. Glycosaminoglycans

Synthesis of the alternating polysaccharide hyaluronan has been discussed in Section C,3 and may occur by an insertion mechanism. However, other glycosaminoglycans (sulfate esters of **chondroitin**, **dermatan**, **keratan**, **heparan**, and **heparin**) grow at their nonreducing ends.[183,184] Their synthesis is usually initiated by the hydroxyl group of serine or threonine side chains at special locations within several secreted proteins.[185] These proteins are synthesized in the rough ER and then move to the Golgi. Addition of the first sugar ring begins in the ER with transfer of single xylosyl residues to the initiating –OH groups.[186–190b] This reaction is catalyzed by the first of a group of special glycosyltransferases of high specificity that form the special terminal units (Chapter 4, Section D,1), that anchor the alternating polysaccharide represented here as $(X–Y)_n$:

$$(X \rightarrow Y)_n \rightarrow 4\,\text{GlcA}\beta 1 \rightarrow 3\,\text{Gal}\beta 1 \rightarrow 3\,\text{Gal}\beta 1 \rightarrow 4\,\text{Xyl}\beta 1 \rightarrow \text{O-Ser / Thr}$$

After transfer of the xylosyl residue from UDP-xylose to the –OH group in the protein,[190a] a second enzyme with proper specificity transfers a galactosyl group from UDP-galactose, joining it in β-1,4 linkage. A third enzyme transfers another galactosyl group onto the first one in β-1,3 linkage. A fourth enzyme, with a specificity different from that used in creating the main chain, then transfers a glucuronosyl group from UDP-glucuronic acid onto the chain terminus to complete the terminal unit.[190c] Then two more enzymes transfer the alternating units in sequence to form the repeating polymer with lengths of up to 100 or more monosaccharide residues. The sequence $(X–Y)_n$ in the preceding formula is:

$$(\rightarrow 4\,\text{GlcA}\beta 1 \rightarrow 3\,\text{GalNAc}\beta 1 \rightarrow)_n$$
for chondroitin[172a,191,192]

$$(\rightarrow 4\,\text{GlcA}\beta 1 \rightarrow 4\,\text{GlcNAc}\alpha 1 \rightarrow)_n$$
for heparan sulfate and heparin

$$(\rightarrow 3\,\text{Gal}\beta 1 \rightarrow 4\,\text{GlcNAc}\beta 1 \rightarrow)_n$$
for dermatan sulfate

Subsequent modifications of the polymers involve extensive formation of *O*-sulfate esters,[190a,193–197] *N*-deacetylation and *N*-sulfation,[198,199] and epimerization at C5.[10] In some tissues almost all GluA is epimerized.[200] The modifications are especially extensive in dermatan, heparan sulfates, and heparin (see also p. 177).[196,201–203b] The modifications are not random and follow a defined order. *N*-Deacetylation must precede *N*-sulfation, and *O*-sulfation is initiated only after *N*-sulfation of the entire chain is complete. The modifications occur within the Golgi (see Fig. 20-7) but not all

of the glycosyltransferases, PAPS (3'-phosphoadenosine 5'-phosphosulfate)-dependent sulfotransferases, and epimerases are present within a single compartment. Nevertheless, an entire glycosaminoglycan chain can be synthesized within 1–3 min.[189]

The completed polymers are modified uniformly. There are clusters of sulfo groups with unusual structures in chondroitin from squid and shark cartilages[204,205] and fucosylated chondroitin from echinoderms.[206] Similar modifications are present less extensively in vertebrates. One of the best known modifications forms the unique pentasaccharide sequence shown in Fig. 4-13, which is essential to the anticoagulant activity of heparin. This sequence has been synthesized in the laboratory as have related longer heparin chains. A sequence about 17 residues in length containing an improved synthetic version of the unique pentasaccharide binds tightly to both thrombin and antithrombin (Chapter 12, Section C,9).[207,208] This opens the door to the development of improved substitutes for the medically important heparin. Heparan sulfate chains are found on proteoglycans throughout the body, but the highly modified heparin does not circulate in the blood. It is largely sequestered in cytoplasmic granules within mast cells and is released as needed.[208a] Heparin binds to many different proteins (p. 177). Among them is the glycoprotein selenoprotein P (p. 824), which may impart antioxidant properties to the extracellular matrix.[208b]

Although glycosaminoglycans are most often attached to *O*-linked terminal units, chondroitin sulfate chains can also be synthesized with *N*-linked oligosaccharides attached to various glycoproteins serving as initiators.[209] At least one form of keratan sulfate, found in the cornea, is linked to its initiator protein via GlcNAc-Man to *N*-linked oligosaccharides of the type present in many glycoproteins (Section D).

At least 25 different proteins that are secreted into the extracellular spaces of the mammalian body carry glycosaminoglycan chains.[183,210,211] Most of these proteins can be described as (1) **small leucine-rich proteoglycans** with 36- to 42-kDa protein cores and (2) **large modular proteoglycans** whose protein cores have molecular masses of 40 to 500 kDa.[210] The most studied of the second group is **aggrecan**, a major component of cartilage. This 220-kDa protein carries ~100 chondroitin chains, each averaging about 100 monosaccharide residues and ~100 negative charges from the carboxylate and sulfate groups. Aggrecan has three highly conserved globular domains near the N and C termini.[212–213a] The G1 domain near the N terminus is a **lectin** (p. 186), which, together with a small link protein that is structurally similar to the G1 domain, binds to a decasaccharide unit of hyaluronan. One hyaluronan molecule of 500- to1000-kDa mass (~2500–5000 residues) may bind 100 aggrecan and link molecules to form an ~200,000-kDa particle such as that shown in Fig. 4-16. These enormous highly negatively charged molecules, together with associated counterions, draw in water and preserve osmotic balance. It is these molecules that keep our joints mobile and which deteriorate by proteolytic degradation in the common **osteoarthritis**.[214,215] The keratan sulfate content of cartilage varies with age, and the level in serum and in synovial fluid is increased in osteoarthritis.[215] Keratan sulfate is also found in the cornea and the brain. Its content is dramatically decreased in the cerebral cortex of patients with Alzheimer disease.[216]

Other modular core proteins[210] include **versican** of blood vessels and skin,[210,213a,217,217a] **neurocam** and **brevican** of brain, **perlecan** of basement membranes,[218] **agrin** of neuromuscular junctions, and **testican** of seminal fluid. However, several of these have a broader distribution than is indicated in the foregoing description. The sizes vary from 44 kDa for testican to greater than 400 kDa for perlecan. The numbers of glycosaminoglycan chains are smaller than for aggrecan, varying from 1 to 30. Another of the chondroitin sulfate-bearing core protein is **appican**, a protein found in brain and one of the splicing variants of the amyloid precursor protein that gives rise to amyloid deposits in Alzheimer disease (Chapter 30).[217a,b]

The core proteins of the leucine-rich proteoglycans have characteristic horseshoe shapes and are constructed from ~28-residue repeats, each containing a β turn and an α helix. The three-dimensional structures are doubtless similar to that of a ribonuclease inhibitor of known structure which contains 15 tandem repeats.[219,220] A major function of these proteoglycans seems to be to interact with collagen fibrils, which have distinct proteoglyan-binding sites,[221] and also with fibronectin.[222] The small leucine-rich proteoglycans have names such as **biglycan**, **decorin**,[222a] **fibromodulin**, **lumican**, **keratoglycan**, **chondroadherin**, **osteoglycin**, and **osteoadherin**.[219,223–223c] The distribution varies with the tissue and the stage of development. For example, biglycan may function in early bone formation; decorin, which has a high affinity for type I collagen, disappears from bone tissue as mineralization takes place. Osteoadherin is found in mature osteoblasts.[223] **Phosphocan**, another brain proteoglycan, has an unusually high content (about one residue per mole) of L-isoaspartyl residues (see Box 12-A).[224]

Proteoglycans bind to a variety of different proteins and polysaccharides. For example, the large extracellular matrix protein **tenascin**, which is important to adhesion, cell migration, and proliferation, binds to chondroitin sulfate proteoglycans such as neurocan.[225] **Syndecan**, a transmembrane proteoglycan, carries both chondroitin and heparan sulfate chains, enabling it to interact with a variety of proteins that mediate cell–matrix adhesion.[185]

The ability of dissociated cells of sponges to aggregate with cells only of a like type (p. 29) depends upon large extracellular proteoglycans. That of *Microciona prolifera* appears to be an aggregate of about three hundred 35-kDa core protein molecules with equal masses of attached carbohydrate. This **aggregation factor** has a total mass of ~2 x 10^4 kDa.[226,227] It apparently interacts specifically, in the presence of Ca^{2+} ions, with a 210-kDa cell matrix protein to hold cells of the same species together.[227]

2. *O*-Linked Oligosaccharides

A variety of different oligosaccharides are attached to hydroxyl groups on appropriate residues of serine, threonine, hydroxylysine, and hydroxyproline in many different proteins (Chapter 4). Such oligosaccharides are present on external cell surfaces, on secreted proteins, and on some proteins of the cytosol and the nucleus.[228–231b] The rules that determine which –OH groups are to become glycosylated are not yet clear.[232] Glycosylation occurs in the ER, and, just as during synthesis of the long carbohydrate chains of proteoglycans, the sugar rings are added directly to an –OH group, either of the protein or of the growing oligosaccharide. The first glycosyl group transferred is most often **GalNAc** for external and secreted proteins[233] but more often **GlcNAc** for cytosolic and nuclear proteins.[228,231,233a–c] Glycosylation of protein –OH groups can occur on either the luminal or cytosolic faces of the ER membranes.[234] The external *O*-linked glycoproteins often have large clusters of oligosaccharides attached to –OH groups of serine or threonine, but cytosolic proteins may carry only a small number of small oligosaccharides.

Of great importance are the **blood group determinants** which are discussed in Box 4-C. The ABO determinants are found at the nonreducing ends of *O*-linked oligosaccharides. Conserved Ser/Thr sites in the epidermal growth factor domains (Table 7-3) of various proteins carry ***O*-linked fucose**.[235]

The secreted **mucins** are unique in having clusters of large numbers of oligosaccharides linked by ***N*-acetylgalactosamine** to serine or threonine of the polypeptide.[236] The following core structures predominate.[237] These may be lengthened or further branched by the particular variety of glycosyltransferases present in a tissue and by their specificities.[233] The human genome contains at least nine mucin genes.[238] The large apomucins contain central domains with tandem repeats rich in Ser, Thr, Gly, and Ala and flanked at the ends by cysteine-rich domains.[239] For example, porcine submaxillary mucins are encoded by a gene with at least three alleles that encode 90, 125, or 133 repeats. The polypeptide may contain as many as 13,288 residues. N-terminal cysteine-rich regions are involved in dimer formation.[240]

core 1 Galβ1 → 3 GalNAc – Ser/Thr

core 2 Galβ1 → 3 GalNAc – Ser/Thr – Ser/Thr
6
↑
GalNAcβ1

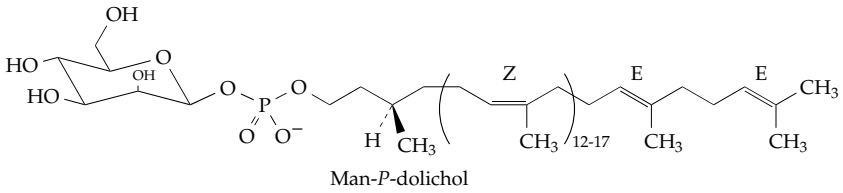
core 3 GlcNAcβ1 → 3 GalNAc – Ser/Thr

core 4 Galβ1 → 4 GlcNAcβ1 → 6 GalNAc– Ser/Thr
3
↑
GlcNAcβ1

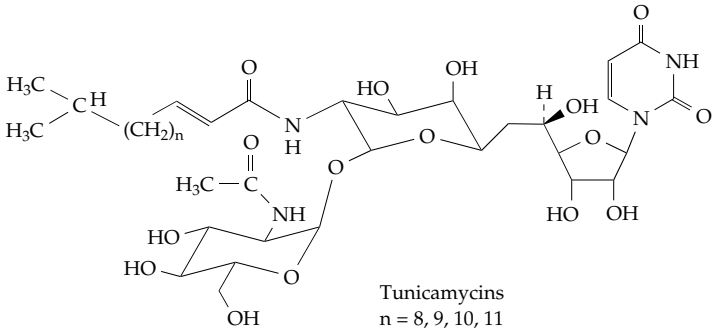
3. Assembly of *N*-Linked Oligosaccharides on Glycosyl Carrier Lipids

In eukaryotes the biosynthesis of the *N*-linked oligosaccharides of glycoproteins depends upon the polyprenyl alcohols known as **dolichols**, which are present in membranes of the endoplasmic reticulum. They contain 16–20 prenyl units, of which the one bearing the OH group is completely saturated as a result of the action of an NADPH-dependent reductase on the unsaturated precursor.[241] The predominant dolichol in mammalian cells contains 19 prenyl units. The structure of its mannosyl phosphate ester, one of the intermediates in the oligosaccharide synthesis, is illustrated below. The fully extended 95-carbon dolichol has a length of almost 10 nm, four times greater than that of oleic acid and twice the thickness of the nonpolar membrane bilayer core. The need for this great length is not clear nor is it clear why the first prenyl unit must be saturated for good acceptor activity.

The assembly of the oligosaccharides that will become linked to Asn residues in proteins occurs on the phosphate head of dolichol-*P*. The process begins on the cytoplasmic face of the membrane and within the lumen of the rough or smooth ER and continues within cisternae of the Golgi apparatus.[234,242–245] The initial transfer of GlcNAc-*P* to dolichol-*P* (Fig. 20-6, step *a*) appears to occur on the cytoplasmic face of the ER and is specifically inhibited by **tunicamycin**.[246,247] As the first "committed reaction" of *N*-glycosylation, it is regulated by a variety of hormonal and other factors.[248,249] The reaction takes place cotranslationally as the still unfolded peptide chain leaves the ribosome.[242] The oligosaccharide, still attached to the dolichol, continues to grow on the cytosolic surface of the ER membrane by transfer of GlcNAc and five residues of mannose from their sugar nucleotide forms (Fig. 20-6, steps *b* and *c*).[249a] The intermediate Dol-*P*-*P*-$GlcNAc_2Man_5$ crosses the membrane bilayer (Fig. 20-6, step *d*), after which mannosyl and glucosyl units are added (steps *e* and *f*). These sugars are carried across the membrane while attached to dolichol.



Man-*P*-dolichol



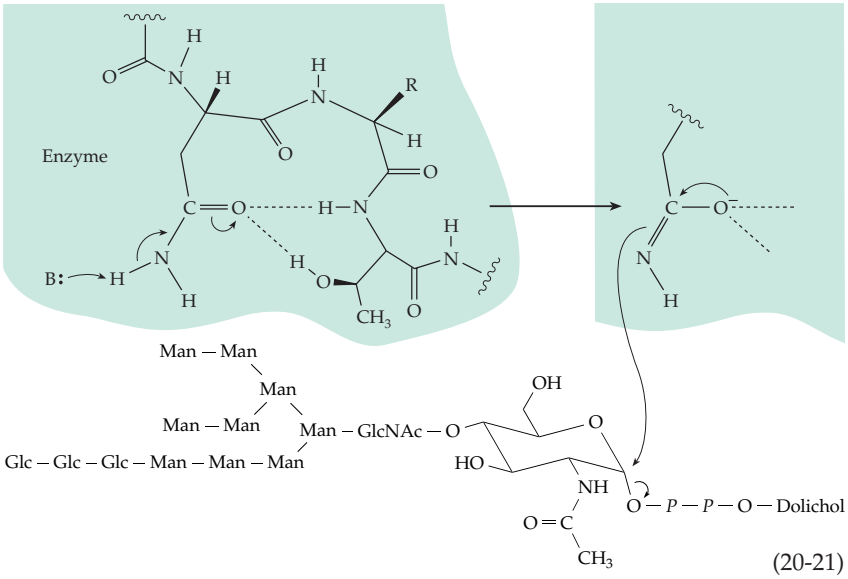
Tunicamycins
n = 8, 9, 10, 11

The completed 14-residue branched oligosaccharide $Glc_3Man_9GlcNAc_2$, with the structure indicated in Fig. 20-6, is then transferred to a suitable asparagine side chain (step *g*). This may be on a newly synthesized protein or on a still-growing polypeptide chain that is being extruded through the membrane into the lumenal space of the rough ER (Eq. 20-21; Fig. 20-6). The glycosylation site is often at the sequence Asn-X-Ser(Thr), which is likely to be present at a beta bend in the folded protein. Bends of the type illustrated in Eq. 20-21 and stabilized by the asparagine side chain are apparently favored.[250] In such a bend the –OH of the serine or threonine helps to polarize the amide group of the Asn side chain, perhaps enolizing it and generating a nucleophilic center that can participate in a displacement reaction[250,251] as indicated in Eq. 20-21. The **oligosaccharyltransferase** that catalyzes the reaction is a multisubunit protein. As many as eight different subunits have been reported for the enzyme in yeast. Genes for at least five of these are essential.[250–252b] One subunit serves to recognize suitable glycosylation sites.

Trimming of glycoprotein oligosaccharides. After transfer to glycoproteins the newly synthesized oligosaccharides undergo trimming, the hydrolytic removal of some of the sugar units, followed by addition of new sugar units to create the finished glycoproteins. The initial glycosylation process ensures that the glycoproteins remain in the lumen of the ER or within vesicles or cisternae separated from the cytoplasm. The subsequent processing appears to allow the cell to **sort** the proteins. Some remain attached to membranes and take up residence within ER, Golgi, or plasma membrane. Others are passed outward into transfer vesicles, Golgi, and secretion vesicles. A third group enter **lysosomes**. A series of specific inhibitors of trimming reactions, some of whose structures are shown in Fig. 20-7, has provided important insights.[253–255] Use of these inhibitors, together with immunochemical methods and study of yeast mutants,[250,252,256] is enabling us to learn many details of glycoprotein biosynthesis.

Whereas the formation of dolichol-linked oligosaccharides occurs in an identical manner in virtually all eukaryotic cells, trimming is highly variable as is the addition of new monosaccharide units.[257–257b] The major pathway for mammalian glycoproteins is shown in Fig. 20-7. Specific hydrolases in the ER remove all of the glucosyl units and one to three mannosyl units.[258] Removal of additional mannosyl residues occurs in the cis Golgi, to give the pentasaccharide core $Man_3GlcNAc_2$ which is common to all of the complex N-linked oligosaccharides. However, partial trimming without additional glycosylation produces some "high mannose" oligosaccharides.[258a] Removal of glucose may be necessary to permit some glycoproteins to leave the ER.

Sulfate groups and in some cases fatty acyl groups[259] may also be added. The exact composition of the oligosaccharides may depend upon the condition of the cell and may be altered in response to external influences.[257] Oligosaccharides attached to proteins that remain in the ER membranes may undergo



(20-21)

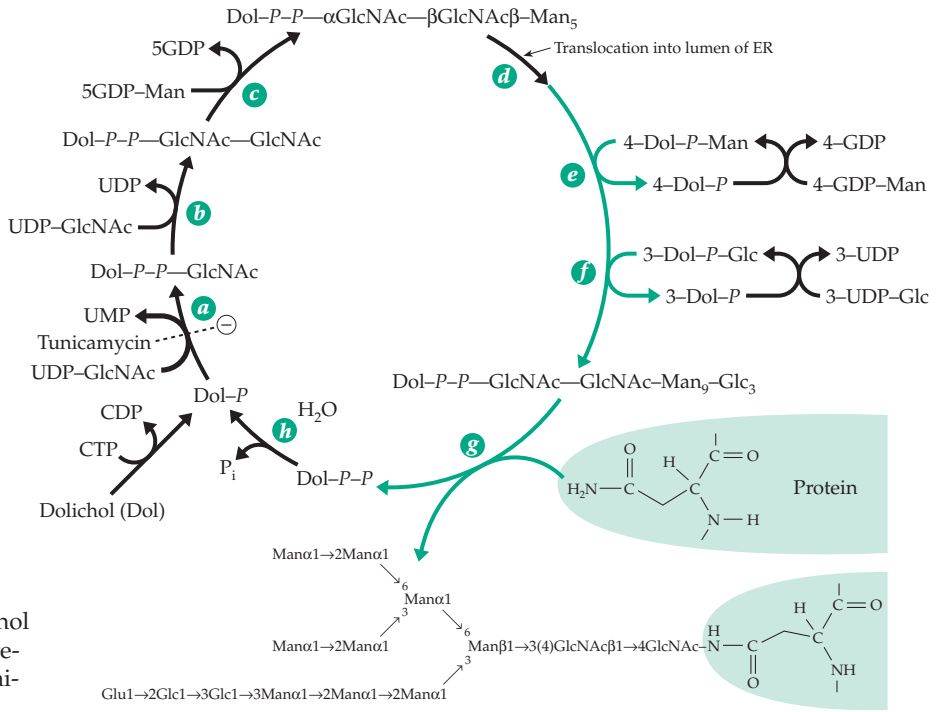


Figure 20-6 Biosynthesis of the dolichol diphosphate-linked oligosaccharide precursor to glycoproteins. The site of inhibition by tunicamycin is indicated.

very little trimming. However, the three glucosyl residues are usually removed by the glucosidases present in the rough ER. Some plant glycoproteins of the high-mannose type undergo no further processing.

Extensions and terminal elaborations. Even before trimming is completed, addition of new residues begins within the medial cisternae.[260] In mammalian Golgi, *N*-acetylglucosamine is added first. Galactose, sialic acid, and often fucose are then transferred from their activated forms to create such elaborate oligosaccharides as that shown in Fig. 4-17. As many as 500 glycosyltransferases, having different specificities for glycosyl donor and glucosyl acceptor, may be involved.[245] Extensions of the basic oligosaccharide structure often contain polylactosamine chains, branches with fucosyl residues, and sulfate groups.[245,261] More than 14 sialyltransferases place sialic acid residues, often in terminal positions, on these cell surface oligosaccharides.[262,263]

The cell wall of the yeast *Saccharomyces* is rich in **mannoproteins** that contain 50–90% mannose.[264] The ~250-residue mannan chains consist of an α-1,6-linked backbone with mono-, di-, and tri-mannosyl branches. These are attached to the same core structure as that of mammalian oligosaccharides. All of the core structures are formed in a similar way.[258,265] The mannoproteins may serve as a "filler" to occupy spaces in a cell wall constructed from β-1,3- and β-1,6-linked glycans and chitin. All of the four components, including the mannoproteins, are covalently linked together.[266] As was emphasized in Chapter 4 (pp. 186–188) glycoproteins serve many needs in biological recognition. The N-linked oligosaccharides play a major role in both animals and plants.[266a–c] Use of mass spectrometry, new automated methods of oligosaccharide synthesis,[266d] and development of new synthetic inhibitors[266c] are all contributing to current studies of what is commonly called "glycobiology."

The perplexing Golgi apparatus. First observed by Camillo Golgi[267,268] in 1898, the stacked membranes, now referred to simply as Golgi, remain somewhat mysterious.[268–271] There are at least three functionally distinct sets of Golgi cisternae, the **cis** (nearest the nucleus), **medial**, and **trans**. An additional series of tubules referred to as the **trans Golgi network** lies between the Golgi and the cell surface and may be the site at which lysosomal enzymes are sorted from proteins to be secreted.[260,272] Immunochemical staining directed toward specific glycosidases and glycosyl transferases suggested that the trimming reactions of glycoproteins start in the ER and continue as the proteins pass outward successively from one compartment of the Golgi to the next (Fig. 20-8). This has been the conventional view since the 1970s. The movement of the glycoproteins between compartments is thought to take place in small vesicles using a rather elaborate system of specialized proteins. Some of these coat the vesicles[273,274] while others target the vesicles to specific locations, e.g., the lysosomes[275] or the plasma membranes where they may be secreted.[273,277] A host of regulatory G-proteins assist these complex processes and drive them via hydolysis of GTP.[271,278]

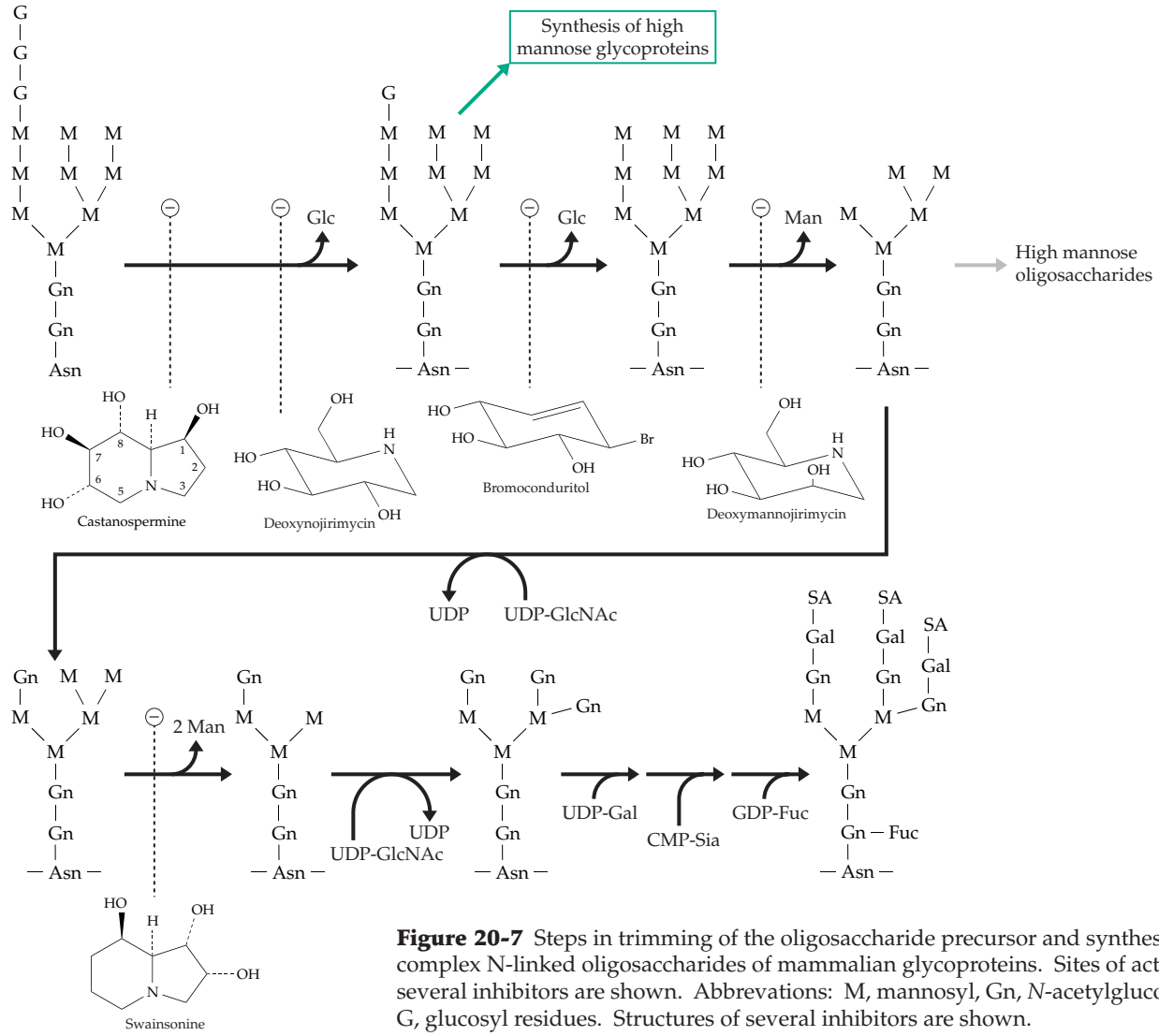


Figure 20-7 Steps in trimming of the oligosaccharide precursor and synthesis of complex N-linked oligosaccharides of mammalian glycoproteins. Sites of action of several inhibitors are shown. Abbrevations: M, mannosyl, Gn, *N*-acetylglucosaminyl, G, glucosyl residues. Structures of several inhibitors are shown.

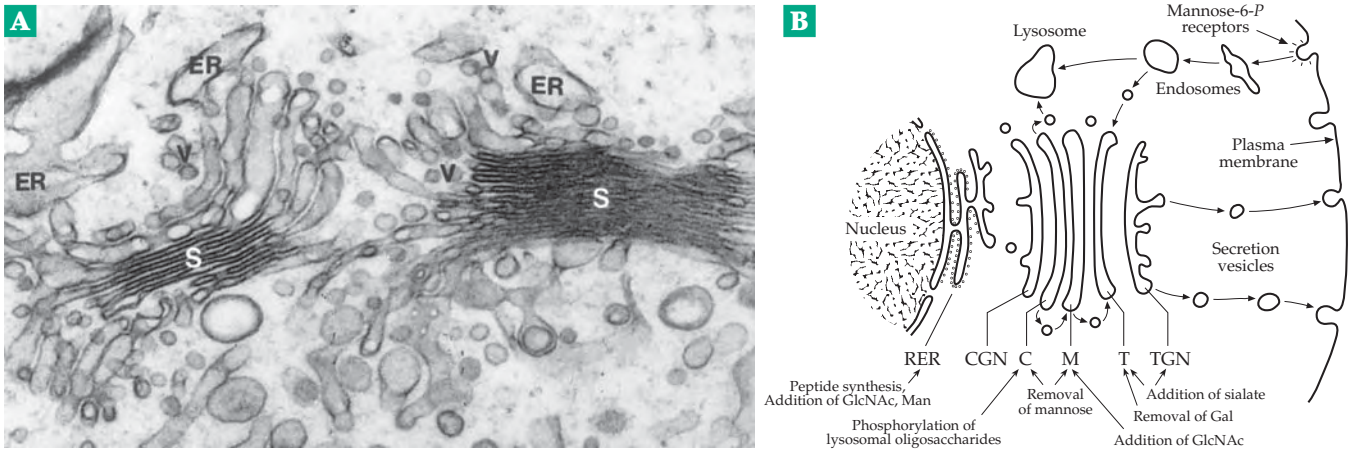
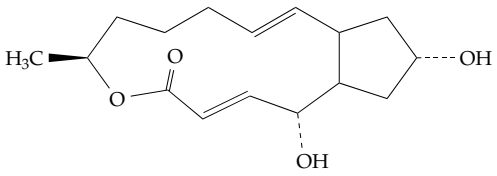


Figure 20-8 (A) Electron micrograph showing a transverse section through part of the Golgi apparatus of an early spermatid. Cisternae of the ER, Golgi stacks (S), and vesicles (V) can be seen. Curved arrows point to associated tubules. Magnification X45,000.[276] Courtesy of Y. Clermont. (B) Scheme showing functions of endoplasmic reticulum, transfer vesicles, Golgi apparatus, and secretion vesicles in the metabolism of glycoproteins.

While most proteins synthesized in the ER follow the exocytotic pathway through the Golgi, some are retained in the ER and some that pass on through the Golgi are returned to the ER.[279] In fact, such **retrograde transport** can carry some proteins taken up by endocytosis through the plasma membrane and through the Golgi to the ER where they undergo *N*-glycosylation. Retrograde transport is essential for recycling of plasma membrane proteins and lipids. The forward flow of glycoproteins and membrane components from the ER to the Golgi can be blocked by the fungal macrocyclic lactone **brefeldin A**. In cells treated with this drug, which inactivates a small



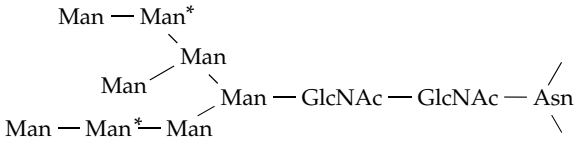
Brefeldin A

CTP-binding protein,[280] the Golgi apparatus is almost completely resorbed into the ER by retrograde transport. Proteins remaining in the ER undergo increased *O*-glycosylation as well as unusual types of *N*-glycosylation.

Although the conventional view of flow through the Golgi is generally accepted, it is difficult to distinguish it from an alternative explanation: *The Golgi compartments may move outward continuously while retrograde transport occurs via the observed vesicles*.[268,272] Some evidence for this **cisternal maturation model** has been known for many years but was widely regarded as reflecting unusual exceptions to the conventional model. In fact, both views could be partially correct; vesicular transport may function in both directions.[280a] High-resolution tomographic images are also altering our view of the Golgi.[280b]

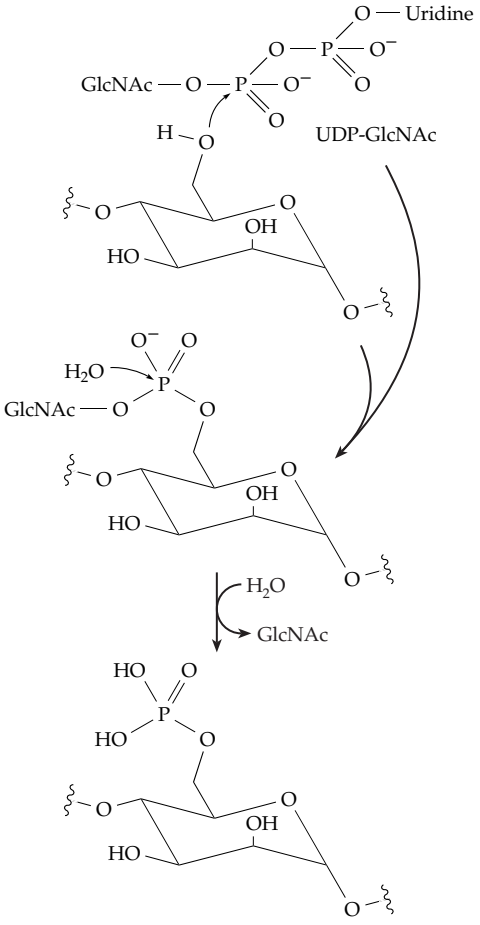
The proteins of Golgi membranes are largely integral membrane proteins and peripheral proteins associated with the cytosolic face. Some of the integral membrane proteins are the oligosaccharide-modifying enzymes, which protrude into the Golgi lumen.[280c,280d] Many other proteins participate in transport,[280d–f] docking, membrane fusion,[280g,h] and acidification of Golgi compartments.[280i] Many of the first studies of vesicular transport were conducted with synaptic vesicles and are considered in Chapter 30 (see Fig. 30-20). Other aspects of membrane fusion and transport are discussed in Chapter 8. A group of specialized Golgi proteins, the **golgins**, are also present. They are designated golgin-84, -95, -160, -245, and -376 (giantin or macrogolgin) and were identified initially as human **autoantigens** (Chapter 31), appearing in the blood of persons with autoimmune disorders such as Sjögren's syndrome.[281,282] Another protein of molecular mass ~130 kDa, and which appears to be specific to the trans-Golgi network, has been found in human serum of patients with renal vasculitis.[283]

Lysosomal enzymes. Various **sorting signals** are encoded within proteins. These include the previously mentioned C-terminal KDEL (mammals) or HDEL (yeast) amino acid sequence, which serves as a retrieval signal for return of proteins from the Golgi to the ER (p. 521).[279,284] Other sorting signals are provided by the varied structures of the oligosaccharides attached to glycoproteins. These sugar clusters convey important biological information, which is "decoded" in the animal body by interaction with various **lectins** that serve as receptors.[285] This often leads to endocytosis of the glycoprotein. An example is provided by the more than 50 proteins that are destined to become lysosomal enzymes and which undergo phosphorylation of 6-OH groups of the mannosyl residue marked by asterisks on the following structure. This is an *N*-linked oligosaccharide that has been partially trimmed. The phosphorylation is accomplished in



two steps by enzymes present in the cis Golgi compartment (Eq. 20-22) An ***N*-acetylglucosaminyl-phosphotransferase** transfers phospho-GlcNAc units from UDP-GlcNAc onto the 6-OH groups of mannosyl residues. These must be recognized by the phosphotransferase as appropriate.[286,287] Then a hydrolase cleaves off GlcNAc.

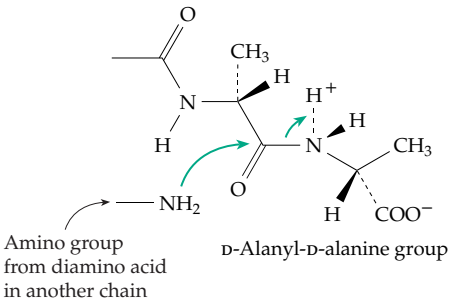
The proteins carrying the mannose 6-phosphate groups bind to one of two different **mannose 6-*P* receptors** present in the Golgi membranes and are subsequently transported in clathrin-coated vesicles to endosomes where the low pH causes the proteins to dissociate from the receptors, which may be recycled.[288–290a] The hydrolytic enzymes are repackaged in lysosomes. The same mannose 6-*P* receptors also appear on the external surface of the plasma membrane allowing many types of cells to take up lysosomal enzymes that have "escaped" from the cell. These proteins, too, are transported to the lysosomes. The mannose 6-*P* receptors have a dual function, for they also remove insulinlike growth factor from the circulation, carrying it to the lysosomes for degradation.[287,290] Most Man 6-*P* groups are removed from proteins once they reach the lysosomes but this may not always be true.[291] Not all lysosomal proteins are recruited by the mannose 6-*P* receptors. Some lysosomal membrane proteins are sorted by other mechanisms.[292]



The hepatic asialoglycoprotein (Gal) receptor. A variety of proteins are taken out of circulation in the blood by the hepatocytes of the liver. Serum glycoproteins bearing sialic acid at the ends of their oligosaccharides (see Fig. 20-7) have relatively long lives, but if the sialic acid is removed by hydrolysis, the exposed galactosyl residues are recognized by the multisubunit **asialoglycoprotein receptor**.[293–295] The bound proteins are then internalized rapidly via the coated pit pathway and are degraded in the lysosomes. Other receptors, including those that recognize transferrin, low-density lipoprotein, α_2 macroglobulin, and T lymphocyte antigens, also depend upon interaction with oligosaccharides.[296]

E. Biosynthesis of Bacterial Cell Walls

The outer surfaces of bacteria are rich in specialized polysaccharides. These are often synthesized while attached to lipid membrane anchors as indicated in a general way in Eq. 20-20.[136,296a] One of the specific biosynthetic cycles (Fig. 20-9) that depends upon undecaprenol phosphate is the formation of the **peptidoglycan** (murein) layer (Fig. 8-29) of both gram-negative and gram-positive bacterial cell walls. Synthesis begins with attachment of L-alanine to the OH of the lactyl group of UDP-*N*-acetylmuramic acid in a typical ATP-requiring process (Fig. 20-9, step *a*).[297] Next D-glutamic acid, *meso*-diaminopimelate (Fig. 8-29), or L-lysine, and D-alanyl-D-alanine are joined in sequence, each in another ATP-requiring step.[298–301d] The entire unit assembled in this way is transferred to undecaprenol phosphate with creation of a pyrophosphate linkage (step *e*). An *N*-acetylglucosamine unit is added by action of another transferase (step *f*), and in an ATP-requiring process ammonia is sometimes added to cap the free α-carboxyl group of the D-glutamyl residue (step *g*). In *Staphylococcus aureus* and related gram-positive bacteria five glycyl units are also added, each from a molecule of glycyl-tRNA (green arrows in Fig. 20-9). The completely assembled repeating unit, together with the connecting peptide chain needed in the crosslinking reaction, is transferred onto the growing chain (step *h*). As in formation of dextrans, growth is by insertion of the repeating unit at the reducing end of the chain. The polyprenyl diphosphate is released, and the cycle is completed by the action of a pyrophosphatase (step *i*). This step is blocked by **bacitracin**, an antibiotic which forms an unreactive complex with the polyprenyl diphosphate carrier. Completion of the peptidoglycan requires crosslinking. This is accomplished by displacement of the terminal D-alanine of the pentapeptide by attack by the $-NH_2$ group of the diaminopimelate or lysine or other diamino acid (see also Fig. 8-29).[301e]



Because the peptidoglycan layer must resist swelling of the bacteria in media of low osmolarity, it must be strong and must enclose the entire bacterium. At the same time the bacterium must be able to grow in size and also to divide. For these reasons bacteria must continuously not only synthesize peptidoglycan but also degrade it.[302,303] The latter is accomplished by hydrolytic cleavage using cell wall enzymes to the ***N*-acetylglucosamine-anhydro-*N*-acetylmuramate-tripeptide** (GlcNAc-1,6-anhydro-MurNAc-L-Ala-D-Glu-A_2pm) fragment.[304,305] A hydrolase cuts the peptide bridge.[305a] This process is probably essential to formation of new growing points for expansion of the murein layer. Most of the peptide fragments that are released in the periplasm are transported back into the cytosol.

The anhydroMurNAc is removed, and new UDP-MurNAc and D-Ala-D-Ala units are added salvaging the tripeptide unit. The repaired UDP-MurNA-pentapeptide can then reenter the biosynthetic pathway (Fig. 20-9).

The O-antigens and lipid A. A cluster of sugar units of specific structure makes up the repeating unit of the "O-antigen" of *Salmonella*. The many structural variations in this surface polysaccharide account for the over two thousand serotypes of *Salmonella* (p. 180).[121,306] As is illustrated in Fig. 8-30, the O-antigen is a repeating block polymer that is attached to a complex lipopolysaccharide "core" and a hydrophobic membrane anchor known as lipid A (Figs. 8-28 and 8-30).[307–308a] Lipid A and the attached core and O-antigen are synthesized inside the bacterial cell by enzymes found in the cytoplasmic membrane.[309] The complete lipopolysaccharide units are then translocated from the inner membrane to the outer membrane of the bacteria. The synthesis of the O-antigen is understood best. Consider the following group E3 antigen, where Abe is abequose (Fig. 4-15) and Rha is rhamnose:

$$\begin{array}{l}\text{Abe}\alpha 1\\ \quad\downarrow\\ \quad 4\\ (\rightarrow 6\,\text{Gal}\beta 1\rightarrow 6\,\text{Man}1\rightarrow 4\,\text{Rha}1\rightarrow)_n\end{array}$$

Assembly of this repeating unit begins with the transfer of a *phosphogalactosyl* unit from UDP-Gal to the phospho group of the lipid carrier undecaprenol phosphate. The basic reaction cycle is much like that in Fig. 20-9 for assembly of a peptidoglycan.

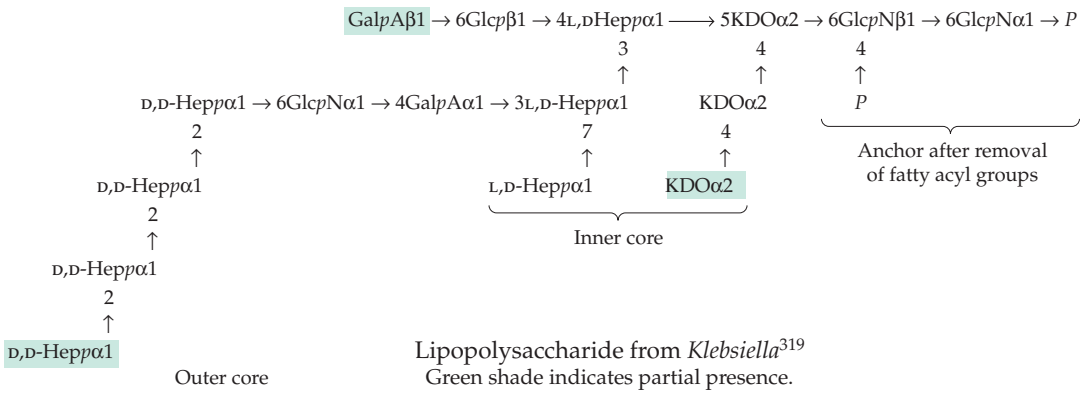
The oligosaccharide repeating unit of the O-antigen is constructed by the consecutive transferring action of three more transferases. For the antigen shown above, one enzyme transfers a rhamnosyl unit, another a mannosyl unit, and another an abequosyl unit from the appropriate sugar nucleotides. Then the entire growing O-antigen chain, which is attached to a second molecule of undecaprenol diphosphate, is transferred onto the end of the newly assembled oligosaccharide unit. In effect, the newly formed oligosaccharide is inserted at the reducing end of the growing chain just as in Fig. 20-9. Elongation continues by the transfer of the entire chain onto yet another tetrasaccharide unit. As each oligosaccharide unit is added, an undecaprenol diphosphate unit is released and a phosphatase cleaves off the terminal phospho group to regenerate the original undecaprenol phosphate carrier. When the O-antigen is long enough, it is attached to the rest of the lipopolysaccharide.

The lipid A anchor is also based on a carbohydrate skeleton. Its assembly in *E. coli*,[307] which requires nine enzymes, is depicted in Fig. 20-10. *N*-Acetylglucosamine 6-*P* is acylated at the 3-position[310] and after deacetylation[311] at the 2-position. As shown in this figure, acylation is accomplished by transfer of hydroxymyristoyl groups from acyl carrier protein (ACP). Two molecules of the resulting UDP-2,3-diacyl-GlcN are then joined via the reactions shown to give the acylated disaccharide precursor to lipid A. Stepwise transfer of KDO, L-*glycero*-D-*manno*-heptose, and other monosaccharide units from the appropriate sugar nucleotides and further acylation follows (Fig. 20-10). The assembled O-antigen chain is transferred from undecaprenol diphosphate onto the lipopolysaccharide core. This apparently occurs on the periplasmic surface of the plasma membrane. If so, the core lipid domain must be flipped across the plasma membrane before the O-antigen chain is attached.[312] Less is known about the transport of the completed lipopolysaccharide across the periplasmic space and into the outer membrane.

The core structures of the lipopolysaccharides vary from one species to another or even from one strain of bacteria to another. All three domains (lipid A, core, and O-antigen) contribute to the antigenic properties of the bacterial surface[313] and to the virulence of the organism.[313,314] Nitrogen-fixing strains of *Rhizobium* require their own peculiar lipopolysaccharides for successful symbiosis with a host plant.[315] However, there are some features common to most lipopolysaccharides. Two to three residues of KDO are usually attached to the acylated diglucosamine anchor, and these are often followed by 3 – 4 heptose rings.[316–318]

The structure of the inner core regions of a typical lipopolysaccharide from *E. coli* is indicated in Fig. 8-30. The complete structure of the lipopolysaccharide from a strain of *Klebsiella* is shown at the top of the next page.[319] Here L, D-Hep*p* is D-*manno*-heptopyranose and D, D-Hep*p* is D-*glycero*-D-*manno*-heptose. As in this case, the outer core often contains several different hexoses. The lipopolysaccharide of *Neisseria meningititis* has sialic acid at the outer end.[320] However, the major virulence factor for this organism, which is a leading cause of bacterial meningitis in young children, is a capsule of poly(ribosyl)ribitol phosphate that surrounds the cell.[321] *Haemophilus influenzae*, a common cause of ear infections and meningitis in children, has no *O*-antigen but a more highly branched core oligosaccharide than is present in *E. coli*.[321a] *Legionella* has its own variations.[321b]

Gram-positive bacteria. Although their outer coatings are extremely varied, all gram-positive bacteria have a peptidoglycan similar to that of gram-negative bacteria but often containing the intercalated pentaglycine bridge indicated in Fig. 8-29. However, the peptidoglycan of gram-positive bacteria is 20–50 nm thick, as much as ten times thicker than that of *E. coli*. Furthermore, the peptidoglycan is intertwined with the anionic polymers known as teichoic acids and



Lipopolysaccharide from *Klebsiella*[319]
Green shade indicates partial presence.

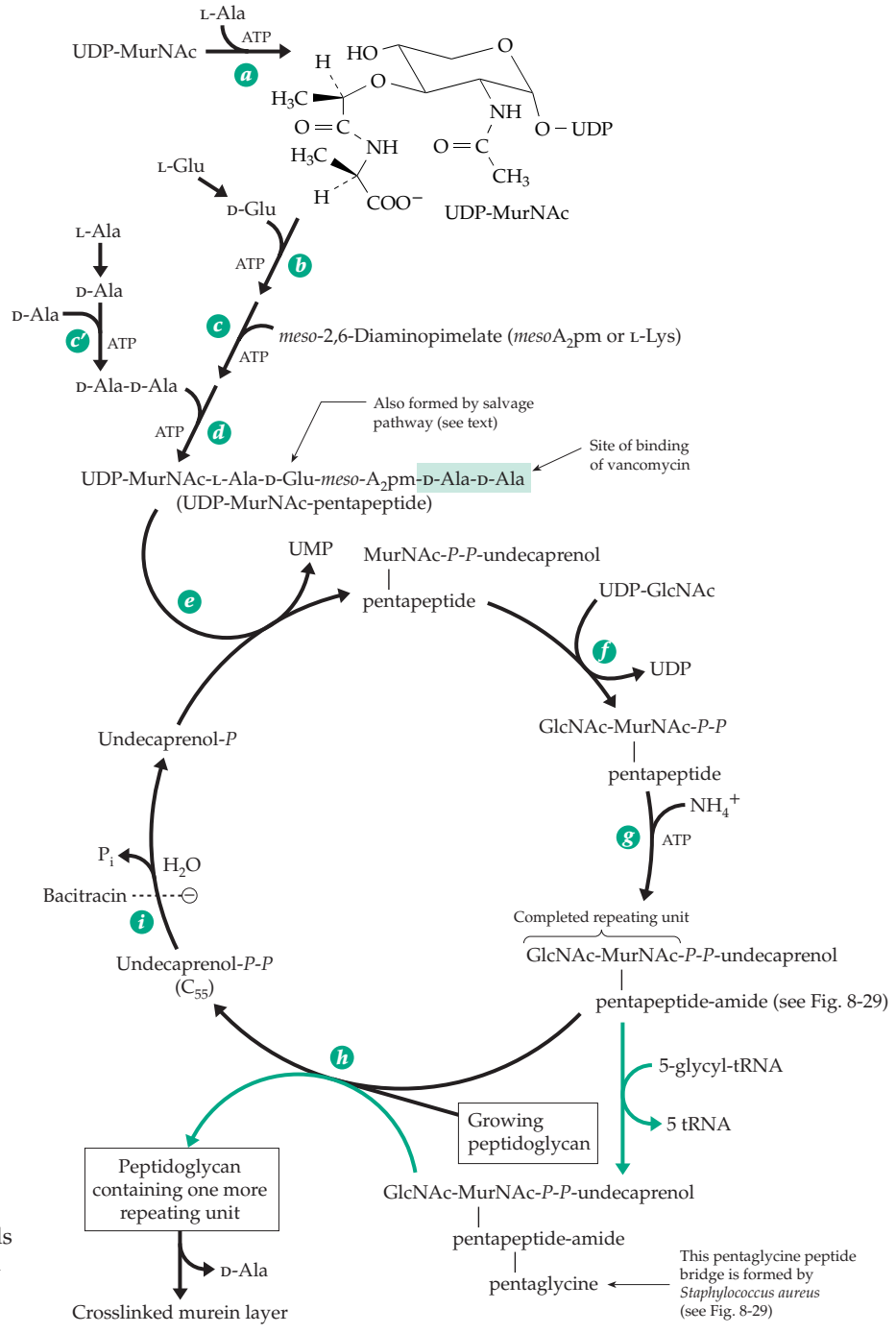


Figure 20-9 Biosynthesis of bacterial peptidoglycans. See Fig. 8-29 for details of the peptidoglycan structures. Green arrows show alternative route used by gram-positive bacteria.

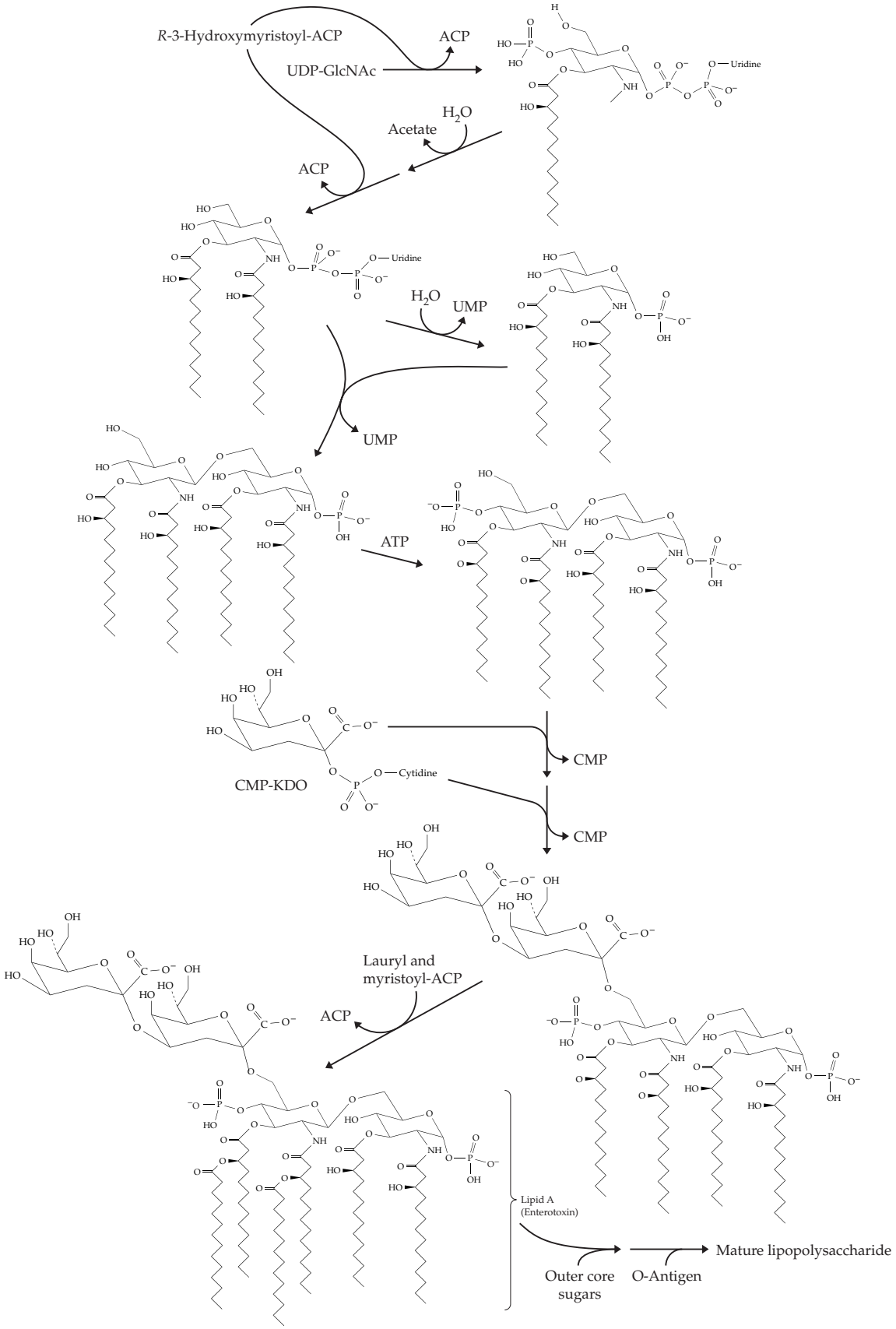
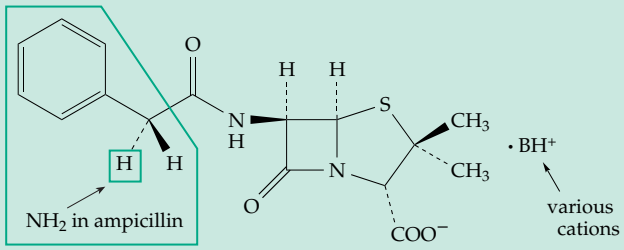


Figure 20-10 Proposed biosynthetic route for synthesis of lipid A and the mature lipopolysaccharide of the *E. coli* cell wall. After C. R. H. Raetz *et al.*[307]

BOX 20-G PENICILLINS AND RELATED ANTIBIOTICS



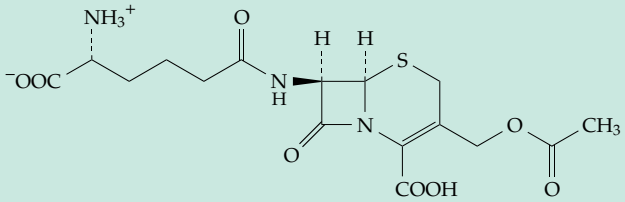
Penicillin G
(Benzylpenicillin)

Many organisms produce chemical substances that are toxic to other organisms. Some plants secrete from their roots or leaves compounds that block the growth of other plants. More familiar to us are the medicinal antibiotics produced by fungi and bacteria. The growth inhibition of one kind of organism upon another was well known in the last century, e.g., as reported by Tyndall[a,b] in 1876. The beginning of modern interest in the phenomenon is usually attributed to Alexander Fleming, who, in 1928, noticed the inhibition of growth of staphylococci by *Penicillium notatum*. His observation led directly to the isolation of penicillin, which was first used on a human patient in 1930. The early history as well as the subsequent purification, characterization, synthesis, and development as the first major antibiotic has been recorded in numerous books and articles.[c–i] During the same time period, Rene Dubos isolated the peptide antibiotics **gramicidin** and **tyrocidine**.[j] A few years later **actinomycin** (Box 28-A) and **streptomycin** (Box 20-B) were isolated from soil actinomycetes (streptomyces) by Waksman, who coined the name **antibiotic** for these compounds. Streptomycin was effective against tuberculosis, a finding that helped to stimulate an intensive search for additional antibacterial substances. Since that time, new antibiotics have been discovered at the rate of more than 50 a year. More than 100 are in commercial production.

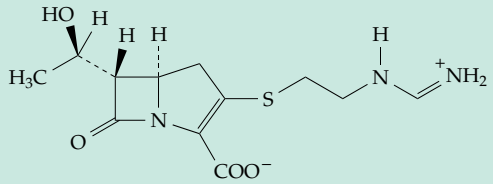
Major classes of antibiotics include more than 200 peptides such as the gramicidins, bacitracin, tyrocidines and valinomycin (Fig. 8-22)[k]; more than 150 **penicillins, cephalosporins,** and related compounds; **tetracyclines** (Fig. 21-10); the **macrolides**, large ring lactones such as the **erythromycins** (Fig. 21-11); and the **polyene** antibiotics (Fig. 21-10).

Penicillin was the first antibiotic to find practical use in medicine. Commercial production began in the early 1940s and benzylpenicillin (penicillin G), one of several natural penicillins that differ in the R group boxed in the structure above, became one of the most important of all drugs. Most effective against gram-positive bacteria, at higher concentrations it also attacks gram-negative bacteria including *E. coli*. The widely used semisynthetic penicillin **ampicillin** (R = D-α-aminobenzyl) attacks both gram-negative and gram-positive organisms. It shares with penicillin extremely low toxicity but some danger of allergic reactions. Other semisynthetic penicillins are resistant to β-lactamases, enzymes produced by penicillin-resistant bacteria which cleave the four-membered β-lactam ring of natural penicillins and inactivate them.

Closely related to penicillin is the antibiotic **cephalosporin C**. It contains a D-α-aminoadipoyl side chain, which can be replaced to form various semisynthetic cephalosporins. **Carbapenems** have similar structures but with CH_2 replacing S and often a different chirality in the lactam ring.



Cephalosporin C



Imipenem, a carbapenem antibiotic of last resort

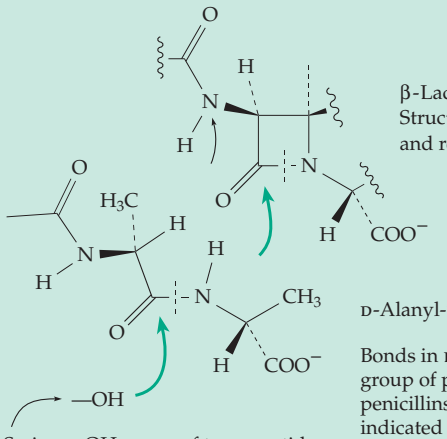
These and other related β-lactams are medically important antibacterial drugs whose numbers are increasing as a result of new isolations, synthetic modifications, and utilization of purified biosynthetic enzymes.[c,l,m]

How do antibiotics act? Some, like penicillin, block specific enzymes. Peptide antibiotics often form complexes with metal ions (Fig. 8-22) and disrupt the control of ion permeability in bacterial membranes. Polyene antibiotics interfere with proton and ion transport in fungal membranes. Tetracyclines and many other antibiotics interfere directly with protein synthesis (Box 29-B). Others intercalate into DNA molecules (Fig. 5-23; Box 28-A). There is no single mode of action. The search for suitable antibiotics for human use consists in finding compounds highly toxic to infective organisms but with low toxicity to human cells.

Penicillin kills only growing bacteria by preventing proper crosslinking of the peptidoglycan

BOX 20-G (continued)

layer of their cell walls. An amino group from a diamino acid in one peptide chain of the peptidoglycan displaces a D-alanine group in a transpeptidation (acyltransferase) reaction. The transpeptidase is also a hydrolase, a DD-carboxypeptidase. Penicillins are structural analogs of D-alanyl-D-alanine and bind to the active site of the transpeptidase.[l,n–p] The β-lactam ring of penicillins is unstable, making penicillins powerful acylating agents. The transpeptidase apparently acts by a double displacement mechanism, and the initial attack of a nucleophilic serine hydroxyl group of the enzyme on penicillin bound at the active site leads to formation of an inactivated, penicillinoylated enzyme.[q,r] More than one protein in a bacterium is derivatized by penicillin.[s] Therefore, more than one site of action may be involved in the killing of bacterial cells.



Several classes of **β-lactamases**, often encoded in transmissible plasmids, have spread worldwide rapidly among bacteria, seriously decreasing the effectivenss of penicillins and other β-lactam antibiotics.[t–y] Most β-lactamases (classes A and C) contain an active site serine and are thought to have evolved from the DD transpeptidases, but the B type[y] has a catalytic Zn^{2+}. The latter, as well as a recently discovered type A enzyme,[z] hydrolyze imipenem, currently one of the antibiotics of last resort used to treat infections by penicillin-resistant bacteria. Some β-lactam antibiotics are also powerful inhibitors of β-lactamases.[u,aa,bb] These antibiotics may also have uses in inhibition of serine proteases[cc,dd] such as elastase. Some antibiotic-resistant staphylococci produce an extra penicillin-binding protein that protects them from beta lactams.[ee] Because of antibiotic resistance the isolation of antibiotics from mixed populations of microbes from soil, swamps, and lakes continues. Renewed efforts are being made to find new targets for antibacterial drugs and to synthesize new compounds in what will evidently be a never-ending battle. We also need better antibiotics against fungi and protozoa.

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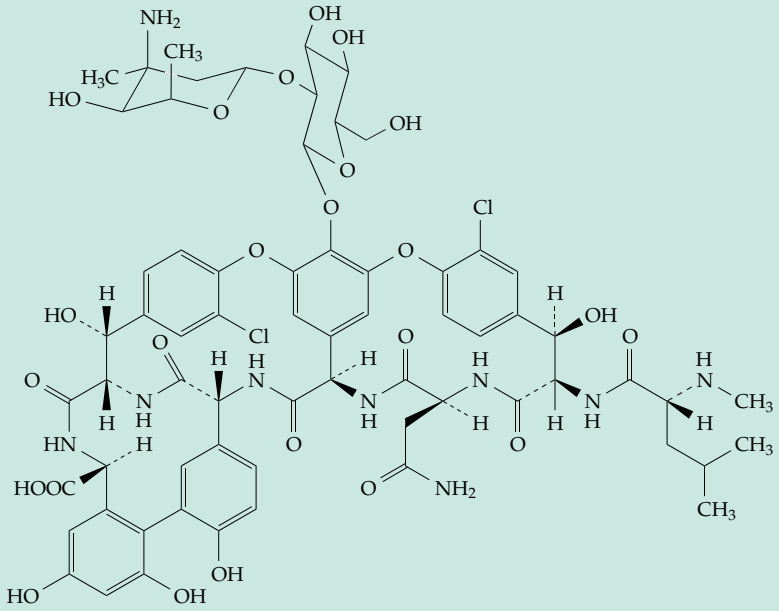
BOX 20-H ANTIBIOTIC RESISTANCE AND VANCOMYCIN



Vancomycin

As antibiotics came into widespread use, an unanticipated problem arose in the rapid development of resistance by bacteria. The problem was made acute by the fact that resistance genes are easily transferred from one bacterium to another by the infectious R-factor plasmids.[a–d] Since resistance genes for many different antibiotics may be carried on the same plasmid, "super bacteria," resistant to a large variety of antibiotics, have developed, often in hospitals.

The problem has reached the crisis stage, perhaps most acutely for tuberculosis. Drug-resistant *Mycobacteria tuberculosis* have emerged, especially, in patients being treated for HIV infection (see Box 21-C). Mechanisms of resistance often involve inactivation of the antibiotics. Aminoglycosides such as streptomycin, spectinomycin, and kanamycin (Box 20-B) are inactivated by en-zymes catalyzing phosphorylation or adenylylation of hydroxyl groups on the sugar rings.[e–g] Penicillin and related antibiotics are inactivated by β-lactamases (Box 20-G). Chloramphenicol (Fig. 25-10) is inactivated by acylation on one or both of the hydroxyl groups.

What is the origin of the drug resistance factors? Why do genes for inactivation of such unusual molecules as the antibiotics exist widely in nature? Apparently the precursor to drug resistance genes fulfill normal biosynthetic roles in nature. An antibiotic-containing environment, such as is found naturally in soil, leads to selection of mutants of such genes with drug-inactivating properties. Never-theless, it is not entirely clear why drug resistance factors have appeared so promptly in the population. Overuse of antibiotics in treating minor infections is one apparent cause.[h,i] Another is probably the widespread use on farms.[j] A nationwide effort to decrease the use of erythromycin in Finland had a very favorable effect in decreasing the incidence of erythromycin-resistant group A streptocci.[h]

Because of the rapid development of resistance, continuous efforts are made to alter antibiotics by semisynthesis (see Box 20-G) and to identify new targets for antibiotics or for synthetic antibacterial compounds.[d] An example is provided by the discovery of vancomycin. Like the penicillins, this antibiotic interferes with bacterial cell wall

teichuronic acids (p. 431). Both proteins and neutral polysaccharides, sometimes covalently bound, may also be present.[303] Like peptidoglycans, teichoic and teichuronic acids are assembled on undecaprenyl phosphates[303] or on molecules of diacylglycerol.[322] Either may serve as an anchor. A "linkage unit" may be formed by transfer of several glycosyl rings onto an anchor unit. For example, in synthesis of ribitol teichoic acid sugar rings are transferred from UDP-GlcNAc, UDP-ManNAC, and CDP-Gal to form the following linkage unit:

$$\text{Lipid}-P-P-\text{GlcNAc}-\text{ManNAc}-(P\text{-Gal})_{2,3}$$

Then many ribitol phosphate units are added by transfer from CDP-ribitol. Finally, the chain is capped by transfer of a glucose from UDP-Glc. Lipoteichoic acids often carry covalently linked D-alanine in ester linkage, altering the net electrical charge on the cell surface.[322a] The completed teichoic acid may then be transferred to a peptidoglycan, releasing the lipid phosphate for reuse.[303] Glycerol teichoic acid may be formed in a similar fashion.[322] Teichuronic acids arise by alternate transfers of *P*-GalNAc from UDP-GalNAc and of GlcA from UDP-GlcA.[303]

Gram-positive bacteria often carry surface proteins that interact with host tissues in establishing human infections. Protein A of *Staphylococcus* is a well-known

BOX 20-H (continued)

synthesis but does so by binding tightly to the D-alanyl-D-alanine termini of peptidoglycans that are involved in crosslinking (Fig. 8-29, Fig. 20-9).[k,l] Like penicillin, vancomycin prevents crosslinking but is unaffected by β-lactamases. Initially bacteria seemed unable to develop resistance to vancomycin, and this antibiotic was for 25 years the drug of choice for β-lactam resistant streptococci or staphylococci. However, during this period bacteria carrying a plasmid with nine genes on the transposon Tn1546 (see Fig. 27-30) developed resistance to vancomycin and were spread worldwide.[l] Vancomycin-resistant bacteria are able to sense the presence of the antibiotic and to synthesize an altered **D-alanine:D-alanine ligase**, the enzyme that joins two D-alanine molecules in an ATP-dependent reaction to form the D-alanyl-D-alanine needed to permit peptidoglycan crosslinking (Fig. 20-9, step *c'*). The altered enzyme adds D-lactate rather than D-alanine providing an –OH terminus in place of $-NH_3^+$. This prevents the binding of vancomycin[l,m] and allows crosslinking of the peptidoglycan via depsipeptide bonds. Another gene in the transposon encodes an oxoacid reductase which supplies the D-lactate.[l] A different resistant strain synthesizes a D-Ala-D-Ser ligase.[n] High-level vancomycin resistance is not attained unless the bacteria also synthesize a D-alanyl-D-alanine dipeptidase.[o]

The D-Ala-D-Ala ligase does provide yet another attractive target for drug design.[p,q] Still another is the D-Ala-D-Ala adding enzyme (Fig. 20-8, step *d*; encoded by the *E. coli MurF* gene).[r,s] Strategies for combatting vancomycin resistance include synthesis of new analogs of the antibiotic[t,u] and simultaneous administration of small molecules that catalyze cleavage of the D-Ala-D-lactate bond formed in cell wall precursors of resistant bacteria.[v]

Another possibility is to use **bacteriophages** directly as antibacterial medicines. This approach was introduced as early as 1919 and has enjoyed considerable success. It is now regarded as a promising alternative to the use of antibiotics in many instances.[w]

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example. After synthesis in the cytoplasm, it enters the secretory pathway. An N-terminal hydrophobic leader sequence and a 35-residue C-terminal sorting signal guide it to the correct destination. There a free amino group of an unlinked pentaglycyl group of the peptidoglycan carries out a transamidation reaction with an LPXTG sequence in the proteins, cutting the chain between the threonine and glycine residues, and anchoring the protein A to the peptidoglycan.[323]

Group A streptococci, which are serious human pathogens, form α-helical coiled-coil threads whose C termini are anchored in the cell membrane. They protrude through the peptidoglycan layers and provide a hairlike layer around the bacteria. A variable region at the N termini provides many antigens, some of which escape the host's immune system allowing infection to develop.[324] Group B streptococci form carbohydrate antigens linked to teichoic acid.[325] Streptococci, which are normally present in the mouth, utilize their carbohydrate surfaces as receptors for adhesion, allowing them to participate in formation of dental plaque.[326]

Cell walls of mycobacteria are composed of a peptidoglycan with covalently attached galactan chains. Branched chains of **arabinan**, a polymer of the furanose ring form of arabinose with covalently attached **mycolic acids**, are glycosidically linked to the galactan.[327] Shorter **glycopeptidolipids**, containing

modified glucose and rhamnose rings as well as fatty acids, contribute to the complexity of mycobacterial surfaces.[328]

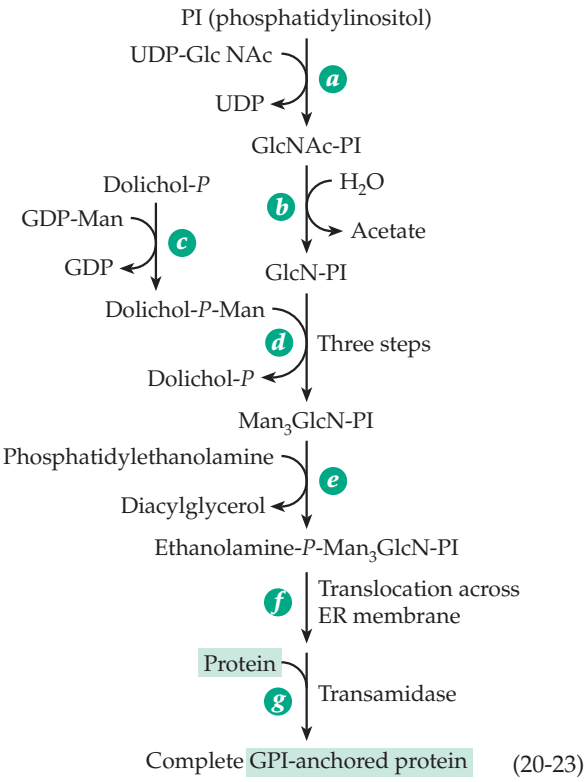
These examples describe only a small sample of the great diversity of cell coats found in the prokaryotic world. Some bacteria also provide themselves with additional protection in the form of external sheaths of crystalline arrays of proteins known as S-layers.[329]

F. Biosynthesis of Eukaryotic Glycolipids

Glycolipids may be thought of as membrane lipids bearing external oligosaccharides. In this sense, they are similar to glycoproteins both in location and in biological significance. Like the glycoproteins, glycolipids are synthesized in the ER, then transported into the Golgi apparatus and eventually outward to join the outer surface of the plasma membrane. Some glycolipids are attached to proteins by covalent linkage.

1. Glycophosphatidylinositol (GPI) Anchors

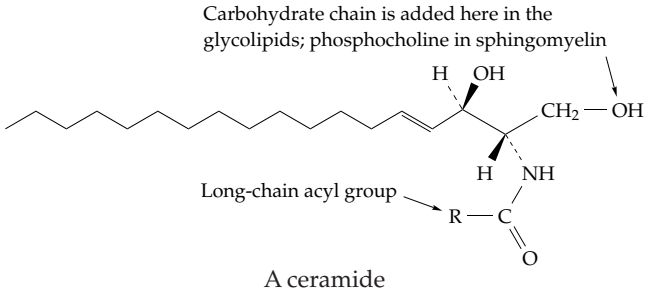
More than 100 different human proteins are attached to phosphatidylinositol anchors of the type shown in Fig. 8-13.[329a] Similar anchors are prevalent in yeast and in protozoa including *Leishmania* and *Trypanosoma*,[330–334b] and *Plasmodium* [334c] where they often bind major surface proteins to the plasma membrane.[335] They are also found in mycobacteria.[336] The structures of the hydrophobic anchor ends are all similar.[337] Two or three fatty acyl groups hold the molecule to the bilayer. Variations are found in the attached glycan portion, both in the number of sugar rings and in the structures of the covalently attached phosphoethanol-amine groups.[337–339] A typical assembly pathway is shown in Eq. 20-23. The first step (step *a* in Eq. 20-23), the transfer of an *N*-acetylglucosamine residue to phosphatidylinositol, is surprisingly complex, requiring at least three proteins.[340] The hydrolytic deacetylation (step *b*) helps to drive the synthetic process. Step *c* provides dolichol-*P*-mannose for the GPI anchors as well as for glycoproteins. The phosphoethanolamine part of the structure is added from phosphatidylethanolamine, apparently via direct nucleophilic displacement.[333] In this way the C terminus of the protein forms an amide linkage with the $-NH_2$ group of ethanolamine in the GPI anchor. Another unexpected finding was that this completed anchor unit undergoes "remodeling" during which the fatty acyl chains of the original phosphatidylinositol are replaced by other fatty acids.[339,339a]



(20-23)

2. Cerebrosides and Gangliosides

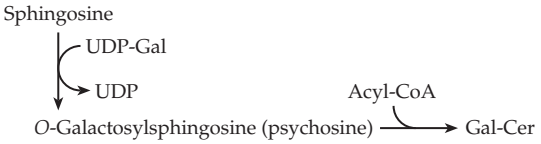
These two groups of glycolipids are derived from the *N*-acylated sphingolipids known as **ceramides**. Some biosynthetic pathways from sphingosine to these substances are indicated in Fig. 20-11. Acyl, glycosyl, and sulfo groups are transferred from appropriate derivatives of CoA, CDP, UDP, CMP, and from PAPS to form more than 40 different gangliosides.[341,342] The biosynthesis of a sphingomyelin is also shown in this scheme but is discussed in Chapter 21.



A ceramide

Each biosynthetic step in Fig. 20-11 is catalyzed by a specific transferase. Most of these enzymes are present in membranes of the ER and the Golgi.[343–346] Furthermore, the sequence by which the transferases act may not always be fixed, and a complete biosynthetic scheme would be far more complex than is shown in the figure. For example, one alternative

sequence is the synthesis of galactosyl ceramide by transfer of galactose to sphingosine followed by acylation (Eq. 20-24). However, the pathway shown in Fig. 20-11 is probably more important.



(20-24)

G. The Intracellular Breakdown of Polysaccharides and Glycolipids

The attention of biochemists has been drawn to the importance of pathways of degradation of complex polysaccharides through the existence of at least 35 inherited **lysosomal storage diseases**.[347–351] In many of these diseases one of the 40-odd lysosomal hydrolases is defective or absent.

1. Mucopolysaccharidoses

There are at least seven mucopolysaccharidoses (Table 20-1) in which glycosaminoglycans such as hyaluronic acid accumulate to abnormal levels in tissues and may be excreted in the urine. The diseases cause severe skeletal defects; varying degrees of mental retardation; and early death from liver, kidney, or cardiovascular problems. As in other lysosomal diseases, undegraded material is stored in intracellular inclusions lined by a single membrane. Various tissues are affected to different degrees, and the diseases tend to progress with time.

First described in 1919 by Hurler, **mucopolysaccharidosis I** (MPS I, the **Hurler syndrome**) leads to accumulation of partially degraded dermatan and heparan sulfates (Fig. 4-11).[347,352,353] A standard procedure in the study of diseases of this type is to culture fibroblasts from a skin biopsy. Such cells cultured from patients with the Hurler syndrome accumulate the polysaccharide, but when fibroblasts from a normal person are cultured in the same vessel the defect is "corrected." It was shown that a protein secreted by the normal fibroblasts is taken up by the defective fibroblasts, permitting them to complete the degradation of the stored polysaccharide. This "Hurler corrective factor" was identified as an **α-L-iduronidase**. In the **Hunter syndrome** (MPS II) dermatan sulfate and heparan sulfate accumulate. The missing enzyme is a **sulfatase** for 2-sulfated iduronate residues.[354,355] The diagram at the bottom of the page illustrates the need for both of these enzymes as well as three others in the degradation of dermatan.[352,356,357]

The **Sanfilippo disease** type A (MPS III) corrective factor is a heparan *N*-sulfatase. However, as is true for many other metabolic diseases, the same symptoms

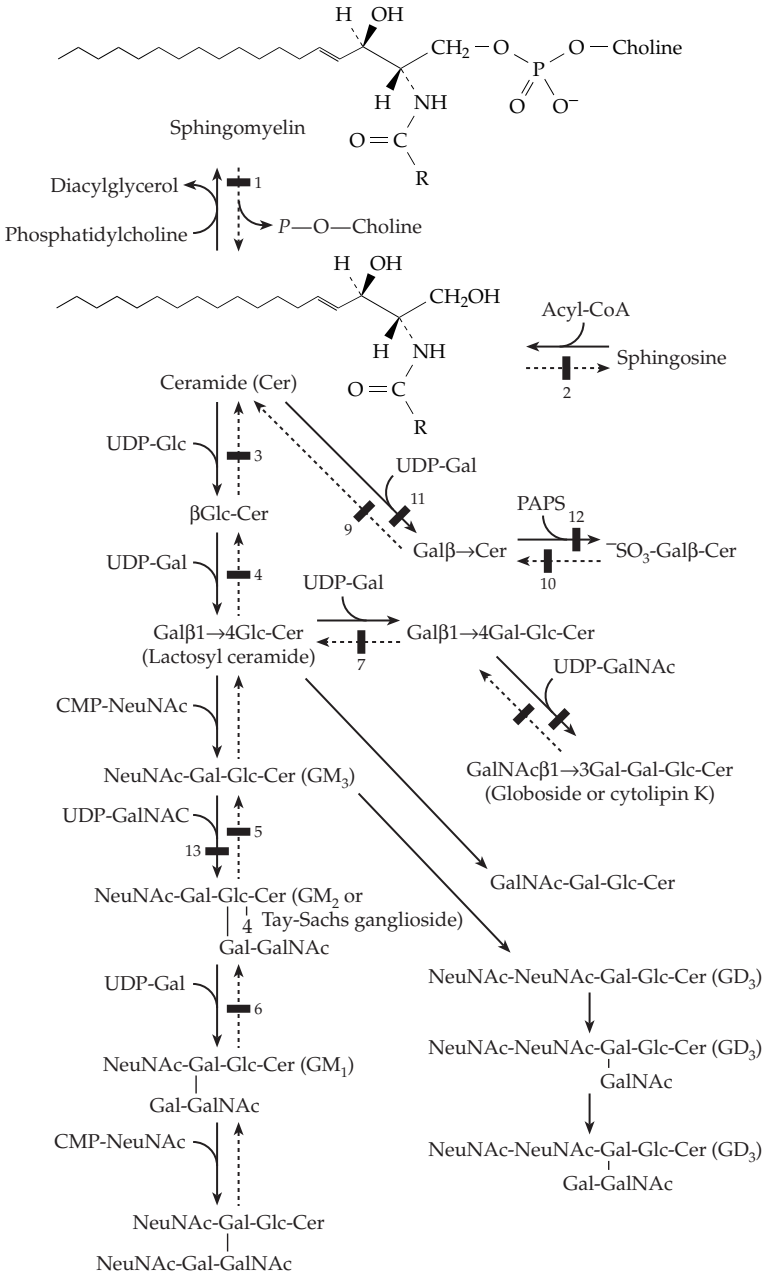


Figure 20-11 Biosynthesis and catabolism of glycosphingolipids. The heavy bars indicate metabolic blocks in known diseases.

may arise from several causes. Thus, Sanfilippo diseases B and D arise from lack of an *N*-acetylglucosaminidase and of a sulfatase for GlcNac-6-sulfate, respectively.[354] In Sanfilippo disease C the missing or defective enzyme is an acetyl transferase that transfers an acetyl group from acetyl-CoA onto the amino groups of glucosamine residues in heparan sulfate fragments. All four of these enzymes are needed to degrade the glucosamine-uronic acid pairs of heparan. The *N*-sulfate groups must be removed by the *N*-sulfatase. The free amino groups formed must then be acetylated before the *N*-acetylglucosaminidase can cut off the GlcNac groups. Remov-al of the 6-sulfate groups requires the fourth enzyme. Completion of the degradation also requires both β-glucuronidase and α-L-iduronidase. Another lysosomal enzyme deficiency, which is most prevalent in Finland, is the absence of **aspartylglucosaminidase**, an N-terminal nucleophile hydrolase (Chapter 12, Section C,3) that cleaves glucosamine from aspartate side chains to which oligosaccharides were attached in glycoproteins.[358–360]

Some hereditary diseases are characterized by lack of two or more lysosomal enzymes. In **I-cell disease** (mucolipidosis II), which resembles the Hurler syndrome, at least ten enzymes are absent or are present at much reduced levels.[350,361] The biochemical defect is the absence from the Golgi cisternae of the *N*-acetylglucosaminyl phosphotransferase that transfers *P*-GlcNAc units from UDP-GlcNAc onto mannose residues (Eq. 20-22) of glycoproteins marked for use in lysosomes.

2. Sphingolipidoses

There are at least ten lysosomal storage diseases, known as sphingolipidoses, that involve the metabolism of the glycolipids. Their biochemical bases are indicated in Fig. 20-11 and in Table 20-1. **Gaucher disease**[362–365] is a result of an autosomal recessive trait that permits glucosyl ceramide to accumulate in macrophages. The liver and spleen are seriously damaged, the latter becoming enlarged to four or five times normal size in the adult form of the disease. In the more severe juvenile form mental retardation occurs. By 1965, it was established that cerebroside is synthesized at a normal rate in the individuals affected, but that a lysosomal hydrolase was missing. This blocked the catabolic pathway indicated by dashed arrows in Fig. 20-11 (block No. 3 in the figure). In many patients a single base change causing a Leu → Pro substitution accounts for the defect. In **Fabry disease** an X-linked gene that provides for removal of galactosyl residues from cerebrosides is defective.[350] This leads to accumulation of the triglycosylceramide whose degradation is blocked at point 7 in Fig. 20-11.

The best known and the commonest sphingolipidosis is **Tay–Sachs disease**.[366–368] Several hundred cases have been reported since it was first described in 1881. A terrible disease, it is accompanied by mental deterioration, blindness, paralysis, dementia, and death by the age of three. About 15 children a year are born in North America with this condition, and the world figure must be 5–7 times this. The defect is in the α subunit of the β-hexosaminidase A (point 7 in Fig. 20-10)[366,366a] with accumulation of ganglioside G_{M2}. Somewhat less severe forms of the disease are caused by different mutations in the same gene[369] or in a protein activator. **Sandhoff disease**, which resembles Tay–Sachs disease, is caused by a defect in the β subunit, which is present in both β-hexosaminidases A and B.[368] Mutant "knockout" mice that produce only ganglioside GM_3 as the major ganglioside in their central nervous system die suddenly from seizures if they hear a loud sound. This provides further evidence of the essential nature of these components of nerve membranes.[369a]

3. Causes of Lysosomal Diseases

The descriptions given here have been simplified. For many lysosomal diseases there are mild and severe forms and infantile or juvenile forms to be contrasted with adult forms. Some of the enzymes exist as multiple isozymes. An enzyme may be completely lacking or

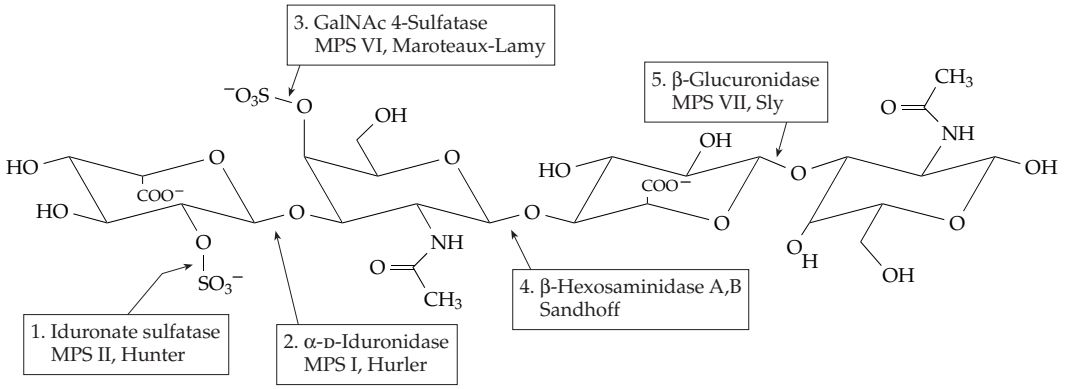


TABLE 20-1
Lysosomal Storage Diseases: Sphingolipidoses and Mucopolysaccharidoses[a]

No. in Fig. 20-11	Name	Defective enzyme
1.	Niemann–Pick disease[b]	Sphingomyelinase
2.	Farber disease (lipogranulomatosis)	Ceramidase
3.	Gaucher disease[c]	β-Glucocerebrosidase
4.	Lactosyl ceramidosis	β-Galactosyl hydrolase
5.	Tay–Sachs disease[c]	β-Hexosaminidase A
6.	G_{M1} gangliosidosis[d]	β-Galactosidase
7.	Fabry disease[c]	α-Galactosidase
8.	Sandhoff disease[e]	β-Hexosaminidases A and B
9.	Globoid cell leukodystrophy	Galactocerebrosidase
10.	Metachromatic leukodystrophy	Arylsulfatase A
13.	Hematoside (G_{M3}) accumulation	G_{M3}-*N*-acetylgalactosaminyltransferase
	Pompe disease[f]	α-Glucosidase
	Hurler syndrome (MPS I)[c]	α-L-Iduronidase
	Hunter syndrome (MPS II)[c]	Iduronate 2-sulfate sulfatase
	Sanfilippo disease[c,g]	
	Type A (MPS III)	Heparan *N*-sulfatase
	Type B	*N*-Acetylglucosaminidase
	Type C	Acetyl-CoA: α-glucosaminide *N*-acetyltransferase
	Type D	GlcNAc-6-sulfate sulfatase
	Maroteaux–Lamy syndrome (MPS VI)[g]	Arylsulfatase B
	Sly syndrome (MPS VII)[g,h]	β-Glucuronidase
	Aspartylglycosaminuria[i]	Aspartylglucosaminidase
	Mannosidosis	β-Mannosidase
	Fucosidosis	α-L-Fucosidase
	Mucolipidosis	α-*N*-Acetylneuraminidase
	Sialidosis[j]	

[a] A general reference is Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., eds. (1995) *The Metabolic and Molecular Bases of Inherited Disease*, 7th ed., Vol. 1, McGraw-Hill, New York (pp. 2427–2879)
[b] Wenger, D. A., Sattler, M., Kudoh, T., Snyder, S. P., and Kingston, R. S. (1980) *Science* **208**, 1471–1473
[c] See main text
[d] Hoogeveen, A. T., Reuser, A. J. J., Kroos, M., and Galjaard, H. (1986) *J. Biol. Chem.* **261**, 5702–5704
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[f] See Box 20-D
[g] Neufeld, E. F., and Muenzer, J. (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. 2465 – 2494, McGraw-Hill, New York
[h] Wu, B. M., Tomatsu, S., Fukuda, S., Sukegawa, K., Orii, T., and Sly, W. S. (1994) *J. Biol. Chem.* **269**, 23681–23688
[i] Mononen, I., Fisher, K. J., Kaartinen, V., and Aronson, N. N., Jr. (1993) *FASEB J.* **7**, 1247–1256
[j] Seppala, R., Tietze, F., Krasnewich, D., Weiss, P., Ashwell, G., Barsh, G., Thomas, G. H., Packman, S., and Gahl, W. A. (1991) *J. Biol. Chem.* **266**, 7456–7461

may be low in concentration. The causes of the deficiencies may include total absence of the gene, absence of the appropriate mRNA, impaired conversion of a proenzyme to active enzyme, rapid degradation of a precursor or of the enzyme itself, incorrect transport of the enzyme precursor to its proper destination, presence of mutations that inactivate the enzyme, or absence of protective proteins. Several lysosomal hydrolases require auxiliary **activator proteins** that allow them to react with membrane-bound substrates.[350,351,365,370]

4. Can Lysosomal Diseases Be Treated?

There has been some success in using enzyme replacement therapy for lysosomal deficiency diseases.[347,371-371b] One approach makes use of the fact that the mannose-6-*P* receptors of the plasma membrane take up suitably marked proteins and transfer them into lysosomes (Section C,2). The missing enzyme might simply be injected into the patient's bloodstream from which it could be taken up into the lysosomes.[372] This carries a risk of allergic reaction, and it may be safer to attempt microencapsulation of the enzyme, perhaps in ghosts from the patient's own erythrocytes.[347] A second approach, which has had limited success, is transplantation of an organ[371] or of bone marrow[373] from a donor with a normal gene for the missing enzyme. This is dangerous and is little used at present. However, new hope is offered by the possibility of transferring a gene for the missing enzyme into some of the patient's cells. For example, the cloned gene for the transferase missing in Gaucher disease has been transferred into cultured cells from Gaucher disease patients with apparent correction of the defect.[374] Long-term correction of the Hurler syndrome in bone marrow cells also provides hope for an effective therapy involving gene transfer into a patient's own bone marrow cells[375] or transplantation of selected hematopoietic cells.[375a]

In the cases of Gaucher disease and Fabry disease, it is hoped that treatment of infants and young children may prevent brain damage. However, in Tay–Sachs disease the primary sites of accumulation of the ganglioside GM_2 are the ganglion and glial cells of the brain. Because of the "blood–brain barrier" and the severity of the damage it seems less likely that the disease can be treated successfully.

The approach presently used most often consists of identifying carriers of highly undesirable genetic traits and offering genetic counseling. For example, if both parents are carriers the risk of bearing a child with Tay–Sachs disease is one in four. Women who have borne a previous child with the disease usually have the genetic status of the fetus checked by **amniocentesis**. A sample of the amniotic fluid surrounding the fetus is withdrawn during the 16th to 18th week of pregnancy. The fluid contains fibroblasts that have become detached from the surface of the fetus. These cells are cultured for 2–3 weeks to provide enough cells for a reliable assay of the appropriate enzymes. Such tests for a variety of defects are becoming faster and more sensitive as new techniques are applied.[376] In the case of Tay–Sachs disease, most women who have one child with the disease choose abortion if a subsequent fetus has the disease.

The diseases considered here affect only a small fraction of the problems in the catabolism of body constituents. On the other hand, fewer cases are on record of deficiencies in biosynthetic pathways. These are more often absolutely lethal and lead to early spontaneous abortion. However, blockages in the biosynthesis of cerebrosides are known in the special strains of mice known as Jimpy, Quaking, and msd (myelin synthesis deficient).[377,378] The transferases (points 11 and 12 of Fig. 20-11) are not absent but are of low activity. The mice have distinct neurological defects and poor myelination of nerves in the brain. A human ailment involving impaired conversion of GM_3 to GM_2 (with accumulation of the former; point 13 of Fig. 20-11) has been reported. Excessive synthesis of sialic acid causes the rare human **sialuria.**[379] This is apparently a result of a failure in proper feedback inhibition.

Animals suffer many of the same metabolic diseases as humans. Among these are a large number of lysosomal deficiency diseases.[380] Their availability means that new methods of treating the diseases may, in many cases, be tried first on animals. For example, enzyme replacement therapy for the Hurler syndrome is being tested in dogs.[381] Bone marrow transplantation for human **α-mannosidosis** is being tested in cats with a similar disease.[382] Mice with a hereditary deficiency of β-glucuronidase are being treated by gene transfer from normal humans.[357]

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Study Questions

1. Constitution of cell surface oligosaccharides or polysaccharides includes the following:
   - D-Glucose
   - D-Mannose
   - D-Galactose
   - L-Arabinose
   - L-Fucose
   - D-Glucuronic acid
   - D-Neuraminic acid

   Outline pathways for biosynthesis of these compounds from glucose.

2. Decarboxylation steps are required for synthesis of UDP-xylulose and UDP-apiose (Fig. 21-1). Propose chemical mechanisms for these reactions.

3. How do animals and plants differ with respect to transport and storage of glucose?

4. Comment on unresolved questions about the biosynthesis of cellulose, amylose, and amylopectin. What glycosyl carrier groups are required?

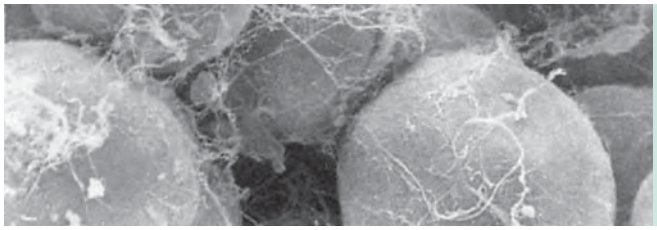
5. Most 5- and 6-membered sugars are found in nature as pyranose ring forms. Why is ribose in RNA in the furanose ring form?

6. If the ratio $[NAD^+]/[NADH]$ in a cell were 500 and the ratio $[NADP^+]/[NADPH]$ were 0.002, what concentrations of fructose and sorbitol would be in equilibrium with 0.1 mM glucose? See Box 20-A and Table 6-4.

7. Write a balanced equation for reaction of boric acid (H_3BO_3) with two sugar rings to give a borate diol ester linkage (Box 20-E).

8. Describe in general terms the process by which *N*-linked oligosaccharides are synthesized and attached to proteins. What are the functions of the ER and the Golgi?

9. What, if any, restrictions do you think should be applied to the use of antibiotics on farms?

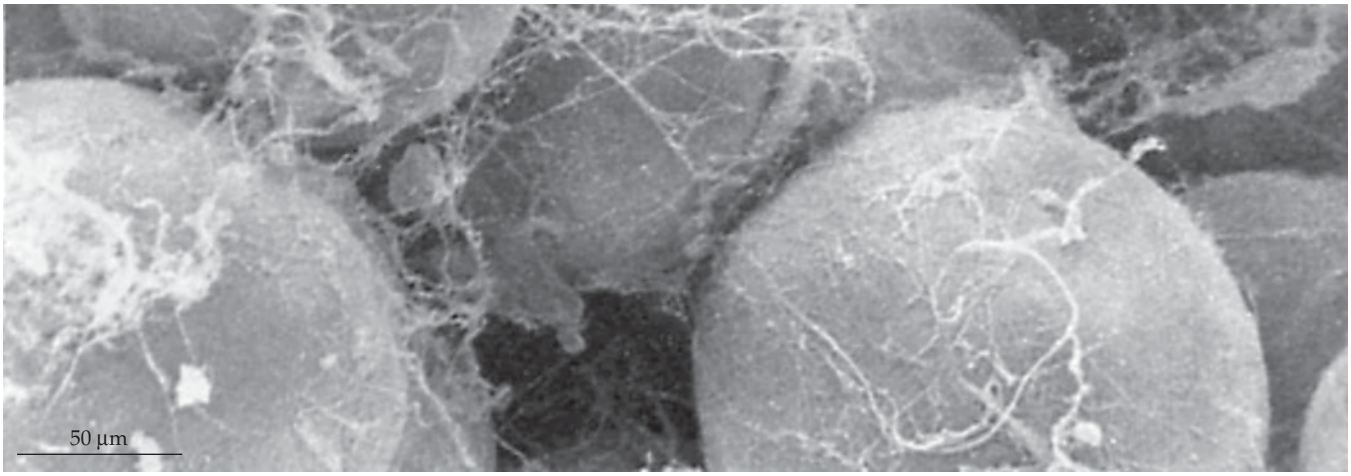


Two large fat-filled adipose cells are seen in the foreground of this scanning electron micrograph. They are part of a larger cluster of cells from rat tissue. Delicate strands of connective tissue fibers intertwine the cells and hold them together. While most of the connective tissue substance has been washed away during preparation of the specimen, the remnants give a realistic impression of the soft, loose nature of the intercellular material. From Porter and Bonneville (1973) *Fine Structure of Cells and Tissues*, Lea and Febiger, Philadelphia, Pennsylvania. Courtesy of Mary Bonneville.

Contents

Specific Aspects of Lipid Metabolism

21



The basic pathways for both synthesis of fatty acids and for their β-oxidation (Fig. 17-1) have been described in Chapter 17. However, there are many variations to these pathways, and additional sets of enzymes are needed to synthesize the complex array of lipids present in most organisms. We will consider these details in this chapter. Like most other organisms, human beings are able to synthesize triacylglycerols (triglycerides), phospholipids, and glycolipids needed for cell membranes. Glucose can serve as the starting material. However, dietary lipids are also a major source. For this reason, we will start with a discussion of the digestion and uptake of lipids and of the distribution by way of the bloodstream of ingested lipids and of lipids synthesized in the liver or in other tissues.

A. Digestion, Synthesis, and Distribution of Triacylglycerols in the Human Body

Digestion of triglycerides begins in the stomach with emulsification and partial digestion by gastric lipase. Within the small intestine the ~100-residue protein called **colipase**[1–2a] binds to the surface of the fat droplets and provides an attachment site for the 449-residue **pancreatic lipase**. This Ca^{2+}-dependent serine esterase cleaves each triglyceride to two molecules of fatty acid and one of a 2-monoacylglycerol.[3–5] These products are emulsified by bile salts (Fig. 22-10) and are then taken up by the cells of the intestinal lining. The fatty acids are converted to acyl-CoA esters which transfer their acyl groups to the monoacylglycerols to resynthesize the triacylglycerols.[6] The latter are incorporated into the very large lipoprotein particles called **chylomicrons** (Table 21-1) and enter the bloodstream via the lymphatic system (Fig. 21-1).[7] Free fatty acids are also transported as complexes with serum albumin.

Synthesis of lipids from carbohydrates is an efficient process, which occurs largely in the liver and also in intestinal epithelial cells.[6] The newly synthesized triacylglycerols, together with smaller amounts of phospholipids and cholesterol, combine with specific **apolipoproteins**, which are also synthesized in the liver, to form **very low density lipoprotein (VLDL)** particles which are secreted into the blood stream. They transport the newly formed triacylglycerols from the liver to other body cells including the adipocytes, which store excess fat (Fig. 21-1).

1. Plasma Lipoproteins

The small particles of plasma lipoprotein, which carry triacylglycerols, can be separated according to their buoyant densities by centrifugation. They have been classified into five groups of increasing density but smaller size as **chylomicrons,** very low density lipoproteins **(VLDL)**, intermediate density lipoproteins (**IDL**), low density **(LDL),** and high density lipoproteins **(HDL)** (Table 21-1 and Fig. 21-2). Each lipoprotein particle contains one or more apolipoproteins (Table 21-2), whose sizes vary from the enormous 4536-residue apoB-100 to apoC-II and apoC-III, each of which contains just 79 residues[7a] and the 57-residue apoC-I.[7b]

The larger lipoproteins are spherical micelles containing a core of triacylglycerols and esters of cholesterol surrounded by a 2- to 3-nm-thick layer

consisting of phospholipid, free cholesterol, and the apolipoprotein components.[8] The size of the lipoprotein particles also varies from a 200- to 500-nm diameter for chylomicrons to as little as 5 nm for the smallest HDL particles. The difference in volume is more impressive. If, as has been estimated,[9] a 22-nm diameter LDL particle contains about 2000 cholesterol and cholesteryl ester molecules and 800 phospholipids, a small HDL particle of 7-nm diameter will have room for only about 60 molecules of cholesterol and 90 of phospholipid, while a chylomicron may carry 10 million molecules of triacylglycerol. HDL particles are quite heterogeneous. As is indicated in Table 21-1, they are sometimes dividing into HDL2 and HDL3 density groups. In addition, there is a pre-HDL with lower phospholipid content and discoid forms low in cholesterol. Models of a reconstituted lipoprotein disc contain two molecules of apoA-I and ~160 phosphatidylcholines that form a bilayer core.[10–10b]

Each apolipoprotein has one or more distinct functions. The apoB proteins probably stabilize the lipoprotein micelles. In addition, apoB-100 is essential to recognition of LDL by its receptors. The 79-residue apoC-II has a specific function of activating the lipoprotein lipase that hydrolyses the triacylglycerols of chylomicrons and VLDL. Lack of either C-II or the lipase results in a very high level of triacylglycerols in the blood.[11]

The large apolipoprotein B-100 is synthesized in the liver and is a principal component of VLDL, IDL, and LDL. It is the sole protein in LDL, accounting for nearly 20% of the mass of LDL particles. Partly because of its insolubility in water, its detailed structure is uncertain. If it were all coiled into an α helix, it would be 680 nm long and could encircle the LDL particle nearly 10 times! While the true structure of apoB-100 is unknown, it is thought to be extended and to span at least a hemisphere of the LDL surface.[12] It consists of at least five domains. Sixteen cysteines are present in the first 25 residues at the N terminus, forming a crosslinked high-cysteine region. There are also 16 *N*-glycosylated sites. Domain IV (residues 3071–4011) is thought to contain the site that binds to its specific receptor, the LDL receptor.[12] Heterogeneity in the amide I band of the infrared absorption spectrum (Fig. 23-3) suggests that about 24% is α helix, 23% β sheet, and that a large fraction consists of turns, and unordered and extended peptide structures.[13]

In intestinal epithelial cells the same apoB gene that is used to synthesize apoB-100 in the liver is used to make the shorter **apoB-48** (48%) protein. This is accomplished in an unusual way that involves "editing" of the mRNA that is formed. Codon 2153 in the mRNA for the protein is CAA, encoding glutamine. However, the cytosine of the triplet is acted on by a deaminase, an editing enzyme, to form UAA, a chain termination codon.[14,15] A third form of apoB is found in **lipoprotein(a)** (Lpa). This LDL-like particle contains apoB-100 to which is covalently attached by a single disulfide linkage (probably to Cys 3734 of apoB-100) a second protein, **apo(a)**. The latter consists largely of a chain of from 11 to over 50 kringle domains resembling the 78-residue kringle-4 of plasminogen (see Fig. 7-30C)[16–19a] as well as a protease domain.[20] This additional chain may cause tighter binding to LDL receptors and may cause lipoprotein(a) to displace plasminogen from cell surface receptors.[21] The amount of Lp(a) varies over 1000-fold among individuals and is genetically determined. The number of kringle domains also varies.[17] Although the presence of high Lp(a) is associated with a high risk of atherosclerosis and stroke,[21a] many healthy 100-year olds also have high serum Lp(a).[22]

Apolipoprotein A-I is the primary protein component of HDL.[23–25b] Most of the 243 residues consist of a nearly continuous amphipathic α helix with kinks at regularly spaced proline residues.[26–28] Two disulfide-linked ApoA-I molecules may form a belt that encircles the discoid lipoprotein.[25b] ApoA-II is the second major HDL protein, but no clearly specialized function has been identified.[29,30] ApoA-I, II, and IV, apoC-I, II, and III, and apoE all have multiple repeats of 22 amino acids with sequences that suggest amphipathic helices. The 391-residue ApoA-IV has 13 tandem 22-residue repeats. Proline and glycine are present in intervening hinge regions.[23] This may enable these proteins to spread over and penetrate the surfaces of the lipoprotein micelles. Most of these proteins are encoded by a related multigene family.[7,30a]

The 299-residue apolipoprotein E plays a key role in metabolism of both triacylglycerols and cholesterol. Like apoB-100 it binds to cell surface receptors.[31–33a] Absence of functional apoE leads to elevated plasma triacylglycerol and cholesterol, a problem that is considered in Chapter 22, Section D. The N-terminal domain, from residues 23 to 164, forms a 6.5 nm-long four-helix bundle, which binds to the LDL receptors.[32] There are three common isoforms of alipoprotein E (apoE2, apoE3, and apoE4). ApoE3 is most common.[33b] The presence of apoE4 is associated with an increased risk of Alzheimer disease (Chapter 30).

The major lipoproteins of insect hemolymph, the **lipophorins**, transport diacylglycerols. The apolipophorins have molecular masses of ~250, 80, and sometimes 18 kDa.[34–37a] The three-dimensional structure of a small 166-residue lipophorin (apolipophorin-III) is that of a four-helix bundle. It has been suggested that it may partially unfold into an extended form, whose amphipathic helices may bind to a phospholipid surface of the lipid micelle of the lipophorin.[35] A similar behavior may be involved in binding of mammalian apolipoproteins. Four-helix lipid-binding proteins have also been isolated from plants.[38] See also Box 21-A. Specialized lipoproteins known as **lipovitellins**

TABLE 21-1
Classes of Lipoprotein Particles

Class	Diameter (nm)	Density (g/ml)	Composition (weight percent)[a]				
			Surface components			Core lipids	
			Protein	Phospholipid	Cholesterol	Cholesteryl esters	Triacylglycerol
Chylomicrons	75–1200	0.93	2	7	2	3	86
VLDL	30–80	0.93–1.006	8	18	7	12	55
IDL	25–35	1.006–1.019	19	19	9	29	23
LDL	18–25	1.019–1.063	22	22	8	42	6
HDL2	9–12	1.063–1.125	40	33	5	17	5
HDL3	5–9	1.125–1.21	45	35	4	13	3
Lp (a); slow pre-β	25–30	1.04–1.09					

[a] Data from Havel, R. J., and Kane, J. P. (1995) in *The Metabolic and Molecular Bases of Inherited Disease*, 7th ed., Vol. II (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., eds), pp. 1841–1852, McGraw-Hill, New York.

These are averages and there is considerable variation.

TABLE 21-2
Properties of Major Plasma Apolipoproteins

Designation	No. residues	Mass (kDa)	Source	Function
A-I	243	29		Major HDL protein
A-II	—	17.4	Liver and intestine	
A-IV	376	44.5		
B-100	4536	513	Liver	VLDL formation; ligand for LDL receptor
B-48	2152	241	Intestine	Chylomicron formation ligand for liver chylomicron receptor
C-I	57	6.6		
C-II	79	8.9	Liver	Cofactor for lipoprotein lipase
C-III	79	8.8		
D	—	31	Many tissues	A lipocalin
E	299	34	Liver, VLDL	Ligand for LDL receptor
(a)	Variable			Ligand for liver chylomicron receptor

store phospholipid in eggs whether from nematodes, frogs, or chickens.[39] There is some sequence similarity to that of human apolipoprotein B-100.

2. Movement of Lipid Materials Between Cells

After the synthesis and release of chylomicrons into the lymphatic circulation, various exchange processes occur by which apolipoproteins, as well as enzymes and other proteins, may be added or removed. These very complex and incompletely understood phenomena are presented in simplified form in Fig. 21-1. Chylomicrons donate apolipoproteins of the A and C families to HDL particles which, in turn, donate apoE and may also return some apoC protein to the chylomicrons.

Both chylomicrons and VLDL particles undergo similar processes in the capillary blood vessels, where their triacylglycerols are hydrolyzed to glycerol and free fatty acids by **lipoprotein lipase**.[40–42a] This enzyme requires for its activity the apolipoprotein C-II which is present in the chylomicrons and VLDL particles. Lipoprotein lipase is also known as the "clearing

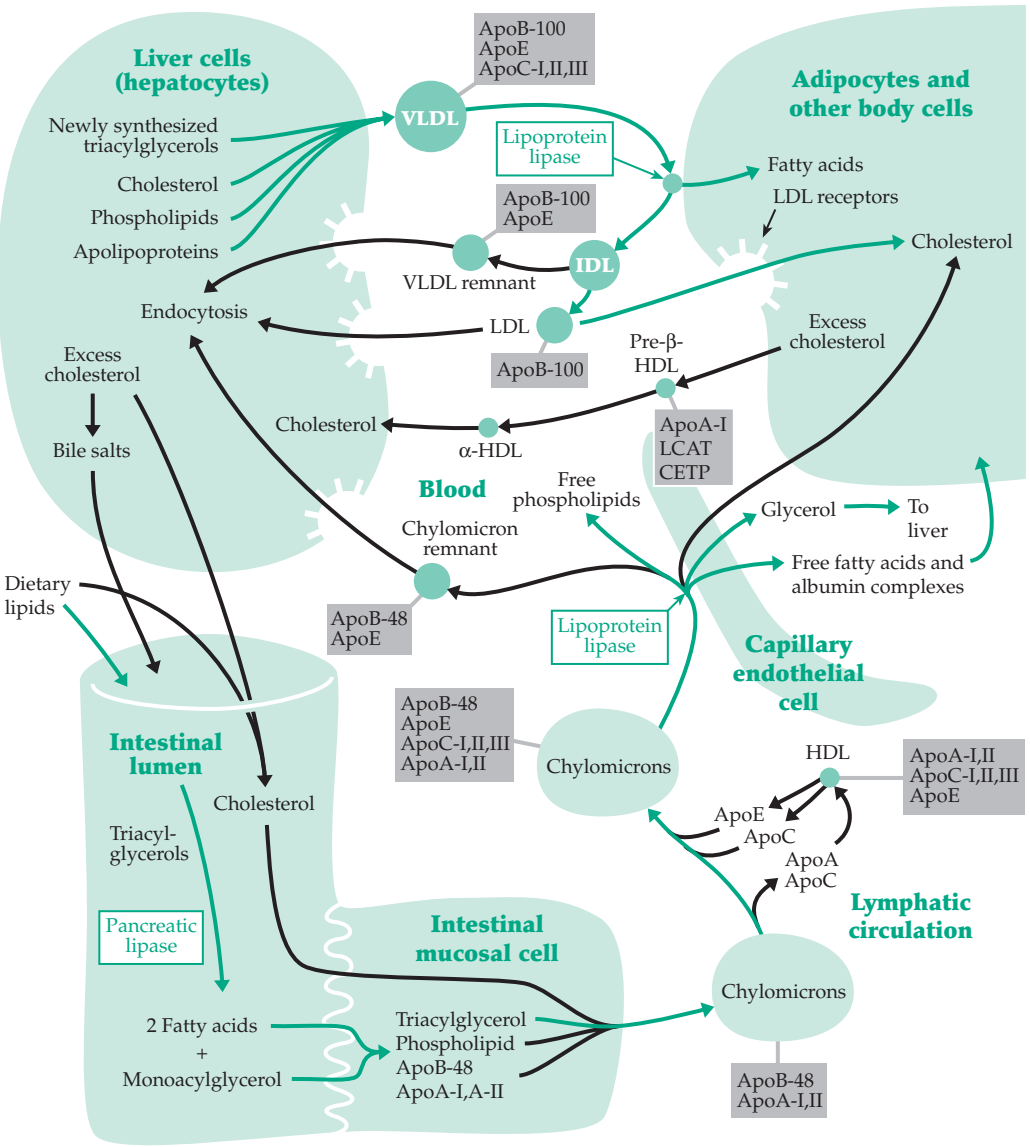


Figure 21-1 Movement of triacylglycerols from liver and intestine to body cells and lipid carriers of blood. VLDL; very low density lipoprotein which contains triacylglycerols, phospholipids, cholesterol, and apolipoproteins B, and C. IDL; intermediate density lipoproteins found in human plasma. LDL; low density lipoproteins which have lost most of their triacylglycerols. ApoB-100, etc., are apolipoproteins listed in Table 21-2. LCAT, lecithin: cholesterol acyltransferase; CETP, cholesteryl ester transfer protein (see Chapter 22).

factor" because it clears the milky chylomicron-containing lymph. It is secreted by adipocytes and other cells and becomes attached to heparan sulfate proteglycans on surfaces of capillary endothelial cells, a major site of its action.[43] Hereditary absence of functional lipoprotein lipase causes **chylomicronemia**, a massive buildup of chylomicrons in plasma.[41,44] The condition does not cause atherosclerosis but may lead to pancreatitis if not treated. Restriction of dietary fat to 20 g / day or less usually prevents problems. Naturally occurring mutations of lipoprotein lipase involving both the aspartate of the catalytic triad (p. 635)[45] and the flexible loop that covers the active site[46] have been discovered.

Both lipoprotein lipase and the less well understood **hepatic lipase** are related structurally to pancreatic lipase.[42,42b] In addition to hydrolysis of the triacylglycerols, the uptake of materials from lipoproteins probably involves shedding of intact phospholipids, perhaps as liposome-like particles.[40]

The free fatty acids and glycerol are taken up by mammalian tissue cells leaving the cholesterol and some of the phospholipids of the VLDL particles as LDL. In humans **intermediate density lipoproteins** (IDL) are formed initially, but some are converted to LDL later. Both LDL particles and the shrunken **chylomicron remnants** and **VLDL remnants** are taken up by endocytosis in coated pits and are degraded by body cells, principally of the liver.[47,48] The best known of these receptors is the 839-residue **LDL receptor,** which has a specific affinity for ApoB-100. The related **VLDL receptor** (apoE receptor) has a higher affinity for apoE[48–50] and may function in uptake of both VLDL and chylomicron remnants. The **LDL receptor-related protein** functions as a third lipoprotein receptor.[51] In addition, a series of **scavenger receptors**, found in abundance in macrophages, take up oxidized lipoproteins and other materials.[51,52] Scavenger receptor B1 (SR-B1), which is also found in liver cells, is involved in uptake of cholesterol from HDL particles by hepatocytes [53] (see also Chapter 22). Liver cells, and other cells as well, contain **lipocalins** and **fatty acid binding proteins** (Box 21-A) that help to carry these relatively insoluble acids to their destinations within the cells. Serum albumin (Box 2-A) is also a major carrier of free fatty acids.[53a] Within the adipocytes the fatty acids are reconverted to triacylglycerols. The low density (LDL) and high density (HDL) lipoproteins are involved primarily in transporting cholesterol to and from cells, a topic that is discussed in Chapter 22, Section D,2.

Fatty acids are carried to tissues for use in synthesis of triacylglycerols, phospholipids, and other membrane lipids. The mobilization of fatty acids from triacylglycerol stores and from cholesterol esters depends upon **hormone-sensitive lipase** (p. 635).[53b, 53c] This enzyme is activated by cAMP-dependent phosphorylation and moves from the cytoplasm to the surfaces of lipid droplets in response to catecholamines and other lipolytic hormones. Fatty acids are a major fuel for aerobic cells. Their conversion to acyl-CoA derivative and oxidation to CO_2 by beta oxidation (Fig. 17-1) and other pathways are discussed in Chapter 17 (pp. 939 – 950).

B. The Biosynthesis of Fatty Acids and Their Esters

The synthesis of fatty acids two carbon atoms at a time from acetyl-CoA has been considered in Chapter 17 and is outlined in Fig. 17-12. In this pathway, which resembles the β oxidation sequence in reverse, the products are saturated fatty acids with an even number of carbon atoms as shown in Fig. 21-2. In this section, we will consider some of the factors that lead to variations in the chain lengths and types of fatty acids.

1. Fatty Acid Synthases

Both bacteria and plants have separate enzymes that catalyze the individual steps in the biosynthetic sequence (Fig. 17-12). The fatty acyl group grows while attached to the small acyl carrier protein (ACP).[54–58] Control of the process is provided, in part, by the existence of isoenzyme forms. For example, in *E. coli* there are three different β-oxoacyl-ACP synthases. They carry out the transfer of any acyl primer from ACP to the enzyme, decarboxylate malonyl-ACP, and carry out the Claisen condensation (steps *b*, *e*, and *f* in Eq. 17-12)[58a – e] One of the isoenzymes is specialized for the initial elongation of acetyl-ACP and also provides feedback regulation.[58c] The other two function specifically in synthesis of unsaturated fatty acids.

In a few bacteria and protozoa and in higher animals the fatty acid synthase consists of only one or two multifunctional proteins. That from animal tissues contains six enzymes and an acyl carrier protein (ACP) domain as well. The human enzyme contains 2504 amino acid residues organized as a series of functional domains.[59–59b] Pairs of the 272-kDa chains associate to form 544-kDa dimers. The complex protein may have arisen via an evolutionary process involving fusion of formerly separate genes.[60] The enzyme contains an ACP-like site with a bound 4'-phosphopantetheine near the C terminus as well as a cysteine side chain near the N terminus in the second acylation site. Since the two –SH groups can be crosslinked by dibromopropanone,[61,62] an antiparallel linear arrangement of the two chains was proposed.[63–65] Locations of the six enzymatic activities in each chain are indicated on page 1187. According to this picture,

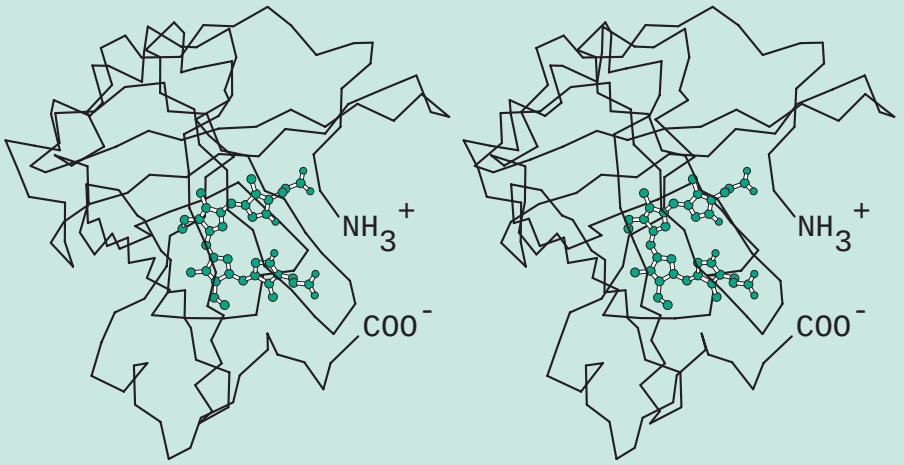
BOX 21-A LIPOCALINS, FATTY ACID-BINDING PROTEINS, AND LIPOPHORINS

Small hydrophobic molecules, which might easily bind in biologically undesirable ways, are chaperoned in animals, plants, and bacteria by binding proteins that provide hydrophobic cavities or crevices appropriate for holding these molecules in readily releasable forms. The **lipocalins**, most of which are extracellular proteins, have a conserved structural motif consisting of an 8-stranded β barrel arranged as two stacked orthogonal sheets with a C-terminal α helix that blocks one end. The other end is able to open and allow a small hydrophobic molecule to bind in the internal cavity[a–c] (see figure). Only three short amino acid sequences are conserved within a large family of lipocalins[b,d] which includes **plasma retinol-binding protein**,[e] mammalian **odorant-binding proteins**,[f] **α-lactalbumin**, **apolipoprotein D**,[a] and the blue biliverdin-binding protein **insecticyanin** of insect hemolymph.[g,h] Most lipocalins are soluble, but some such as the plasma **α1-microglobulin**,[i] which plays a role in the immune system (Chapter 31), have additional functions that require them to bind to other proteins or to cell surfaces.[j] The **gelatinase-associated lipocalin** of human neutrophils binds bacterially derived *N*-formylpeptides that act as chemotactic agents (Chapter 19) and induces release of materials from intracellular granules.[c] A few lipocalins have enzymatic activity. For example, **prostaglandin D synthase** is both an enzyme and a carrier of bile pigments and thyroid hormones.[k] Most lipocalins have been found in higher animals, but at least a few bacterial proteins belong to the family.[d] One is the 77-residue *E. coli* outer membrane lipoprotein.[a]

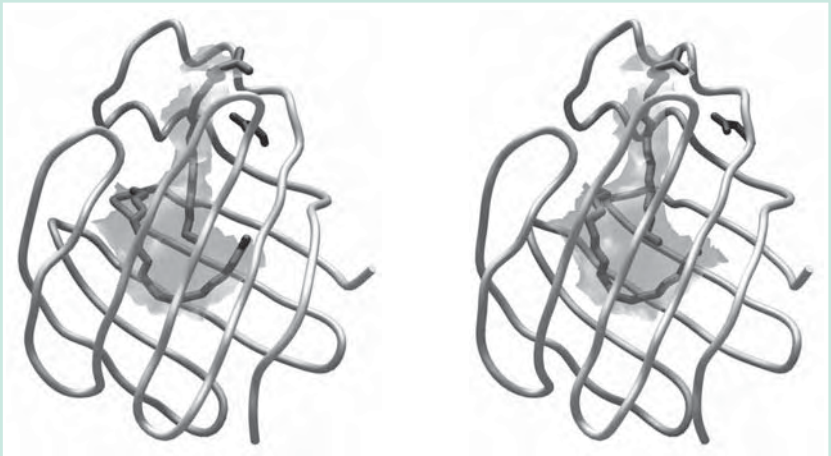
A related family of proteins are represented by **fatty acid-binding proteins**[l–o] and by the intracellular **retinol-** and **retinoic acid-binding proteins** (see also Box 22-A).[p] These are 10-stranded antiparallel β-barrels with two helices blocking an end (see Figure).

A third group of lipid-binding proteins have a four-helix bundle structure. They include the insect **lipophorins**, which transport diacylglycerols in the hemolymph (see main text), and nonspecific lipid carriers of green plants.[q] An 87-residue four-helix protein with a more open structure binds acyl-coenzyme A molecules in liver.[r]

A small 98-residue sterol-binding protein from



Stereoscopic view of an α-carbon model of an insecticyanin subunit with the bound biliverdin. The N and C termini are labeled NH_3^+ and COO^-, respectively. The positions of several amino acid residues are indicated. From Holden *et al.*[g] Courtesy of Hazel Holden.



Structure of a crystalline fatty acid-binding protein from liver with two molecules of bound oleate (dark rods). The lower molecule is more deeply embedded in the protein and more tightly bound than the second molecule, which is closer to the outer surface of the protein. Semitransparent grey marks the solvent-accessible surface of the binding cavity. An unknown molecule, perhaps butanoic acid (as modeled), binds also at top of the protein. See Thompson *et al.*[n] Courtesy of Leonard Banaszak.

BOX 21-A (continued)

the fungus *Phytophthora*, an agriculturally important plant pathogen, has a very different folding pattern. The sterol binds into a cavity formed by six helices and two loops. The protein is not only a sterol carrier but an **elicitin**, which induces a defensive response in the invaded plant. The function of the protein for the invader may be to acquire sterols for the fungus, which is unable to synthesize them.[s]

Many larger lipid carrier proteins are known. The 476-residue plasma cholesteryl ester transfer protein is discussed briefly in Chapter 22. Plasma phospholipid transfer proteins are of similar size.[t,u] A 456-residue human phospholipid-binding protein interacts with the lipopolysaccharide of the surfaces of gram-negative bacteria (Fig. 8-30) and participates in the immune response to the bacteria. It has an elongated boomerang shape with two cavities, both of which bind a molecule of phosphatidylcholine. Other plasma lipid transfer proteins may have similar structures.[v]

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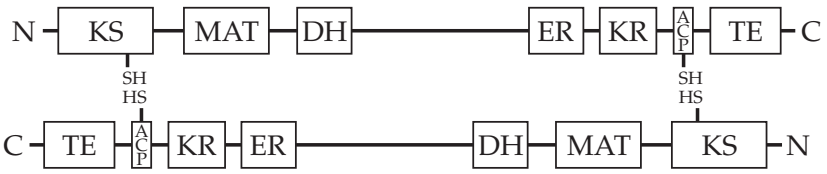
the ACP domain of one chain would cooperate with the β-oxacyl synthase (KS) domain of the second chain. However, more recent studies indicate greater flexibility with the ACP, MAS, and KS domains of a single chain also being able to function together.[58,62] Animal fatty acid synthases produce free fatty acids, principally the C_{16} palmitate. The final step is cleavage of the acyl-CoA by a thioesterase, one of the six enzymatic activities of the synthase.

Yeast fatty acid synthase[66,67] has an $\alpha_6\beta_6$ structure where the 208-kDa α subunit contains the ACP-like site, the active site –SH, and three catalytic activities. The 220-kDa β subunit has five catalytic activities. The yeast enzyme contains the FMN thought to act as $FMNH_2$ in the second reduction step. As in bacteria, the products of the complex are molecules of acyl-CoA of chain lengths C_{14}, C_{16}, and C_{18}.[68]



Abbreviation	Enzymatic activity	Residue numbers
KS	β-Oxoacyl (ketoacyl) synthase	1 – 406
MAT	Malonyl and acetyl transferase	428 – 815
DH	Dehydratase	829 – 969
Central region	Structural core (?)	970 – 1629
ER	Enoyl reductase	1630 – 1850
KR	β-Oxoacyl (ketoacyl) reductase	1870 – 2100
ACP	Acyl carrier protein	2114 – 2190
TE	Thioesterase	2200 – 2505

Organization of eukaryotic fatty acid synthase. From Joshi *et al.*[61]

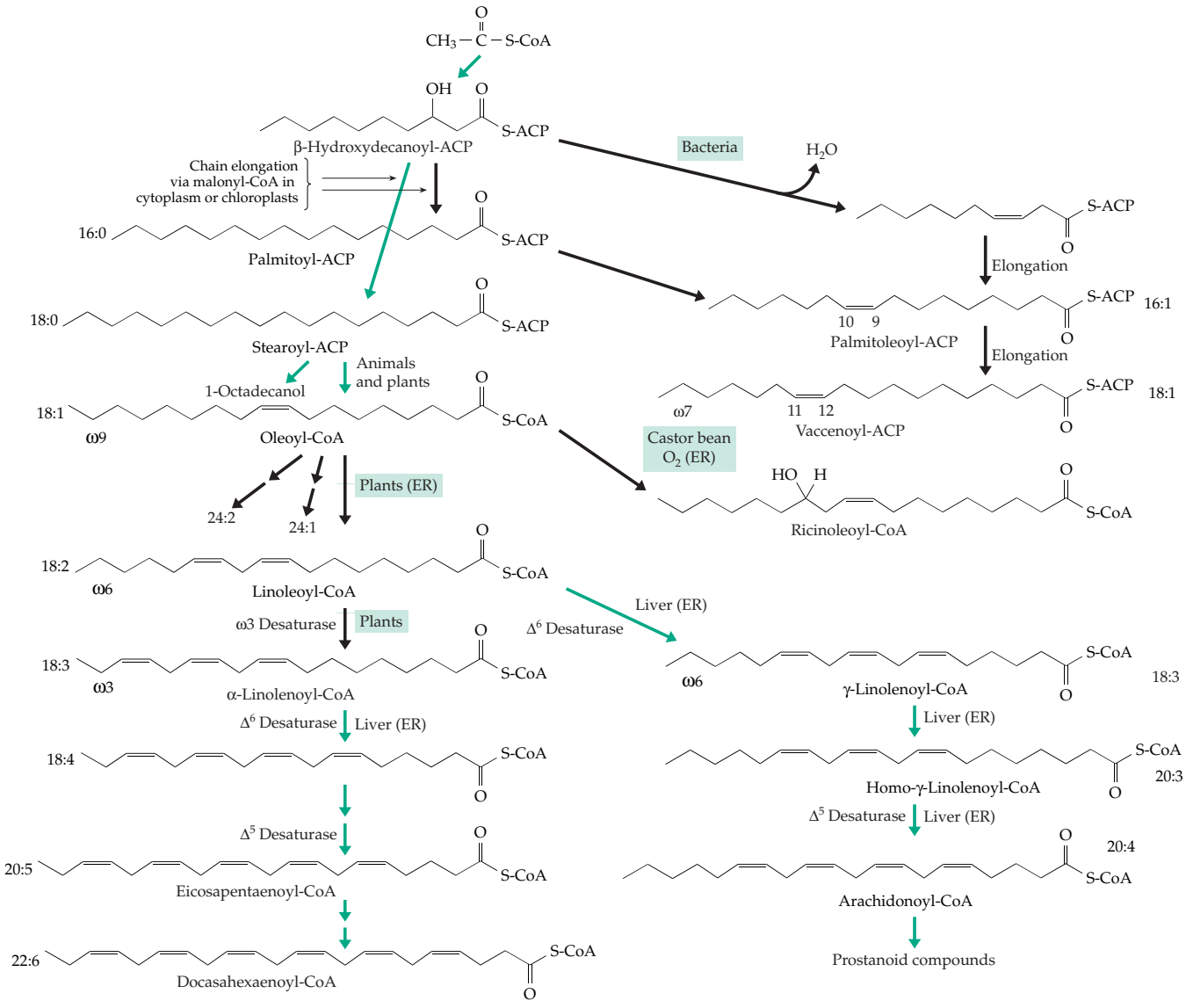


Figure 21-2 Some biosynthetic reactions of fatty acids. Green arrows indicate transformations carried out by the human body.

2. Control of Chain Length

The length of fatty acid chains is controlled largely by the enzymatic activity that releases the fatty acyl-CoA molecules or the free fatty acids from the synthase complex. In the animal enzymes the thioesterase, which is built into the synthase molecule, favors the release primarily of the 16-carbon saturated (16:0) palmitic acid. However, in mammary glands and in the uropygial glands (preen glands) of waterfowl shorter chain fatty acids predominate. These are released from the synthase by reaction with a second thioesterase, a 29-kDa protein[69,70] that catalyzes the otherwise premature release of shorter fatty acids. Cow's milk contains significant amounts of C_4-C_{14} acids as well as those with longer chains, whereas rabbit's milk contains largely C_8 to C_{10} fatty acids.[71]

In plants most biosynthesis occurs in the chloroplasts or in the protoplastids of seeds.[72–75] There are two different synthase systems in chloroplasts, one that forms primarily the 16:0 palmitoyl-ACP and the other the 18:0 stearoyl-ACP. Hydrolysis of the palmitoyl-ACP releases palmitate, one major product of chloroplasts. However, the stearoyl-ACP is desaturated to oleoyl-ACP[75a] before hydrolysis to free oleate or conversion to oleoyl-CoA. In many species oleic acid is almost the sole fatty acid exported by the chloroplasts. However, it undergoes a variety of modification reactions in the plant cytosol.

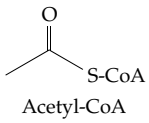
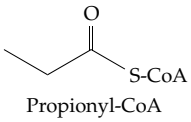
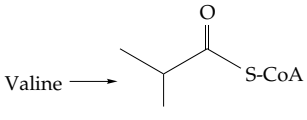
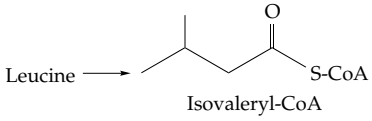
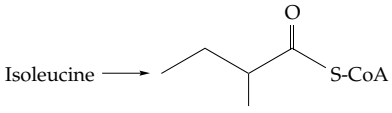
Plants, animals, and fungi all have fatty acid elon-

gation systems in the endoplasmic reticulum. Using malonyl-CoA and NADPH,[76,77] chain lengths of fatty acids may be increased to C_{20} to C_{26}. Elongation of fatty acids can also occur in mitochondria by reactions that are essentially the reverse of β oxidation. The only deviation from an exact reversal of oxidation is the use of NADPH as the reductant for enoyl-CoA reductase. Elongation of fatty acids in the *outer* membrane of mitochondria, followed by transport of the elongated chains into the mitochondria, may even constitute another shuttle for transport of reducing equivalents from NADH into mitochondria (Chapter 18, Section D).[78] Elongation reactions may also occur in peroxisomes.[78a]

3. Starter Pieces and Branches

Acetyl-CoA is most often the primer or starter piece for fatty acid synthesis, but butyryl-CoA is a better primer for rabbits. Butyryl-CoA arises from acetyl-CoA by a reversal of β oxidation, the necessary enzymes occurring in significant amounts in the cytosol.[79] If either acetyl-CoA or butyryl-CoA is the starter piece, chain elongation via malonyl-CoA (Fig. 18-12) leads to fatty acids with an even number of carbon atoms. However, degradation of the branched chain amino acids valine, isoleucine, and leucine creates a series of branched starter pieces (Table 21-3), whose utilization leads to formation of branched fatty acids of the iso and anteiso series. These are found in bacteria, in the lipids of tobacco and wool, in the "sound lens" of echo-locating porpoises,[80] and in many other materials.[81] Propionyl-CoA serves as an intermediate for introduction (via methylmalonyl-CoA) of branches at various other points in a fatty acid chain.[82] For example, 2*R*- and 4*R*-methylhexanoic acids, 2,4,6,8-tetramethyldecanoic acid, and a variety of other branched chain acids are esterified with long- chain alcohols (mainly 1-octadecanol) to form the waxes of the preen glands of ducks and geese.[83] The C_{32} **mycocerosic acid** of *Mycobacterium tuberculosis* is also formed using both malonyl-CoA and methylmalonyl-CoA for chain elongation.[84] This acid is present in mycobacterial cell walls esterified with long-chain diols (Box 21-C).[85]

TABLE 21-3
Starter Pieces for Biosynthesis of Fatty Acids

Starter piece	Fatty acid products
 Acetyl-CoA	Acid with even number of carbon atoms
 Propionyl-CoA	Acid with odd number of carbon atoms
 Isobutyryl-CoA	Iso series (even)
 Isovaleryl-CoA	Iso series (odd)
	Anteiso series (odd)

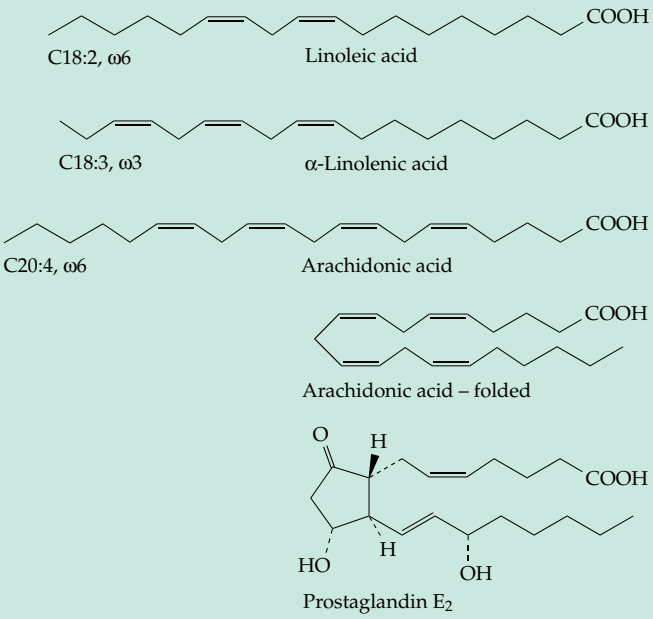
4. Synthesis by the Oxoacid Chain Elongation Process

The carbon skeleton of leucine is derived from that of valine by elongation of the corresponding oxoacid by a single carbon atom that is derived from acetyl-CoA (Fig. 17-18). At least in plants some branched-chain fatty acids of medium length are formed via the same oxoacid elongation process, which extends the chain one carbon atom at a time using 2-oxobutyrate as a starting compound. The same process can be used to form medium length straight-chain fatty acids (up to ~C_{12}) with either an odd or an even number of carbon atoms.[86]

2-Oxoglutarate can also serve as a starter piece for elongation by the oxoacid pathway. Extension by three carbon atoms yields 2-**oxosuberate** (Eq. 21-1). This dicarboxylate is converted by reactions shown in Eq. 24-39 into biotin and in archaebacteria into the coenzyme 7-mercaptoheptanoylthreonine phosphate (HTP), Eq. 21-1.[87] Lipoic acid is also synthesized from a fatty acid, the eight-carbon octanoate.[88,89] A fatty acid synthase system that utilizes a mitochondrial ACP may have as its primary function the synthesis of octanoate for lipoic acid formation.[90] The mechanism of insertion of the two sulfur atoms to form lipoate (Chapter 15) is uncertain. It requires an iron–sulfur protein[91,91a,b] and is probably similar to the corresponding process in the synthesis of biotin (Eq. 24-39)[92-93a] and in formation of HTP (Eq. 21-1). One component of the archaebacterial cofactor **methanofuran** (Chapter 15) is a tetracarboxylic acid that is formed from 2-oxoglutarate by successive condensations with two malonic acid units as in fatty acid synthesis.[94]

BOX 21-B THE ESSENTIAL FATTY ACIDS

In 1930, George and Mildred Burr reported that the C18:2 ($\Delta^{9,12}$) **linoleic acid**, a fatty acid of exclusively plant origin, cured a disease condition observed in rats raised on a highly purified fat-free diet.[a,b] These animals grew poorly, developed a scaly dermatitis, and suffered kidney damage and impaired fertility. The symptoms could be prevented if 1% of the dietary energy was provided by linoleic acid. This C18:2 fatty acid can be converted in animals into a series of other fatty acids by chain elongation and desaturation. All of this series have a double bond six carbon atoms from the $-CH_3$ terminus and form an ω6 (or n-6) family.[c]



ω6: C18:2 → C18:3 → C20:3 → C20:4 → C22:4 → C22:5

Arachidonate

↓ ↓

PGE_1, $PGF_{1\alpha}$ PGE_2, $PGF_{2\alpha}$

The major known essential function of linoleic acid is conversion to the C20:4 ($\Delta^{5,8,11,14}$) **arachidonic acid**, the major precursor to prostaglandins and other prostanoid compounds (Section D). This conversion occurs in infants as well as adults,[d] but the rate may not always be adequate, and arachidonic acid is usually classified as an essential fatty acid. It is not clear whether linoleic acid has any essential role of its own. However, while arachidonic acid can be converted into the prostaglandins designated PGE_2 and $PGF_{2\alpha}$, linoleic acid can also give rise, via the C20:30 **dihomolinolenic acid**, to PGE_1 and $PGF_{1\alpha}$ (see Eq. 21-16). The C18:3 ($\Delta^{9,12,15}$) **α-linolenic acid**, another plant acid, can partially replace linoleic acid and can be converted into PGE_3 and $PGF_{3\alpha}$.

ω3: C18:3 → C18:4 → C20:4 → C20:5 → C22:5 → C22:6

↓ ↓ ↓

PGE_3, $PGE_{3\alpha}$ PG_4 PG_5

Thus, it is not surprising that the three acids are not completely equivalent.[e–g]

Recent interest has focused on the C20:5 **eicosapentaenoic acid** (EPA) and the C22:6 **docosahexaenoic acids** (DHA). These ω3 (or n-3) polyunsaturated acids are formed from linolenic acid by marine algae and are found in fish oils.[h] The C22:5 and C22:6 acids can be converted to prostaglandins of the PG_4 and PG_5 series. DHA together with the ω6 C22:4 acid constitutes over 30% of the fatty acids in brain phospholipids. In the retina DHA accounts for over 60% of the total in the rod outer segments (Fig. 23-40). DHA may be formed in the human body from α-linolenic acid obtained from plant sources (Fig. 21-2). However, the rate of synthesis may be inadequate especially in infants and in persons of old age.[i–m] Deficiency of either EPA or DHA may lead to poor brain development during prenatal and infant life. Formation of new synapses between neurons as well as growth of new neurons in some areas of the brain is associated with thinking and with memory formation (Chapter 30). Therefore, a lack of the essential ω3 and ω6 fatty acids may contribute to mental deterioration in older adults. Eskimo populations, which consume large amounts of fish, have a very low incidence of coronary heart disease. An inverse relation between fish consumption and heart disease has also been demonstrated in other populations.[n] Inclusion of fish oils to 20–30% of total caloric intake in the diet causes a marked decrease in plasma triacylglycerols and very low density lipoproteins (VLDL).[o] This effect has been attributed to the altered composition of the prostanoid compounds known as thromboxanes and prostacyclins (PGI). For similar reasons the ω3 acids may have an antiinflammatory effect.[p,q] Administration of fish oil to patients with kidney disease has proved beneficial[r] and may decrease risk of some cancers.[s] However, a diet high in ω3 fatty acids has also been reported to increase cancer risk.[f] Long-chain ω3 fatty acids may protect against sudden death from heart disease.[t] They may promote lateral phase separation within membranes to form regions low in cholesterol (see references 95a and 119d).

BOX 21-B (continued)

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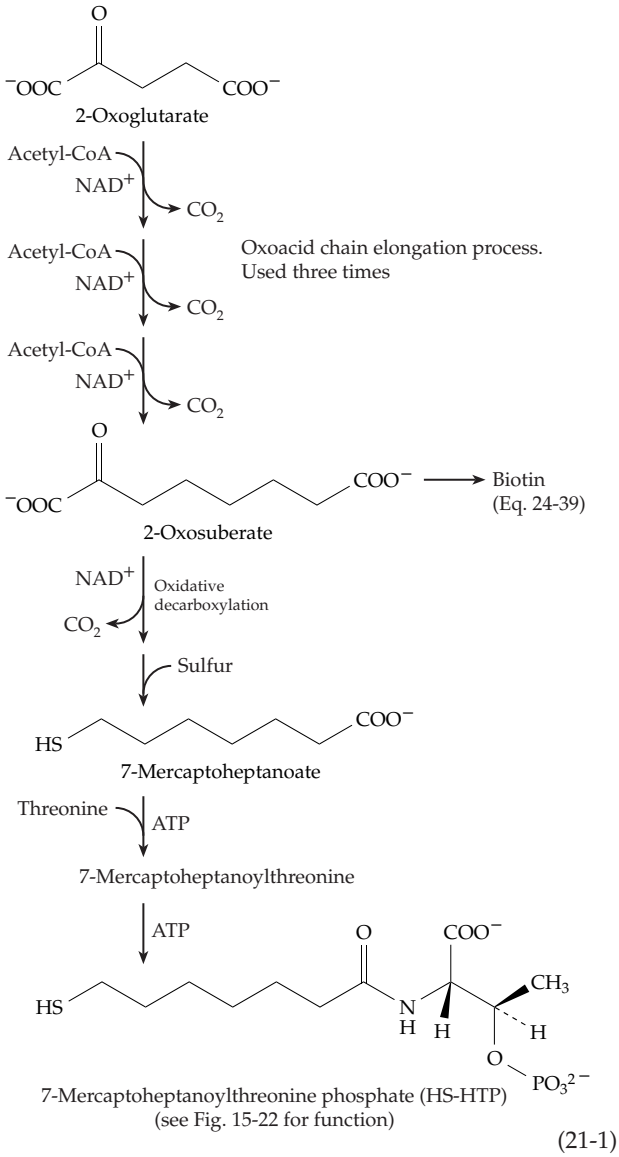
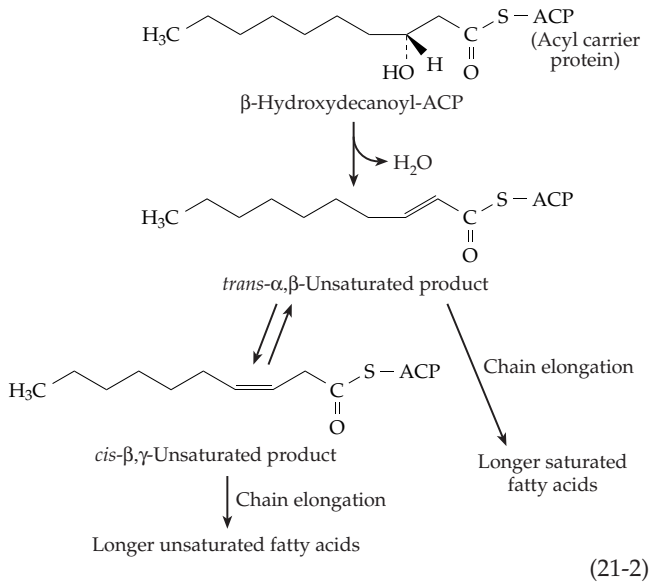


(21-1)

5. Unsaturated Fatty Acids

Fatty acids containing one or more double bonds provide necessary fluidity to cell membranes[95,95a] and serve as precursors to other components of cells. Significant differences in the methods of introduction of double bonds into fatty acids are observed among various organisms. Bacteria such as *E. coli* that can live anaerobically often form **vaccenic acid** as the principal unsaturated fatty acid. It is formed by chain elongation after introduction of a cis double bond at the C_{10} stage of synthesis. The bacteria possess a **β-hydroxydecanoyl thioester dehydratase,** which catalyzes elimination of a β-hydroxyl group to yield primarily the *cis*-β,γ rather than the *trans*-α,β-unsaturated product (Eq. 21-2).[96] The mechanism may resem-



(21-2)

ble that of enoyl hydratase (Eq. 13-7), the indicated *trans*-α,β-unsaturated intermediate (enzyme bound) being isomerized to the *cis*-β,γ-unsaturated product through an allylic rearrangement. The product can then be elongated to the C_{16} **palmitoleoyl-ACP** and C_{18} **vaccenoyl-ACP** derivatives (Fig. 21-2, right side). However, dehydration of β-hydroxydecanoyl-ACP lies at a branch point in the biosynthetic sequences. The *trans*-α,β-unsaturated fatty acyl compound lies on the usual route of chain elongation to palmitoyl-CoA (left side, Fig. 21-2).

In higher plants, animals, protozoa, and fungi, saturated fatty acids are acted upon by **desaturases** to introduce double bonds, usually of the cis (Z) configuration. The substrates may be fatty acyl-ACP, fatty acyl-CoA molecules, membrane phospholipids,[97] or glycolipids.[98] The Δ^9 desaturase, isolated from liver or from yeast, converts stearoyl-CoA to oleoyl-CoA (Eq. 21-3).[99–102] This membrane-associated enzyme system utilizes NADH as a reductant, passing electrons via cytochrome b_5 reductase and cytochrome b_5 itself to the desaturase.[103,104] The pro-*R* hydrogens are removed at both C-9 and C-10.

$$O_2 + 2\ H^+ + 2\ e^- + \text{stearoyl-CoA} \rightarrow \text{oleoyl-CoA} + 2\ H_2O \qquad (21\text{-}3)$$

In plants a similar enzyme catalyzes formation of the first double bond in a fatty acyl group converting stearoyl-ACP into oleoyl-ACP in the chloroplasts.[72,75a,105–108] The soluble Δ^9 stearoyl-ACP desaturase has a diiron-oxo active site (Fig. 16-20, B, C).[109,110] Electrons are donated from light-generated reduced ferredoxin (see Chapter 23). In addition to the Δ^9 desaturase both plants and cyanobacteria usually desaturate C_{18} acids also at the Δ^{12} and Δ^{15} positions and C_{16} acids at the Δ^7, Δ^{10}, and Δ^{13} (ω3) positions.[111,112] Desaturation of oleate occurs primari-

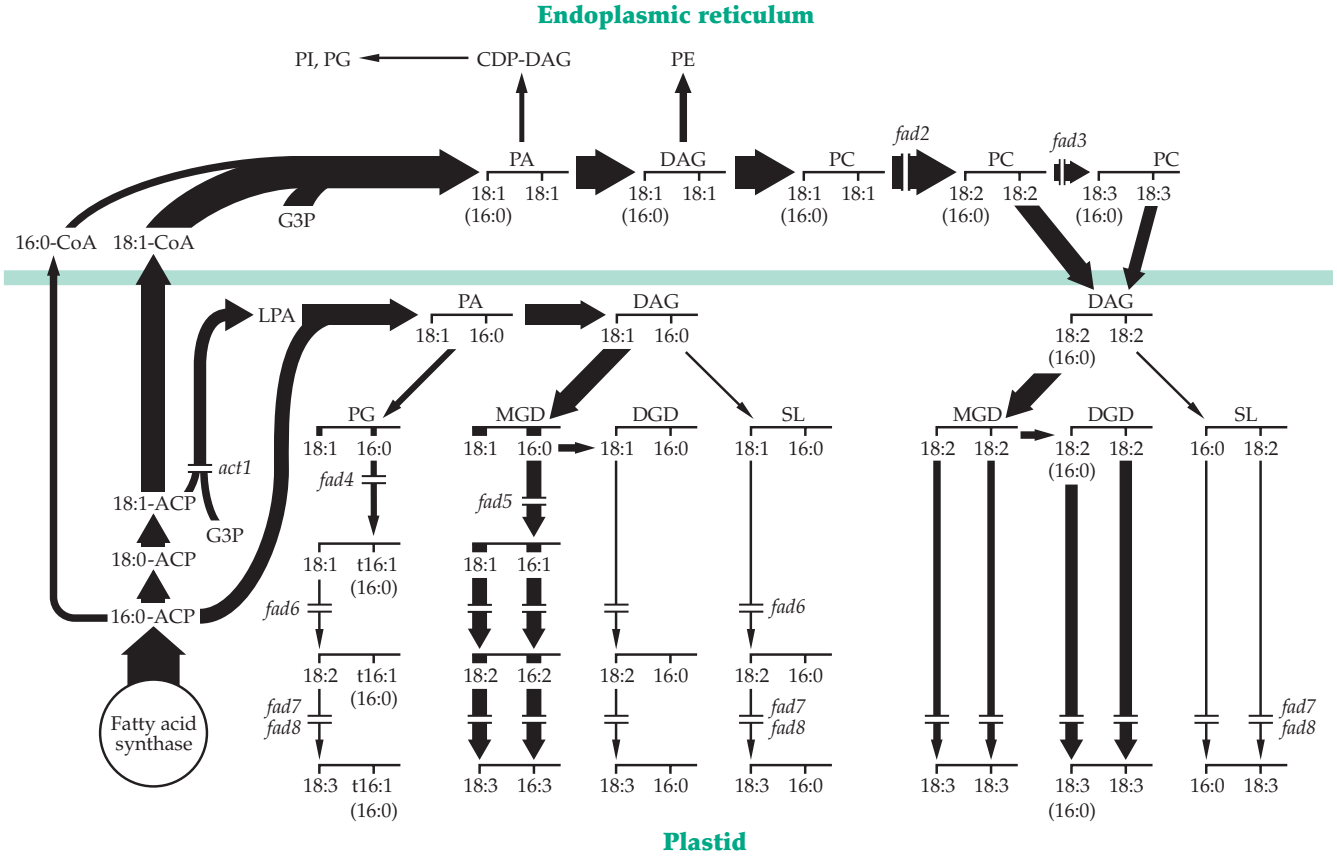


Figure 21-3 Major pathways of synthesis of fatty acids and glycerolipids in the green plant *Arabidopsis*. The major site of fatty acid synthesis is chloroplasts. Most is exported to the cytosol as oleic acid (18:1). After conversion to its coenzyme A derivative it is converted to phosphatidic acid (PA), diacylglycerol (DAG), and the phospholipids: phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylglycerol (PG), and phosphatidylethanolamine (PE). Desaturation also occurs, and some linoleic and linolenic acids are returned to the chloroplasts. See text also. From Sommerville and Browse.[106] See also Figs. 21-4 and 21-5. Other abbreviations: monogalactosyldiacylglycerol (MGD), digalactosyldiacylglycerol (DGD), sulfolipid (SL), glycerol 3-phosphate (G3P), lysophosphatidic acid (LPA), acyl carrier protein (ACP), cytidine diphosphate-DAG (CDP-DAG).

ly in the ER after conversion of the free acid to its coenzyme A derivative or to phosphatidylcholine. Consecutive introduction of two double bonds forms linoleoyl-CoA (18:2, $\Delta^{9,12}$) and linolenoyl-CoA (18:3, $\Delta^{9,12,15}$; see Fig. 21-3).[108] All double bonds are cis. The membrane lipids of chloroplasts contain both linoleic and linolenic acids, which have apparently been returned to the chloroplasts from the cytosol[72] as indicated in Fig. 21-3. Plants grown at colder temperatures have a higher content of these trienoic acids than those grown at higher temperature.[72a]

The origin of ricinoleic acid, an abundant constituent of castor beans, is also shown in Fig. 21-2. It is formed by an **oleate hydroxylase** that has an amino acid sequence similar to those of oleate desaturases.[113] Both hydroxylation and desaturation are reactions catalyzed by diiron centers.[114] Other fatty acid hydroxylases act on the alpha[115] and the omega positions. The latter are members of the cytochrome P450 family.[116,117]

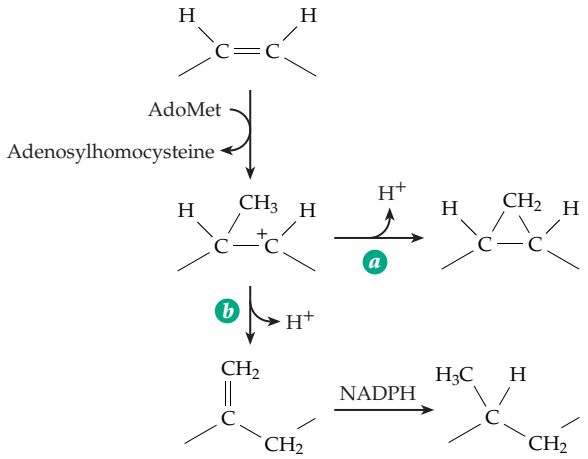
The conversion of oleoyl-CoA to linoleoyl-CoA is accomplished by some insects[118] but does not take place in most animals. As a result of this biosynthetic deficiency, polyunsaturated fatty acids such as linoleic, linolenic, and the C_{20} arachidonic acid are necessary in the diet (Box 21-B). One essential function of linoleic acid is to serve as a precursor of **prostaglandins** and related **prostanoids** (Section D). Dietary linoleate is converted to its CoA derivative and then by sequential Δ^6 desaturation,[119] elongation, and then Δ^5 desaturation, to the 20:4 ($\Delta^{5,8,11,14}$) arachidonoyl-CoA (Fig. 21-2, lower right). These acids are referred to as ω6 because of the position of the last double bond. Linolenic acid can be converted in an analogous fashion to the CoA derivative of the 20:5 ($\Delta^{5,8,11,14,17}$ ω6) eicosapentaenoic acid (**EPA**). The 22:6 docasahexaenoic acid (**DHA**; Fig. 21-2) is apparently formed by elongation of the 22:5 acyl-CoA to 24:5, desaturation, transfer to a peroxisome or mitochondrion, and β oxidation to shorten the chain.[95a]

These acids are very important in human nutrition.[119a–d] (See also Box 21-B.) In the absence of adequate essential fatty acids oleate is desaturated and elongated in a similar sequence to the unusual 20:3 ($\Delta^{5,8,11}$ ω9) acid. Vertebrate tissues also carry out desaturation at the Δ^4 and Δ^3 positions.[111] Lepidoptera, which synthesize a great diversity of pheromones, are rich in unusual desaturases such as the gland-specific acyl-CoA Δ^{11} desaturase of cabbage looper moths.[120]

Polyunsaturated fatty acids, containing 4, 5, or 6 double bonds and chain lengths of up to C_{36}, are found in phosphatidylcholine of vertebrate retinas.[121] Although the double bonds are rarely in conjugated positions in food fats and in animal bodies, some plants convert oleic or linoleic acid into fatty acids with as many as three or four conjugated double bonds.[121a] **Conjugated linoleic acid** (9*c*, 11*t* 18:2) can be formed from 11-*trans*-octadecenoate in the human body.[121b] This compound is also found in meat and dairy products. It has been reported to have anticancer properties[121c] and may be another beneficial dietary constituent. An isomerase isolated from red algae converts polyunsaturated acids into forms with conjugated double bonds. For example, arachidonic acid (5*Z*, 8*Z*, 11*A*, 14*Z*) is converted to (5*Z*, 7*E*, 9*E*, 14*Z*)-eicosatetraenoic acid.[122,123]

6. Cyclopropane Fatty Acids and Mycolic Acids

Fatty acids containing one or more cyclopropane rings are present in many bacteria (p. 381).[124,125] The extra carbon of the cyclopropane ring is added from *S*-adenosylmethionine (AdoMet) at the site of a cis double bond in a fatty acyl group of a phosphatidylethanolamine molecule in a membrane (Eq. 21-4).[126,126a] The same type of intermediate carbocation can yield either a cyclopropane fatty acid (Eq. 21-4, step *a*) or a methenyl fatty acid (Eq. 21-4, step *b*). The latter can be reduced to a branched fatty acid. This is an alternative way of introducing methyl branches that is used by some bacteria.[127]



(21-4)

Mycobacteria are rich in cyclopropane-containing fatty acids. These **mycolic acids** are major components of the cell walls and may account for 30% of the dry weight of the cells.[128] The most abundant mycolic acid of *M. tuberculosis* consists of C_{52} fatty acid containing two cyclopropane rings joined via a Claisen-type condensation with a C_{26} carboxylate fatty acid (Eq. 21-5). A similar mycolic acid formed by *M. smegmatis* has double bonds instead of cyclopropane rings as indicated below Eq. 21-5.[128,129] There are other variations. In place of a double bond or cyclopropane group there may be –OH, $-OCH_3$, C=O, epoxide, or CH_3[127]

Cyclopropane fatty acids are catabolized via β oxidation,[130] which is modified as in Eq. 21-6 when the chain degradation reaches the cyclopropane ring. The

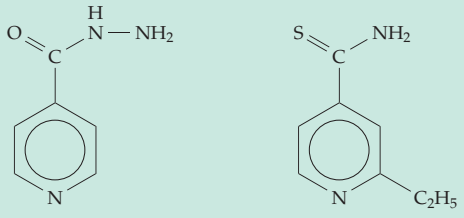
BOX 21-C TUBERCULOSIS

As many as one-third of the inhabitants of the earth are infected by *Mycobacterium tuberculosis*. For most the infection is dormant, but in some the slow-growing bacteria cause a progressive and deadly destruction of the lungs. There are still about three million deaths annually, and *M. tuberculosis* now, as in the past, kills more people then any other pathogen.[a,b] The development of drug-resistant strains of the bacterium and the threat of a world-wide resurgence of tuberculosis[c,d] has spurred new efforts to understand the unusual metabolism of mycobacteria and to develop new drugs. The complete 4.4 million base-pair sequence of the circular genome is known.[c] An unusually large fraction of the genes encode enzymes involved in synthesis and breakdown of lipids, including the synthesis of **mycolic acids** (Eq. 21-5) which are characteristic of mycobacteria.

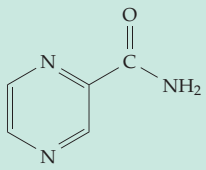
The mycobacterial cell wall, discussed in Chapter 8, contains mycolic acids bound covalently at the nonreducing ends of arabinogalactans that are attached to the inner peptidoglycan layer,[e] as well as phosphatidylinositol-anchored lipoarabinomannans. Other unusual lipids that are also present and account for some of the difficulty of treatment with antibiotics include esters of **mycocerosic acid** with long-chain diols known as phenolphthiocerols and phthiocerols.[f,fg]

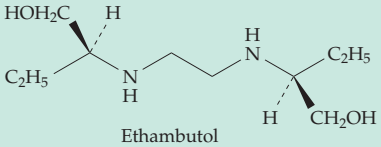
Streptomycin (Boxes 20-B, 20-H) was introduced into clinical use against tuberculosis in about 1943. However, resistant mutants always survived until newer drugs were developed. Isonicotinylhydrazide (**isoniazid**) is especially effective in combinations with suitable antibiotics and other drugs.[g] The four-drug combination isoniazid, rifampicin (Box 28-A), pyrazinamide, and ethambutol is often used. Nevertheless, bacteria resistant to all of these have developed.

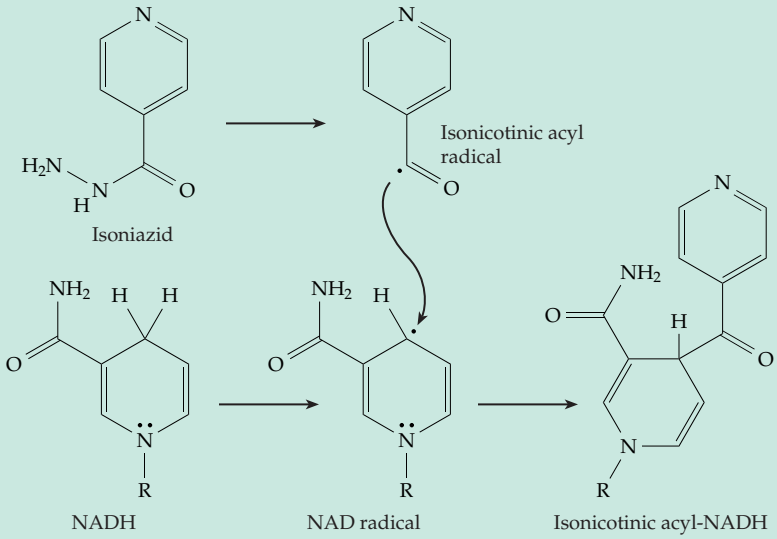
Although isoniazid has been in use for about 45 years, the enzyme that it inhibits has been recognized only recently. It is a specific NADH-dependent **enoyl reductase** involved in synthesis of mycolic acids.[h,i] The isoniazid must be activated by action of a bacterial catalase-peroxidase.[j,k] This enzyme may convert the drug to a reactive radical that combines with a NADH-derived radical to form an adduct in the active site of the enzymes. One possible reaction sequence follows.[h] However, the mechanisms are not clear.

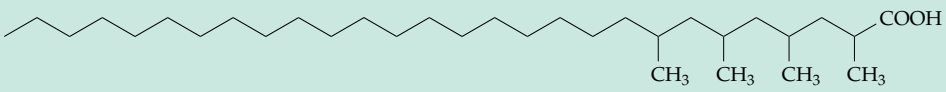

Isonicotinylhydrazide (Isoniazid)

Ethionamide


Pyrazinamide

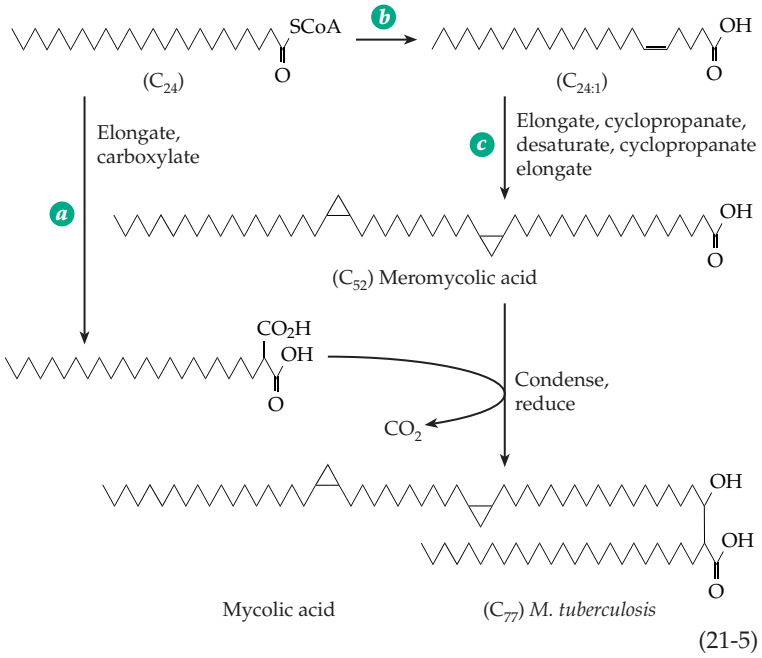

Ethambutol



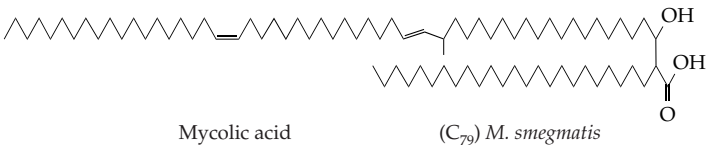

2*R*,4*R*,6*R*,8*R*-Tetramethyloctacosanoic acid (mycocerosic acid)

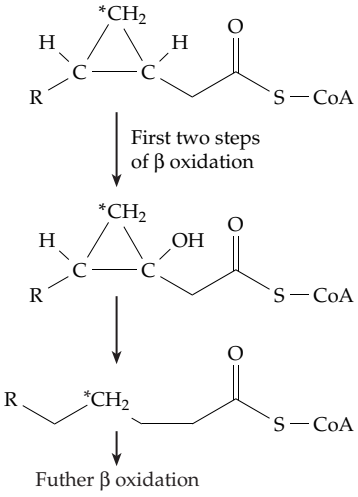
BOX 21-C (continued)

[a] Young, D. B. (1998) *Nature (London)* **393**, 515–516
[b] Venisse, A., Rivière, M., Vercauteren, J., and Puzo, G. (1995) *J. Biol. Chem.* **270**, 15012–15021
[c] Cole, S. T., and and 41 other authors. (1998) *Nature (London)* **393**, 537–544
[d] Iseman, M. D. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 2428–2429
[e] Scherman, M. S., Kalbe-Bournonville, L., Bush, D., Xin, Y., Deng, L., and McNeil, M. (1996) *J. Biol. Chem.* **271**, 29652–29658
[f] Fitzmaurice, A. M., and Kolattukudy, P. E. (1998) *J. Biol. Chem.* **273**, 8033–8039
[fg] Patterson, J. H., McConville, M. J., Haites, R. E., Coppel, R. L., and Billman-Jacobe, H. (2000) *J. Biol. Chem.* **275**, 24900–24906
[g] Blanchard, J. S. (1996) *Ann. Rev. Biochem.* **65**, 215–239
[h] Rozwarski, D. A., Grant, G. A., Barton, D. H. R., Jacobs, W. R. J., and Sacchettini, J. C. (1998) *Science* **279**, 98–102
[i] Baldock, C., Rafferty, J. B., Stuitje, A. R., Slabas, A. R., and Rice, D. W. (1998) *J. Mol. Biol.* **284**, 1529–1546
[j] Sherman, D. R., Mdluli, K., Hickey, M. J., Arain, T. M., Morris, S. L., Barry, C. E., III, and Stover, C. K. (1996) *Science* **272**, 1641–1643
[k] Wengenack, N. L., Lopes, H., Kennedy, M. J., Tavares, P., Pereira, A. S., Moura, I., Moura, J. J. G., and Rusnak, F. (2000) *Biochemistry* **39**, 11508–11513



(21-5)

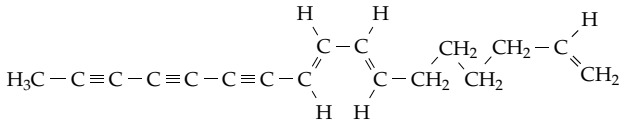




(21-6)

ring opening of the cyclopropanol derivatives occurs readily, even with mild nonenzymatic acid–base catalysis.

Another alteration of unsaturated fatty acids is the formation of acetylenic groups (–C≡C–). This apparently occurs by dehydrogenation of –CH=CH–. Examples of naturally occurring acetylenes are **crepenynic acid** (p. 381), **alloxanthin** (p. 1240), and the following remarkable hydrocarbon from the common cornflower *Centaurea cyanus*[131]:



7. The Lipids of Skin and Other Surfaces

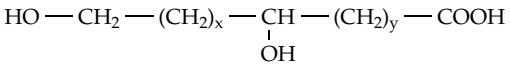
Special fatty materials are often secreted to form external surfaces of organisms.[81,132] An example already mentioned is the secretion of the uropygial glands (preen glands) of water fowl. In the goose 90% of this material is a wax consisting of monoesters of various acids with predominantly **1-octadecanol** as the long-chain fatty alcohol.[81] The latter is formed by reduction of stearoyl-CoA as indicated in Fig. 21-2. Waxes are also important constituents of marine environments, where they are not limited to surfaces. For example, copepods, which constitute a major component of marine zooplankton, may contain up to 70% of their dry weight as wax esters. Some marine animals, such as sperm whales, accumulate the same esters in major amounts as energy stores.[133]

Among the compounds present in the lipids of human skin are a variety of branched fatty acids, both free and combined. They may play a role in maintaining the ecological balance among microorganisms of the skin, and they also impart to each individual a

distinct odor or "chemical fingerprint."[132] Some of the skin lipids are incorporated into the cornified outer skin surface (Box 8-F).[134] See also Section C, 3.

Surface lipids of plants. The thick cuticle (Fig. 1-6) that covers the outer surfaces of green plants consists largely of waxes and other lipids but also contains a complex polymeric matrix of **cutin** (stems and leaves) or **suberin** (roots and wound surfaces).[135,135a] Plant waxes commonly have $C_{10} - C_{30}$ chains in both acid and alcohol components. Methyl branches are frequently present. A major function of the waxes is to inhibit evaporation of water and to protect the outer cell layer. In addition, the methyl branched components may inhibit enzymatic breakdown by microbes. Free fatty acids, free alcohols, aldehydes, ketones, β-diketones, and alkanes are also present in plant surface waxes. Chain lengths are usually $C_{20} - C_{35}$.[136] Hydrocarbon formation can occur in other parts of a plant as well as in the cuticle. Thus, normal **heptane** constitutes up to 98% of the volatile portion of the turpentine of *Pinus jeffreyi*.[81]

Cutin is largely a polyester with a high content of ω-hydroxypalmitic acid and related fatty acids, which are also hydroxylated at a second position:



Cutin monomers. C_{16} acids in which y = 8, 7, 6, or 5 and x + y = 13

This allows branching of the polymer. Monomers of other chain lengths as well as aromatic components related to lignin are also present and polymerized into a high molecular mass branched structure. Suberin is a more complex ligninlike polymer with a high content of phenolic constituents[135] such as vanillin (Fig. 25-8).

Formation of hydrocarbons. Alkanes and alkenes occur in plants, in preen gland secretions, and in insects. The alkanes of plant cuticle are thought to be formed by elongation of a C_{16} acid followed by loss of the carboxyl group. The mechanisms are not obvious. However, these hydrocarbons are often two carbon atoms shorter than the starting fatty acid. The pathway between them might begin by α-oxidation to form an α-peroxy acid which would decarboxylate to form an aldehyde, a reaction similar to that of Eq. 15-36. Alternatively, a long-chain acyl-CoA may be reduced directly to an aldehyde. In fact, when suitable inhibitors are present aldehydes do accumulate in tissues that are forming hydrocarbons.[137] Conversion of an aldehyde intermediate to an alkane may occur by **decarbonylation** (loss of CO). This has been demonstrated in pea (*Pisum sativum*) leaves,[138] in uropygial glands,[139] in flies, and in a colonial green alga, *Botyrococcus braunii*.[138] In the last case 32% of the dry weight of the cells is C_{27}, C_{29}, and C_{31} hydrocarbons. They appear to be formed by action of a decarbonylase that apparently contains a cobalt porphyrin.[137] Plants require cobalt for growth, but an enzymatic function has not previously been established.

In contrast, the sex pheromone of the female housefly is (Z)-9-tricosene, a hydrocarbon apparently formed by an oxidative decarboxylative process from a precursor aldehyde by an enzyme that requires NADPH and O_2 and is apparently a cytochrome P450.[140] Oxidative deformylation by a cytochrome P450 converts aldehydes to alkenes, presumably via a peroxo intermediate.[117] Formation of an alkene by decarboxylation has also been proposed,[141] but a mechanism is not obvious.

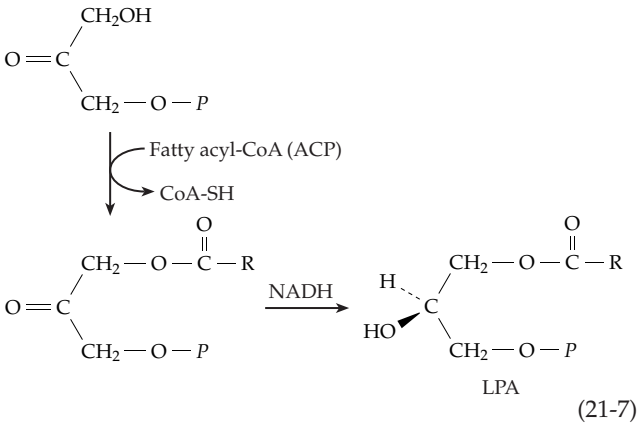
Insect waxes, hydrocarbons, and pheromones. Surface lipids of many insects contain esters of long-chain (as long as 66 carbon atoms) alcohols and long-chain acids.[142] On the other hand, waxes of the tobacco hornworm consist largely of 11- and 12-oxo derivatives of a C_{28} alcohol, which may be esterified to short-chain acids.[142] A major hydrocarbon of cockroaches is 6,9-heptacosadiene.[81]

Insects communicate through the use of a great variety of volatile pheromones. As mentioned in Chapter 8, Section A,1, some moths utilize acetate esters of various isomers of Δ^7 and Δ^{11} unsaturated C_{14} fatty acids as sex pheromones. Some other moths convert the *trans*-11-tetradecenyl acetate into the corresponding C_{14} aldehyde or alcohol, while others use similar compounds of shorter ($C_{11} - C_{12}$) chain length.[143] Some ants use ketones, such as 4-methyl-3-heptanone, as well as various isoprenoid compounds and pyrazines as volatile signaling compounds.[144] Other insects also utilize isoprenoids,[145] alkaloids,[146] and aromatic substances as pheromones.

C. Synthesis of Triacylglycerols, Glycolipids, and Phospholipids

Reduction of dihydroxyacetone phosphate yields *sn*-glycerol 3-phosphate, the starting compound for formation of the glycerol-containing lipids (Fig. 21-4 step *a*).[146a,b] Transfer of two acyl groups from ACP or CoA to the hydroxyl groups of this compound (steps *b* and *c*) yields 1,2-diacylglycerol 3-phosphate (phosphatidic acid). Two different acyltransferases are required.[147] Unsaturated fatty acids are incorporated preferentially into the 2-position. The intermediate 1-acyl-*sn*-glycero-3-phosphate, often called **lysophosphatidic acid** (LPA), is formed in excess in activated platelets and has a variety of signaling activities.[148,149] LPA for signaling is derived by turnover of existing phospholipids. An alternative route of LPA formation in liver is the transfer of one acyl group onto dihy-

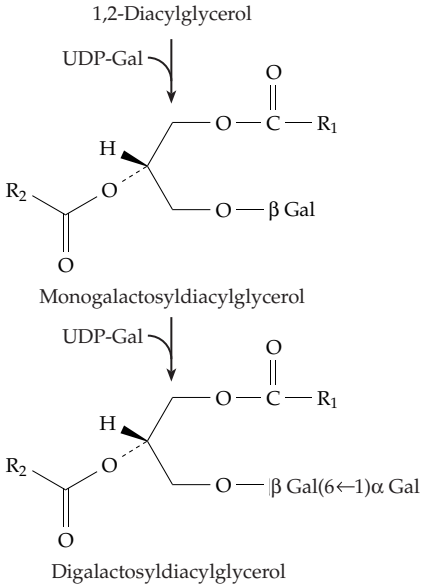
droxyacetone phosphate and reduction prior to addition of the second acyl group (Eq. 21-7).



(21-7)

Phosphatidic acid lies at a metabolic branch point. On the one hand, the phospho group can be removed by a specific phosphatase (step *d*)[150] and another acyl group (most often an unsaturated acyl group) may be transferred onto the resulting diacylglycerol (DAG, diglyceride, step *e*)[150a,b] to form a **triacylglycerol** (triglyceride). Alternatively, the phosphatidic acid may be converted to a **CDP-diacylglycerol** (step *g*), a key intermediate in phospholipid synthesis both in eukaryotes and in bacteria.[151] Not only can phosphatidic acid be hydrolyzed to 1,2-diacylglycerols, but the reverse process can occur by action of a kinase. This presumably permits recycling of the diacylglycerol formed by turnover of membrane phospholipids.[152]

Diacylglycerols can also be converted to a variety of glycolipids such as the **galactolipids** of chloroplasts (Eq. 21-8). See also Chapter 8. These are the major lipids of photosynthetic membranes.[98,153–155] Some bacteria, e.g., the mycoplasma *Acholeplasma laidlawii*, contain both monoglucosyl- and diglucosyl-DAG. Changes in the ratio of these two membrane components may regulate the phase equilibrium between bilayer and nonbilayer forms.[156] 1,2-Diacylglycerol can also react with UDP-sulfoquinovose (Eq. 20-12) to form the characteristic sulfolipid of chloroplasts.[154]



(21-8)

In animals a principal regulatory point for lipid synthesis is in the activation of acetyl-CoA carboxylase by citrate (Fig. 17-20).[156a,b] Beyond that, a complex hormonal control is exerted on both biosynthesis and the catabolism of triglycerides stored in liver and adipose tissues.[157] For example, adrenaline and glucagon, by stimulating production of cAMP, stimulate acetyl-CoA carboxylase,[158] activate lipases that cleave triacylglycerols, and mobilize depot fats.[159] Insulin, on the other hand, promotes lipid storage. It increases the activity of the enzymes of lipogenesis from the ATP-dependent citrate cleavage enzyme (Eq. 13-39) and inhibits cAMP production, thus blocking lipolysis within cells. At the transcriptional level sterols bind to activator proteins (**sterol regulatory element binding proteins,** SREBPs) and activate genes for acetyl-CoA carboxylase[158] and for stearoyl-CoA desaturases.[160] Fatty acid synthases, which play a central role in lipid formation, are controlled by both hormonal and nutritional factors at the transcriptional[161] and translational[162] levels. Environmental factors also have indirect effects. For example, the Δ^9 fatty acid desaturase activity of poikilothermic (cold-blooded) animals is increased at low temperatures. The resulting increased synthesis of unsaturated fats leads to increased fluidity of the membrane bilayer.[163] As mentioned on p. 1193; the same is true for green plants.

1. Phospholipids

Bacterial and also some eukaryotic phospholipids are formed following conversion (Fig. 21-4, step *g*) of phosphatidic acids to CDP-diacylglycerols, which are able to react with a variety of nucleophiles with displacement of CMP.[164–166] Reaction with L-serine (step *h*)[167] leads to **phosphatidylserine,** and reaction with glycerol 3-phosphate (step *i*),[168] which enters cells via a special transporter,[168a] produces **phosphatidylglycerol 3-*P*.** The enzyme catalyzing the formation of phosphatidylserine appears to occur naturally as an integral membrane protein of the ER. Some is also bound to ribosomes and to mitochondria.[169,169a] In contrast, most of the other enzymes of phospholipid formation are closely associated with or embedded in the cytoplasmic membrane. One of these, a pyruvoyl group dependent enzyme (Chapter 14, Section F), catalyzes decarboxylation of phosphatidylserine to **phosphatidylethanolamine** (PE, step *j*, Fig. 21-4).[170] This reaction had been thought unimportant in animals, but

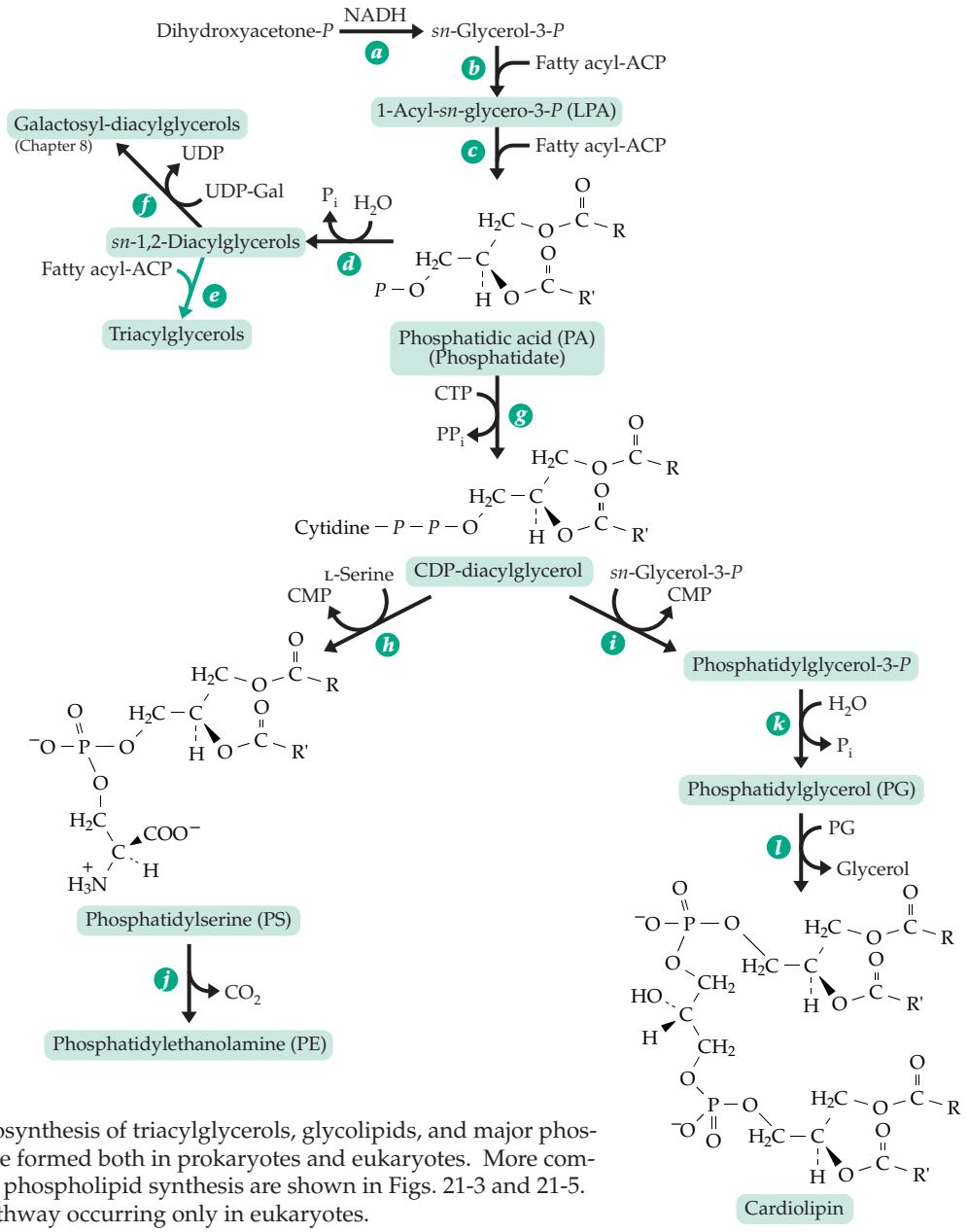


Figure 21-4 Biosynthesis of triacylglycerols, glycolipids, and major phospholipids that are formed both in prokaryotes and eukaryotes. More complete schemes of phospholipid synthesis are shown in Figs. 21-3 and 21-5. Green arrow: pathway occurring only in eukaryotes.

results with cultured cells show that decarboxylation of phosphatidylserine is often the major route of formation of phosphatidylethanolamine in mammalian cells.[171,172] This phospholipid also accounts for 75% of total phospholipid of the *E. coli* cell envelope. It is synthesized on the cytosolic side of the inner membrane, but it is also translocated to the outer membrane, where it is a major constituent of the inner bilayer leaflet.[173] PE is essential for viability of *E. coli* cells.[174] It provides dipolar ionic head groups and apparently serves as a chaperone for folding of some membrane proteins.[175]

After removal of a phosphate from phosphatidylglycerol 3-*P*, the resulting phosphatidylglycerol can be converted to **diphosphatidylglycerol** (known as **cardiolipin**). One manner in which this is accomplished in bacteria is indicated by step *l* of Fig. 21-4. One molecule of glycerol is displaced as two molecules of phosphatidylglycerol are coupled. The alternative pathway of Eq. 21-9 is followed in eukaryotic mitochondria and perhaps in some bacteria. The entire phosphatidic acid group is transferred from CDP-diacylglycerol to phosphatidylglycerol with displacement of CMP.[176–178] Gram-negative bacteria also synthesize a second set of membrane phospholipids, compounds such as **lipid A** (Figs. 8-30, 20-10) that are based on acylated glucosamine.[165]

Phosphatidylcholine, which is rarely present in bacteria, is formed in eukaryotes from phosphatidylethanolamine by three consecutive steps of methylation

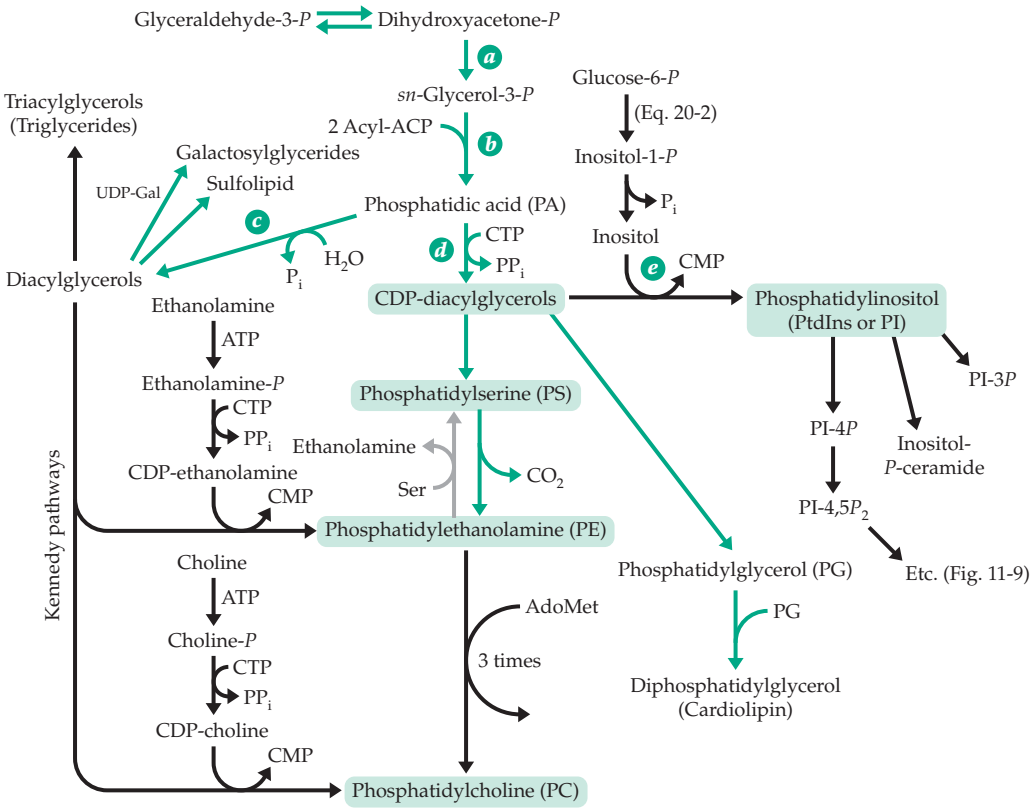
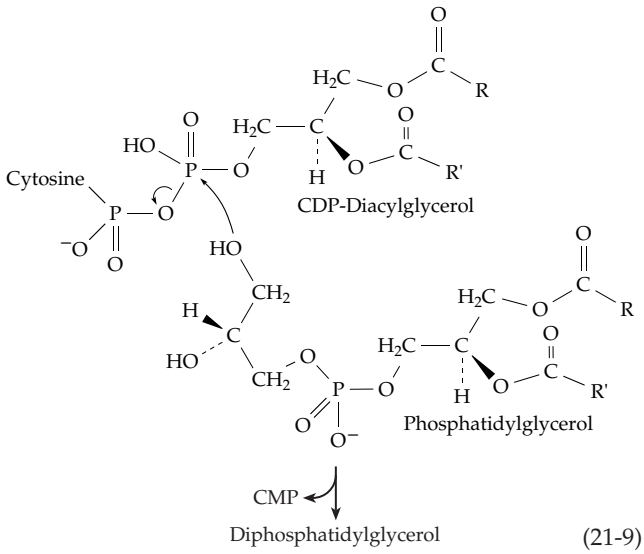


Figure 21-5 A more complete outline of the biosynthesis of triacylglycerols, glycolipids, and phospholipids including characteristic eukaryotic pathways. Green lines indicate pathways utilized by both bacteria and eukaryotes. Structures of some of the compounds are shown in Fig. 21-4. The gray arrows show the formation of phosphatidylserine by exchange with ethanolamine (Eq. 21-10).

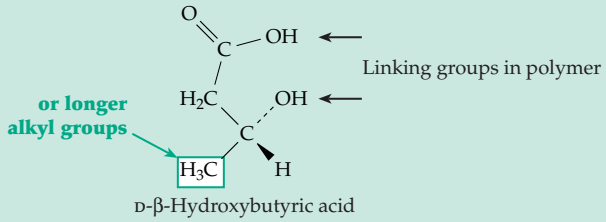


(21-9)

by *S*-adenosylmethionine (Fig. 21-5). This pathway is of major importance in eukaryotic cells.[179,180] However, alternative pathways (the Kennedy pathways),[166] which are represented by black lines on the left side of Fig. 21-5, are also used for formation of both phosphatidylcholine and phosphatidylethanolamine. In both cases, the free base, choline, or ethanolamine[180a,b] is phosphorylated with ATP. Choline phosphate formed in this manner is then converted by reaction with CTP to CDP-choline (Eq. 17-58).[181] Phosphatidylcholine is formed from this intermediate[181a,b] while CDP-ethanolamine is used to form phosphatidylethanolamine (Fig. 21-5). These synthetic reactions occur within cell nuclei as well as on surfaces of cytoplasmic membranes.[181c]

The formation of phosphatidylserine and possibly other phospholipids in animal tissues may also be accomplished by exchange reactions (Eq. 21-10, step *a*).[182,183] At the same time, decarboxylation of phosphatidylserine back to phosphatidylethanolamine (Eq. 21-10, step *b*) also takes place, the net effect being a catalytic cycle for decarboxylation of serine to ethanolamine. The latter can react with CTP to initiate synthesis of new phospholipid molecules or can be converted to phosphatidylcholine (step *c*). However, unless there is an excess of methionine and folate in the diet, choline is an essential human nutrient.[184]

BOX 21-D POLY-β-HYDROXYBUTYRATE AND BIODEGRADABLE PLASTICS

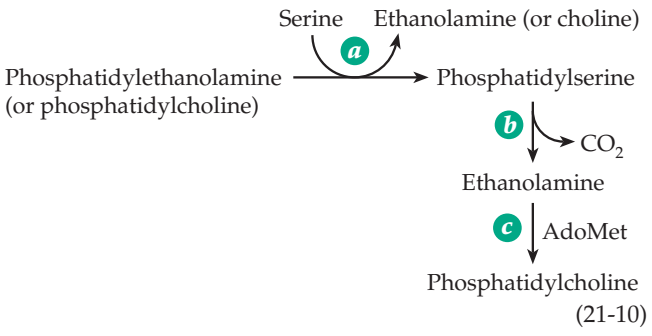


D-β-Hydroxybutyric acid

The important bacterial storage material polyhydroxybutyric acid is related metabolically and structurally to the lipids. This highly reduced polymer is made up of D-β-hydroxybutyric acid units in ester linkage, about 1500 residues being present per chain. The structure is that of a compact right-handed coil with a twofold screw axis and a pitch of 0.60 nm.[a] Within bacteria it often occurs in thin lamellae ~5.0 nm thick. Since a chain of 1500 residues stretches to 440 nm, there must be ~88 folds in a single chain. Present in both cytoplasmic granules and in membranes,[b] polyhydroxybutyrate can account for as much as 50% of the total carbon of some bacteria.[c] In *E. coli* and many other bacteria polyhydroxybutyrate is present in a lower molecular mass form bound to calcium polyphosphates, proteins, or other macromolecules.[d,e] It has also been extracted from bovine serum albumin and may be ubiquitous in both eukaryotes and prokaryotes.[d,e] The polymer may function in formation of Ca^{2+} channels in membranes.[b,d]

Biosynthesis occurs from 3-hydroxybutyryl-CoA. Some bacteria incorporate other β-hydroxyacids into the polymer.[f] Apparently various hydroxyacyl-CoAs can be diverted from the β oxidation pathway to polymer synthesis,[g] and synthases that will accept a variety of β-hydroxyacyl-CoA substrates have been isolated.[h,i] More than 80 different hydroxyacyl groups can be incorporated into the polymer.[i] A bacterially produced copolymer of β-hydroxybutyrate and β-hydroxyvalerate resembles polypropylene but is biodegradeable. It not only can be used for sutures and other medical implants[j] but also could compete with petroleum-derived plastics[h] and be derived from renewable sources. To this end the synthase genes have been cloned, engineered, and transferred into other microorganisms and plants.[k–n] Transgenic cotton plants incorporate polyhydroxybutyrate granules into the cotton fibers altering the properties of the fibers.[m] The polyhydroxybutyrate synthases apper to be related mechanistically to bacterial lipases.[o]

[a] Okamura, K., and Marchessault, R. H. (1967) in *Conformation of Biopolymers*, Vol. 2 (Ramachandran, G. N., ed), pp. 709–720, Academic Press, New York
[b] Reusch, R. N., and Sadoff, H. L. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 4176–4180
[c] Jacob, G. S., Garbow, J. R., and Schaefer, J. (1986) *J. Biol. Chem.* **261**, 16785–16787
[d] Reusch, R. N., Huang, R., and Bramble, L. L. (1995) *Biophys. J.* **69**, 754–766
[e] Huang, R., and Reusch, R. N. (1996) *J. Biol. Chem.* **271**, 22196–22202
[f] Peoples, O. P., and Sinskey, A. J. (1989) *J. Biol. Chem.* **264**, 15298–15303
[g] de Waard, P., van der Wal, H., Huijberts, G. N. M., and Eggink, G. (1993) *J. Biol. Chem.* **268**, 315–319
[h] Müh, U., Sinskey, A. J., Kirby, D. P., Lane, W. S., and Stubbe, J. (1999) *Biochemistry* **38**, 826–837
[i] Gerngross, T. U., and Martin, D. P. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 6279–6283
[j] Pool, R. (1989) *Science* **245**, 1187–1189
[k] Poirier, Y., Dennis, D. E., Klomparens, K., and Somerville, C. (1992) *Science* **256**, 520–523
[l] Mittendorf, V., Robertson, E. J., Leech, R. M., Krüger, N., Steinbüchel, A., and Poirier, Y. (1998) *Proc. Natl. Acad. Sci. U.S.A.* **95**, 13397–13402
[m] John, M. E., and Keller, G. (1996) *Proc. Natl. Acad. Sci. U.S.A.* **93**, 12768–12773
[n] García, B., Olivera, E. R., Minambres, B., Fernández-Valverde, M., Canedo, L. M., Prieto, M. A., García, J. L., Martínez, M., and Luengo, J. M. (1999) *J. Biol. Chem.* **274**, 29228–29241.
[o] Crandall, W. V., and Lowe, M. E. (2001) *J. Biol. Chem.* **276**, 12505–12512



(21-10)

Apparently the synthesis via serine and phosphatidylserine cannot provide an adequate amount of choline, which is present in the body not only in phosphatidylcholine but in plasmalogens, sphingomyelins, and the neurotransmitter **acetylcholine**.[185]

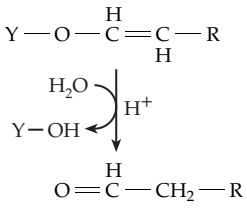
Phosphatidylinositol (PI), a major component of membrane lipids, is formed by displacement of CMP from CMD-dialylglycerol by *myo*-inositol.[186] It is also converted into a variety of less abundant phosphorylated derivatives that engage in signaling activities (see Fig. 11-9). In addition, PI is a component of the glycosylphosphatidylinositol (GPI) membrane anchors for suface proteins (Fig. 8-13). Free GPI anchors, lacking bound proteins, are also present in membranes.

They are especially abundant in many parasitic protozoa and may carry additional glycosyl groups.[186a]

Regulation of phospholipid synthesis, which has been studied in detail in yeast,[187–191] is complex but highly coordinated. The committed step in the synthesis of PE and PC is the hydrolysis of phosphatidate (PA) by a phosphatase to generate diacylglycerols (Fig. 21-5, step *c*). Reaction of PA with CTP (step *d*) also affects synthesis of the other major phospholipids. Much of the coordinate regulation arises at the transcriptional level. For example, genes for the synthesis of PC or PI are repressed by inositol alone and in combination with choline.[187,188] Regulation of CTP synthetase controls the formation of CDP-diacylglycerols.[190] In mammalian cells PC synthesis appears to occur only during the S-phase of the cell cycle (Fig. 11-15).[191] The CTP: phosphocholine cytidyltransferase that catalyzes CDP-choline formation is controlled by storage in a resevoir in the nucleus from which it is transferred to ER membranes.[181b]

2. The Ether-Linked Lipids

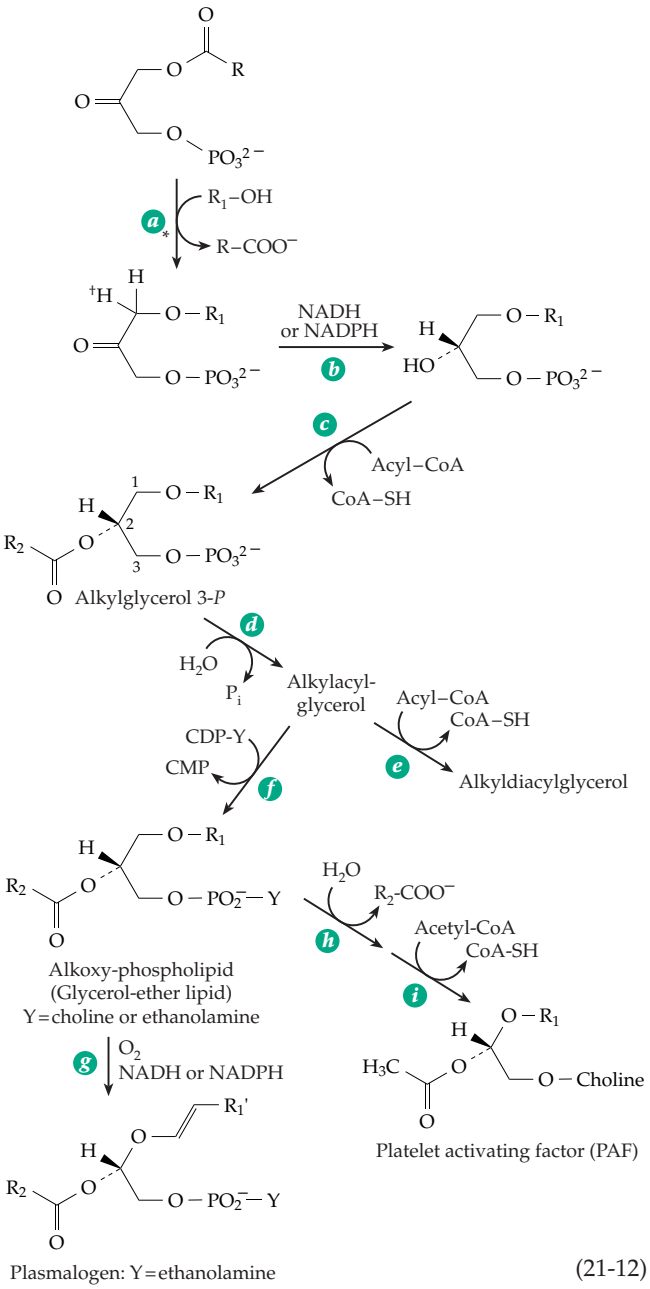
Closely related to both the triacylglycerols and phospholipids, the ether-linked lipids contain in place of one ester group an *alkoxy* (–OR) or *alkenyl* (–O–CH=CH–R) group.[192] Phospholipids containing the alk-1-enyl group, the **plasmalogens**, were first recognized in 1924 by Feulgen and Voit, who were developing histological staining procedures. They observed that treatment of tissue slices with acid resulted in the liberation of aldehydes, which were later shown to be formed by breakdown of the alkenyl lipids (Eq. 21-11). Over 10% of the lipid in the human central nervous system is plasmalogen and about 1% is alkoxy lipid. Among the latter is the **platelet activating factor** (Box 8-A).[193-194a] In many mammalian cells the ethanolamine plasmalogen **plasmenylethanolamine** represents the major storage depot of arachidonic acid.[195]



(21-11)

Ether-linked lipids constitute up to 35% of the total phospholipid in molluscs. Although they are usually regarded as animal constituents, small amounts of ether-linked lipids have been identified in plants. The major phospholipids of archaebacterial membranes are ether-linked derivatives of the polyprenyl phytanyl group and of the dimeric biphytanyl group (Chapter 8).[196–198]

Biosynthesis of ether lipids begins with formation of fatty acyl derivatives of dihydroxyacetone phosphate. The acyl group is then displaced, along with the oxygen atom to which it is attached, by an alkoxy group of a long-chain fatty alcohol (Eq. 21-12, step *a*), which is formed by reduction of the corresponding acyl-CoA.[199] The oxygen of the alcohol (designated by an asterisk) is retained in the product.[200,201] The reaction differs significantly from displacements discussed in Chapter 12. The pro-*R* hydrogen atom (marked by the dagger, †) at C-1 exchanges with the medium during the reaction suggesting that enolization of the dihydroxyacetone phosphate takes place. A possible mechanism would be to add the incoming R–O⁻, generated as in serine proteases, to the double bond of



(21-12)

the enol at C-1. This would generate a transient carbanion on C-2. It could then eliminate the carboxylate containing the original C-1 acyl group, and the enol could then ketonize.

Once an alkoxy derivative of dihydroxyacetone is formed, reduction to the 2-OH form, further acylation, and conversion to various alkyl phospholipids and neutral lipids can occur. The pathways (Eq. 21-12, steps *b*–*f*) are closely akin to those of Fig. 21-4. The conversion of alkoxy lipids to plasmalogens occurs by oxidative desaturation (Eq. 21-12, step *f*).[202] The initial steps in the synthesis of ether-linked lipids take place principally in the peroxisomes. Enzymes catalyzing both the acylation of dihydroxyacetone phosphate and the synthesis of alkyl-dihydroxyacetone-*P* (step *a*, Eq. 21-12) are found in high amounts in animal peroxisomes. In the rare autosomal recessive disorder known as the **Zellweger syndrome** peroxisomes are completely lacking.[203] Both the synthesis of ether-linked lipids[204] and the β oxidation of very-long-chain fatty acids are depressed. These acids, principally C26:0 and C26:1, accumulate in tissues[205,206] of patients with this severe disease, which is usually fatal during the first four months of life.

The platelet activating factor (PAF, Box 8-A) is formed in neutrophils and macrophages from alkylacyl-*sn*-glycero-3-phosphocholine by the action of phospholipase A_2. This enzyme removes the C2 acyl group, which is then replaced by an *acetyl group* transferred from acetyl-CoA to form PAF. Alternatively, a phosphocholine group may be transferred onto 1-alkyl-2-acyl-*sn*-glycerol from CDP-choline as in the formation of phosphatidylcholine (Fig. 21-5). PAF can undergo hydrolytic removal of its acetyl group in tissues but can also transfer it to such acceptors as lysoplasmalogens or sphingosine.[194] Hydrolytic loss of the acetyl group from PAF destroys biological activities including induction of allergic and inflammatory responses.[194a] The various signaling activities of PAF arise from binding to G-protein linked receptors in many cells and tissues.[207]

3. Sphingolipids

Sphinoglipids are phospholipids and glycolipids derived from **sphingosine** and other "long-chain bases."[208] At least 60 bases of this type have been identified.[209] They vary in chain length from C_{14} to C_{26} and include members of the iso and anteiso series. Up to two double bonds may be present. The C_{18} compound, usually called sphingosine, is derived from condensation of palmitoyl-CoA with serine.[209a] Carbon dioxide is lost from the serine during the condensation reaction (Fig. 21-6, step *a*; Chapter 14), and the resulting ketone is reduced with NADPH (step *b*) to form **sphinganine**, a common component of animal sphingolipids. It may be hydroxylated to phytosphingosine in plants and fungi (step *c*).[210,211] Sphinganine is converted to long-chain amides by acyl transfer from acyl-CoA (step *d*) and then undergoes desaturation (step *e*)[212–213a] to form **ceramides**, the precursors to more than 100 gangliosides (Fig. 20-11),[214,215] to the phospholipid **sphingomyelins** (Fig. 21-6, step *g*), and also to free sphingosine (step *h*). This last reaction is degradative and on the pathway of breakdown of gangliosides (Fig. 20-11). Further catabolism of sphingosine is thought to take place by a PLP-mediated chain cleavage to palmitaldehyde.[216]

The essential functions of sphingolipids, including the complex gangliosides, are only now being clarified.[215,217,218] The latter are abundant in brain and are thought to function in cell–cell recognition. On blood cell surfaces they carry blood group antigens (Box 4-C). They play an essential role in spermatogenesis[218] and may function in various signaling processes.[218a] In the outer cornified layers of skin, ceramides with very long chain (C_{28}–C_{36}) fatty acyl groups undergo ω hydroxylation (Fig. 21-6) and become esterified to glutamate side chains of specific skin proteins called **involucrins**. The long hydrocarbon chains are thought to pass entirely through the lipid bilayer to form rigid lamellae of a water-impermeable outer skin barrier.[134] An important hypothesis is that sphingolipids associate with cholesterol to form "lipid rafts," which float in a sea of glycerolipids and serve as bases for various signaling processes. The long hydrophobic acyl chains of the sphingolipids pack well with cholesterol to form a rigid lipid structure of high melting temperature.[218b,c]

4. Complex Lipids in Signaling

While pathways of synthesis of complex lipids have been described, we are far from understanding the dynamics of the synthesis and turnover of the membranous structures built from them. The fact that the lipid bilayer of a cell membrane is so thin means that any sudden changes in composition at a particular location will cause changes in physical properties and a wave of diffusion that will travel along the membrane. The membrane seems to be ideally structured to receive and propagate messages from outer surface or internal receptors, or messages sent along the bilayer.

One of the most studied examples of signaling with membrane lipids is provided by the **phosphoinositide cascade**, which is pictured in Fig. 11-9. Six or more phosphate esters of phosphatidylinositol (PI) are generated by the action of kinases.[219,220] More than 100 extracellular signaling molecules activate specific isozyme forms of **phospholipase C**,[221–224] releasing 20 or more different inositol phosphates from these

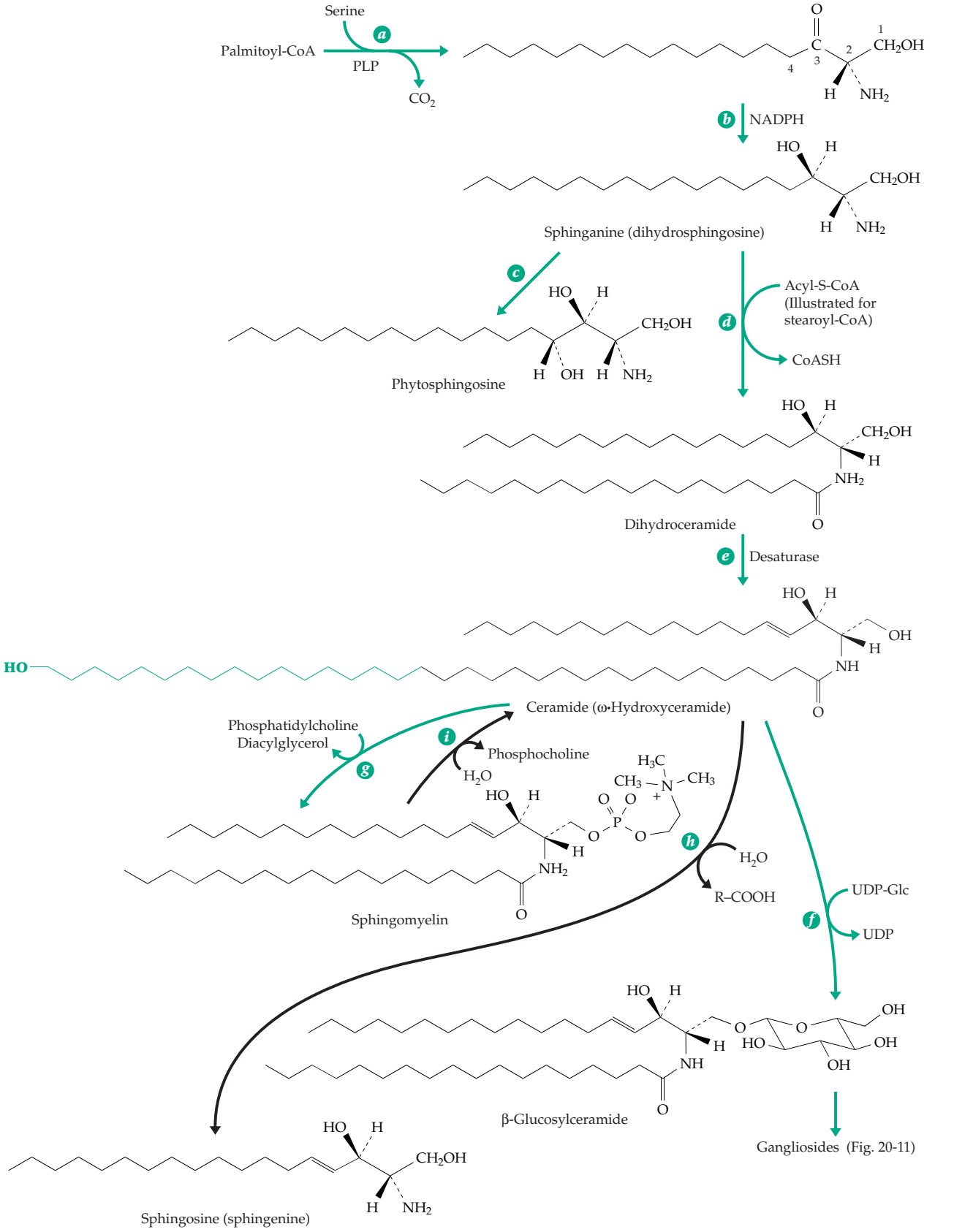


Figure 21-6 Pathways of synthesis and metabolism of sphingolipids. Gray arrows indicate catabolic pathways. See also Fig. 20-11. The green extension on the ceramide structure is that of a long-chain ω-hydroxyceramide that is covalently bound to protein in human skin.

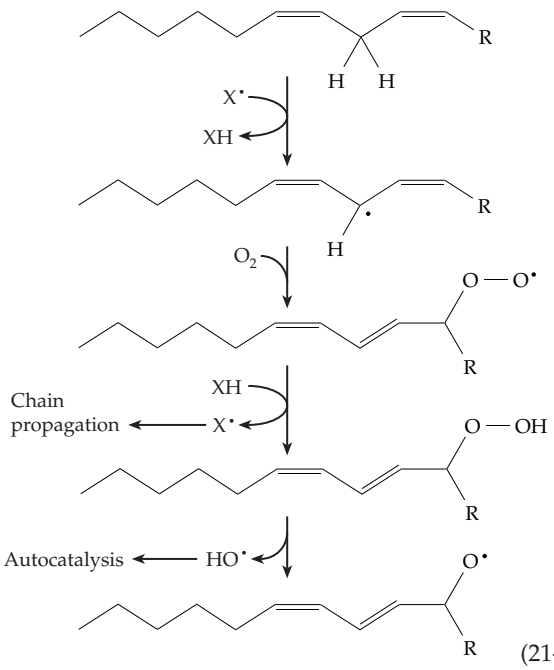
phosphoinositide esters. The released inositol phosphates, which act as water-soluble messengers, are further modified by the action of several phosphatases (Fig. 11-9).[225] At the same time, **diacylglycerols** are left in the membrane. With loss of the negative charges of the PI phosphates there will be immediate electrostatic effects in the membrane, which may alter the ionic environment, open ion channels, etc. The diacylglycerols, which diffuse within the membrane, may lose arachidonic acid from the *sn*-2 position to supply substrate for the arachidonate cascades described in Section D (Eq. 21-16). Diacylglycerols also activate the 11 isozyme forms of **protein kinase C**.[226–228] Some of these enzymes not only are activated by diacylglycerols, but also require **phosphatidylserine** for activity.

Other lipid-based signaling cascades arise from reactions that modify phosphatidylcholine molecules. Unsaturated fatty acids in the *sn*-2 position are readily oxidized by free radicals with cleavage of the hydrocarbon chains to form alcohols, aldehydes, and carboxylic acids. These mimic PAF in their biological activities.[194a,228a] Phosphatidate molecules with saturated or monounsaturated fatty acids in the *sn*-2 position arise from breakdown of phosphatidylcholine catalyzed by **phospholipase D**.[229] Phosphatidate may also be formed by a family of lipid **diacylglycerol kinases**.[230,230a] Phosphatidates containing saturated or monounsaturated fatty acids also have a variety of signaling activities.[230a] An arachidonoyl-diacylglycerol kinase is thought to function in many processes. An example is a PI-mediated cycle in invertebrate vision.[231] Sphingomyelin breakdown (black arrows in Fig. 21-6) releases diffusible ceramides that have been implicated as signaling molecules in cell proliferation, differentiation, growth arrest, and apoptosis.[232–237a] Sphingosine and sphingosine 1-*P* also have signaling functions.[211,237b-e]

Phospholipids have been shown to exchange between different membranes, e.g., of mitochondria and the ER. Exchanges of phosphatidylcholine, phosphatidylinositol, and sphingomyelin are catalyzed by specific **exchange proteins** (Box 21-A).[238,238a] These proteins may also participate in signaling, but their major function may be to transport the phospholipids from their sites of synthesis to the various membranes of the cell.

5. Peroxidation of Lipids and Rancidity

Storage of fats and oils leads to **rancidity**, a largely oxidative deterioration that causes development of unpleasant tastes, odors, and toxic compounds.[239] Similar chemical changes account for the "drying" of oil-based paints and varnishes. These reactions occur most readily with polyunsaturated fatty acids, whether free or in ester linkage within triacylglycerols. The reactions are initiated by free radicals, which may be generated by oxidative enzymes within or outside of cells, or by nonenzymatic reactions catalyzed by traces of transition metals or by environmental pollutants. Characteristic of rancidity is an autocatalytic chain reaction (Eq. 21-13).[239–241]



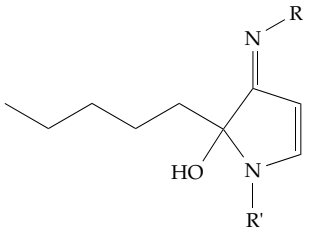
(21-13)

Radical X•, which initiates the reaction, is regenerated in a chain propagation sequence that, at the same time, produces an organic peroxide. The latter can be cleaved to form two additional radicals, which can also react with the unsaturated fatty acids to set up the autocatalytic process. Isomerization, chain cleavages, and radical coupling reactions also occur, especially with polyunsaturated fatty acids. For example, reactive unsaturated aldehydes can be formed (Eq. 21-14).

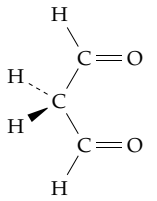
$$\mathrm{R{-}CH{=}CH{-}\overset{\bullet O}{\overset{|}{C}}H{-}R' \rightarrow R{-}CH{=}CH{-}CHO + {}^{\bullet}R'}$$

(21-14)

An intermediate in Eq. 21-13 may be converted to **4-hydroxy-2-nonenal**, a prominent product of the peroxidation of arachidonic or linoleic acids (Eq. 21-15).[242–243a] However, other biosynthetic pathways to this compound are possible.[244,244a] 4-Hydroxy-2-nonenal can react with side chains of lysine, cysteine, and histidine[245] to form fluorescent products such as the following cyclic compound generated by an oxidative reaction.[246]



Polyenoic acids also give rise to malondialdehyde, a reactive mutagenic compound, which can be reduced



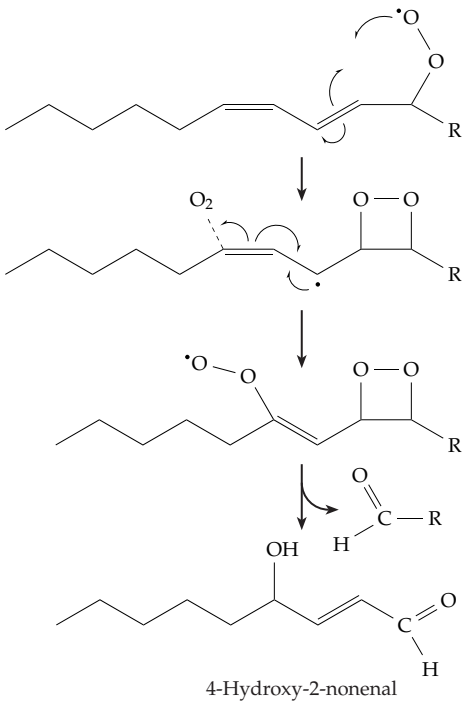
Malondialdehyde

and dehydrated to acrolein, a toxic compound which also reacts with both lysine and serine to

CH_2=CH–CHO

Acrolein

produce products excreted in the urine.[247,248] More dangerous are similar reactions of these aldehydes with proteins of the body in conditions such as diabetes or renal insufficiency.[242] The bifunctional malondialdehyde forms Schiff bases with protein amino groups and acts as a crosslinking agent.[249] **Age pigments** (also called **lipofuscin**), which tend to accumulate within neurons and other cells, are thought to represent precipitated lipid-protein complexes resulting from such reactions.[250] The reactions are similar to those of proteins with the products of sugar breakdown (glycation; p. 69).[250a] Organisms have developed multiple enzymatic mechanisms for detoxification of products of both glycation and oxidative degradation.[243a]



4-Hydroxy-2-nonenal (21-15)

The oxidative degradation represented by the foregoing reactions is referred to as peroxidation. Peroxidation 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.

Catalytic hydrogenation of vegetable oils is widely used to form harder fats and to decrease the content of polyunsaturated fatty acyl groups. The products have a greatly increased resistance to rancidity. However, they also contain fats with trans double bonds as well as isomers with double bonds in unusual positions.[251–253] Such compounds may interfere with normal fatty acid metabolism and also appear to affect serum lipoprotein levels adversely. Trans fatty acids are present in some foods. One hundred grams of butter contain 4–8 g, but hydrogenated fats often contain much more. It has been estimated that in the United States trans fatty acids account for 6–8% of total dietary fat.[253]

6. Some Nutritional Questions

While many of the poorer people on earth starve to death the problems of atherosclerosis and obesity affect many in wealthier societies.[253a–c] The fat content of foods is often blamed, and, as discussed in Boxes 21-B and 22-B, the quality of fatty acids in the diet is very important. However, like fatty acids, carbohydrates are also metabolized via acetyl-CoA and can readily be converted to both fatty acids and cholesterol.[253d] Obesity is largely a problem of excessive total caloric intake.

Why do some people stay slim while others become obese? What are the regulatory mechanisms that affect appetite and body composition? The human body weight tends to be stable or to increase slowly during adult life.[253e] Is there a natural set point for each individual? No, an apparent set point is just a result of action of a multitude of factors including genetic variations[253f] and psychological factors that affect exercise levels, eating habits, etc.[253g] It is worthwhile to recognize that the basal metabolic rate, which is also affected by many factors, accounts for a very high fraction of a person's energy expenditure (p. 283).

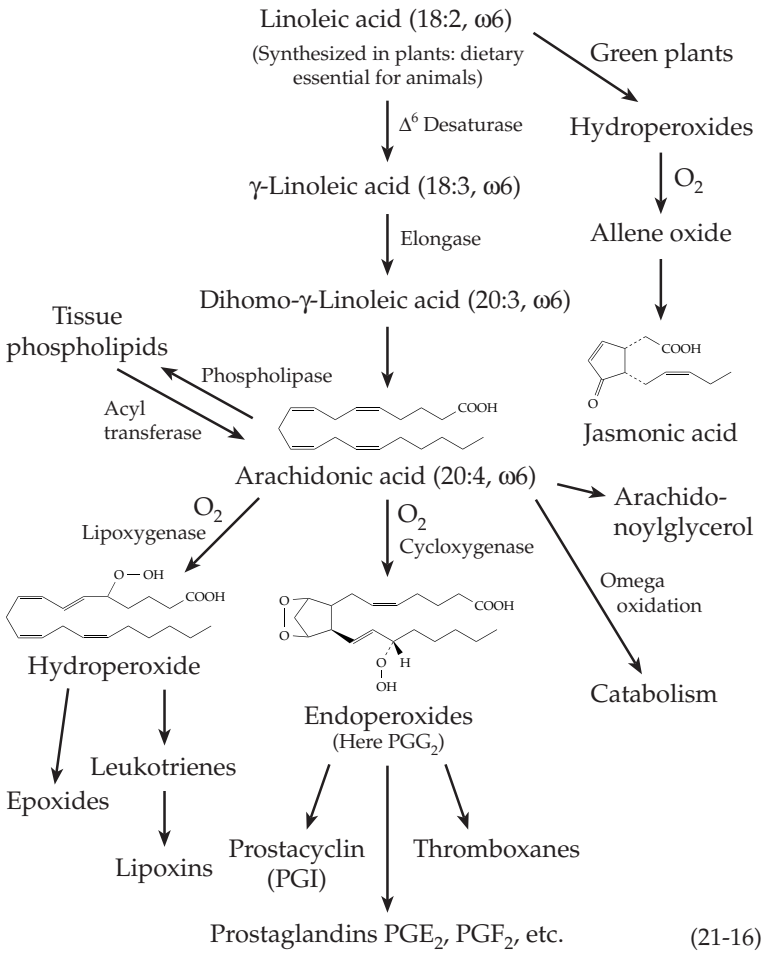
The following are among specific biochemical

factors that act on the energy balance: the activity of acetyl-CoA carboxylase and the associated level of malonyl-CoA[253h,i]; the activity of mitochondrial uncoupling proteins (Box 18-C)[253j,k]; actions of the hormone leptin[253l] (which have been hard to interpret)[253m,n]; and other hormones including cholecystokinins and neuropeptide Y (Chapter 30).

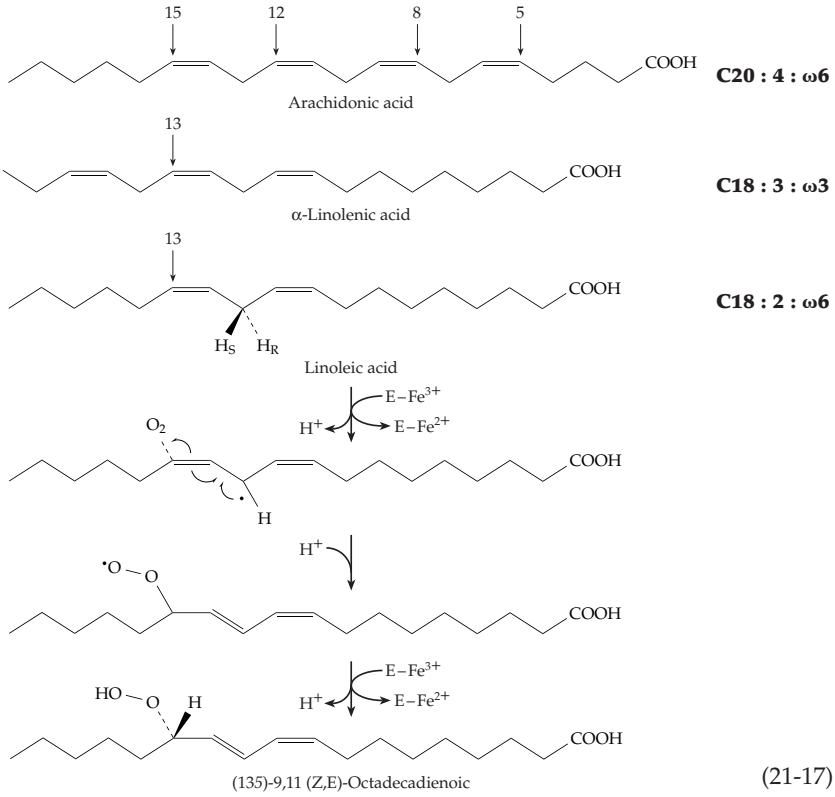
D. Prostaglandins and Related Prostanoid Compounds

Lipid peroxidation has often been regarded simply as an undesirable side reaction, but it is also a normal part of metabolism. Initiated by enzymatically generated radicals, peroxidation occurs as specific metabolic pathways, such as the **arachidonate cascade**, which leads to a variety of local hormones and other substances (Eq. 21-16).[254–256a]

As early as 1930, it was recognized that seminal fluid contains materials that promote contraction of uterine muscles. The active compounds, the **prostaglandins**, were isolated and crystallized in 1960 and were identified shortly thereafter.[257,258] As many as 14 closely related compounds are found in human seminal fluid, one of the richest known sources. Prostaglandins are present in seminal fluid at a total concentration of ~1 mM, but their action on smooth muscles has been observed at a concentration as low as 10^{-9} M. The structures and biosynthetic pathways of several of the prostaglandins are indicated in Fig. 21-7. Prostaglandins are usually abbreviated PG with an additional letter and numerical subscript added to indicate the type. The E type are β-hydroxyketones, the F type 1,3-diols, and the A type α, β-unsaturated ketones. Series 2 prostaglandins arise from arachidonic acid, while series 1 and 3 arise from fatty acids containing one fewer or one more double bond, respectively (Fig. 21-7). Additional forms are known.[257,259]



(21-16)



(21-17)

1. Metabolism of the Prostaglandins

Prostaglandins are not stored by cells but are synthesized in response to external stimuli. Arachidonic acid and other polyenoic acids are present in relatively small amounts (e.g., ~1% of total plasma fatty acids), but they are concentrated in the 2-position of phospholipids. This is in part a result of phospholipid "remodeling." Acyl groups are hydrolyzed from the *sn*-2 position by action of phospholipase A_2. An acyltransferase with a preference for arachidonoyl groups then transfers esterified arachidonic acid from

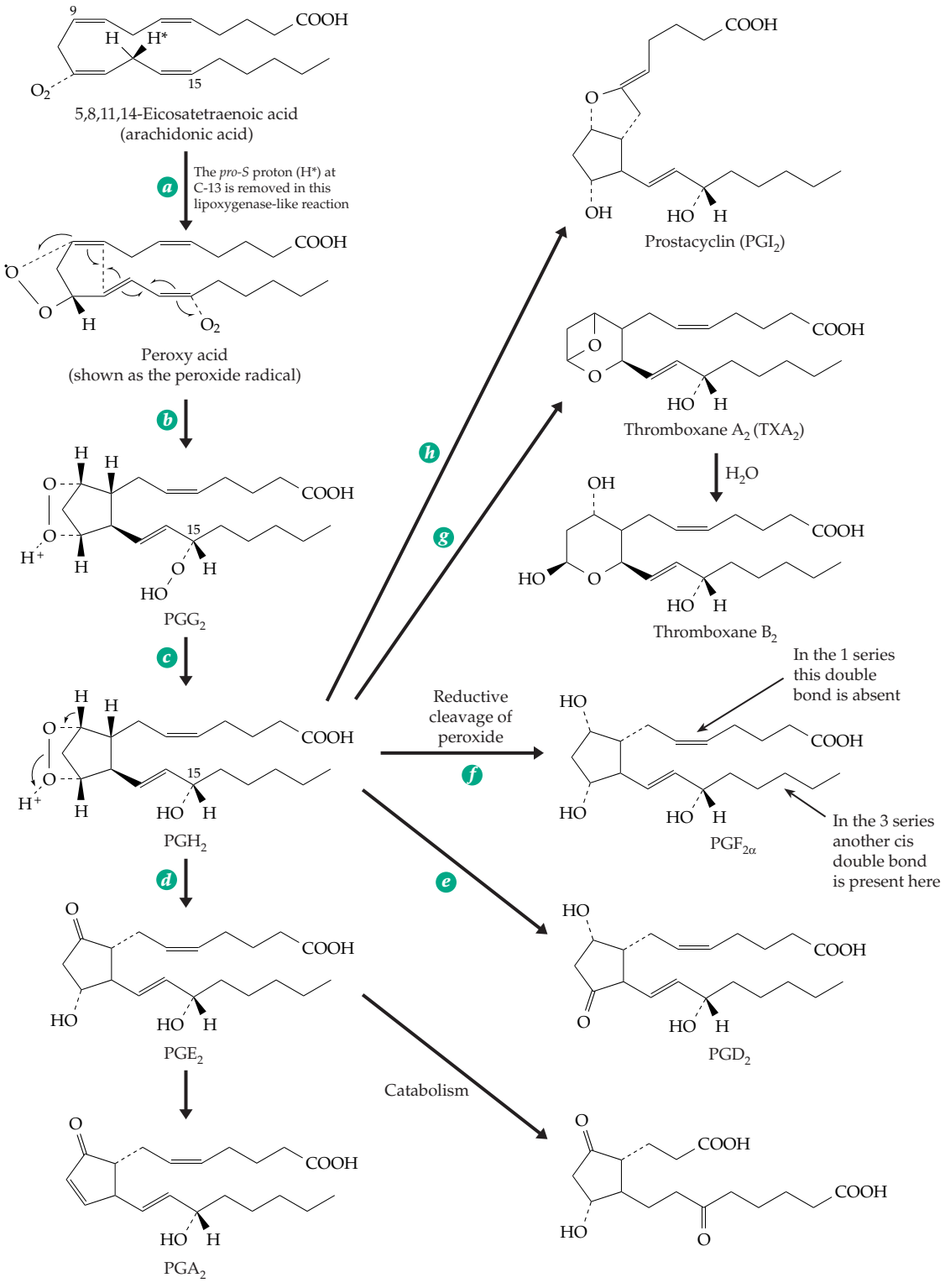


Figure 21-7 Pathway of synthesis and catabolism of the prostaglandins.

phosphatidylcholine and other phospholipids to the lyso forms of the phospholipids, which lack a 2-acyl group. The enzyme has a strong preference for the lyso-ethanolamine plasmalogens. As a consequence, in the plasmenylethanolamine of platelets arachidonoyl groups account for 66% of the acyl residues at the 2-position.[260] An arachidonate-specific acyl-CoA synthetase rapidly reconverts any free arachidonate that is not used for prostanoid synthesis back into phospholipids.[261]

The synthesis of prostaglandins, which was elucidated by Samuelsson,[258–260] begins with the release of arachidonate and other polyenoic acid precursors from phospholipids through the action of phospholipase A_2. The released arachidonate is then acted upon by **prostaglandin H synthases,** which catalyze two consecutive reactions at adjacent but distinct sites in a single protein.[262–262d] The first, **cyclooxygenase** or prostaglandin endoperoxide synthase reaction, forms PGG_2 from arachidonate and the second, a **peroxidase** reaction, generates PGH_2. There are two major mammalian isozymes of prostaglandin synthase, which are often called cyclooxygenase-1 (COX-1) and cyclooxygenase-2 (COX-2). From studies of stereospecifically synthesized ^3H-containing fatty acid precursors, it was established that the first step in cyclooxygenase action involves removal of the *pro*-S proton at C-13 of the fatty acid (step *a*, Fig. 21-7). The O_2-requiring cyclooxygenase resembles lipoxygenase (Eq. 21-17).[181] The product is a peroxy acid, possibly in the form of the peroxide radical shown in Fig. 21-7. This radical (or peroxide anion) undergoes cyclization with synchronous attack by a separate O_2 molecule at C-15 (Fig. 21-7, step *b*) to give the endoperoxide PGG. Reduction of the latter to an OH group by the NADPH-dependent peroxidase (step *c*) yields PGH. The entire sequence is catalyzed by the single 70-kDa PGH synthase, which contains a single heme prosthetic group.[263] During the cyclooxygenase reaction the enzyme appears to accept electrons from cytochrome b_5. During the peroxidase step the heme group undergoes formation of the characteristic peroxidase intermediate compounds I and II[263] (Fig. 16-14). It has been suggested that a tyrosyl radical is generated in the peroxidase active site (on Y385 of COX-1) and is used to form an arachidonate radical that reacts with O_2 in the cyclooxygenase reaction.[264–266b] Alternatively, a carbocation mechanism is also possible.[267]

PGH can break down in three ways to give the E and F series of prostaglandins.[268] In one the proton at C-9 is eliminated (step *d*) as indicated by the small arrows by the PGH_2 structure of Fig. 21-7. An alternative isomerization (step *e*) gives PGD_2. The F prostaglandins are formed by reductive cleavage of the endoperoxide (step *f*). The A series and other prostaglandins arise by secondary reactions, one of which is shown in Fig. 21-7.

A biochemical characteristic of the prostaglandins is rapid catabolism. The product shown in Fig. 21-7 (lower right) arises by oxidation of the 15-OH to a carbonyl group, permitting reduction of the adjacent trans double bond. Two steps of β oxidation as well as ω oxidation are also required[269] to produce the dicarboxylic acid product shown. However, a series of products appears, and the distribution varies among species. Catabolism of prostaglandins is especially active in the lungs, and any prostaglandins entering the bloodstream are removed by a single pass through the lungs. This observation has led to the conclusion that prostaglandins are not hormones in the classical sense but act on a more local basis.

2. Thromboxanes and Prostacyclins

In blood platelets and in some other tissues PGG is also transformed to another series of compounds, the **thromboxanes**,[270] which were identified in 1975. Labile hemiacetals, the thromboxanes A (TXA, Fig. 21-7), are derived by rearrangement of PGH (step *g*). Thromboxane synthase,[271–273] which catalyzes the reaction, has characteristics of a cytochrome P450. Cytochromes P450 are known to react with peroxides as well as with O_2, and the endoperoxide of PGH may be opened by the synthase prior to rearrangement to TXA.[273] Thromboxane A_2 is so unstable that its half-life at 37°C in water is ~36 s. It is spontaneously converted to TXB_2 (Fig. 21-7), which contains an –OH group at C-15. The thromboxanes B are much more stable than TXA but are not very active physiologically.

By 1976, Vane and associates had identified another prostanoid compound, **prostacyclin** (or PGI_2).[274–275a] This compound also arises from PGH_2 by action of a cytochrome P450-like prostacyclin synthase (Fig. 21-7).[273,275,276] It is thought to be an important vasoprotective molecule. As with the thromboxanes, prostacyclin undergoes rapid inactivation[277] by hydrolysis to the physiologically inactive 6-oxo-$PGF_1\alpha$.

3. Lipoxygenases

Lipoxygenases, of which the enzyme from soy beans has been studied the most, also catalyze oxidation of polyunsaturated fatty acids in lipids as indicated in Eq. 21-17. Formation of the hydroperoxide product is accompanied by a shift of the double bond and conversion from cis to trans configuration. Soybean lipoxygenase is a member of a family of related lipoxygenases that are found in all eukaryotes. All

appear to have similar iron- or manganese-containing active sites and to act by similar mechanisms.[278–280e] The major substrate in animals is arachidonic acid (probably as the arachidonate ion). As marked on the structures above Eq. 21-17, there are 5-, 8-, 12-, and 15-lipoxygenases, which catalyze reaction with dioxygen at the indicated places.[281,282] Linoleic and linolenic acids are the primary substrates in plants. Soybean lipoxygenase acts on the 13-position of linoleic acid as shown in Eq. 21-17. However, this enzyme is often referred to as a *15-lipoxygenase* because it acts on arachidonate at C15. The 100-kDa enzyme from

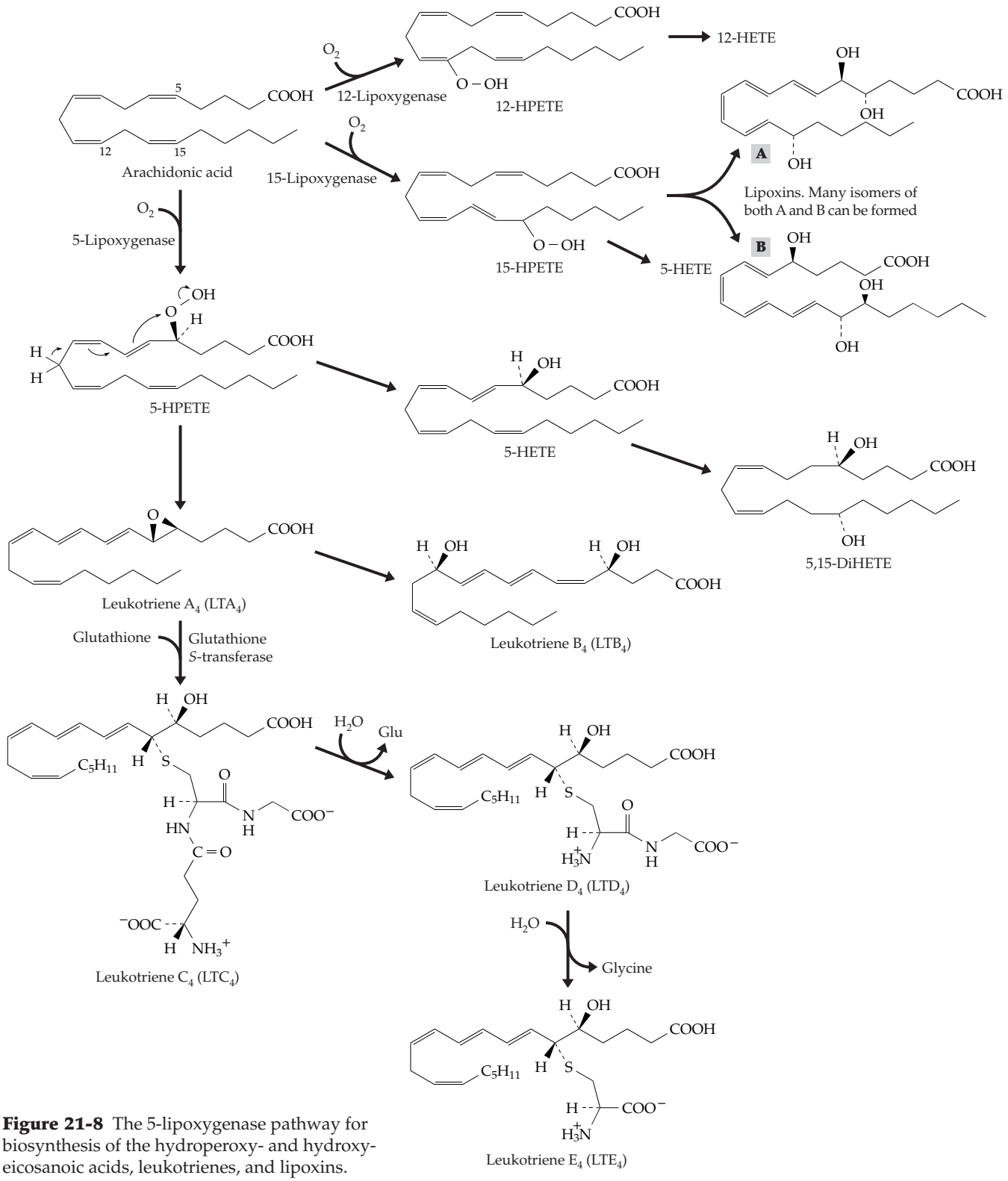


Figure 21-8 The 5-lipoxygenase pathway for biosynthesis of the hydroperoxy- and hydroxy-eicosanoic acids, leukotrienes, and lipoxins.

soybeans contains one atom of Fe(II), which is bound by a cluster of His and Tyr side chains.[283] It must be oxidized to the Fe(III) state before becoming active.[279,284] The initial reaction with O_2 may occur via an intermediate radical.

4. Leukotrienes, Lipoxins, and Related Compounds

Yet another series of products results from the action on arachidonate of tissue lipoxygenases, which compete with the prostaglandin-forming cyclooxygenases. The 5-lipoxygenase (Fig. 21-8) produces the unstable peroxide **5-hydroperoxy 6,8,11,14-eicosatetraenoic acid** (usually abbreviated **5-HPETE**). This enzyme requires ATP and Ca^{2+} and appears to be regulated by a series of other metabolites.[285] The 12- and 15-lipoxygenases, whose distribution varies among different mammalian organs and tissues, form the corresponding 12- and 15-HPETES as well as 5, 15-, 8,15-, 14,15-diHPETEs.[255,286] Some of these peroxides have physiological effects of their own, but they are largely transformed by peroxidases to more stable compounds such as the corresponding alcohols (hydroxy-icosatetraenoic acids or HETEs; Fig. 21-8).

The **leukotrienes** are formed from 5-HPETE (Fig. 21-8).[287,288] Dehydration of HPETE produces the unstable epoxide **leukotriene A_4** (LTA_4), which can be hydrolyzed enzymatically by leukocytes to the diol **leukotriene B_4** (LTB_4).[289,290] Alternatively leukotriene synthase, present in many cells, catalyzes the addition of glutathione (Box 11-B) to the LTA. This is a ring-opening reaction of the epoxide that can be visualized as a nucleophilic displacement by the thiolate anion of glutathione at C-6 (Fig. 21-8). The product is **leukotriene C_4** (LTC_4),[291] which can undergo consecutive removal of glutamate and glycine to form **leukotrienes D** and **E** (LTD_4, LTE_4), respectively. Removal of the glutamate occurs by the action of γ-glutamyl transpeptidase (Box 11-B), whereas removal of the glycine is hydrolytic. LTC_4 and the more potent LTD_4 have been identified as the **slow-reacting substance of anaphylaxis** (SRS-A), a long-sought mediator of bronchial asthma.[292,293] Leukotrienes can be formed from polyunsaturated acids other than arachidonic acid. Thus, eicosapentaenoic acid yields LTC_5 and LTD_5.[294] A lipoxygenase-derived product from the C18:2 linoleic acid is 13-hydroxylinoleic acid, which is made principally by endothelial cells that line blood vessels. It may contribute to resistance to blood clotting.[294]

Products of the 15-lipoxygenase pathway include a group of trihydroxytetraenes formed by leukocytes.[295] Several routes of biosynthesis, which may involve epoxide intermediates, are known.[296,297] The structures of two of these compounds, **lipoxin A** and **lipoxin B,** are shown in Fig. 21-8. Several stereoisomers and cis–trans isomers can be formed. These compounds can all arise from 15-HPETE, either by enzyme action or nonenzymatically. In fact, the entire series of prostanoid compounds arise by reactions related to but more specific than those that occur during nonenzymatic autoxidation of arachidonate.[255,298] Cytochrome P450-catalyzed reaction with O_2 can convert arachidonic acid into four different **epoxytrienoic acids** (**EETs**), which may also exist as stereoisomers. They are vasodilators which affect a variety of signaling pathways.[298a,b]

5. Physiological Effects of the Prostanoids

The release of arachidonate and initiation of the arachidonate cascade is induced by hormones, various inflammatory and immunological stimuli, and even mechanical agitation. Tissues do not all behave the same in response to the arachidonate cascade.[299] Blood platelets form largely thromboxane A_2, whereas tissues of the aorta form prostacyclin. Prostaglandin D_2 is a major prostanoid in the central nervous system.[300] Biological functions of prostanoids are also varied.[256a,301] The **primary prostaglandins** PGE and PGF were first recognized as mediators of inflammation. However, PGE_2 and PGF_2 sometimes have opposite effects. The unstable precursors PGG_2 and PGH_2, which have half-lives of only a few minutes, are much more powerful than the more stable PGEs and PGFs. Prostaglandin D_2, which is released in lungs during attacks of asthma, is thought to be a major bronchoconstrictor, but it may also serve as a neurotransmitter.[300] Thromboxanes released from platelets cause smooth muscle contraction and aggregation of the platelets, the first step in blood clot formation. Thromboxanes have half life-times of only seconds but are extremely potent not only in inducing platelet aggregation but also in causing contraction of blood vessels. Prostacyclin has the opposite effect, being a potent vasodilator that causes relaxation of smooth muscle. Upon release from blood vessel walls it acts to prevent clot formation.

The lipoxygenase pathway (Fig. 21-8) leading to the leukotrienes, lipoxins, and other products is especially active in leukocytes and in mast cells.[302] The leukotrienes promote inflammation, but lipoxins A_4 and B_4 are antiinflammatory.[303] The release of leukotrienes LTC_4, LTD_4, and LTE_4 in lung tissue is correlated with the long-lasting contractions of smooth muscle of the bronchi that are characteristic of asthma.[304] Leukotriene LTC_4 is ~1000 times more powerful than histamine in inducing such contraction. Leukotrienes have also been found in the central nervous system.[305]

Some effects of prostaglandins are mediated through cell surface G-protein coupled receptors (see Chapter 11).[306] Some other prostanoids bind to and activate nuclear peroxisome proliferator-activated receptors.[306] PGJ_2 may inhibit fatty acid synthesis and fat deposition in adipose tissue through these receptors. Some of the prostanoid derivatives enter membranes and may become incorporated into phospholipids and exert their effects there.

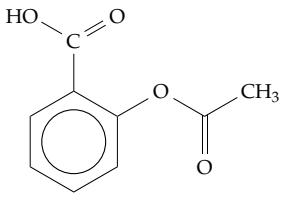
A number of medical uses of prostaglandins have been discovered and more will probably be developed. While prostaglandins may be required for conception, small amounts of PGE_2 or $PGF_{2\alpha}$ induce abortion. $PGF_{2\alpha}$ is also used to induce labor. Prostaglandins are widely employed to control breeding of farm animals, to synchronize their estrus cycles, and to improve the efficiency of artificial insemination.[307]

6. Inflammation

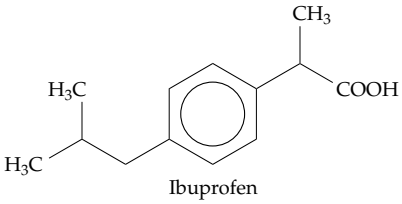
Special interest in the prostaglandins has focused on pain of inflammation and allergic responses. The medical significance is easy to see. Five million Americans have **rheumatoid arthritis**, an inflammatory disease. Bronchial asthma and other allergic diseases are equally important. Our most common medicine is **aspirin**, an anti-inflammatory drug. Both the inflammatory response and the immune response are normal parts of the defense mechanisms of the body, but both are potentially harmful, and it is their regulation that is probably faulty in rheumatoid arthritis and asthma. Overproduction of prostaglandins may be a cause of menstrual cramps.[308]

Prostaglandins have been implicated both in the induction of inflammation and in its relief. In inflammation small blood vessels become dilated, and fluid and proteins leak into the interstitial spaces to produce the characteristic swelling (edema). Many polymorphonuclear leukocytes attracted by chemotactic factors that include LTB_4[309] (Chapter 19) migrate into the inflamed area, engulfing dead tissue and bacteria. In this process lysosomes of the leukocytes release phospholipase A, which hydrolyzes phospholipids and initiates the arachidonate cascade. The leukotrienes that are formed promote the inflammatory response. However, cAMP can suppress inflammation, and PGE_2 has a similar effect. Indeed, E prostaglandins, when inhaled in small amounts, relieve asthma.

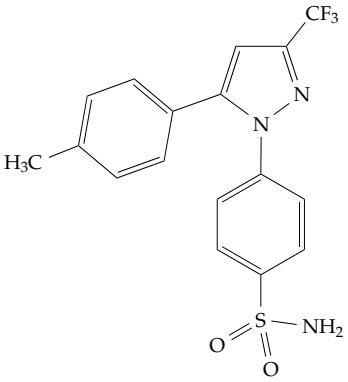
The synthesis of prostaglandins is inhibited by aspirin[310] and many other analgesic drugs. Aspirin is an acetylating reagent, and the inhibition has been traced to acetylation of the side chain –OH group of a single serine residue, Ser 530 of COX-1 or Ser 516 of COX-2 in the arachidonate binding channel.[311–313] Other nonsteroidal antiinflammatory drugs (NSAIDs),



Aspirin (acetylsalicylic acid)



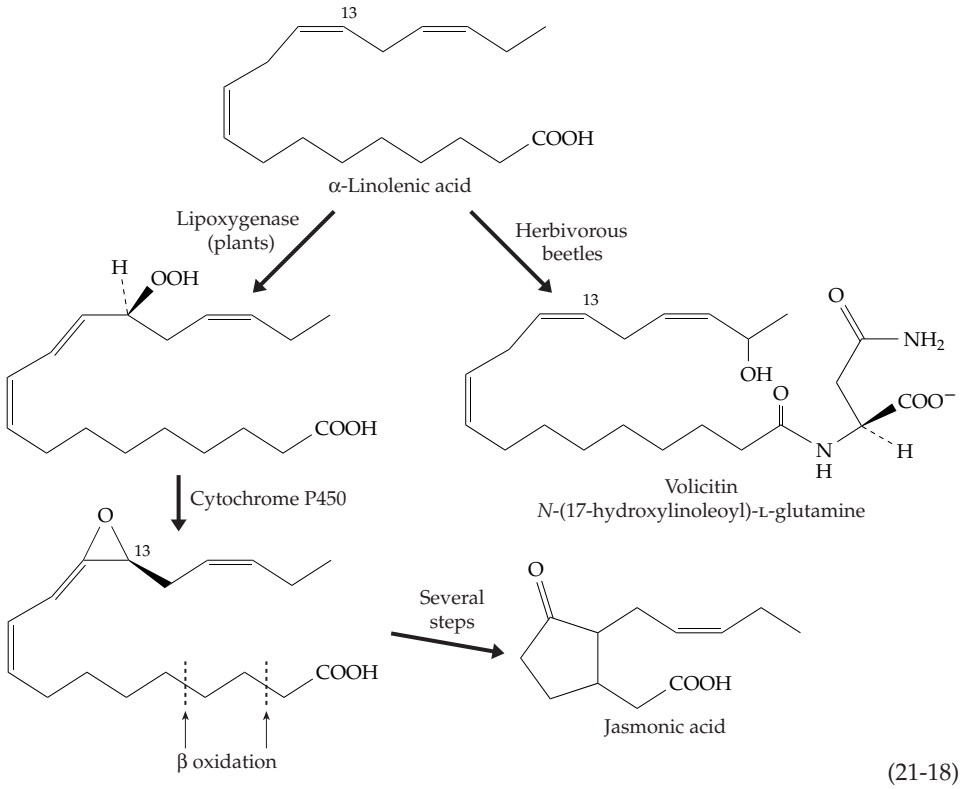
Ibuprofen



Celecoxib, a new COX-2 inhibitor

e.g., ibuprofen, are competitive inhibitors of the cyclooxygenases.[314] COX-3, a variant form of COX-1, may be the target for acetaminohen (Box 18-E).[314a] However, the same drugs also inhibit activation of neutrophils and may thus exercise their anti-inflammatory action in more than one way.[315] Since PGE_1 is a potent **pyrogen** (fever-inducing agent), a relationship to the ability of aspirin to reduce fever is also suggested. Unfortunately, all of these drugs inhibit COX-1 of platelets. Small regular doses of aspirin may be useful in preventing blood clots in persons with arterial disease, but they can be disastrous. Thousands of people die annually of hemorrhage caused by aspirin.[316,317]

Recently it was recognized that COX-1 provides eicosinoids for homeostatic purposes, while it is COX-2 that is inducible and generates prostoglandins for production of leukotrienes and induction of the inflammatory response. Now there is a major effort, with the first drugs already in use, to develop specific inhibitors for COX-2, which do not inhibit COX-1. It is hoped that these will be safer than aspirin.[256a,266a,311,317,317a-c] However, these drugs can also cause dangerous side effects.[317d] COX-2 of macrophages is also inhibited by γ-tocopherol, a major form of vitamin E.[317e]



(21-18)

7. Plant Lipoxygenases and Jasmonic Acid

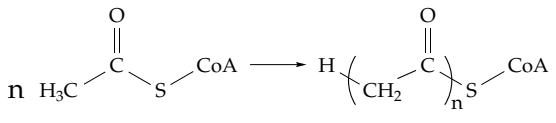
An octadecenoid signaling pathway (Eq. 21-18), which resembles the arachidonate cascade in some respects, plays an important role in green plants.[318–320] Alpha-linolenic acid is acted upon by a lipoxgenase in plastids to form a 13-hydroperoxy derivative. This is dehydrated and cyclized by allene oxide synthase. Although this doesn't appear to be an oxidation–reduction process, the enzyme seems to be a cytochrome P450 and to initiate the cyclization to the unstable epoxide **allene oxide** by homolytic cleavage of the peroxy group.[321,321a] Allene oxide synthase and the cyclase that acts in the next step may be cytosolic, while the β oxidation that shortens the chain occurs in plants exclusively in peroxisomes or glyoxysomes.[319]

Jasmonic acid is a plant growth regulator that affects many aspects of plant development as well as responses to environmental signals. A very important function is mobilization of plant defenses in response to damage by herbivores, by bacterial or fungal pathogens, or by ultraviolet light.[322,323] The synthesis of protease inhibitors as well as phytoalexins is induced. A curious variant of the jasmonate pathway is the acquisition of α-linoleic acid from plants by chewing caterpillars. The linoleic acid is hydroxylated by the insect, and conjugated with glutamine to form **volicitin** (*N*-(17-hydroxylinoleoyl)-L-glutamine; Eq. 21-18). Some of this compound reenters the plant from material regurgitated into wounds by the caterpillars. Volicitin induces the plant to release volatile terpenes and other compounds. The value to the caterpillars is not clear, but not only does volicitin induce defensive reactions in plants but also the released volatile compounds attract wasps that parasitize the caterpillars.[324,325] Plants also form a group of **isoprostanes** E_1 from α-linolenic acid.[326]

Allene oxides are unusual biological products. However, they are formed from arachidonic acid by some corals and are evidently precursors to prostaglandin esters, which may be present in high concentrations.[327] Allene oxides are also present in starfish oocytes.[321,328]

E. The Polyketides

In 1907, Collie proposed that polymers of ketene ($CH_2=C=O$) might be precursors of such compounds as **orsellinic** acid, a common constituent of lichens. The hypothesis was modernized in 1953 by Birch and Donovan, who proposed that several molecules of acetyl-CoA are condensed (Eq. 21-19) but *without the two reduction steps required in biosynthesis of fatty acids* (Fig. 17-12).[329] As we now know they were correct in assuming that the condensation occurs via malonyl-CoA and an acyl carrier group of an enzyme. The resulting **β-polyketone** can react in various ways to give the large group of compounds known as polyketides.



Acetyl-CoA β-Polyketone (21-19)

β-Polyketones can be stabilized by ring formation through ester or aldol condensations. Remaining carbonyl groups can be reduced (prior to or after cyclization) to hydroxyl groups, and the latter can be eliminated as water to form benzene or other aromatic rings. Figure 21-9 illustrates two ways in which cyclization can occur. One involves a Claisen ester condensation during which the enzyme and its SH group are eliminated. Enolization of the product gives a trihydroxy-acetophenone. The second cyclization reaction is the aldol condensation. Following the condensation water is eliminated, and the product is hydrolyzed and enolized to form orsellinic acid. Another product of fungal metabolism is **6-methylsalicylic acid,** which lacks one OH group of orsellinic acid. This synthesis can be explained by assuming that the carbonyl group at C-5 of the original β-polyketone was reduced to an OH group at some point during the biosynthesis. Elimination of two molecules of water together with enolization of the remaining ring carbonyl gives the product (Fig. 21-9).[330]

By allowing a few variations in the basic polyketone structure, the biosynthesis of a large number of unusual compounds can be explained. Extra oxygen atoms can be added by hydroxylation, and methyl groups may be transferred from *S*-adenosylmethionine to form methoxyl groups.[331] Occasionally a methyl group may be transferred directly to the carbon chain. Glycosyl groups may also be attached.[332,333] Many starter pieces other than acetyl-CoA may initiate polyketide synthesis. These include the branched-chain acids of Table 21-3, nicotinic and benzoic acids, 4-coumaroyl-CoA, and a 14:1 Δ^9-ACP. The last of these starter pieces is formed by desaturation of the corresponding 14:0-ACP and is converted via polyketide synthesis to one of a family of **anacardic acids,** which provide pest resistance to a variety of dicotyledenous plants (Fig. 21-10, bottom).[334] The CoA derivative of malonic acid amide is the starter piece for synthesis of the antibiotic **tetracycline** as indicated in Fig. 21-10).[335] Polyketide origins of some other antibiotics are also indicated in this figure.

The cloning and sequencing of genes for enzymes involved in synthesis of polyketides of fungi and actinomycetes has shown that these enzymes are closely related to the fatty acid synthases and, like the latter, have a multidomain structure (Fig. 21-11). The possibilities of engineering these genes, together with the urgent need for new antibiotics, has led to an

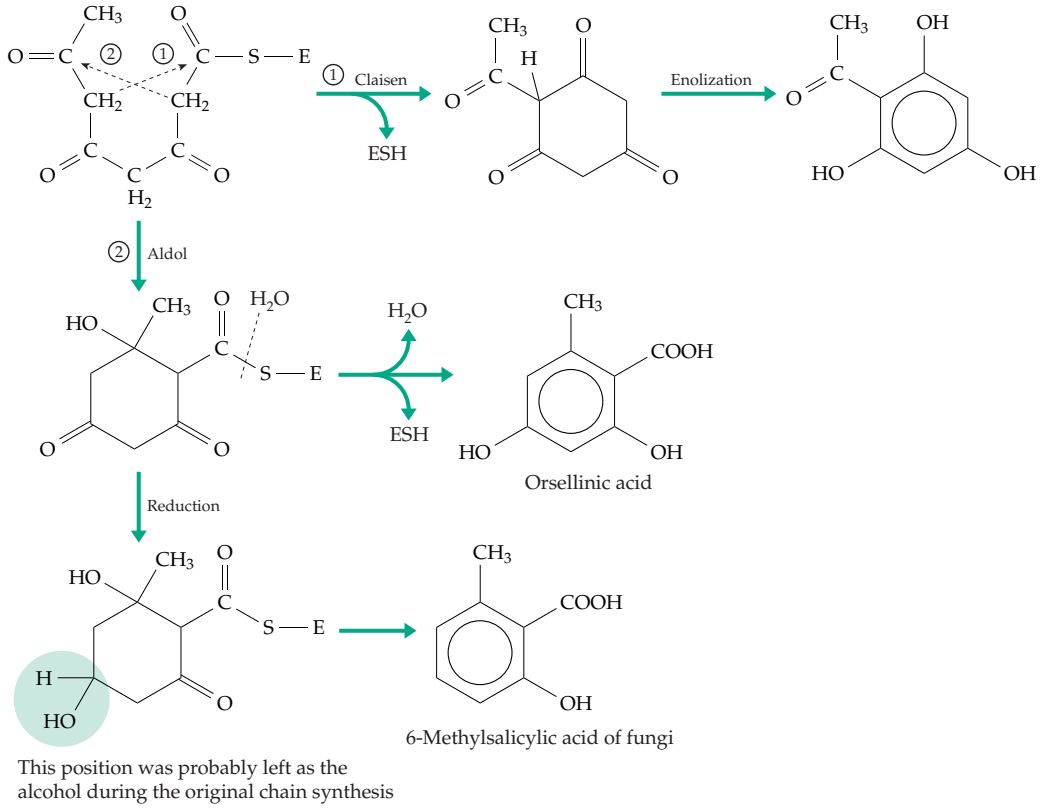
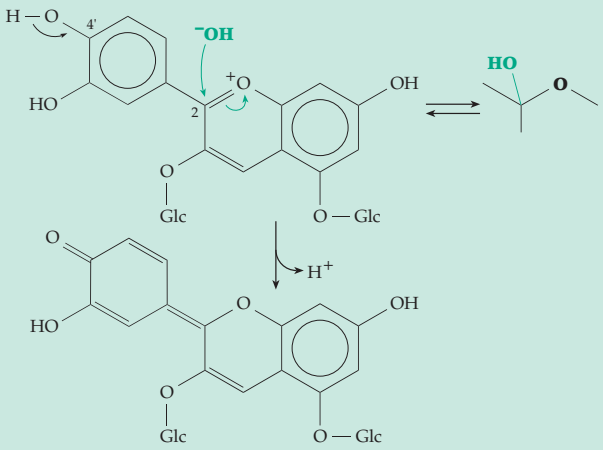


Figure 21-9 Postulated origin of orsellinic acid and other polyketides.

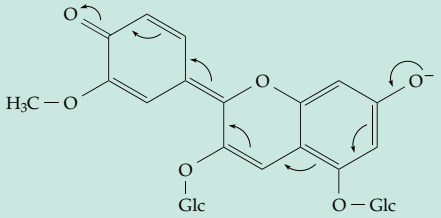
BOX 21-E HOW THE FLOWERS MAKE THEIR COLORS

Most of the pigments of flowers arise from a single polyketide precursor. Phenylalanine is converted to ***trans*-cinnamic acid** (Eq. 14-45) and then to cinnamoyl-CoA. The latter acts as the starter piece for chain elongation via malonyl-CoA (step *a* in the accompanying scheme). The resulting β-polyketone derivative can cyclize in two ways. The aldol condensation (step *b*) leads to **stilbenecarboxylic acid** and to such compounds as **pinosylvin** of pine trees. The Claisen condensation (step *c*) produces **chalcones**, **flavonones**, and **flavones**. These, in turn, can be converted to the yellow **flavonol pigments** and to the red, purple, and blue **anthocyanidins**.[a–c]

At the bottom of the synthetic scheme on the next page the structures and names of three common anthocyanidins are shown. The names are derived from those of flowers from which they have been isolated. The colors depend upon the number of hydroxyl groups and on the presence or absence of methylation and glycosylation. In addition to the three pigments indicated in the diagram, three other common anthocyanidins are formed by methylation. **Peonidin** is 3'-methylcyanidine. Methylation of delphinidin at position 3' yields **petunidin**, while methylation at both the 3' and 5' positions gives **malvidin**. There are many other anthocyanidins of more limited distribution. Anthocyanidins are nearly insoluble, but they exist in plants principally as glycosides known as **anthocyanins**. The number of different glycosides among the many species of flowering plants is large. Both the 3 and 5 –OH groups may be glycosylated with Glc, Gal, Rha, Ara, and by a large variety of oligosaccharides. The colors of the anthocyanins vary from red to violet and blue and are pH dependent. For example, **cyanin** (diglucosyl cyanidin) is red in acid solution and becomes violet upon dissociation of the 4'-hydroxyl group:

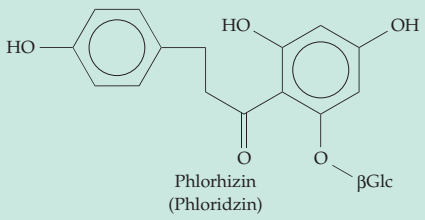


Dissociation of the 7–OH generates an anion with an extended conjugated π electron system, which will favor absorption of long-wavelength light (Chapter 23) and a blue color. Notice that a large number of resonance structures can be drawn for both the anthocyanin and the dissociated forms. Formation of complexes of Mg^{2+} or other metal ions with the 4' $-O^-$ and adjacent OH groups may also stabilize blue colored forms.[d]

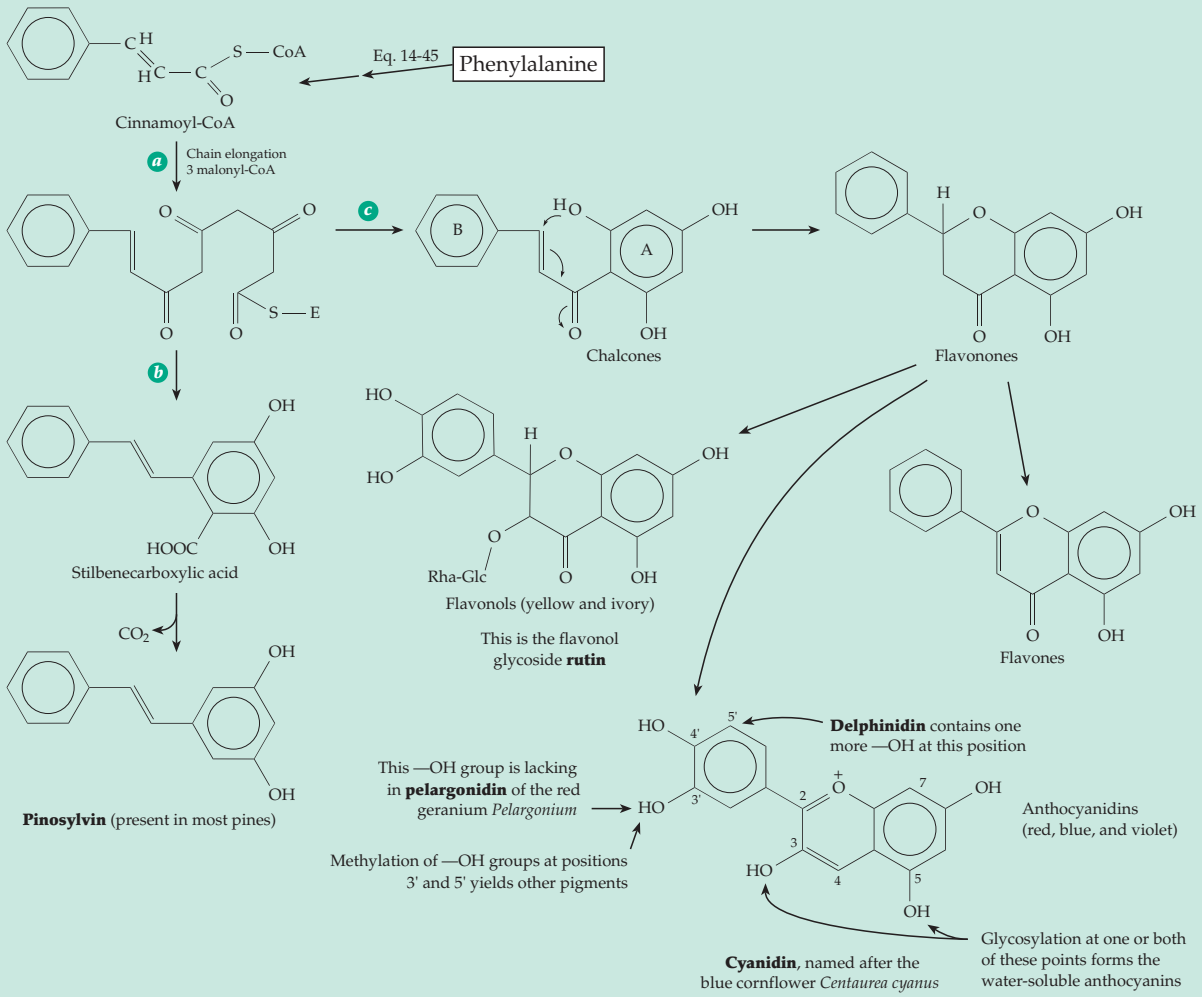


Most blue flower pigments are based on delphinidin,[b] but the "heavenly blue" of the morning glory is a peonidin with a complex caffeoylglucose-containing glycosyl group on the 3-position. Its blue color has been attributed to the relatively high pH of ~7.7 in vacuoles.[e] The aromatic rings within the glycosyl group of this and other complex anthocyanins may fold over the primary chromophore and stabilize the colored forms. A competing reaction, which is indicated in green on the first structure in this box, is the addition of a hydroxyl ion at C-2 to give a nearly colorless adduct.[f]

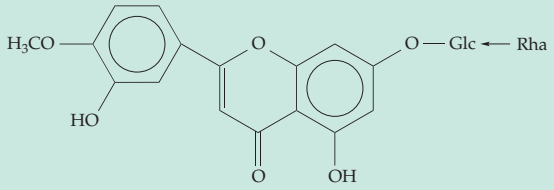
The yellow pigments of flowers are usually flavonols. The most common of all is **rutin**, the 3α-rhamnosyl-D-glucosyl derivative of **quercitin** (see diagram). An extraordinary number of other flavonols, flavones, and related compounds are found throughout the plant kingdom.[g] One of these is **phlorhizin**, a dihydrochalcone found in the root bark of pears, apples, and other plants of the rose family. Phlorhizin specifically blocks resorption of glucose by kidney tubules. As a result, the drug induces a strong glucosuria. The biochemical basis is uncertain, but the action on kidney tubules may be related to inhibition of mutarotase.[h]

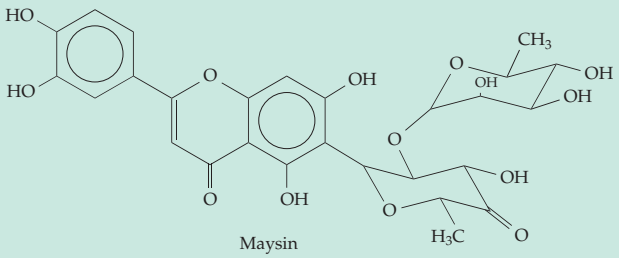


BOX 21-E (continued)



The flavone glycoside **hesperidin** makes up 80% of the dry weight of orange peels. It has been claimed (but not proved) that this compound, also known as **vitamin P** and **citrus bioflavonoid**, is essential to good health. Another flavone, **maysin**, is a resistance factor for the corn earworm and is present in silks of resistant strains of *Zea mays*.[i]





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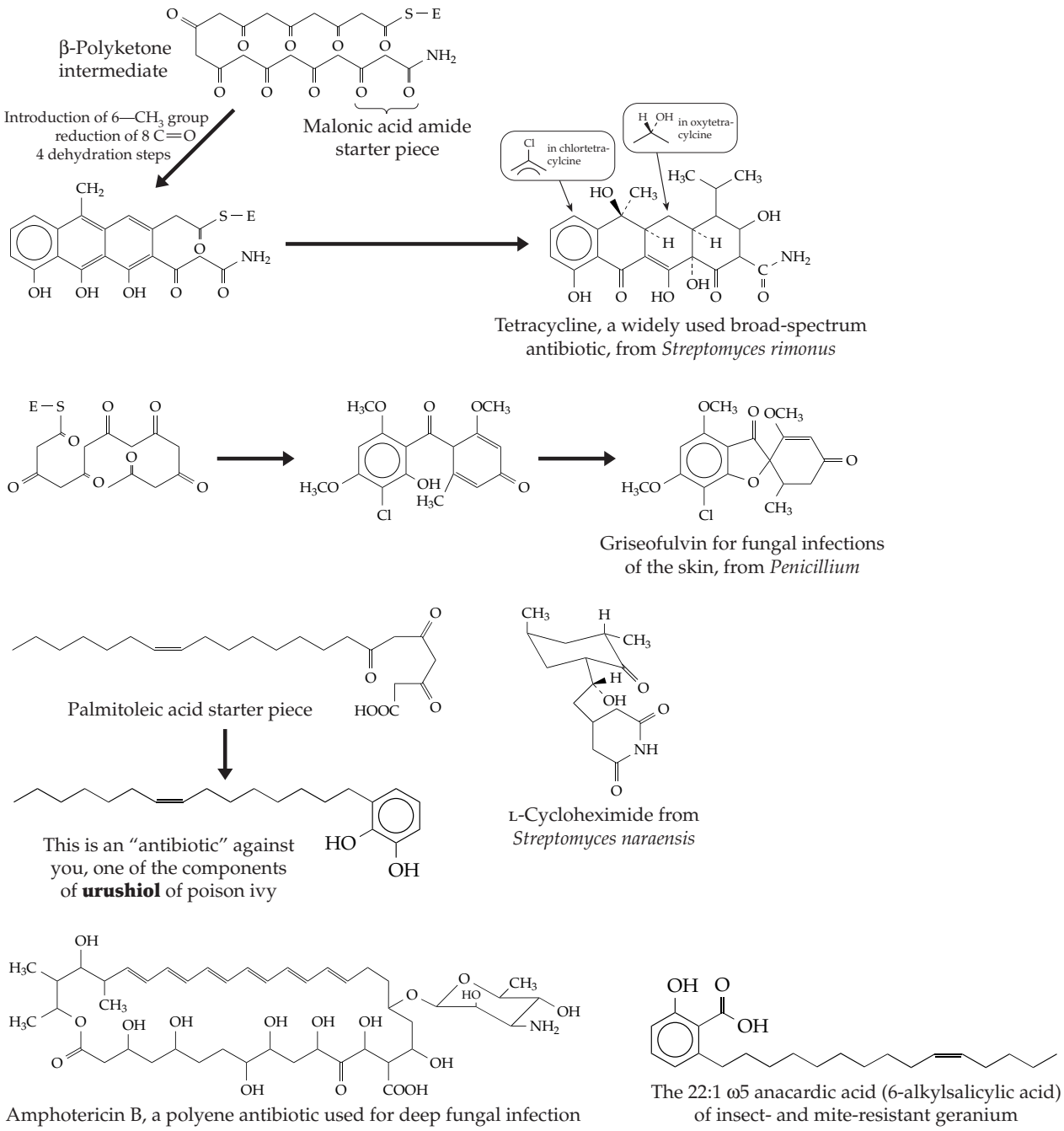

Figure 21-10 Some important polyketide antibiotics and plant defensive compounds.

explosion of information about polyketide synthases.[336,336a–c]

A 26-kb gene cluster encoding enzymes for synthesis of the blue antibiotic **actinorhodin** by *Streptomyces coelicolor* has been cloned and sequenced.[332,337] The three large ~10-kb genes required for formation of the broad-spectrum antibiotic **erythromycin** by *Saccharopolyspora erythraea* have also been cloned and sequenced.[337–339] In both cases, the genes encode large proteins with structures resembling those of the eukaryotic fatty acid synthases (Section B,1). However, a new feature is evident. As shown in Fig. 21-11, each of the three polypeptides of the deoxyerythronolide synthase, which synthesizes the aglycone of erythromycin, consists of two multidomain modules, each able to catalyze one round of reaction with a new molecule of malonyl-CoA. When reduction of an oxo group or dehydration and

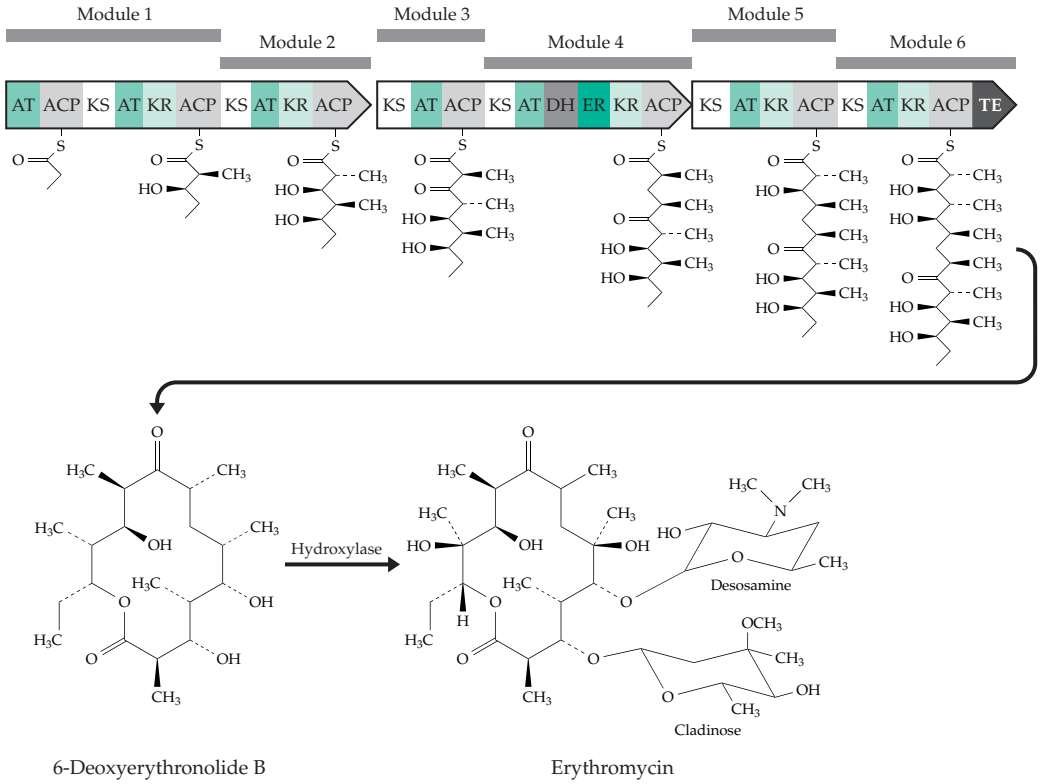

Figure 21-11 Catalytic domains within three polypeptide chains of the modular polyketide synthase that forms 6-deoxyerythronolide B, the aglycone of the widely used antibiotic erythromycin. The domains are labeled as for fatty acid synthases; AT, acyltransferase; ACP, acyl carrier protein; KS, β-ketoacyl-ACP synthase; KR, ketoreductase; DH, dehydrase; ER, enoylreductase; TE, thioesterase. After Pieper *et al.*[338] Courtesy of Chaitan Khosla.

reduction of an enoyl-CoA are not needed in a round, the KR, DH, and ER domains are absent (as in module 3 of Fig. 21-11). A final domain contains a thioesterase that releases and cyclizes the product. The "assembly line" sequence of synthetic steps, beginning with a propionyl group from propionyl-CoA, is pictured in Fig. 21-11. Two hydroxylation steps[340] and transfer of two unusual glycosyl groups complete the synthesis of the antibiotic.

Other medically important polyketides include the antibiotics **doxorubicin** (14-hydroxydaunomycin; Fig. 5-23),[341] rifamycin (Box 28-A),[342] and the antifungal **pimaricin**,[343] **griseofulvin**, and **amphotericin** (Fig. 21-10), the HMG-CoA reductase inhibitor **lovastatin**,[344] the 2-butanyl-4-methylthreonine of cyclosporin A (Box 9-F),[345] and other immunosuppressants such as **rapamycin**.[346] Many characteristic plant products, including **stilbenes**[347] and **chalcones**[348,348a] (Box 21-E), are polyketides. A variety of different polyketides serve as phytoalexins.[349] Some such as **aflatoxin**[350] are dangerous toxins. Ants and ladybird beetles make toxic polyamine alkaloids using a polyketide pathway.[350a]

Avermectin (Fig. 30–25), a widely used antibiotic against canine heartworms, is formed by a polyketide synthase with an unusually broad specificity for starter units. More than 40 alternative carboxylic acids are accepted. By grafting the first multidomain module of the erythromycin-forming synthase (of Fig. 21-11) onto the wide-specificity loading module of the avermectin-forming synthase, a whole new series of antibiotics have been created.[351] This is only one of many steps being taken to create new aliphatic and aromatic, linear and macrocyclic polyketides by genetic engineering.[336,352,353] Combinatorial biosynthesis (see Chapter 3) is also being developed[336,354] and has even been discovered in nature.[355]

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Study Questions

1. Outline possible pathways of metabolism of dietary fats. Consider digestion, transport of fatty acids, storage, conversion to prostaglandins, steroids, etc. Will any of the fat be converted into glucose?

2. What are the functions in the human body of the following?

   Pancreatic lipase
   Lipocalins
   Lipoprotein lipase
   Very low density lipoprotein (VLDL)
   Hormone-sensitive lipase
   Chylomicrons
   Apolipoproteins

3. Describe two different types of fatty acid synthase. Compare the basic chemical reactions that are involved. Also, compare these with the reactions of fatty acid oxidation.

4. Discuss the different types of fatty acids found in the human body and the synthetic pathways by which they are formed.

5. What mechanisms are utilized for incorporation of double bonds into fatty acids? Propose a mechanism that makes use of polyketide synthase domains (Fig. 21-11) in the synthesis of polyunsaturated fatty acids. See Metz *et al.*[356]

6. In what locations would you expect to find the following?

   Tripalmitin
   Mycolic acids
   Arachidonic acid
   Propionic acid
   Docosahexaenoic acid

7. Substitution of a small percentage of ω6 fatty acids in the diet of insulin-resistant rodents with ω3 unsaturated fatty acids normalized insulin action.[357] Can you suggest possible mechanisms? Is this result significant to human nutrition?

8. Formation of the 3-hydroxymyristoyl groups of lipid A (Fig. 8-30) requires O_2. Comparisons of amino acid sequences suggest that an Fe^{2+}/2-oxoglutarate-dependent oxygenase is involved.[358] Write a balanced equation for this reaction.

9. Phosphatidylcholine can be formed by two pathways as described on pp. 1198–1199. A third pathway, used by some bacteria, involves a direct one-step reaction of choline with CDP-diacylglycerol.[359] Write a reasonable chemical mechanism.

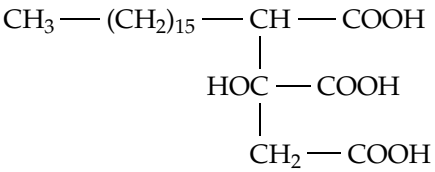
Study Questions

10. ^{14}C-Carboxyl labeled palmitic acid is fed to a fasted rat. There is no increase in liver glycogen, but the glucose units of the glycogen contain ^{14}C.
    a) Outline, using appropriate equations, the reaction sequence by which the carbon atoms of glucose become labeled.
    b) Explain why there is no net synthesis of glycogen from the fatty acid.

11. a) Write the reactions that most *dietary* tripalmitin will undergo in the body of an adult human in order to be deposited in adipose tissue as tripalmitin.
    b) What is the minimum amount of ATP (high energy bonds) normally required to deposit the one mole of dietary tripalmitin in adipose tissue? Count only ATP involved in tripalmitin metabolism and consider the source of glycerol in the adipose tissue.

12. Describe the biochemical effects on lipid metabolism of injecting into a normal animal
    a) insulin
    b) glucagon
    c) epinephrine

13. Suggest a biosynthetic pathway for formation of the fungal metabolite **agaricic acid**:



14. The ketone **palmiton** $CH_3(CH_2)14 - *CO - (CH_2)_{14}CH_3$ is formed by mycobacteria. The carbon marked by an asterisk was found to be labeled after feeding of [1-^{14}C]palmitic acid. Suggest a biosynthetic pathway.

15. The following reaction occurs in the biosynthesis of fatty acids.

    Crotonyl-ACP + NADPH + H^+ $\rightarrow$ butyryl-ACP + $NADP^+$

    The reduction half-reactions for crotonyl-ACP and NADPH are

    Crotonyl-ACP + 2 H^+ + 2 e^- $\rightarrow$ butyryl-ACP
    $E^{o'} = -0.015$ V
    $NADP + 2\ H^+ + 2\ e^- \rightarrow NADPH + H$
    $E^{o'} = -0.320$ V

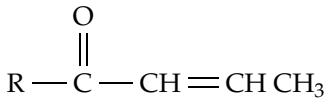
    What is $\Delta G^{o'}$ for this reaction? What is the equilibrium constant for the reaction?

16. How does the inhibition of citrate synthase affect fatty acid synthesis?

17. Malonyl CoA is an allosteric effector of carnitine acyl transferase. What kind of effector is it, i.e., activator or inhibitor, and what is the logic behind the interaction?

18. Compound II is formed in a series of enzymatic reactions from compound I. Propose a mechanistically realistic sequence, showing by name any cofactors required.

I

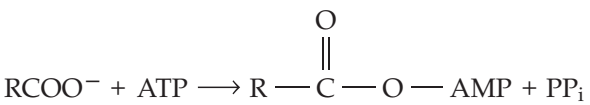


II

19. Fatty acid biosynthesis requires NADPH. Where does the NADPH come from?

20. An individual has been found who is missing malic enzyme in his cytoplasm. He has instead an enzyme that converts the oxalacetate made from the citrate lyase reaction directly to pyruvate and CO_2. Discuss this patient in terms of the likely effect of these changes on his ability to synthesize fatty acids.

21. The $\Delta G^{o'}$ values for the hydrolysis of any P – O – P bond of ATP, inorganic pyrophosphate, or any acyl CoA thiolester are all about –34 kJ/mole, while the corresponding figure for the hydrolysis of a mixed carboxylic phosphate anhydride is about –55 kJ/mole. Calculate the value of $\Delta G^{o'}$ for the following reaction describing the activation of fatty acids to the fatty acyl adenylate.



Study Questions

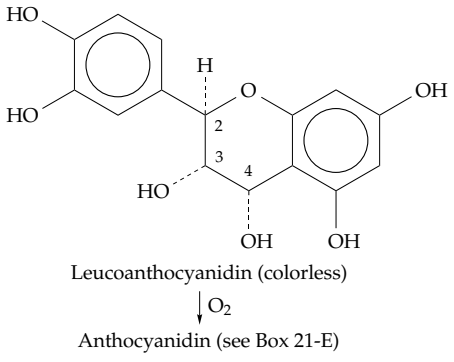
22. Fatty acid biosynthesis is made irreversible by the specific input of energy. Name the reactions or give equations for those steps in the pathway that require ATP. It is important that you consider both the mitochondrial and cytosolic components of the pathway.

23. The fatty acid biosynthesis pathway communicates with at least three other metabolic pathways either by sharing common intermediates or by regulatory mechanisms. Fill in the table below. List four molecules that have this function. You should name the additional pathway where each of these is found and briefly describe what it does in this second pathway. Do **not** use a redox cofactor as one of your choices.

Molecule	Other Pathway	Role in Second Pathway

24. The endogenous cannabinoid 2-arachidonoylglycerol is thought to play important roles both in the brain (Chapter 30) and in the immune system (Chapter 31). Leukocyte 12-oxygenase acts on this compound.[360] What products would be expected?

25. In the synthesis of anthocyanidins (Box 21-E) another Fe^{2+}/2-oxoglutarate-dependent oxygenase acts on the colorless leukoanthrocyanidin, which is then converted to the colored anthocyanidin:

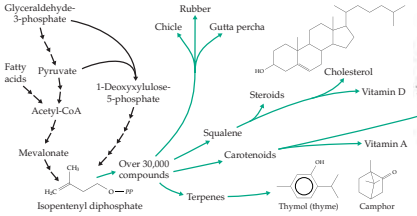


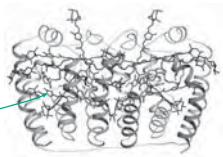
Propose a reasonable sequence for this reaction. See Nakajima *et al*.[361]

26. Why does a dietary deficiency of choline in humans induce a fatty liver, i.e., a liver in which the hepatocytes contain excess triglycerides?

27. Name two important functions of citrate in the conversion of acetate to palmitate by adipose tissues.

28. Outline the steps by which linoleic acid can be converted to prostaglandins in the human body.





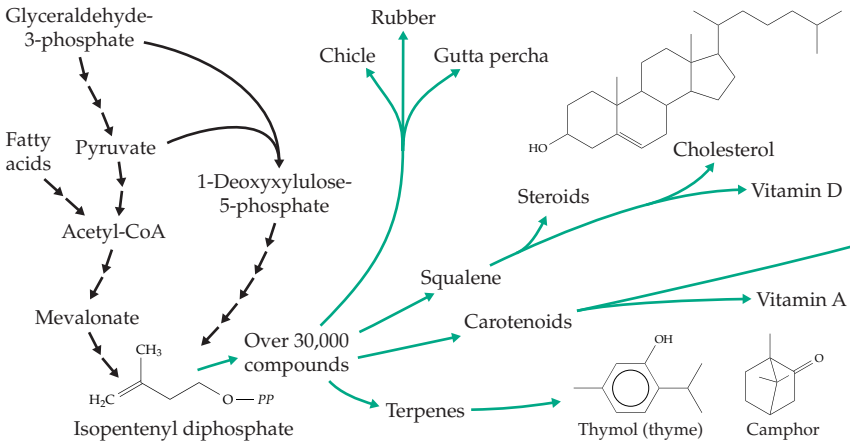
Peridinin, a carotenoid (8 molecules) embedded in a light-collecting complex from a dinoflagellate. See stereo view, Fig. 23-29

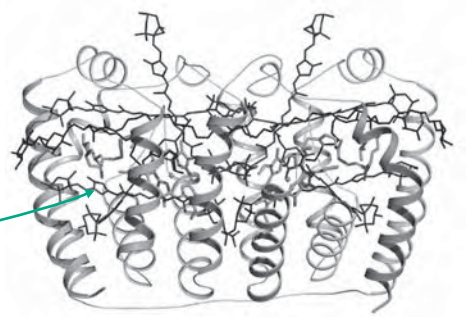
Starting with the simple compounds acetyl-CoA, glyceraldehyde-3-phosphate, and pyruvate, which arise via the central pathways of metabolism, the key intermediate **isopentenyl diphosphate** is formed by two independent routes. It is then converted by bacteria, fungi, plants, and animals into thousands of different naturally occurring products. These include high polymers, such as rubber, as well as vitamins, sterols, carotenoids, and over 30,000 different terpenes and related compounds. Many of the latter are found only in specific plants where they may function as defensive compounds or pheromones.

Contents

Polyprenyl (Isoprenoid) Compounds

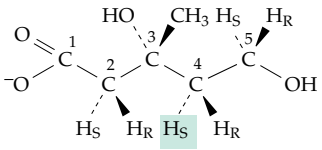
22



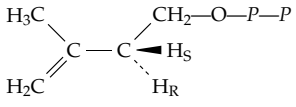


Peridinin, a carotenoid (8 molecules) embedded in a light-collecting complex from a dinoflagellate. See stereo view, Fig. 23-29

The **terpenes**, **carotenoids**, **steroids**, and many other compounds arise in a direct way from the prenyl group of **isopentenyl diphosphate** (Fig. 22-1).[1–6a] Biosynthesis of this five-carbon branched unit from **mevalonate** has been discussed previously (Chapter 17, Fig. 17-19) and is briefly recapitulated in Fig. 22-1. Distinct isoenzymes of 3-hydroxy-3-methylglutaryl-CoA synthase (HMG-CoA synthase) in the liver produce HMG-CoA destined for formation of ketone bodies (Eq. 17-5) or mevalonate.[7,8] A similar cytosolic enzyme is active in plants which, collectively, make more than 30,000 different isoprenoid compounds.[9,10] However, many of these are formed by an alternative pathway that does not utilize mevalonate but starts with a thiamin diphosphate-dependent condensation of glyceraldehyde 3-phosphate with pyruvate (Figs. 22-1, 22-2).



3(*R*) Mevalonate



Isopentenyl diphosphate

The two-step reduction of HMG-CoA to mevalonate (Fig. 22-1, step *a*)[11–15] is highly controlled, a major factor in regulating cholesterol synthesis in the human liver.[12,16,17] The N-terminal portion of the 97-kDa 888-residue mammalian HMG-CoA reductase is thought to be embedded in membranes of the ER, while the C-terminal portion is exposed in the cytoplasm.[16] The enzyme is sensitive to feedback inhibition by cholesterol (see Section D, 2). The regulatory mechanisms include a phosphorylation–dephosphorylation cycle and control of both the rates of synthesis and of proteolytic degradation of this key enzyme.[14,15,18–20]

A. Isopentenyl Diphosphate and Polyprenyl Synthases

In animals all isoprenoid compounds are apparently synthesized from mevalonate, which is converted by the consecutive action of two kinases[21–23] into mevalonate 5-diphosphate (Fig. 22-1, step *b*). Mevalonate kinase is found predominantly in peroxisomes, which are also active in other aspects of steroid synthesis in humans.[21,24] A deficiency of this enzyme is associated with mevalonic aciduria, a serious hereditary disease in which both blood and urine contain very high concentrations of mevalonate.[23] Mevalonate diphosphate kinase, which is also a decarboxylase, catalyzes phosphorylation of the 3-OH group of mevalonate (step *c*, Fig. 22-1) and decarboxylative elimination of phosphate (step *d*)[25] to form isopentenyl diphosphate.

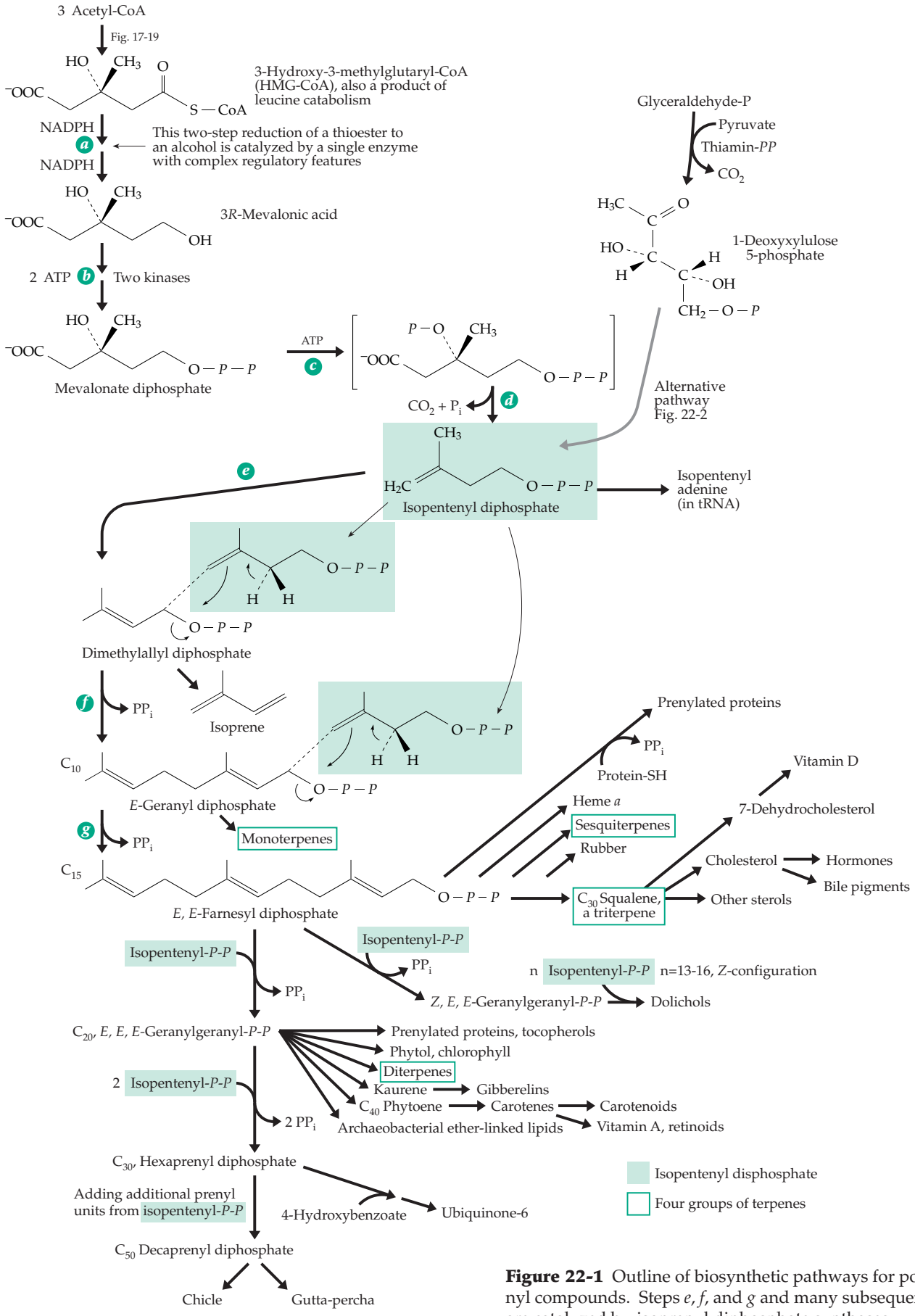


Figure 22-1 Outline of biosynthetic pathways for polyprenyl compounds. Steps *e*, *f*, and *g* and many subsequent steps are catalyzed by isoprenyl diphosphate synthases.

1. An Alternative Pathway for Isoprenoid Synthesis

It is generally agreed that mevalonate is the precursor to sterols in higher plants as well as in animals and is also the precursor to plant carotenoids. However, it is poorly incorporated into monoterpenes and into some diterpenes such as those of the taxane group.[26,27] The alternative **glyceraldehyde 3-phosphate:pyruvate pathway** explains this result. The pathway also operates in some bacteria and apparently is the sole source of isoprenoid compounds for the unicellular alga *Scenedesmus*.[28] The pathway is outlined in Fig. 22-2. Pyruvate is decarboxylated by a thiamin diphosphate-dependent enzyme,[29] and the resulting enamine is condensed with D-glyceraldehyde 3-phosphate to form 1-deoxyxylulose 5-phosphate.[28,30–31a] The latter undergoes an isomeroreductase rearrange-

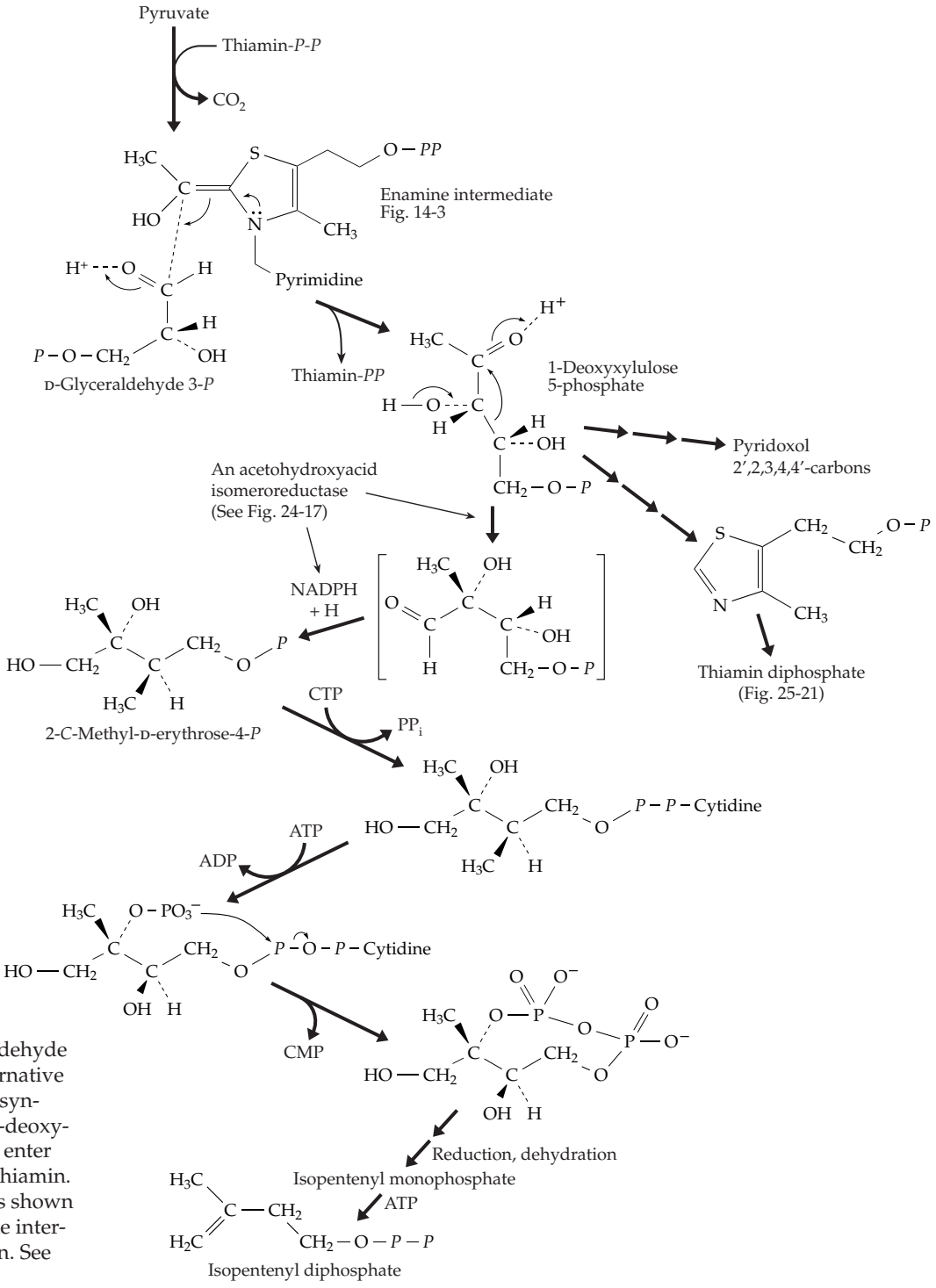
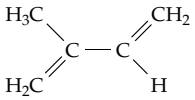


Figure 22-2 The glyceraldehyde 3-phosphate:pyruvate alternative pathway of isoprenoid biosynthesis. The intermediate 1-deoxyxylulose 5-phosphate may enter terpenes, vitamin B_6, and thiamin. Isopentenyl diphosphate is shown as the final product, but the intermediate steps are uncertain. See Lange *et al*.[32g]

ment of the type that occurs in the biosynthesis of valine and isoleucine (Fig. 24-17),[32] but the additional steps on to isopentenyl diphosphate are not obvious. However, some intermediate compounds have been identified as is indicated in Fig. 22-2.[32a–g] 1-Deoxyxylulose 5-phosphate has also been identified as an intermediate in the biosynthesis of vitamin B_6 in *E. coli*.[32a,h,33] It gives rise to the 2', 2, 3, 4, and 4' carbon atoms of pyridoxine and also to the pyrimidine ring of thiamin. See Chapter 25.

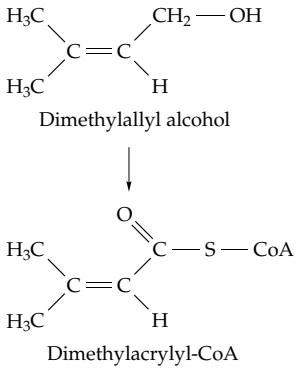
2. Isomerization and Isoprene Formation

Before polymer formation begins, one molecule of isopentenyl diphosphate must be isomerized to **dimethylallyl diphosphate** (Fig. 22-1, step *e*, Eq. 13-56).[10,33a,b] In this process the hydrogen that was in the 4-*pro-S* position of mevalonic acid (the *pro-R* position of isopentenyl pyrophosphate) is lost. Dimethylallyl diphosphate is unstable and can undergo acid-catalyzed elimination of PP_i to form isoprene, apparently by a carbocation mechanism.



Isoprene

This evidently accounts for the presence of isoprene in the breath.[34] Isoprene is also formed by many plants and is released into the atmosphere in large amounts, which contribute to photochemical formation of haze. A Mg^{2+}-dependent enzyme catalyzes the elimination of pyrophosphate.[35] Isoprene emissions rise with increasing temperature, and it has been suggested that the isoprene may dissolve in chloroplast membranes and in some way confer increased heat resistance.[36,37] Hydrolytic dephosphorylation can lead to dimethylallyl alcohol, which is oxidized in the liver to dimethylacrylyl-CoA (Eq. 22-1).

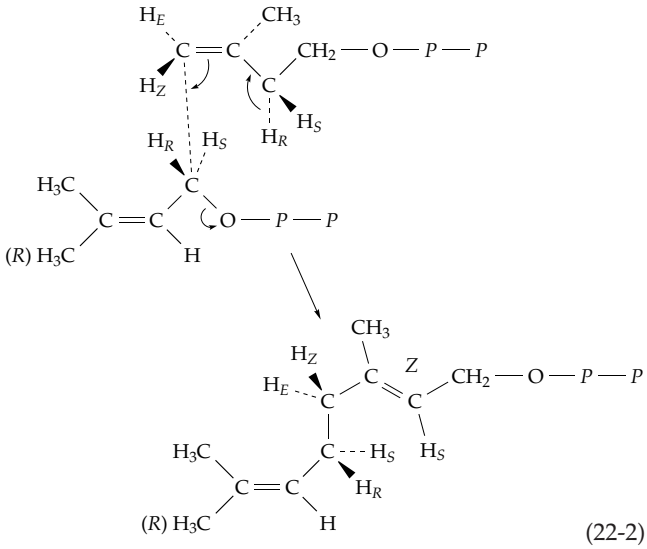


(22-1)

The latter is also a catabolite of leucine and can be converted back to HMG-CoA via a biotin-dependent carboxylation (see Fig. 24-18). This provides a means of recycling the dimethylallyl alcohol back to the polyprenyl pathway.[38]

3. Polyprenyl Compounds

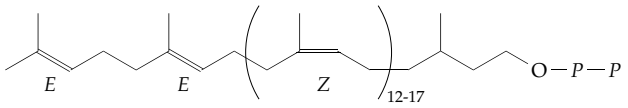
Dimethylallyl diphosphate serves as the starter piece for most polyprenyl compounds. Additional prenyl units are added, with elimination of pyrophosphate, by the action of **polyprenyl diphosphate synthases** as indicated in Fig. 22-1. Many of the products have all-trans (*E*) double bonds. A substantial number of these synthases are known and are distinguished by their chain length specificity and stereochemical properties.[39–46b] The most studied is farnesyl diphosphate synthase. The three-dimensional structure of an avian form is known.[47,47a] It catalyzes steps *f* and *g* of Fig. 22-1, joining three prenyl groups with the *E,E* (trans, trans) configuration. This protein, which consists almost entirely of packed α helices, has a large central cavity with conserved lysines and two aspartate-rich sequences (DDXXD) along its walls. These polar groups, together with magnesium ions, probably bind the pyrophosphate groups of the substrates.[48] Aspartates 224 and 225 of a bacterial form of the enzyme appear to be essential for catalytic activity.[49] The reaction is thought to be initiated by elimination of PP_i to form a carbocation to which the second prenyl unit adds as in Eq. 22-2.[50,51] For each prenyl unit a hydrogen atom that was originally the 4-*pro-S* hydrogen of mevalonic acid is lost as a proton.[52,53] Addition of another prenyl unit gives *E,E,E* geranylgeranyl diphosphate.[46a]



(22-2)

The chain length of the polyprenyl compounds appears to be determined by the protein structure of

the synthase.[45,54,54a,b] Polymerization of prenyl units can continue with the formation of high molecular weight polyprenyl alcohols such as the **dolichols** and bacterial decaprenols (Chapter 20) or of the high polymers **rubber** (all *Z* configuration), chicle, and **gutta-percha**.[6a] Dolichols, which function in the biosynthesis of glycoproteins, consist of 16–21 prenyl units and are synthesized in the endoplasmic reticulum as the diphosphates.[55–57] Farnesyl diphosphate is elongated to *Z*,*E*,*E* geranylgeranyl diphosphate, and polymerization continues with addition of 13–18 more units, all with the *Z* (cis) configuration.[46b,57a,b] However, after dephosphorylation[58] the double bond of the last unit added becomes saturated.[59] Partial absence of the required reductase causes a serious human deficiency disease involving faulty glycoprotein synthesis.[60]



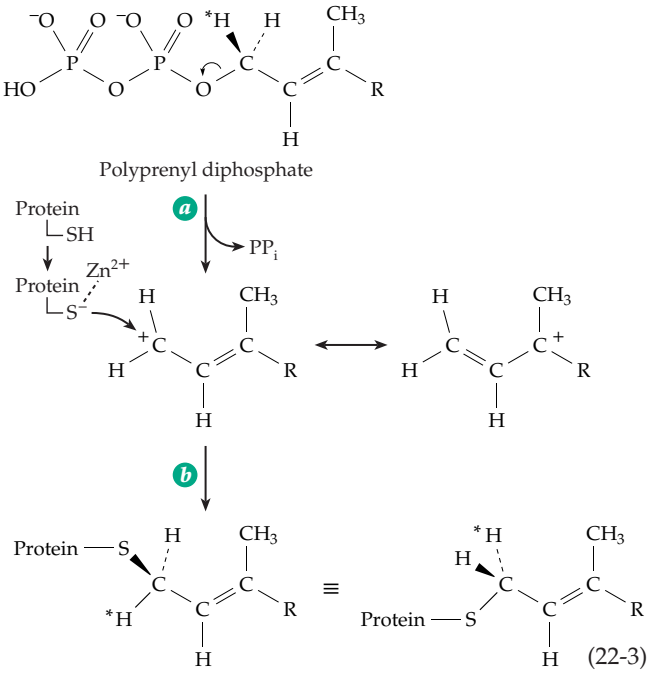
A fully extended 19-unit dolichol (dolichol-19) would have a length of about 10 nm, twice that of the bilayer in which it is dissolved. It has been suggested that the central part of the molecule has a helical structure, while the ends are more flexible. Dolichols also appear to increase the fluidity of membrane bilayers.[61] Bacterial undecaprenyl diphosphate, which has a similar function, contains only one *E* and ten *Z* double bonds[62–63a] (see p. 1152).

Rubber also contains almost entirely *Z* double bonds. Consistent with this fact is the finding that the prenyltransferases catalyzing formation of rubber promote loss of the *pro-R* proton rather than the *pro-S* proton of mevalonic acid (see Eq. 22-2). There appear to be two types of prenyltransferase in animal mitochondria giving rise to *E* and *Z* double bonds, respectively.[64] In contrast, the rubber tree contains a 137-residue protein, the **rubber elongation factor**. This small protein binds to *E* prenyltransferases causing them to form *Z* double bonds.[65] The bacterium *Micrococcus luteus* synthesizes all *E* polyprenyl alcohol diphosphates up to the C_{45} nonaprenyl compound **solanesyl diphosphate**.[66]

Chain elongation during polymerization of prenyl units can be terminated in one of a number of ways. The pyrophosphate group may be hydrolyzed to a monophosphate or to a free alcohol. Alternatively, two polyprenyl compounds may join "head to head" to form a symmetric dimer. The C_{30} terpene **squalene**, the precursor to cholesterol, arises in this way from two molecules of farnesyl diphosphate as does **phytoene**, precursor of the C_{40} carotenoids, from *E*,*E*,*E* geranylgeranyl diphosphate. The phytanyl groups of archaebacterial lipids (p. 385) arise rather directly from geranylgeranyl diphosphate by transfer of the polyprenyl group to the $-CH_2OH$ group of *sn*-glycerol 3-phosphate.[67,67a] This is followed by hydrogenation of the double bonds. Formation of diphytanyl group (p. 388) must involve additional crosslinking reactions.

4. Prenylation of Proteins and Other Compounds

Polyprenyl groups are often transferred onto thiolate ions of cysteine side chains of certain proteins that bind to membranes (p. 559).[68,69] We have previously considered the Ras family (Chapter 11). Recoverin, an important protein in the visual cycle (Fig. 23-43), is another example of a prenylated protein. Both **farnesyltransferases**[70–76f] and **geranylgeranyltransferases**[72,77–78b] have been characterized, and the three- dimensional structure of the former has been established.[73,75–76] The two-domain protein contains a seven-helix crescent-shaped hairpin domain and an α,α-barrel similar to that in Fig. 2-29. A bound zinc ion in the active site may bind the $-S^-$ group of the substrate protein after the farnesyl diphosphate has been bound into the active site.[76,79] These enzymes are thought to function by a carbocation mechanism as shown in Eq. 22-3 and with the indicated inversion of configuration.[71]



Inhibition of these prenyltransferases blocks growth of tumor cells. Many prenyltransferase inhibitors are apparently nontoxic to normal cells and are undergoing human clinical trials as anticancer drugs.[76a, 79a,b] Among other important polyprenyl

compounds are the side chains of vitamin K, the ubiquinones, plastoquinones, tocopherols, and the phytyl group of chlorophyll. In all cases, a diphosphate of a polyprenyl alcohol serves as an alkyl group donor. Introduction of the polyprenyl chain into aromatic groups, such as those of the quinones (Fig. 15-24), occurs at a position ortho to a hydroxyl group in the reduced quinone (hydroquinone). The reader should be able to propose a reasonable prenyltransferase mechanism involving participation of the hydroxy group. The monoprenyl compound dimethylallyl diphosphate prenylates the N^6 position of adenine in a specific site in many tRNA molecules (Fig. 5-33)[80] as well as the C-4 position of L-tryptophan in the synthesis of ergot alkaloids.[81]

B. Terpenes

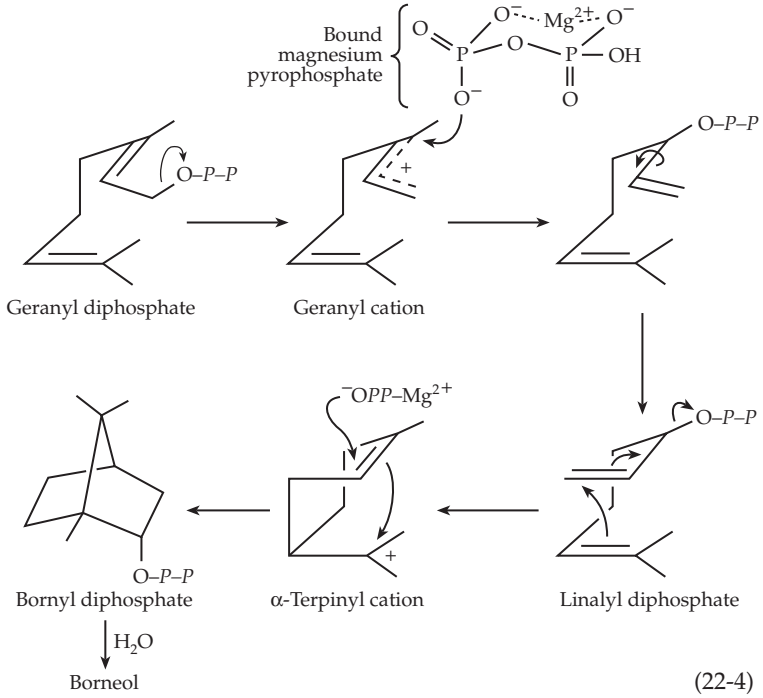
The number of small compounds that arise from isopentenyl diphosphate and are found in plants, animals, and bacteria is staggering. Just a few of these "terpenes" are shown in Figs. 22-3 and 22-4. The biosynthetic pathways have been worked out by "feeding" radioactively labeled acetate to plants and studying the characteristic labeling patterns in the terpenes. Many of the enzymes involved have been identified and studied. A given plant usually contains a large number of different terpenes, which are often concentrated in specialized "oil glands" or resinous duct tissues. Lesser amounts, often as glycosides of terpene alcohols, may be present within cells. Some terpenes occur in truly enormous amounts. For example, turpentine may contain 64% of **α-pinene** and juniper oil 65% **α-terpineol**.[82] The large quantities of α-pinene released into the air from pine trees are a major cause of photochemical smog.[83]

Terpenes have a variety of functions. Plant terpenes may deter herbivores and attract pollinators. They may participate in competition among plants and may act as antibiotics, called **phytoalexins**, to protect plants from bacteria and fungi.[84] In invertebrate animals terpenes serve as hormones, pheromones, and defensive repellants (Figs. 22-3, 22-4). The terpene squalene is the precursor to sterols. Some terpenes are toxic. For example, thujone (Fig. 22-3), which is present in the liqueur absinthe, causes serious chronic poisoning.[84]

1. Biosynthesis of Monoterpenes

The compounds of Figure 22-3 each contain ten carbon atoms and are called monoterpenes. They occur largely in plants, but some function in arthropods as pheromones. As with chain elongation, the cyclization of geranyl diphosphate to the various monoterpenes appears to occur through loss of pyrophosphate (as PP_i) with formation of an intermediate carbocation such as that depicted in Equation 22-3.[85–88a] Similar mechanisms initiate cyclization of sesquiterpenes and diterpenes. Numerous terpene cyclases have been isolated, and several have been studied carefully. A stereochemical view of the formation of borneol is illustrated in Eq. 22-4. Both linalyl-*PP* and bornyl-*PP* are intermediates. Croteau and associates suggested that a tight ion pair between carbocation and magnesium pyrophosphate is maintained at each stage.[86,89]

As is indicated in Fig. 22-3, the same intermediate cation can yield a variety of end products. For example, pure geranyl diphosphate: pinene cyclase catalyzes formation of several other terpenes in addition to α-pinene.[89] Another aspect of terpene synthesis is that insects may convert a plant terpene into new compounds for their own use. For example, **myrcene**, which is present in pine trees, is converted by bark beetles to **ipsenol** (Fig. 22-3), a compound that acts as an aggregation pheromone.[90]



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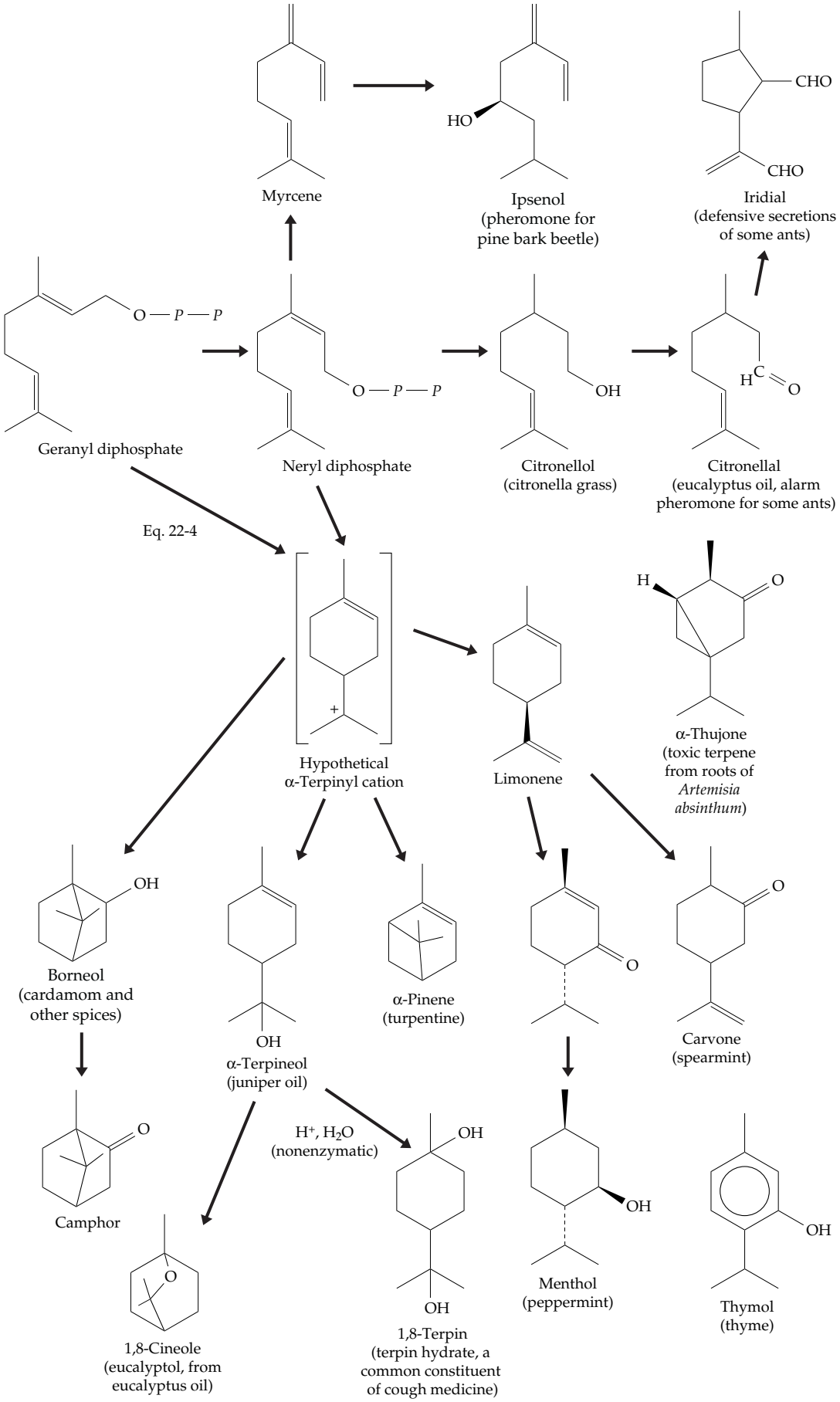
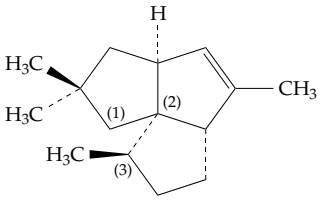


Figure 22-3 Probable biosynthetic pathways of some monoterpenes and related substances. Some of the natural sources are indicated.

2. Sesquiterpenes and Diterpenes

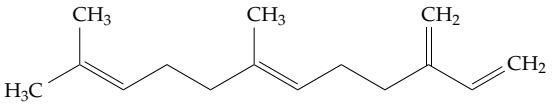
Most of the compounds shown in Figure 22-4 are derived from the C_{15} farnesyl diphosphate. There are more than 300 known cyclic structures among these **sesquiterpenes,** and many sesquiterpene synthases have been characterized.[91,91a] **Aristolochene** is formed by the action of a 38-kDa cyclase that has been isolated from species of *Penicillium* and *Aspergillus*.[92–94] Notice that the synthesis must involve two cyclization steps and migration of a methyl group. Three-dimensional structures are known for at least two terpene synthases,[95,96] and comparison of gene sequences suggests that many others have similar structures. The **5-epi-aristolochene synthase** of tobacco makes the 5-epimer of aristolochene (Fig. 22-4). It binds the diphosphate group within a central cavity using two Mg^{2+} ions held by carboxylates, some of which are in the DDXXD sequence found also in polyprenyl diphosphate synthases. The enzyme active sites of both the epi-aristolochene synthase and a **pentalenene synthase** from *Streptomyces*[96] are rich in polar groups that form hydrogen-bonded networks and which participate in proton abstraction and donation during the rearrangement reactions that must occur.



Pentalenene

Aromatic groups that are also present may assist in stabilizing intermediate carbocationic species. Deprotonation by an aspartate side chain in the epi-aristolochene synthase has been proposed to assist the cyclization; subsequent reprotonation by an Asp•Tyr•Asp triad would generate a new carbocation and promote the necessary methyl group migration. A detailed step-by-step mechanism has been proposed.[95] The fungal pentalenene synthase has an active site histidine, which is proposed to serve as proton acceptor and donor for the several steps of the reaction of farnesyl diphosphate.[96] The carbon atoms originating from C1 to C3 of the precursor have been marked on the pentalenene structure as well as on the aristolochene structure in Fig. 22-4. We now see how synthases can guide the terpene cyclase reactions to give specific products.

Another sesquiterpene synthase forms **trichodiene**, the parent compound for a family of mycotoxins and antibiotics.[97,97a,b] A different sesquiterpene synthase, present in peppermint and also in a wide range of other plants and animals, forms the acyclic **(E)-β-farnesene**.[98] It serves as alarm pheromone for aphids, has a variety of signaling functions in other insects, and is a urinary pheromone in mice.[98]



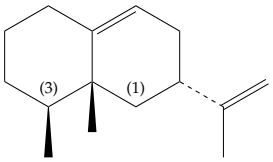
(*E*)-β-Farnesene

Abscisic acid, one of five known types of plant hormone of general distribution throughout higher plants, is not regarded as a true terpene because it arises by degradation of a carotenoid.[99–99b] However, its structure (Fig. 22-4) is that of a sesquiterpene.

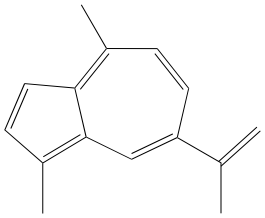
The C_{20} **diterpenes** are derived from geranylgeranyl-*PP*. Among the best known members are another group of plant hormones, the **gibberellins**.[100–102a] The first gibberellin was isolated as a product of plants infected with a *Fusarium* fungus. The rice plants grew in an abnormally tall, weak form. Subsequently, this multimembered class of over 50 highly modified diterpenes have been shown to have a variety of regulatory functions in all higher plants. For example, gibberellins are essential for stem elongation.

Equation 22-5 gives an abbreviated biosynthetic sequence for gibberellin A_1. The ring closure of step *a*, Eq. 22-5, may be initiated by protonation of the double bond at the left of the first structural formula. The resultant carbocation could initiate the consecutive closure of the two rings and the loss of a proton from a methyl group (step *b*) to yield copalyl-*PP*. Steps *c* and *d*, Eq. 22-5, each represent several reactions. In step *c*, pyrophosphate is eliminated, and the methyl group that becomes a methylene in *ent*-kaurene (enantiotopic kaurene) undergoes migration.[102b] Step *d* involves several hydroxylation and oxidation steps as well as a ring contraction through which one of the original methylene groups ends up as a carboxyl group in the final product.[102,103,103a] Deactivation of the hormone is initiated by a 2-oxoglutarate-dependent dioxygenase.[103b]

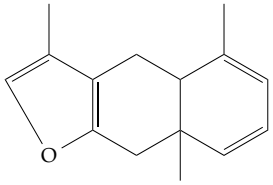
The **juvenile hormone** of insects (Fig. 22-4) is also of polyprenyl origin.[104,105] However, two of the methyl groups have been converted to (or replaced by) ethyl groups. The isolation and identification of the structure of the juvenile hormone was a difficult task. After its completion it was a surprise to researchers to discover that a large variety of synthetic compounds, sometimes with only a small amount of apparent structural similarity, also serve as juvenile hormones, keeping insects in the larval stage or preventing insect eggs from hatching. Furthermore, a number of plant products such as **juvebione** (Fig. 22-4),[106] which was originally isolated from paper, have the same effect. Thus, in nature products of plant metabolism have a profound effect upon the development of insects that



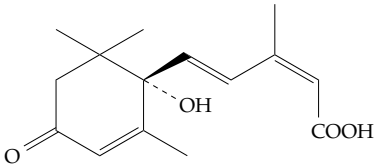
Aristolochene, parent hydrocarbon of many fungal toxins



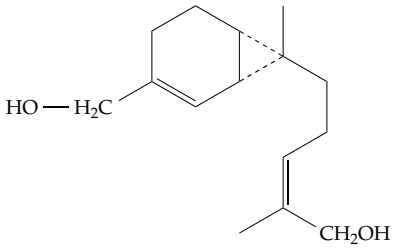
Lactarazulene, dye from the mushroom *Lactarius deliciosus*



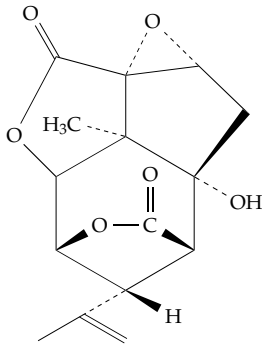
Furanoeudesma-1,3-diene, a sesquiterpene from myrrh with analgesic properties



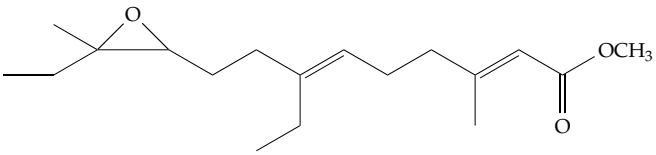
Abscisic acid, an important hormone of green plants



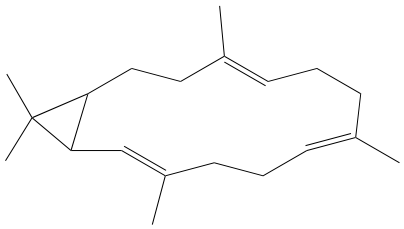
Sirenin, a sperm attractant produced by female gametes of the phycomycete *Allomyces*



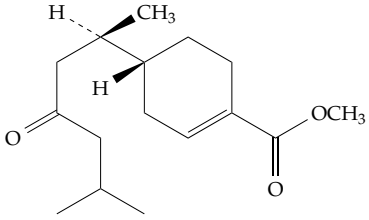
Picrotoxin, a plant toxin sometimes used to kill fish



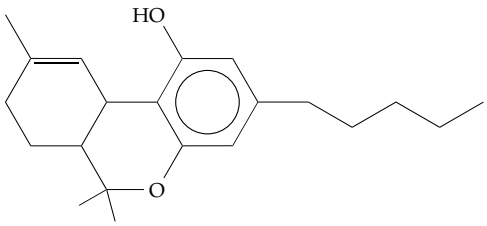
Juvenile hormone, a substance acting to maintain the larval stage of insects



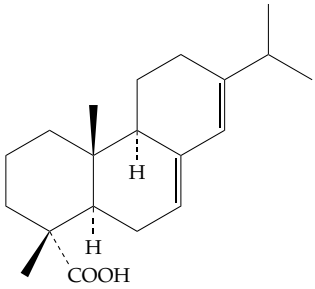
Casbene, a macrocyclic phytoalexin



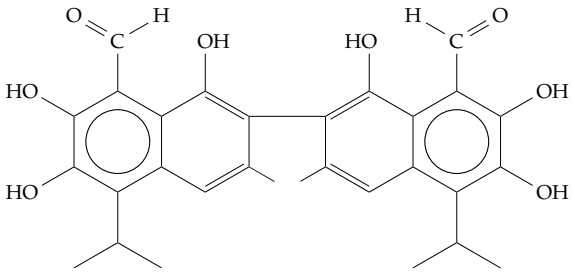
Juvebione, a product of the balsam fir with juvenile hormone activity



Δ'-3,4-*trans*-Tetrahydrocannabinol (Δ'-THC) a psychotropic component of marijuana

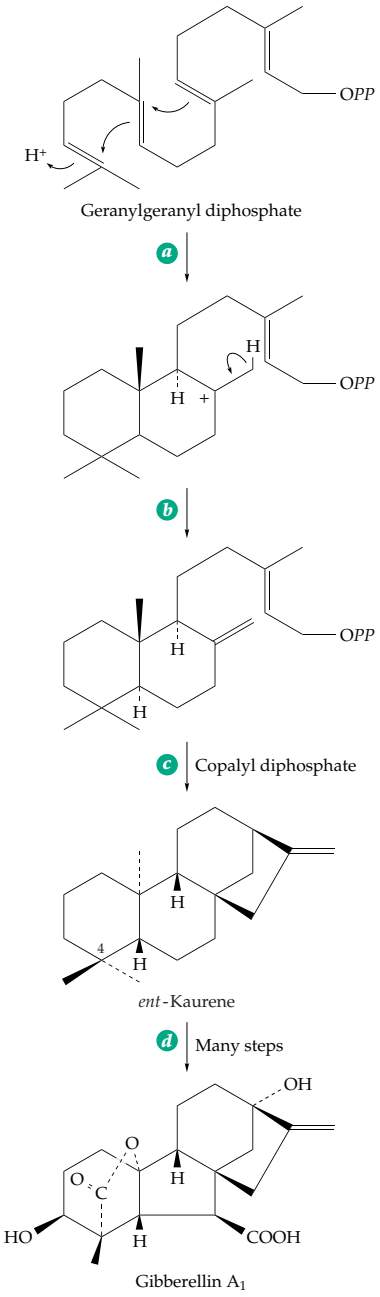


Abietic acid, a component of rosin, from conifers



Gossypol, toxic, dimeric sesquiterpene from cottonseed

Figure 22-4 More terpenes and related substances. The numbers in parentheses on the aristolochene structure are those of atoms in the precursor farnesyl diphosphate.



(22-5)

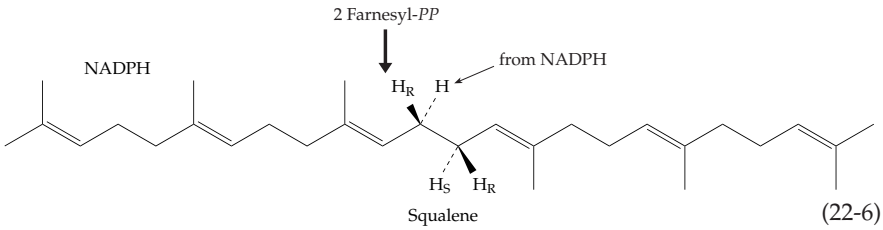
eat the plants. There is interest in the possible use of juvenile hormone, or of synthetic compounds mimicking its action, as insecticides.

Many conifers secrete **oleoresin** (pitch) in response to attack by bark beetles. The oleoresin contains approximately equal amounts of turpentine (a mixture of monoterpenes and sesquiterpenes) and diterpenoid rosin including **abietic acid** (Fig. 22-4).[91,107,107a] The oleoresin is toxic to beetles and, after evaporation of the turpentine, forms a hard rosin seal over the wounds. **Casbene** (Fig. 22-4) is a diterpene produced by castor beans as a phytoalexin (Chapter 31), an antifungal antibiotic.[108] The synthesis of the anticancer compound taxol (Box 7-D) from geranylgeranyl diphosphate involves extensive oxidative and other modification.[109]

3. Formation of the Symmetric Terpenes, Squalene and Phytoene

Two molecules of the C_{15} farnesyl diphosphate can be joined "head to head" to form the C_{30} squalene (Eq. 22-6). Similarly, two C_{20} **geranylgeranyl-*PP*** molecules can be joined to form the C_{40} phytoene (Fig. 22-5), a precursor of carotenoid pigments of plants.[110] In the synthesis of squalene both pyrophosphate groups are eliminated from the precursor molecules, and one proton from C-1 of one of the molecules of farnesyl-*PP* is lost. The other three C-1 hydrogens are retained. At the same time, one proton is introduced from the *pro-S* position of NADPH. Squalene synthase has been difficult to obtain,[111,112] and its mechanism has been uncertain. However, there is strong evidence in favor of carbocationic intermediates.[113–114b] The first step (Eq. 22-7, step *a*) involves reaction of the initial carbocation with the double bond of the second farnesyl-*PP* to form the cyclopropane derivative **presqualene alcohol-*PP***, which was first isolated from yeast as the free alcohol by Rilling and associates.[115] The loss of the second pyrophosphate (Eq. 22-7, step *b*) generates a cyclopropylcarbinyl cation, which can rearrange (step *c*) to a more stable tertiary cation.[112,113] The latter is reduced by NADPH with opening of the cyclopropane ring (Eq. 22-7, step *d*). Once formed squalene diffuses within and between membranes with the help of cytosolic protein carriers.[116]

Phytoene (Fig. 22-5) is apparently formed from geranylgeranyl-*PP* via **prephytoene-*PP*,** whose structure is entirely analogous to that of presqualene-*PP*.[44,117] However, no reduction by NADH is required (Eq. 22-8). It is known that the 5-*pro-R* hydrogen atoms of mevalonate are retained in the phytoene as indicated by a shaded box in Eq. 22-8. Elimination of the other (*pro-S*) hydrogen yields 15,15'–*Z* phytoene (*cis*-phytoene), while elimination of the *pro-R* hydrogen yields all-*E* (trans) phytoene. Higher plants and fungi form mostly *cis*-phytoene, but some bacteria produce the all-*E* isomer.[118]



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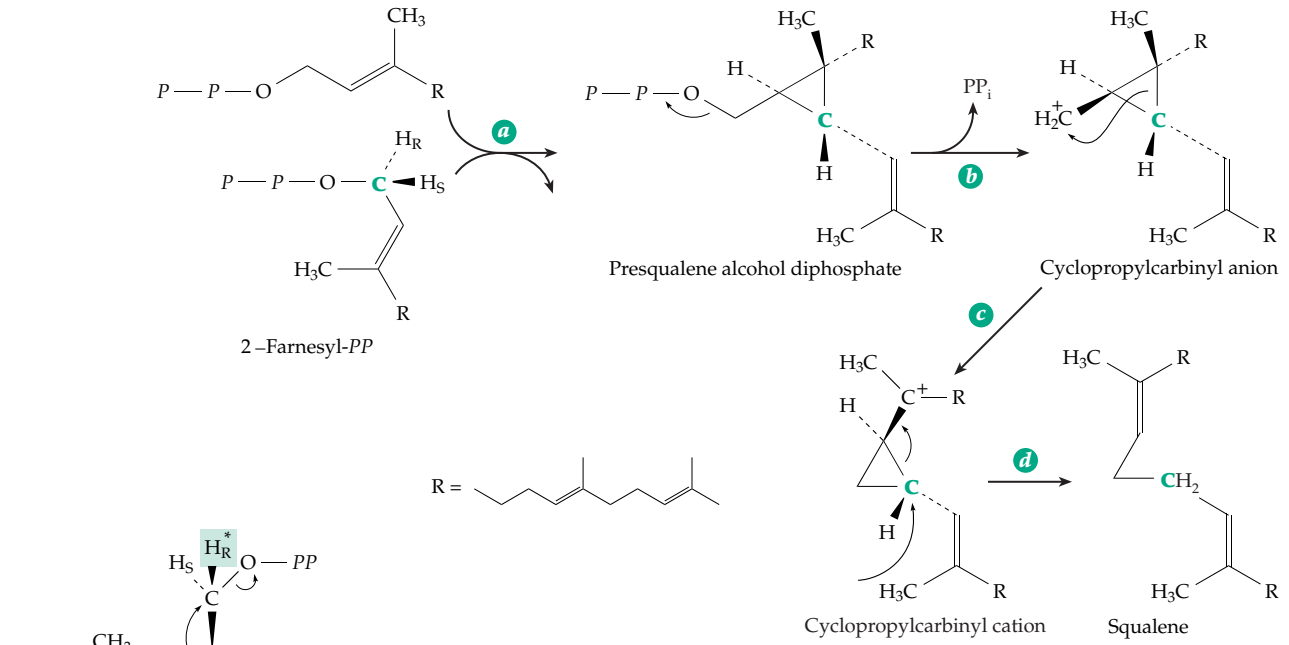

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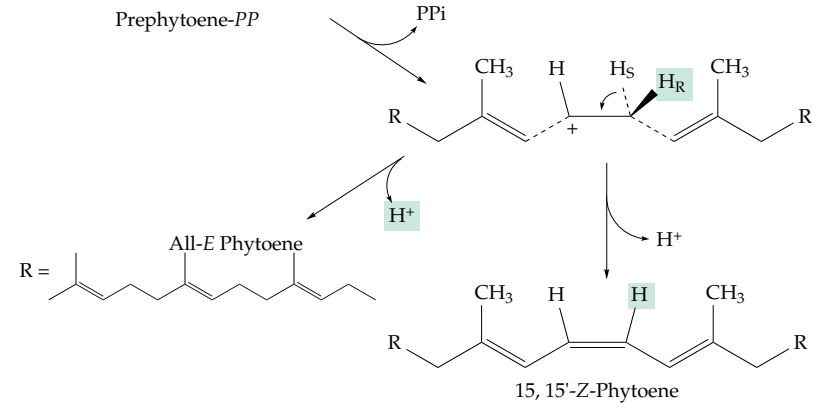
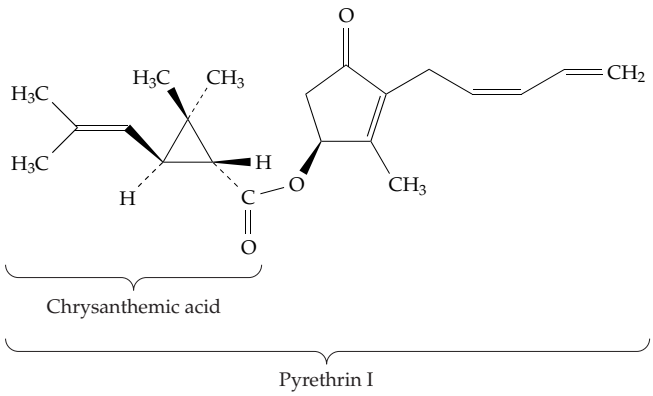


(22-8)

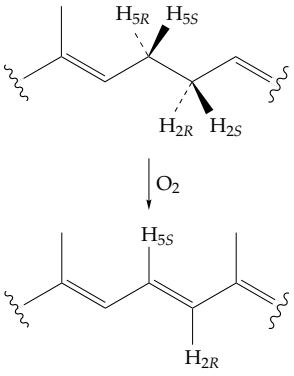
Another polyprenyl compound formed by a head to head condensation is **chrysanthemic acid**. This monoterpene component of the pyrethrum insecticides is formed by chrysanthemums from two molecules of dimethyallyl-*PP* via an intermediate analogous to presqualene alcohol-*PP*.[118a]



A quite different "tail to tail" condensation, whose chemistry is still obscure,[118b] must occur in archaebacteria whose lipids contain the C_{40} diphytanyl alcohol. An example is the diglyceryltetraether,[119,120] whose structure is shown on p. 388.

C. Carotenes and Their Derivatives

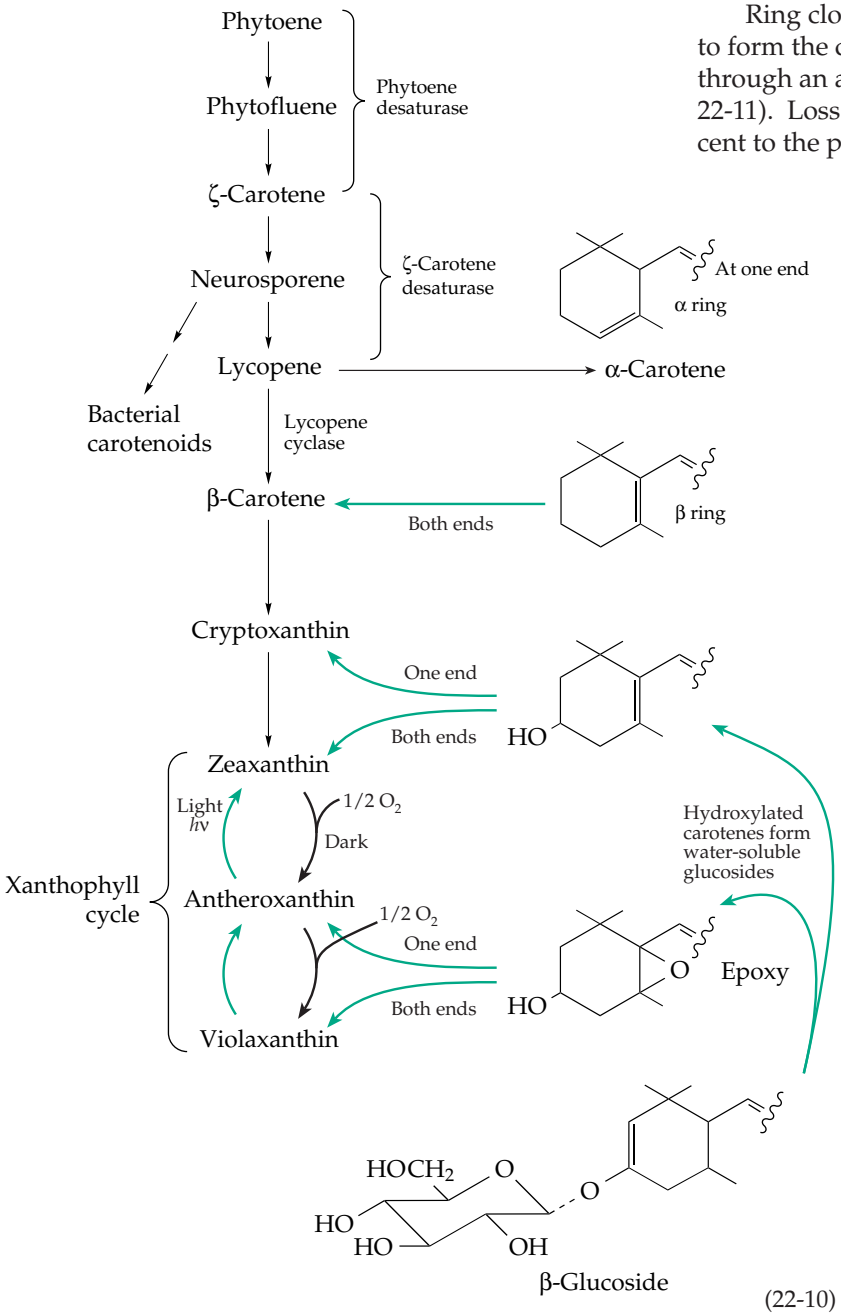
Phytoene can be converted to the carotenes by pathways indicated in part in Fig. 22-5 and Eq. 22-10. One of the first products is **lycopene**, the red pigment of tomatoes and watermelons, which is an all-trans compound. If 15-*Z* phytoene is formed, it must, at some point, be isomerized to an all-*E* isomer, and four additional double bonds must be introduced. The isomerization *may* be nonenzymatic. The double bonds are created by an oxygen-dependent **desaturation,** which occurs through the trans loss of hydrogen atoms.



(22-9)

Desaturation takes place in a stepwise fashion, and many intermediate compounds with fewer double bonds are known (Eq. 22-10).[118,121–123] The enzymes required have not been characterized well until recently. Plant enzymes are present in small amounts, and isolation has been difficult. However, the genes for carotenoid biosynthesis in such bacteria as the purple photosynthetic *Rhodobacter*,[118,124] *Rhodospirillum*,[125] and *Rubrivarax*,[126] the cyanobacterium *Synechococcus*,[127] and the nonphotosynthetic *Erwinia*[44,118] have been cloned, sequenced, and used to produce enzymes in quantities that can be studied. Matching genes from higher plants have also been cloned and expressed in bacteria.[123]

Ring closure at the ends of the lycopene molecule to form the carotenes can be formulated most readily through an acid-catalyzed carbocation mechanism (Eq. 22-11). Loss of one or the other of two protons adjacent to the positive charge leads to the β ring of β-carotene or to the α ring of α-carotene.[110,123a] Compounds with only one ring may also be formed.[123b] In many bacteria



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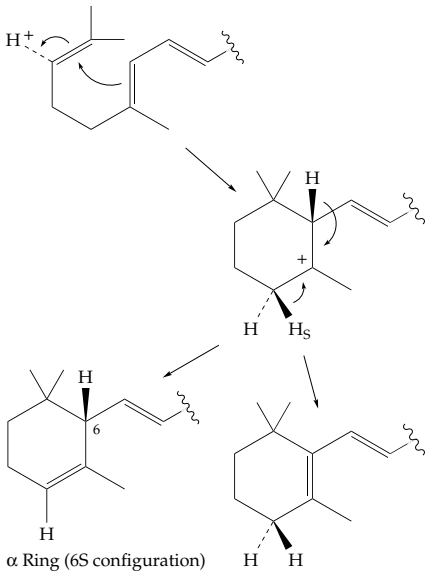


(22-11)

these rings are not formed at all, but the open-chain (acyclic) cartenoids may be modified in ways similar to those of higher plants.[118,124,125]

A genetic engineering success is the transfer of genes for synthesis of β-carotene into rice. The resulting "golden rice" contains enough carotene in its endosperm to make a significant contribution to the vitamin A needs of people for whom

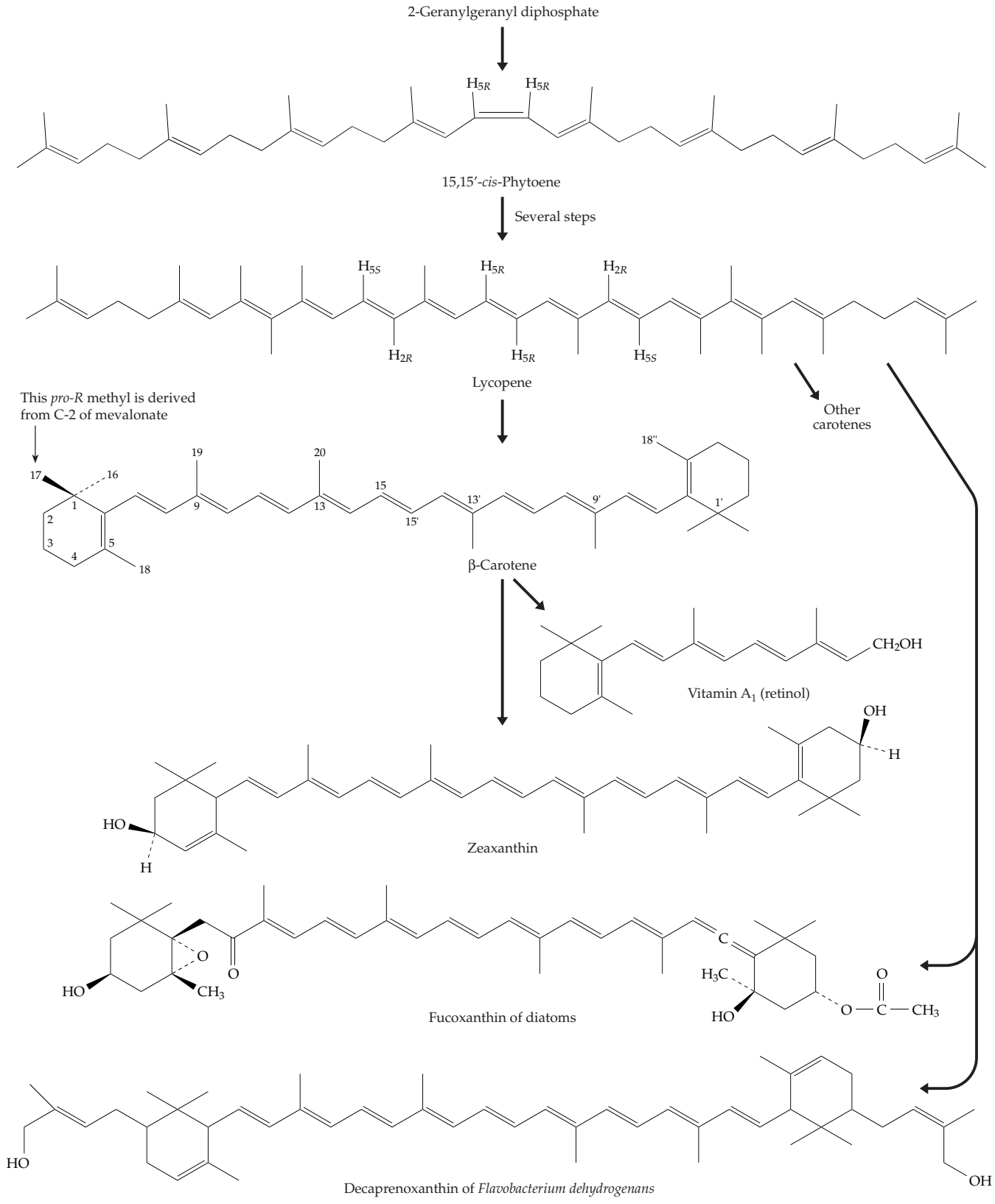
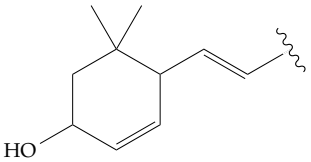


Figure 22-5 Structures and partial biosynthetic pathways for a few of the more than 600 known carotenoid compounds. The origin of some hydrogen atoms from mevalonate is shown, using the numbering for mevalonate. The numbering system for C_{40} carotenoids is also indicated.

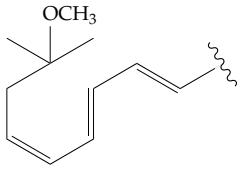
rice is a major food. There are estimated to be over 100 million vitamin A-deficient children in the world. As many as one-quarter of a million of these go blind each year.[127a] It is hoped that the golden rice will help to alleviate the problem. At the same time, an ongoing program is supplying vitamin A, which is stored in the liver, at regular intervals of time to many children.[127b]

1. Xanthophylls and Other Oxidized Carotenes

Carotenes can be hydroxylated and otherwise modified in a number of ways.[110,128–131] The structure of zeaxanthin, one of the resulting **xanthophylls**, is indicated in Fig. 22-5. Some other xanthophylls are shown in Eq. 22-10. Lutein resembles zeaxanthin, but the ring at one end of the chain has been isomerized by a shift in double bond position to the accompanying structure. The photosynthetic bacterium *Rhodospirillum rubrum* has its own special carotenoid spirilloxanthin, which has the accompanying structure at both ends of the chain.



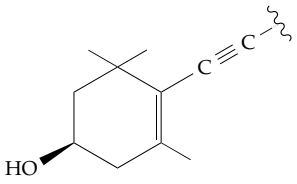
Lutein (structure at one end of molecule)



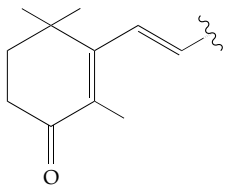
Spirilloxanthin

Fucoxanthin (Fig. 22-5) is the characteristic brown pigment of diatoms. One end of the molecule has an epoxide, also formed by the action of O_2, while the other end contains an **allene** structure rare in nature. Even so, fucoxanthin may be the most abundant carotenoid of all. The structure of the allene-containing end of the fucoxanthin molecule (turned over from that shown in Fig. 22-5) is also given in Eq. 22-12. Figure 22-5 does not indicate the stereochemistry of the allene group correctly; the carotenoid chain protrudes behind the ring as drawn in the equation. **Violaxanthin** contains epoxide groups in the rings at both ends of the molecule (Eq. 22-12). An isomerase in algae converts violaxanthin into **neoxanthin** (Eq. 22-12), which contains the allene structure at one end. Subsequent acetylation yields fucoxanthin.

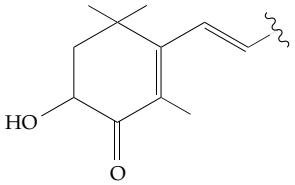
Other algal carotenoids contain acetylenic triple bonds. For example, **alloxanthin** has the following structure at both ends of the symmetric molecule. The symmetric carotenoids **canthaxanthin** and **astaxanthin** have oxo groups at both ends:



Alloxanthin



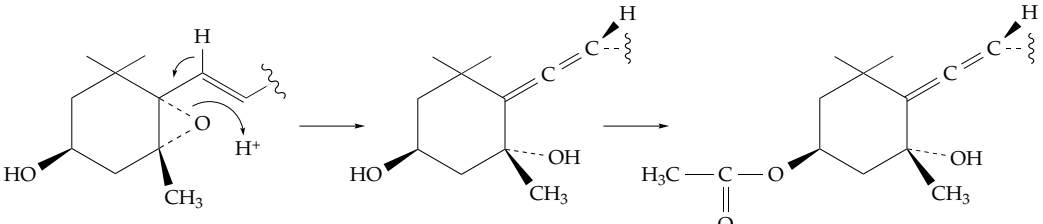
Canthaxanthin



Astaxanthin

These carotenoids have a limited distribution and occur as complexes, perhaps in Schiff base linkage, with proteins. Astaxanthin–protein complexes with absorption maxima ranging from 410 nm to 625 nm or more provide the color to the lobster's exoskeleton.[130,132] Whereas most naturally occurring carotenoids have all-*E* double bonds, mono-*Z* isomers of canthaxanthin are found in the colored carotenoproteins of the brine shrimp *Artemesia*.[133]

Some bacteria synthesize C_{50} carotenoids such as decaprenoxanthin (Fig. 22-5), the extra carbon atoms at each end being donated from additional prenyl groups, apparently at the stage of cyclization of lycopene.[134] Thus, a carbocation derived by elimination of pyrophosphate from dimethylallyl-*PP* could replace the H^+ shown in the first step of Eq. 22-11. The foregoing descriptions deal with only a few of the many known structural modifications of carotenoids.[2,135,136]



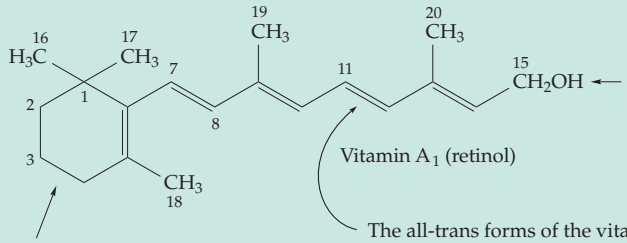
Violaxanthin (ring structure present at both ends)

Neoxanthin (this structure occurs at one end only)

Fucoxanthin

(22-12)

BOX 22-A VITAMIN A



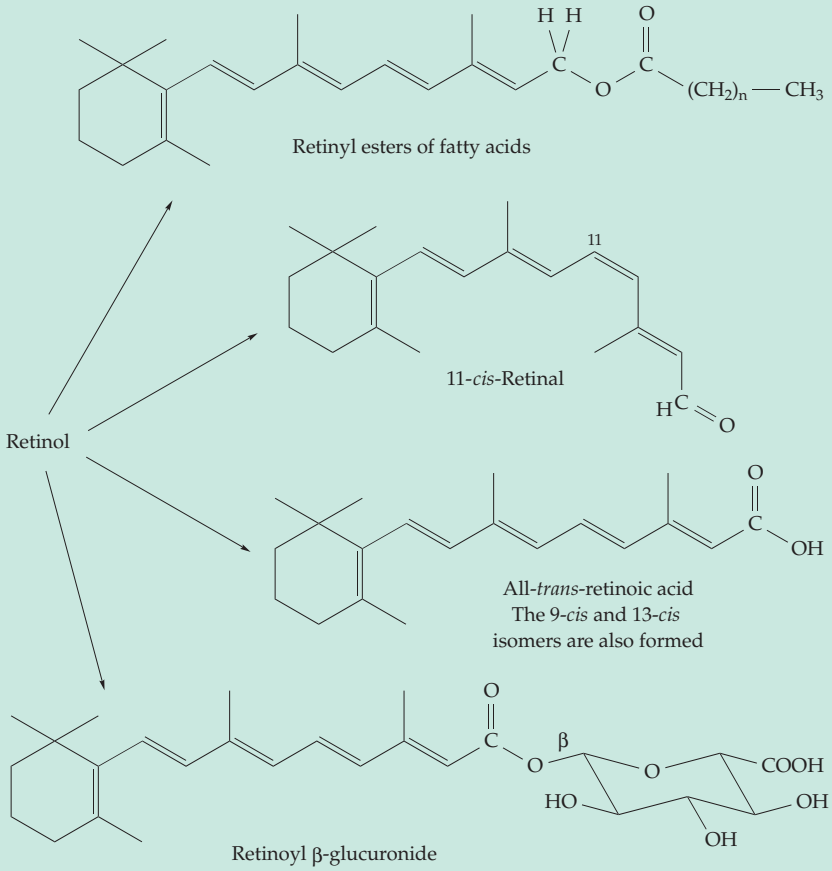
The recognition in 1913 of vitamin A (Box 14-A) was soon followed by its isolation from fish liver oils.[a–c] Both vitamin A_1 (**retinol**) and vitamin A_2 are 20-carbon polyprenyl alcohols. They are formed by cleavage of the 40-carbon β-carotene (Fig. 22-5) or other carotenoids containing a β-ionone ring. While the carotenes are plant products, vitamin A is produced only in animals, primarily within cells of the intestinal mucosa.[d–f] The carotene chains are cleaved in the center, and to some extent in other positions,[g] by oxygenases; β-carotene yields as many as two molecules of the vitamin A aldehyde **retinal**.[h–i] The retinal is reduced by NADH to retinol which is immediately esterified, usually with saturated fatty acids, by transfer of an acyl group from a fatty acyl-CoA or from phosphatidylcholine. The resulting retinyl esters are transported in chylomicrons. They remain in the chylomicron remnants (see Fig. 21-1), which are taken up by the liver where both hydrolysis and reesterification occur.[j] Vitamin A is one of the few vitamins that can be stored in animals in relatively large quantities. It accumulates in the liver, mainly as retinyl palmitate, in special storage cells termed stellate cells.[f] The human body usually contains enough vitamin A to last for several months.

Free retinol is released from the liver as a 1:1 complex of retinol with the 21-kDa **retinol-binding protein**.[k,l] This protein is normally almost saturated with retinol and is bound to another serum protein, the 127-residue **transthyretin** (prealbumin).[m,n] Some of the retinol is oxidized to **retinoic acid**. Both all-*trans* and 13-*cis*-retinoic acids as well as 5,6-epoxyretinoic acid are found in tissue.[f,o,p,pq]

Another metabolite, which may be very important, is **retinoyl β-glucuronide**.[q,r,rs]

Cell surfaces of body tissues appear to contain receptors for the retinol-binding protein. Many cells also contain cytoplasmic retinol-binding proteins[s–u] as well as proteins that bind retinoic acid.[u–y] These proteins are members of the large superfamily of hydrophobic transporter molecules described in Box 21-A. This includes the milk protein **β-lactoglobulin,** which also forms a complex with retinol.[z,aa]

A strikingly early symptom of vitamin A deficiency is **night blindness**. A variety of other symptoms include dry skin and hair, conjunctivitis of the eyes, retardation of growth, and low resistance to infection. The skin symptoms are particularly noticeable in the internal respiratory passages and alimentary canal lining. About 0.7 mg/day of vitamin A is required by an adult. The content of vitamin A in foods is often expressed in terms of international units: 1.0 mg of retinol equals 3333 I.U.



BOX 22-A VITAMIN A (continued)

Vitamin A, as retinal, has a clearly established role in vision (Chapter 23) and apparently has a specialized function in reproduction. In vitamin A deficiency no sperm cells are formed in males, and fetal resorption occurs in females. Rats deprived of vitamin A but fed retinoic acid become blind and sterile but otherwise appear healthy.[e,bb] Evidently either the alcohol or the aldehyde has an essential function in reproduction, whereas bone growth and maintenance of mucous secretions requires only retinoic acid. Indeed, retinoic acid is 100 to 1000 times more active than other forms of vitamin A in these differentiation functions.[r]

In vitamin A deficiency the internal epithelial surfaces of lungs and other tissues, which are usually rich in mucous secreting cells and in ciliated cells, develop thick layers of keratinizing squamous cells similar to those on the external surface of the body. The synthesis of some mannose- and glucosamine-containing glycoproteins consequently decreases.[cc] The major effects of retinoic acid is evidently through regulation of transcription (Chapter 28). In developing lungs retinoic acid promotes the transformation of undifferentiated epithelial cells into mucus-secreting cells.[dd]

Do we know all of the special chemistry of vitamin A that is involved in its functions? Retinal could form Schiff bases with protein groups as it does in the visual pigments. Redox reactions could occur. Conjugative elimination of water from retinol to form **anhydroretinol** is catalyzed nonenzymatically by HCl. Anhydroretinol occurs in nature and

Anhydroretinol

may serve as an inhibitor of the action of 14-hydroxy-*retro*-retinol in lymphocyte differentiation.[ee,ff]

Much recent interest has been aroused by the fact that retinoid compounds, including both retinol and retinoic acid, reduce the incidence of experimentally induced cancer. In addition, 13-*cis*-retinoic acid taken orally is remarkably effective in treatment of severe cystic acne.[gg] However, both vitamin A and retinoic acid in large doses are **teratogenic**, i.e., they cause fetal abnormalities. The use of 13-*cis*-retinoic acid during early phases of pregnancy led to a high incidence of major malformations in infants born.[hh]

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2. Properties and Functions of Carotenes

The most characteristic property of carotenoids is the striking color, most often yellow to red, which is used by birds as a sexual attractant and by plants to attract pollinators.[137,138] The associated light absorption fits these compounds for a role in photosynthetic light-harvesting,[139] in photoprotection, and in photoreception,[140] matters that are dealt with in Chapter 23. One aspect of photoprotection, which involves the **xanthophyll cycle,** is also indicated in Eq. 22-10. The cycle allows green plants to adjust to varying light intensity by altering the amount of zeaxanthin available for quenching excessive amounts of photoexcited chlorophyll (Chapter 23). Zeaxanthin undergoes epoxidation by O_2 to form antheroxanthin and violaxanthin as shown in Eq. 22-10. The process requires NADPH and reduced ferredoxin.[131] When light intensity is high the process is reversed by an ascorbate-dependent violaxanthin **de-epoxidase**.[99,128,141]

Violaxanthin also functions as a precursor to the plant hormone abscisic acid. Compare the structure of the latter (Fig. 22-4) with those of carotenoids. Oxidative cleavage of violaxanthin or related epoxy-carotenoids initiates the pathway of synthesis of this hormone.[142,143]

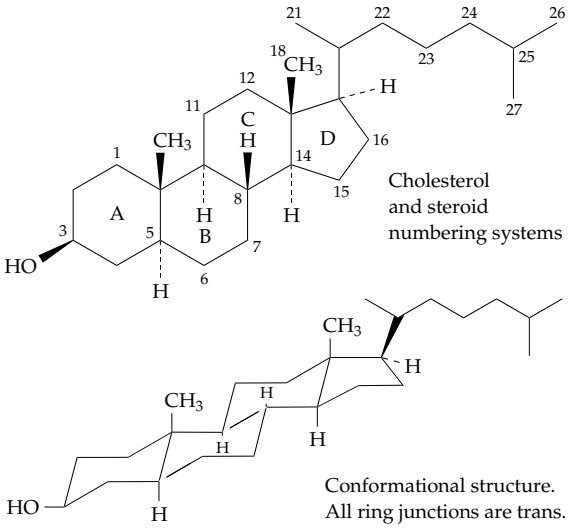
The system of conjugated double bonds responsible for carotenoid colors also helps to impart specific shapes to these largely hydrophobic molecules and ensures that they occupy the appropriate niches in the macromolecular complexes with which they associate. Information on stereochemistry is provided in a short review by Britton.[138]

β-Carotene, which can serve as an antioxidant at low oxygen pressures and can quench singlet oxygen,[144,145] has been associated with a reduced incidence of lung cancer.[137,146] While most animals do not synthesize carotenoids, they use them to make vitamin A and related retinoids and also as colorants. Yellow and red pigments of bird feathers[147] and the colors of tissues of salmon and of lobsters and other invertebrates are derived from dietary carotenoids, which are often modified further by the new host. The lobster accumulates astaxanthin, as a blue protein complex,[148] and the flamingo uses the astaxanthin of shrimp to color its feathers.[130]

Dietary carotenes and carotenoids are absorbed and transported in the plasma of humans and animals by lipoproteins.[149] The conversion of carotenes to vitamin A (Box 22-A) provides the aldehyde **retinal** for synthesis of visual pigments (Chapter 23) and **retinoic acid**, an important regulator of gene transcription and development (Chapter 32).[150–152c] See also Section E,5.

D. Steroid Compounds

The large class of **steroids** contains a characteristic four-ring nucleus consisting of three fused six-membered rings and one five-membered ring.[153] **Cholestanol** (dihydrocholesterol) may be taken as a representative steroid alcohol or **sterol**. Most sterols, including cholestanol, contain an 8- to 10-carbon side chain at position 17. The polyprenyl origin of the side chain is suggested by the structure. Steroid compounds usually contain an oxygen atom at C-3. This atom is present in an –OH group in the sterols and

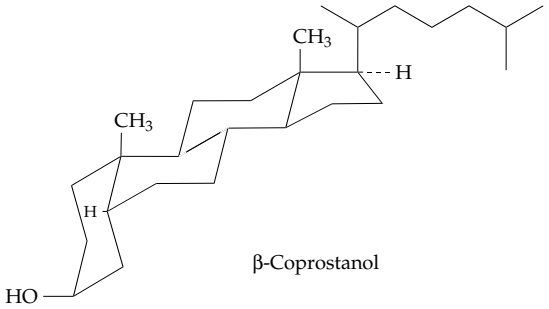


frequently in a carbonyl group in other steroids. Most steroids contain two axially oriented methyl groups, the "angular methyl groups,"which are attached to the ring system and numbered C-18 and C-19. In the customary projection formulas they are to be thought of as extending forward toward the viewer. In the same manner, the equatorially oriented 3-OH group of cholestanol and the side chain at C-17 also project forward toward the viewer in the projection formula.

The angular methyl groups, the 3-OH groups, and the side chain of cholestanol are all on the same side of the steroid ring in the projection formula and are all said to have a **β orientation**. Substituents projecting from the opposite side of the ring system are **α oriented**. While the methyl groups (C-18 and C-19) almost always have the β orientation, the 3-OH group has the α orientation in some sterols. Dashed lines are customarily used to connect α-oriented substituents, and solid lines are used for β-oriented substituents in structural formulas. Cholesterol is chiral and its enantiomer does not support life for *C. elegans* and presumably for other organisms.[153a]

In cholestanol the ring fusions between rings A and B, B and C, and C and D are all trans; that is, the hydrogen atoms or methyl groups attached to the

bridgehead carbon atoms project on opposite sides of the ring system. This permits all three of the six-membered rings to assume relatively unstrained chair conformations. However, the introduction of a double bond alters the shape of the molecule significantly. Thus, in cholesterol the double bond between C-5 and C-6 (Δ^5) distorts both the A and B rings from the chair conformation. In some steroid compounds the junction between rings A and B is cis. This greatly alters the overall shape of the steroid from the relatively flat one of cholestanol to one that is distinctly bent. An example is **β-coprostanol**, a product of bacterial action on cholesterol and a compound occurring in large amounts in the feces. In some sterols, notably the estrogenic hormones, ring A is completely aromatic and the methyl group at C-19 is absent.



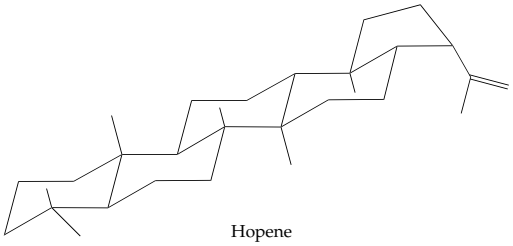
β-Coprostanol

1. Biosynthesis of Sterols

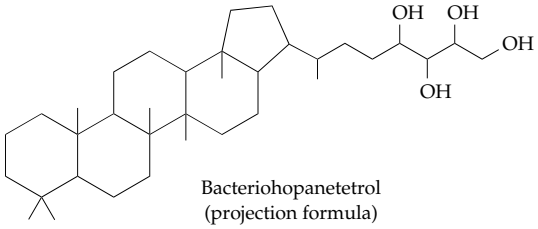
Most animal steroids arise from cholesterol, which in turn is derived from squalene. This C_{30} triterpene, whose biosynthesis is described in Section B, is named after the dogfish *Squalus* in whose liver it accumulates as a result of blockage in oxidation to cholesterol. Squalene is also a prominent constituent of human skin lipids. Its conversion to **cholesterol**, which takes place in most animal tissues,[117,154–156] is initiated by a microsomal enzyme system that utilized O_2 and NADPH to form **squalene 2,3-oxide** (Fig. 22-6, step *a*). The subsequent cyclization reaction, which probably takes place through a carbocation created by attack of a proton on the oxygen atom of the epoxide ring (Fig. 22-6, step *b*), is catalyzed by the large 70- to 80-kDa **oxidosqualene cyclase**.[157–159] The enzyme from rat liver consists of 733 residues and contains a highly conserved sequence with two consecutive aspartates that are thought to be at the active site. The sequence is somewhat similar to that of prenyltransferases and sesquiterpene cyclases.[157] The cyclization step appears to require that the enzyme hold the substrate in a rigid conformation as indicated in Fig. 22-6. The flow of electrons effects the closure of all four rings. The carbocation created at C-2 of squalene (C-4 of the sterol ring that is formed) by opening of the epoxide ring reacts with electrons from the 6,7 double bond to close ring A leaving a carbocation at C-6. This in turn reacts with the 10,11 double bond leaving a carbocation at C-10, etc. At the end of this cascade a carbocation is left on C-19 of squalene, which is numbered C-20 in the incipient sterol. The closures of rings A and B both follow the Markovnikov rule by generating relatively stable tertiary carbocations. Thus, the natural chemical reactivities of the substrates are followed in these enzymatic reaction steps. However, this is not the case in the closure of ring C to form a 6-membered ring instead of a 5-membered ring. This presumably happens because the enzyme imposes the correct geometry for a 6-membered ring on the squalene and the correct stereochemistry on the ring closure.[160]

The rearrangement of this initially created C-20 carbocation to **lanosterol** (Fig. 22-6, step *c*) is also a remarkable reaction that requires the shift of a hydride ion and of two methyl groups, as indicated by the arrows in the figure. In addition, a hydrogen at C-9 (sterol numbering) is lost as a proton. Lanosterol is named for its occurrence in lanolin, the waxy fat in wool. Although the principal component of lanolin is cholesterol, lanosterol is its precursor both in sheep and in all other animals. Cholesterol is in turn the precursor to other animal sterols. The cholesterol biosynthetic pathway also provides cells with a variety of important signaling molecules.[160a]

In green plants, which contain little or no cholesterol, **cycloartenol** is the key intermediate in sterol biosynthesis.[161–162a] As indicated in Fig. 22-6, step *c'*, cycloartenol can be formed if the proton at C-9 is shifted (as a hydride ion) to displace the methyl group from C-8. A proton is lost from the adjacent methyl group to close the cyclopropane ring. There are still other ways in which squalene is cyclized,[162,163,163a] including some that incorporate nitrogen atoms and form alkaloids.[163b] One pathway leads to the **hopanoids**. These triterpene derivatives function in bacterial membranes, probably much as cholesterol does in our membranes. The three-dimensional structure of a bacterial hopene synthase is known.[164,164a] Like glucoamylase (Fig. 2-29) and farnesyl transferase, the enzyme has an $(\alpha,\alpha)_6$-barrel structure in one domain and a somewhat similar barrel in a second domain. The active site lies in a large interior cavity. The properties of the hopene synthase are similar to those of oxidosqualene synthase, and it appears to function by a similar mechanism, which resembles that of Fig. 22-6 but does not depend upon O_2. Hopene lacks polar groups, but these are provided in the hopanoids by a polyol side chain. One of these compounds, **bacteriohopanetetrol**, may be one of the most abundant compounds on earth.[160,165,166] Hopanoids appear to originate from mevalonate synthesized via the 1-deoxyxylulose pathway (Fig. 22-2). The polyol side chain is probably formed from ribose.[166]



Hopene



Bacteriohopanetetrol
(projection formula)

Formation of cholesterol. The conversion of lanosterol to cholesterol requires at least 19 steps,[167,168] which are catalyzed by enzymes bound to membranes of the ER. The removal of the three methyl groups of lanosterol, the migration of the double bond within the B ring, and the saturation of the double bond in the side chain may occur in more than one sequence, two of which are indicated in Fig. 22-7. The predominant pathway in many organisms including humans is the oxidative demethylation at the C / D ring junction (C-14) by a cytochrome P450 called **lanosterol 14α-demethylase**. This single enzyme catalyzes three consecutive O_2 and NADPH-dependent reactions that convert the methyl group to hydroxymethyl, formyl, and then free formate (right side of Fig. 22-8).[169–172b] Steps *a* and *b* are typical cytochrome P450 oxygenation reactions. In step *b* a geminal diol is formed and is dehydrated to the formyl derivative. The third step is atypical. Shyadehi *et al.* proposed the sequence depicted in steps *c-f* in which an Fe(III) peroxo intermediate reacts as shown.[172] This mechanism is supported by the fact that both ^{18}O present in the formyl group and ^{18}O from $^{18}O_2$ appear in the liberated formate.

The corresponding reactions of the methyl groups at C-4 on the A ring[167,168,173] are depicted on the left side of Fig. 22-8. The 4α methyl group is first hydroxylated by a microsomal (ER) system similar to cytochrome P450 but able to accept electrons from NADH and cytochrome b_5 rather than NADPH.[173] The two-step oxidation of the resulting alcohol to a carboxylic acid is catalyzed by the same enzyme. A second enzyme catalyzes the dehydrogenation of the 3-OH group to a ketone allowing for efficient β-decarboxylation (Fig. 22-8, steps *j* and *k*).[173a] Inversion of configuration at C-4, assisted by the 3-carbonyl group (step *l*), places the second 4-methyl group in the α orientation. After reduction of the 3-carbonyl by a third enzyme the sequence is repeated on this second methyl group.

In addition to the enzymes that are embedded in the membranes of the ER, conversion of lanosterol to cholesterol depends upon soluble cytoplasmic carrier proteins.[174] See also Box 21-A. Other sterols formed in

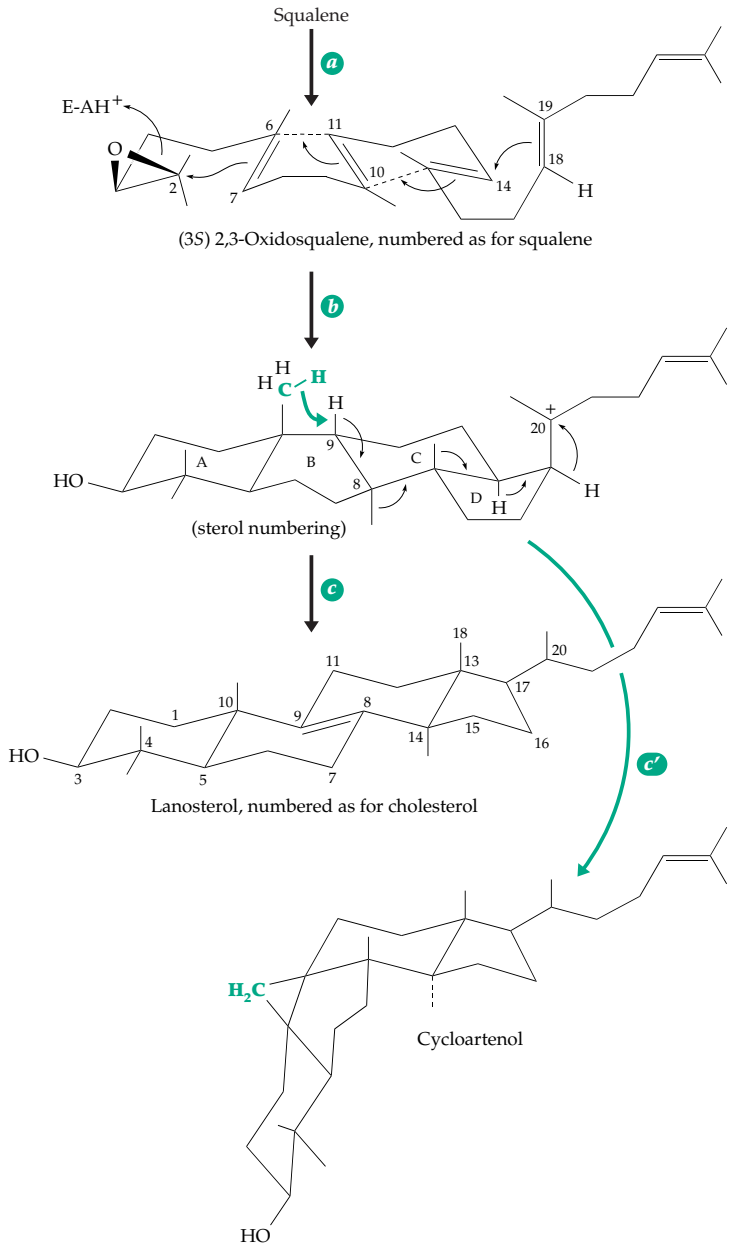
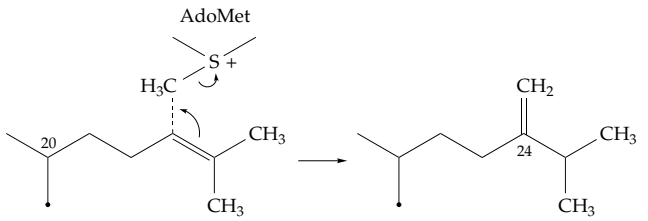


Figure 22-6 The cyclization of all-*trans* squalene to lanosterol and cycloartenol.

the animal body are **7-dehydrocholesterol**, prominent in skin and a precursor of vitamin D. Both β-cholestanol and its isomer β-coprostanol are formed by bacteria in the intestinal tract, and small amounts of cholesterol are converted to cholestanol within tissues. **Ergosterol**, the most common sterol in fungi, contains the $\Delta^{5,7}$ ring system of 7-dehydrocholesterol as well as an extra double bond in the side chain. It arises from zymosterol (Fig. 22-7).[173,174a]

As indicated previously, plant sterols are thought to be formed in most cases through cycloartenol which is often converted to **24-methylenecycloartenol**, a substance present in grapefruit peel and in many other plants. The methylene carbon is donated by *S*-adenosylmethionine (AdoMet) as shown in Eq. 22-13, which implies a transient intermediate carbocation. Saturation of the side chain and oxidative demethylation similar to that shown in Fig. 22-8[175] and introduction of a double bond[176] leads to **campesterol** (Fig. 22-9). It has the Δ^5-unsaturated ring of cholesterol but, like many other plant sterols, the side chain has one additional methyl group, which is also donated from *S*-adenosylmethionine.[161,177,178] Several more steps are



Side chain of cycloartenol — 24-Methylenecycloartenol

(22-13)

required to convert campesterol into the plant steroid hormone **brassinolide**.[179–180] Among higher plants, **sitosterol** and **stigmasterol** are the most common sterols. Each contains an extra ethyl group in the side chain. Sitosterol is formed by the methylation (by AdoMet) of ergosterol. For the guinea pig stigmasterol is a vitamin, the"antistiffness factor" necessary to prevent stiffening of the joints. Some other plant steroids arise without addition of the extra carbons at C-23 or C-24 but usually via a different cyclization of squalene. Of these, the cucurbitacins (Fig. 22-9) are among the bitterest substances known.[181]

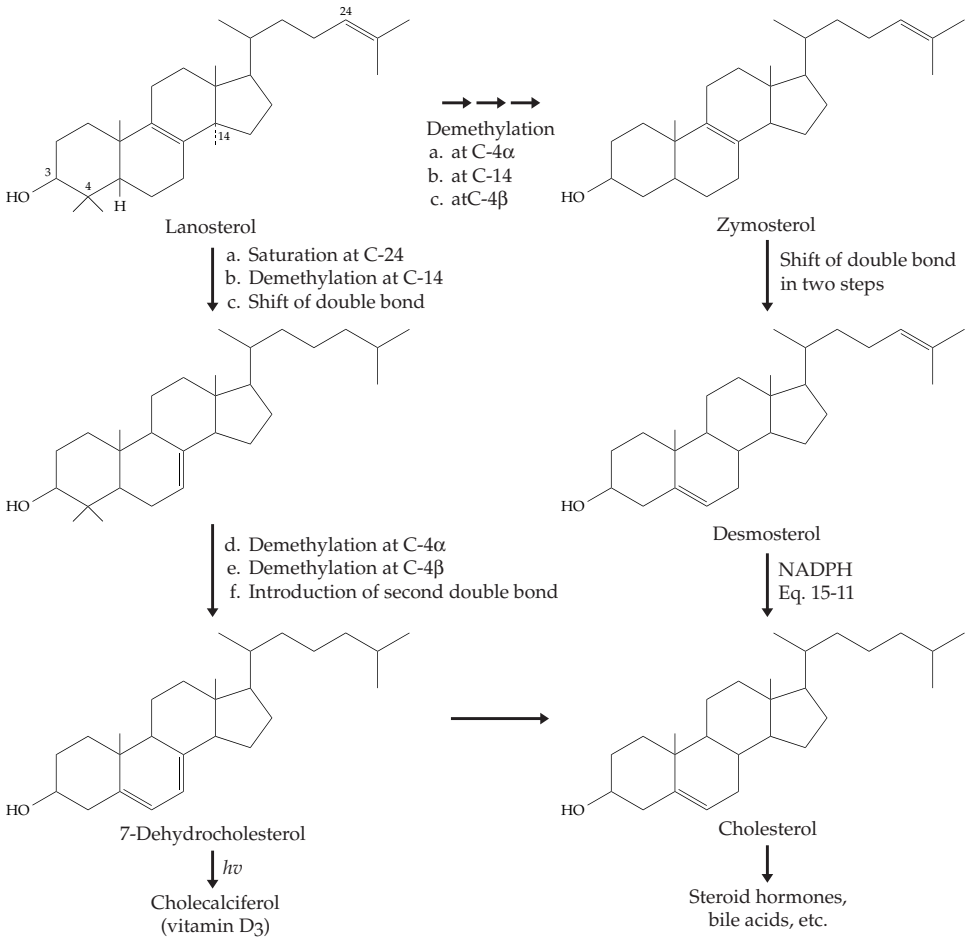


Figure 22-7 Conversion of lanosterol to cholesterol. Two of many possible sequences are shown.

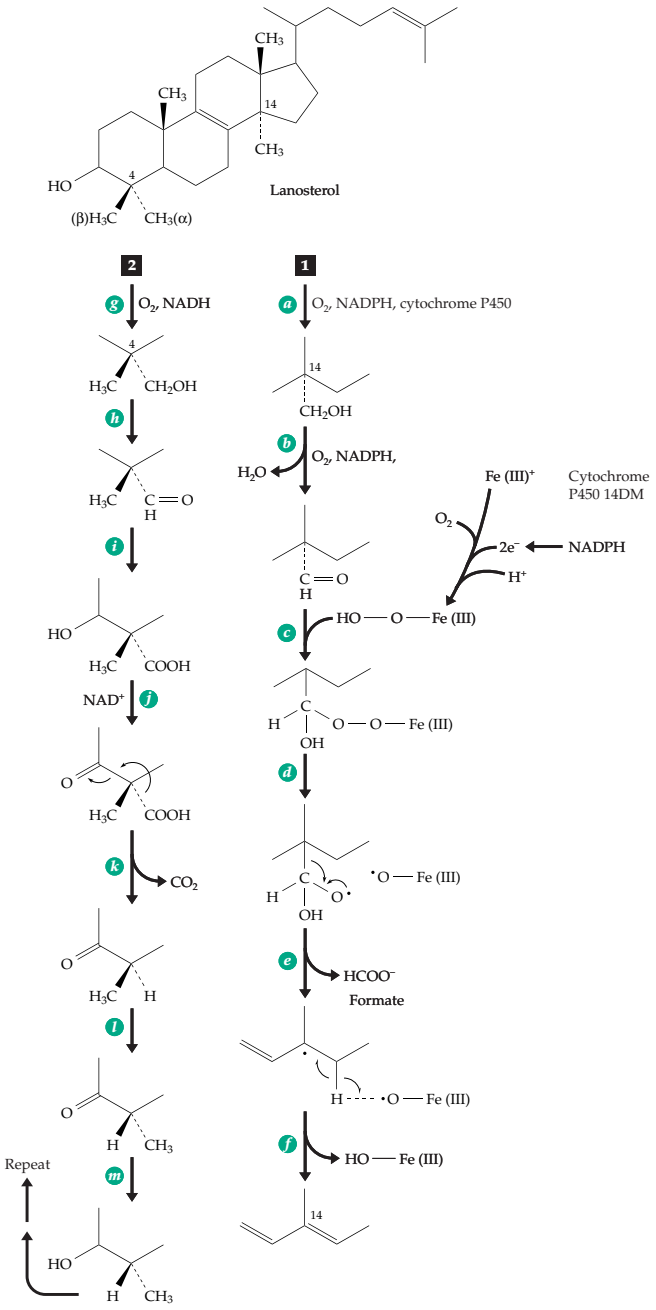


Figure 22-8 Steps in the demethylation of lanosterol. The most frequent sequence, labeled [1], begins with demethylation at C-14 by the action of a cytochrome P450 and is followed [2] by the successive demethylation of the α-CH_3 and β-CH_3 at C-4 by an NADH-dependent oxygenase.

2. Metabolism of Cholesterol in the Human Body

Cholesterol is both absorbed from the intestinal tract and synthesized from acetate via squalene, principally in the liver. The quantities produced are substantial. Daily biosynthesis is ~600 mg, and dietary uptake may supply another 300 mg.[182] Not only is there a large amount of cholesterol in the brain and other nervous tissues but also about 1.7 g of cholesterol per liter is present in blood plasma, about two-thirds of it being esterified principally to unsaturated fatty acids. The cholesterol content of plasma varies greatly with diet, age, and sex. By age 55 it averages 2.5 g / liter and may be considerably higher. Women up to the age of menopause have distinctly lower blood cholesterol than do men. Cholesterol regulates its own abundance by a variety of feedback mechanisms.[183,184] These include inhibition of the synthesis by means of reduced activities (step *a* of Fig. 22-1) of HMG-CoA reductase, farnesyl diphosphate synthase (step *g* of Fig. 22-1), and squalene synthase. All of these reactions are essential steps in cholesterol synthesis.[16,185] On the other hand, cholesterol induces an increase in acyl-CoA:cholesterol acyltransferase.

Dietary cholesterol, together with triacylglycerols, is absorbed from the intestinal tract and enters the large lipoprotein chylomicrons (see Fig. 21-1). Absorption of cholesterol is incomplete, usually amounting to less than 40% of that in the diet. Absorption requires bile salts and is influenced by other factors.[186] As it is needed cholesterol is taken from the plasma lipoproteins into cells by endocytosis. Much of the newly absorbed cholesterol is taken up by the liver. The liver also secretes cholesterol, in the form of esters with fatty acids, into the bloodstream.

Cholesterol is synthesized in the ER and other internal membranes by most cells of the body.[187,188] Newly formed cholesterol is sorted from the ER into the various membranes of the cell, the greatest abundance being in plasma membranes where cholesterol plays an essential role in decreasing fluidity. Cholesterol also aggregates with sphingolipids to form rigid lipid "rafts" floating in the plasma membrane. These rafts are thought to have important functions in signaling, in distribution of lipid materials,[188a] and in influencing protein translocation.[188b] Caveolae in cell surfaces may also arise from cholesterol-rich rafts.[188c] However, cholesterol must also be able to move out of the internal membranes back into the interior ER of the cell to provide for homeostasis and to allow formation of cholesteryl esters for transport, bile acids for excretion by liver, and the steroid hormones.[183,184,189] Movement between organelles occurs with the aid of sterol carrier proteins.[190–190c]

Liver and some intestinal cells export cholesterol into the bloodstream, together with triacylglycerols and phospholipids in the form of VLDL particles, for uptake by other tissues (see Fig. 21-1). Cholesteryl esters are formed in the ER by **lecithin:cholesterol acyltransferase** (LCAT), an enzyme that transfers the central acyl group from phosphatidylcholine to the hydroxyl group of cholesterol.[191,191a] This enzyme is also secreted by the liver and acts on free cholesterol in lipoproteins.[192] Tissue acyltransferases also form cholesteryl esters from fatty acyl-CoAs.[192a]