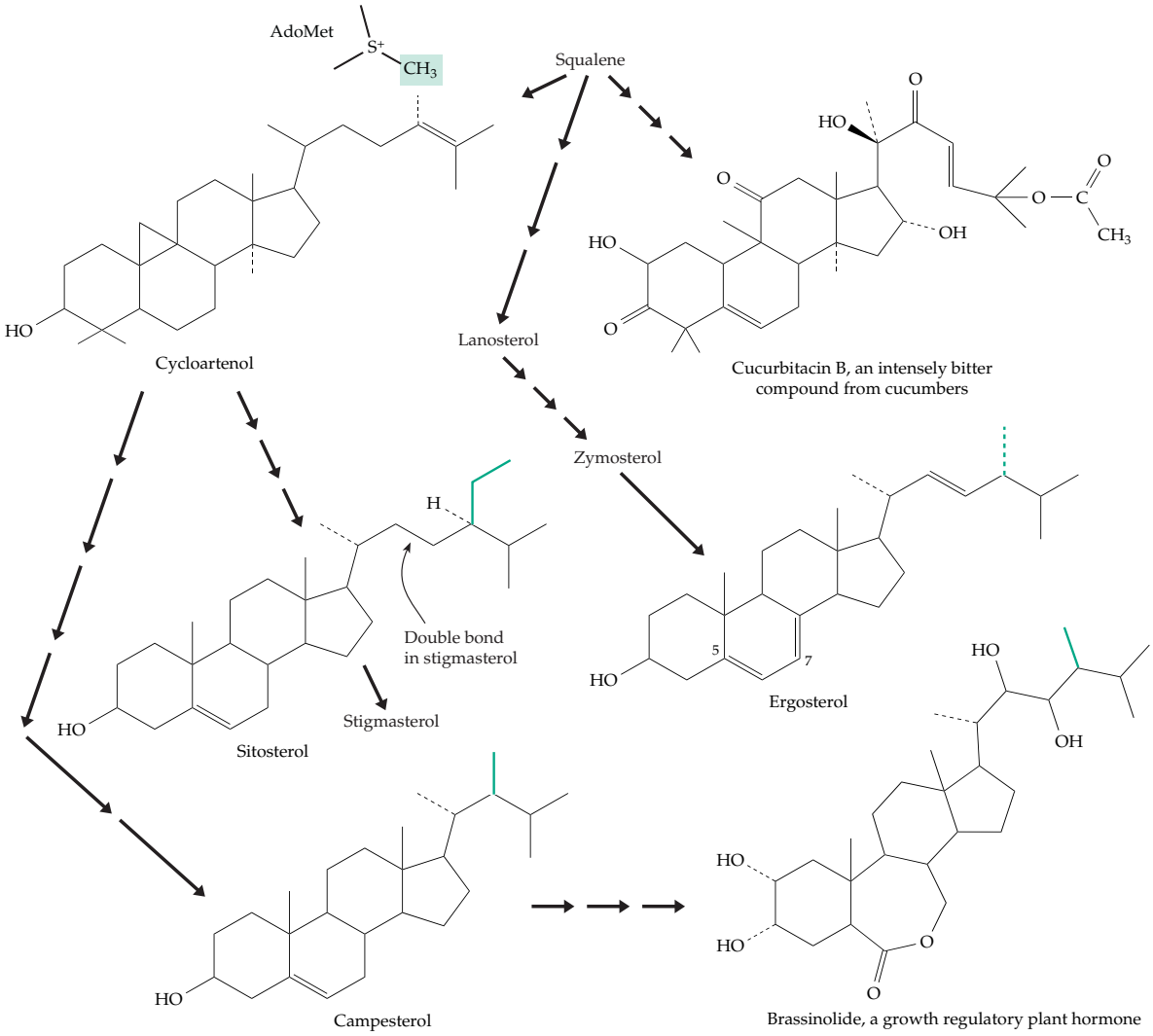



**Figure 22-9** Structures and routes of biosynthesis for a few plant steroids.

In the brain a complex of cholesterol with apolipoprotein E (Table 21-2) promotes the formation of new synapses (Chapter 30). Synthesis of cholesterol for this purpose appears to occur within glial cells.[192b,c]

***Serum cholesterol.*** Most cholesterol is carried in the blood by low density lipoprotein (LDL, Tables 21-1, 21-2), which delivers the cholesteryl esters directly to cells that need cholesterol. Both a 74-kDa **cholesteryl ester transfer protein**[193–195a] and a **phospholipid transfer protein**[196,196a] are also involved in this process. **Cholesterol esterases**, which release free cholesterol, may act both on lipoproteins and on pancreatic secretions.[197–199]

The LDL–cholesterol complex binds to LDL receptors on the cell surfaces.[167,168,200–202] These receptors are specific for apolipoprotein B-100 present in the LDL. The occupied LDL–receptor complexes are taken up by endocytosis through coated pits; the apolipoproteins are degraded in lysosomes, while the cholesteryl esters are released and cleaved by a specific **lysosomal acid lipase**[203,204] to form free cholesterol.

While the primary role of LDL appears to be the transport of esterified cholesterol to tissues, the high density lipoproteins (HDL) carry excess cholesterol away from most tissues to the liver.[205–207] The apoA-I present in the HDL particle not only binds lipid but activates LCAT, which catalyzes formation of cholesteryl esters which migrate into the interior of the HDL and are carried to the liver.

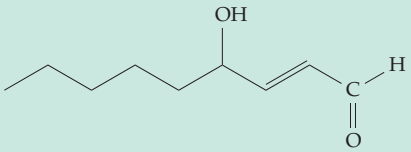
Unlike other lipoproteins, HDL particles are assembled outside of cells from lipids and proteins, some of which may be donated from chylomicrons (see Fig. 21-1) or other lipoprotein particles. HDL has a higher protein content than other lipoproteins and is more heterogeneous. The major HDL protein is apolipoprotein A-I, but many HDL particles also contain A-II,[205,208–210] and apolipoproteins A-IV, D, and E may also be present. A low plasma level of HDL cholesterol is associated with a high risk of atherosclerosis.[205,207]

## BOX 22-B ATHEROSCLEROSIS

Our most common lethal disease is atherosclerosis, which causes constriction and blockage of arteries of the heart, brain, and other organs. In the United States, Europe, and Japan half of all deaths can be attributed to this ailment.[a,b] There seems to be a variety of causes. However, there is agreement that the disease begins with injury to the endothelial cells that form the inner lining of the arteries.[a,c,d] This is followed by the aggregation of blood platelets at the sites of injury and infiltration of smooth muscle cells, which may be attracted by 12-hydroxyeicosotetraenoic acid and other chemoattractants formed by activated platelets.[c] **"Foam cells"** laden with cholesterol and other lipids appear, and the lesions enlarge to become the characteristic plaques (**atheromas**).

The best understood cause of atherosclerosis is the genetic defect **familial hypercholesterolemia**, an autosomal dominant trait carried by one person in 500 all over the world.[e] Males with the defective gene tend to develop atherosclerosis when they are 35–50 years of age. The approximately one in a million persons *homozygous* for the trait develop coronary heart disease in their teens or earlier. Cultured fibroblasts from these patients have 40- to 60-fold higher levels of HMG-CoA reductase (Eq. 15-9) than are present normally, and the rate of cholesterol synthesis is increased greatly. The LDL level is very high and, as shown by Brown and Goldstein,[f–i] the LDL receptor gene is defective. Genetic defects associated with a low HDL level are also associated with atherosclerosis[b,j–l] as is a genetic variant of the metalloproteinase **stromelysin**.[m]

Other factors favoring development of atherosclerosis include hypertension and smoking. Chickens infected with a herpes virus (Marek disease virus) develop the disease after infection, and it is possible that artery damage in humans can also be caused by viruses[n] or bacterial infections.[o] In recent years it has been established that oxidative modification of the phospholipids in LDL induces the uptake of LDL by scavenger receptors of macrophages. This appears to trigger the development of foam cells and atherosclerotic plaques.[c,p,q] The initial damage is thought to be caused by lipid peroxides in the diet or generated by lipoxygenases in platelets and other cells.[c,p,r] Unsaturated fatty acids in lipoproteins can undergo oxidation (Chapter 21), especially in the presence of $Cu^{2+}$ ions,[s,t] to yield malondialdehyde, 4-hydroxynonenal (Eq. 21-15), and other reactive compounds, which may damage the lipoproteins and cause them to have too high an affinity for their receptors in the smooth muscle cells of artery walls.[p,u] The 17β-hydroperoxy derivative of cholesterol has also been found in atherosclerotic lesions and may account for some of the toxicity of oxidized LDL.[r] Ascorbic acid may help to prevent formation of these oxidation products.[p,q,v] Chlorinated sterols may also be produced by the myeloperoxidase of the phagocytic macrophages that are abundant in atherosclerotic plaque.[w] Trans fatty acids, which are abundant in some margarines, and other hydrogenated fats raise both cholesterol and LDL levels.[x] Another cause of artery disease may be the presence of excessive homocysteine,[y,z] which can accumulate as a result of marginal deficiencies of folate, vitamin $B_6$, or vitamin $B_{12}$.



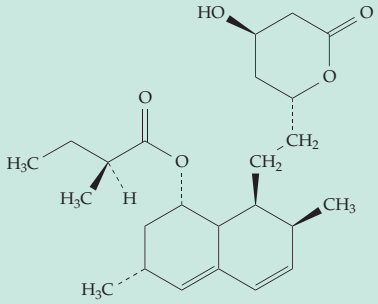
4-Hydroxy-2-nonenal

What can be done to prevent atherosclerosis? For persons with a high LDL level there is little doubt that a decreased dietary intake of cholesterol and a decrease in caloric intake are helpful. While such dietary restriction may be beneficial to the entire population, controlled studies of the effect of dietary modification on atherosclerosis have been disappointing and confusing.[aa] A diet that is unhealthy for some may be healthy for others. For example, an 88-year old man who ate 25 eggs a day for many years had a normal plasma cholesterol level of 150–200 mg / deciliter (3.9–5.2 mM)![bb] Comparisons of diets rich in unsaturated fatty acids, palmitic acid, or stearic acid have also been confusing.[cc,cd,dd] Can it be true that palmitic acid from tropical oils and other plant sources promotes atherogenesis, but that both unsaturated fatty acids and stearic acid from animal fats are less dangerous?

One of the best therapeutic approaches may be to prevent absorption of cholesterol from the intestines by inclusion of a higher fiber content in the diet.[ee] Supplementation with a cholesterol-binding resin may provide additional protection. Plant sterols also interfere with cholesterol absorption. Incorporation of esters of **sitostanol** into margarine provides an easy method of administration.[ff] Supplemental vitamin E may also be of value.[q] Another effective approach is to decrease the rate of cholesterol synthesis by administration of drugs that inhibit the synthesis of cholesterol. Inhibitors of HMG-CoA reductase,[gg,hh] (e.g., vaLostatin) isopentenyl-*PP* isomerase, squalene synthase (e.g.,

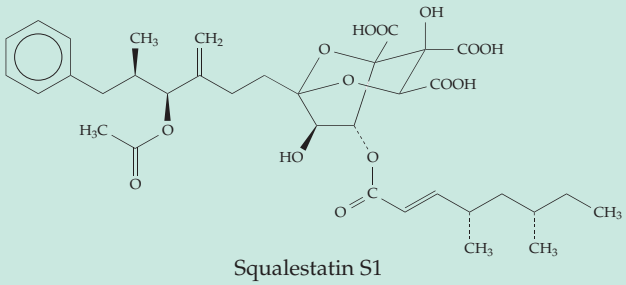
## BOX 22-B ATHEROSCLEROSIS (continued)

squalestatin S1),[ii] and other enzymes in the biosynthetic pathway are targets for drug treatment.



Lovastatin
(Mevinolin)

Questions of possible long-term toxicity remain. Since 1976 there has been a greater than 25% decrease in the incidence of ischemic heart disease in the United States.[jj] Increased exercise, a decreased severity of influenza epidemics, and fluoridation of water[kk] have been suggested as explanations.



Squalestatin S1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***The LDL and related receptors.*** The LDL receptor gene extends over 50 kb of DNA and appears to be a mosaic of exons shared by several other genes that seem to have nothing to do with cholesterol metabolism.[201,211] The 839-residue receptor protein consists of five structural domains. The N-terminal domain that binds the LDL consists of seven repeated ~40-residue cysteine-rich modules.[212,213] This is followed by a large domain that is homologous to a precursor of the epidermal growth factor, a 48-residue domain containing many *O*-glycosylated serine and threonine residues, a 27-residue hydrophobic region that spans the membrane, and a 50-residue C-terminal cytoplasmic domain.[200,214–216] Synthesis of LDL receptors is regu-

lated by a feedback mechanism, the cholesterol released within cells inhibiting the synthesis of new receptors.

As mentioned in Chapter 21, there are several related receptors with similar structures. Two of them have a specificity for apolipoprotein E and can accept remnants of VLDL particles and chylomicrons.[216–220] The LDL receptor-related protein is a longer-chain receptor.[216,221] LDL particles, especially when present in excess or when they contain oxidized lipoproteins, may be taken up by endocytosis into macrophages with the aid of the quite different **scavenger receptors**.[221–225] The uptake of oxidized lipoproteins by these receptors may be a major factor in promoting development of atherosclerosis (Box 22-B). On the other hand, **scavenger receptor SR-B1**, which is also present in liver cells, was recently identified as the receptor for HDL and essential to the "reverse cholesterol transport" that removes excess cholesterol for excretion in the bile.[213,213a]

***Abnormalities of cholesterol metabolism.*** A variety of genetic problems have been identified, many of them being associated with atherosclerosis (Box 22-B).[218,226–230] In the commonest form of **familial hypercholesterolemia** a mutation in the LDL receptor protein prevents normal synthesis, binding, clustering into coated pits, or uptake of LDL and its cholesteryl esters. Over 600 mutations have been identified.[229,229a] Some of these are present in a $Ca^{2+}$-binding region of the 5th cysteine-rich module.[230] Other disorders that raise the plasma LDL level include a defective apoB-100 protein (see p. 1182)[230a] and deficiency of a protein that seems to be involved in incorportation of LDL receptors into clathrin-coated pits during endocytosis or in receptor recycling.[229a] In a **cholesteryl ester storage disease** the lysosomal lipase is lacking. Absence of lecithin:cholesterol acyltransferase from plasma causes corneal opacity and often kidney failure.[231]

In the very rare and fatal **Niemann–Pick C1** disease lysosomes in cells of the central nervous system and the viscera accumulate LDL-derived cholesterol. Study of the DNA of patients led to discovery of a 1278-residue integral membrane protein, which may be required for the Golgi-mediated transport of unesterified cholesterol from lysosomes to the ER.[189,232–234c]

Some people with elevated lipoprotein levels have VLDL that migrates on electrophoresis in the β band rather than the pre-β band (see Box 2-A). The presence of the β-VLDL is associated with a high incidence of artery disease,[218] which is most likely to develop in persons homozygous for a genetic variant of apolipoprotein E. The problem may arise because apo-E is required for receptor-mediated uptake of VLDL, which interacts both with tissue LDL receptors and with hepatic apo-E receptors. Genes for many of the apolipoproteins are polymorphic, and numerous alleles are present in a normal population.[218,235] In the rare **Tangier disease** apolipoprotein A-I is catabolized too rapidly, and the HDL level is depressed, resulting in accumulation of cholesterol esters in macrophages, Schwann cells, and smooth muscles. Orange-yellow enlarged tonsils are characteristic of the disease.[236] An ABC type transporter that allows cholesterol to leave cells is defective.[236a,b,c] The faulty component is known as the **cholesterol-efflux regulatory protein**. Another ABC transporter, apparently encoded by a pair of genes, which are expressed predominantly in liver and intestinal cells, prevents excessive accumulation of plant sterols such as sitosterol (Fig. 22-9).[236d,e]

***Bile acids.*** Among the metabolites of cholesterol the bile acids (Fig. 22-10)[182,237,238] are quantitatively the most important (100–400 mg / day). These powerful emulsifying agents are formed in the liver and flow into the bile duct and the small intestine. A large fraction is later reabsorbed in the duodenum and is returned to the liver for reuse.[238a] Formation of the bile acids involves the removal of the double bond of cholesterol, inversion at C-3 to give a 3α-hydroxyl group, followed by hydroxylation and oxidation of the side chain.[238b–f] The principal human bile acids are **cholic acid** and **chenodeoxycholic acid** (Fig. 22-10). The free bile acids are then converted to CoA derivatives and conjugated with glycine and taurine to form **bile salts**, such as **glycocholic** and **taurocholic acids**.[238d] Several rare lipid-storage diseases are associated with defective bile acid formation.[239–241] In one of these, **cerebrotendinous xanthomatosis**, cholestanol is deposited both in tendons throughout the body and in the brain. Oxidation of the cholesterol side chain is incomplete with excretion, as glucuronides, of large amounts of bile alcohols (precursors to the bile acids). The synthesis of bile acids is regulated by feedback inhibition by the bile acids, but in this disease the inhibition is absent and the rates of both cholesterol biosynthesis and oxidation are increased. The problem is not one of storage of cholesterol but of the cholestanol that arises as a minor product of the pathway. A proper ratio of bile salt, phosphatidylcholine, and cholesterol in the bile is important to prevention of **cholesterol gallstones**.[207]

A variety of other oxidative modifications of cholesterol take place in tissues to give small amounts of diols.[242] Hydroperoxides of cholesterol may also be formed.[243] Some of the products are probably toxic, but others may be essential. One of these is 26-hydroxycholesterol, a minor component of plasma but a major neonatal excretion product.[244]

The body contains sulfate esters of cholesterol and other sterols,[245] sometimes in quite high concentrations relative to those of unesterified steroids. These esters are presumably soluble transport forms. They

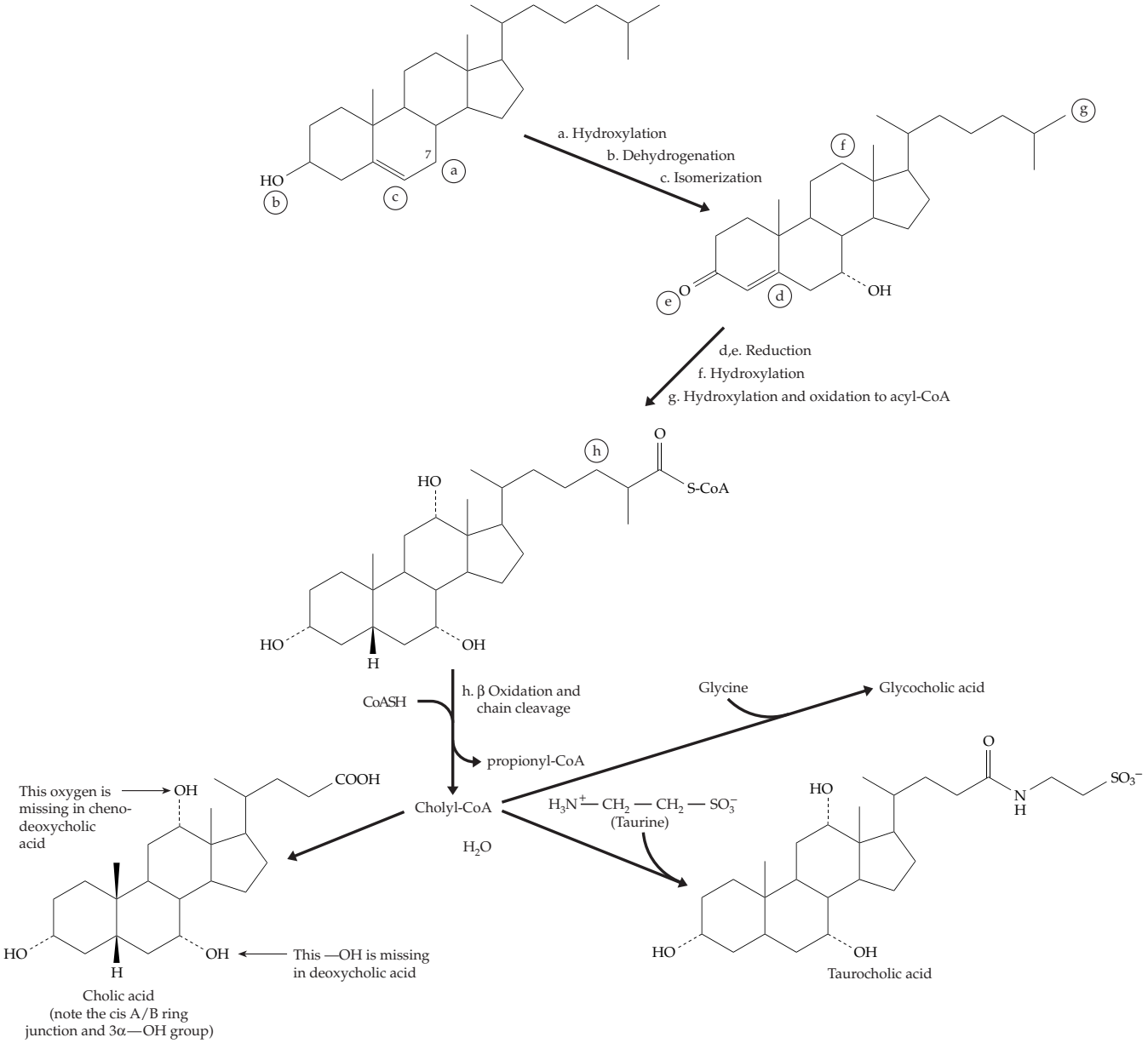



**Figure 22-10** Formation of the bile acids.

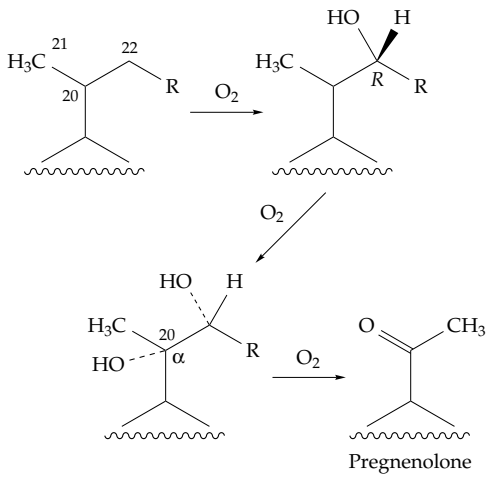
are hydrolyzed by a steroid sulfatase present within cells and whose absence causes **X-linked ichthyosis**, one of the commoner forms of scaly skin. The defect may also lead to corneal opacity but appears not to cause any other problems.[246] Glucuronides of sterols are another group of water-soluble metabolites.[247] We tend to think of cholesterol as an inert structural component of cell membranes. However, it has been found in ester linkage to a signaling protein of the "hedgehog" family, important in embryo development.[248]

## E. The Steroid Hormones

In the animal body three important groups of hormones are formed by the metabolism of cholesterol: the **progestins**, the **sex hormones**, and the **adrenal cortical hormones**.[249] Their synthesis occurs principally in mitochondria of the adrenal cortex and the gonads. Steroid hormone synthesis is regulated by hormones, such as **corticotropin** (ACTH), from the anterior pituitary[250] (see Chapter 30) and is also dependent upon the recently discovered **steroidogenic acute regulatory protein,** which in some way promotes the movement of stored cholesterol into mitochondrial membranes.[251,252] Some major pathways of

biosynthesis are outlined in Fig. 22-11. The side chain is shortened to two carbon atoms through hydroxylation and oxidative cleavage to give the key intermediate **pregnenolone**. The reaction is initiated by the mitochondrial **cytochrome P450$_{ssc}$**, which receives electrons from NADPH and adrenodoxin (Chapter 16).[253] Hydroxylation occurs sequentially on C-22 and C-20 (Eq. 22-14). The chain cleavage is catalyzed by the same enzyme, an overall 6-electron oxidation occurring in three $O_2$-dependent steps. Dehydrogenation of the 3-OH group of pregnenolone to C=O is followed by a shift in the double bond, the oxosteroid isomerase reaction (Eq. 13-30, step *b*). In bacteria these two steps are catalyzed by different proteins, but a single human 3β-hydroxysteroid / $\Delta^5$-$\Delta^4$ isomerase catalyzes both reactions.[254,255] The product is the α,β-unsaturated ketone **progesterone**.

Most steroid hormones exist in part as sulfate esters and may also become esterified with fatty acids.[256] The fatty acid esters may have relatively long lives within tissues.[256] A special sex hormone-binding globulin transports sex hormones in the blood and regulates their access to target cells.[256a,b]

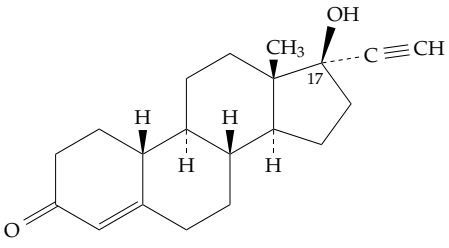



(22-14)

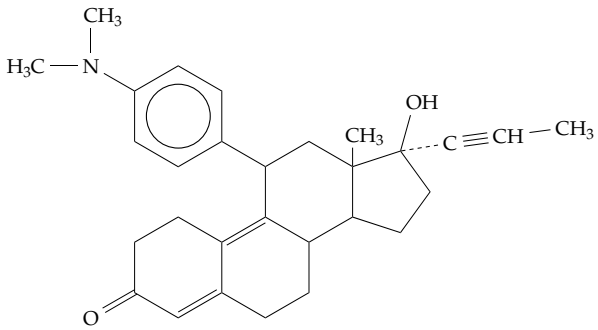
## 1. Progestins

Progesterone is the principal hormone of the **corpus luteum**, the endocrine gland that develops in the ovarian follicle after release of an ovum. Progesterone is also formed in the adrenals, testes, and placenta. It is metabolized rapidly, largely by reduction to alcohols, which may then be conjugated and excreted as glucuronides (see Eq. 20-16).[247] Reduction of the double bond within the A ring of progesterone leads to complete loss of activity, an indication that the α,β-unsaturated ketone group may play an essential role in the action of the hormone. Progesterone has a special role in the maintenance of pregnancy, and together with the estrogenic hormones it regulates the menstrual cycle. It is also essential for reproduction in lower animals such as birds and amphibians.[257,258]

The synthetic progesterone agonist **norethindrone** is widely used as one component of contraceptive pills. Having an opposite effect is **mifepristone** (also known as RU 486), a powerful antagonist of both progesterone and glucocorticoids. It is capable of inducing abortion and has other medical uses.[258–260] It is an effective emergency contraceptive agent that prevents implantation of an embryo.[261,262]




Norethindrone
(19-Nor-17α-ethynyltestosterone)




Mifepristone (RU 486)

## 2. Adrenal Cortical Steroids

Within the adrenal cortex (the outer portion of the adrenal glands) progesterone is converted into two groups of hormones of which **cortisol** and **aldosterone** are representative.[263] Two different cytochrome P450 hydroxylases, found in the ER and specific for C-21 and C-17α, respectively, together with a mitochondrial cytochrome P450 specific for C-11β (Eq. 18-55) participate in formation of cortisol.[264] Two of the same enzymes together with additional hydroxylases are required to form aldosterone.

Absence of the C-21 hydroxylase is one of the commonest of hereditary metabolic defects and is one of several enzymatic deficiencies that lead to **congenital adrenal hyperplasia**.[265–269] Cortisol, the synthesis of which is controlled by ACTH, is secreted by the adrenals in amounts of 15–30 mg daily in an adult. The hormone, which is essential to life, circulates in the blood, largely bound to the plasma protein **transcortin**. Cortisol, in turn, exerts feedback inhibi-

tion on ACTH production, and it is this feedback loop that fails when the C-21 hydroxylase is missing. Normally the circulating cortisol binds to receptors in both the pituitary and the hypothalamus of the brain to inhibit release of both ACTH and its hypothalamic releasing hormone (corticotropin-releasing hormone, CRF; see also Chapter 30). Girls are especially seriously affected by adrenal hyperplasia because, as ACTH production increases, the adrenal glands swell and produce an excessive amount of androgens. This occurs during the prenatal period of androgen release that initiates sexual differentiation. Girls with this deficiency are born with a masculine appearance of their external genitalia and continue to develop a masculine appearance. For reasons that are not clear the gene for the 21-hydroxylase is located within the HLA region (Chapter 31) of human chromosome 6.

Cortisol is a glucocorticoid which promotes gluconeogenesis and the accumulation of glycogen in the liver (Chapter 17). While it induces increased protein synthesis in the liver, it inhibits protein synthesis in muscle and many other tissues and leads to breakdown of fats to free fatty acids in adipose tissue.

Cortisol and its close relative **cortisone**, which was discovered by Kendall and Reichstein in the late 1940s, are probably best known for their anti-inflammatory effect in the body.[268,270] The effect depends upon several factors including inhibition of protein synthesis by fibroblasts, neutrophils, and antibody-forming cells. Migration of neutrophils into the inflamed area is also suppressed. Because of this action cortisone and synthetic analogs such as prednisolone and dexamethasone are among the modern "wonder drugs." They are used in controlling acute attacks of arthritis and of serious inflammations of the eyes and other organs. However, prolonged therapy can have serious side effects including decreased resistance to infections, wasting of muscle, and resorption of bone. The last results from a specific inhibition of calcium absorption from the gastrointestinal tract, glucocorticoids being antagonistic to the action of vitamin D (Box 22-C).

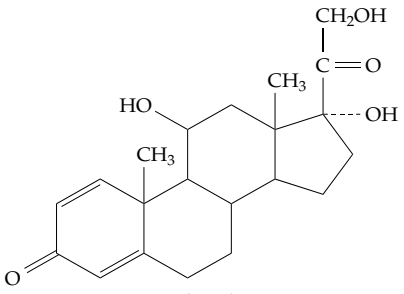
**Aldosterone**, which is classified as a **mineralocorticoid**, is produced under the control of the **renin–angiotensin** hormone system (Box 22-D), which is stimulated when sodium ion receptors in the kidneys detect an imbalance. It is synthesized in vascular cells of the body as well as in the adrenal cortex.[271] Aldosterone promotes the resorption of sodium ions in the kidney tubules and thus regulates water and electrolyte metabolism.[267,268] Glucocorticoids also have weak mineralocorticoid activity, and most patients with adrenocortical insufficiency (**Addison's disease**) can be maintained with glucocorticoids alone if their salt intake is adequate. Addison's disease develops when the adrenals are destroyed, most often by autoimmune disease or by tuberculosis.

## 3. Androgens

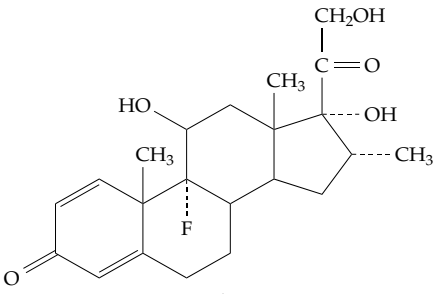
The principal **androgenic** or male sex hormone is **testosterone** formed from pregnenolone through removal of the side chain at C-17. The first step in the conversion is 17α-hydroxylation by a cytochrome P450 which may also mediate the further oxidative cleavage to **$\Delta^4$-androstenedione** (Fig. 22-11). Reduction of the 17-carbonyl group forms testosterone. About 6–10 mg are produced daily in men, and smaller amounts (~0.4 mg) are synthesized in women. Testosterone is carried in the blood as a complex with a β-globulin and affects a variety of target tissues including the reproductive organs. Another striking effect is stimulation of the growth of the beard. Testosterone also causes premature death of follicles of head hair in genetically susceptible individuals. However, a bald man can usually grow a full beard, and follicles of the beard type, when transplanted to the head, remain immune to the action of androgen. No one knows what regulatory differences explain this fact. Baldness might be cured by use of suitable antagonists of the androgenic hormones, but the beard might fall out and sexual interest could be lost.

Androgen synthesis in the human male fetus begins at the age of about 70 days when the testes enlarge and go through an important period of activity that begins the conversion of the infant body to a male type. Other bursts of testosterone synthesis occur during infancy, but there is little further synthesis until the onset of puberty.[268,272]

Within many target tissues testosterone is converted by an NADPH-dependent 5α-reductase into **5α-dihydrotestosterone**. That this transformation is important is shown by the fact that absence of one of the two 5α-reductase isomers causes a form of **pseudohermaphroditism** in which male children are often mistaken as female and raised as girls. However, at puberty they become unmistakably male.[268,273–275] Many other metabolites of testosterone are known. These include the

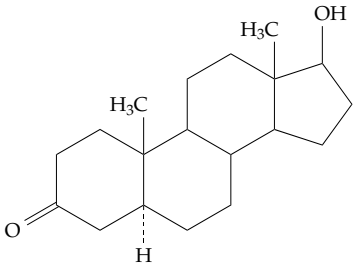


Prednisolone

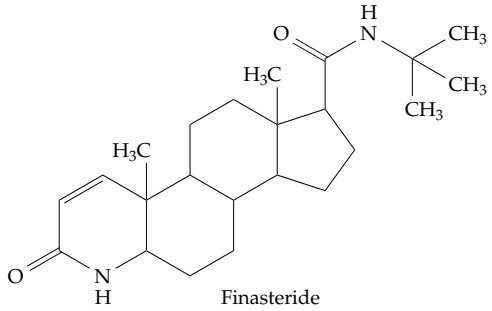


Dexamethasone

isomeric 5β-dihydrotestosterone and 5α-androstane-3β, 17β-diol which arises by reduction of the carbonyl group of 5α-dihydrotestosterone. Testosterone and dihydroxytestosterone have distinct roles in the body.




5α-Dihydrotestosterone

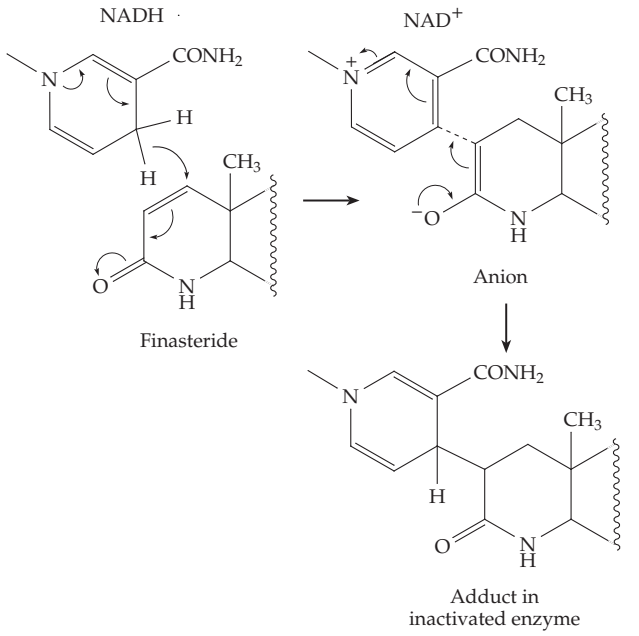



Finasteride

For example, testostosterone is required for sperm cell formation, voice deepening, and growth of pubic hair while dihydroxytestosterone stimulates development of the prostate gland and male pattern hair growth.[276]

Males with deficiency of the 5α-reductase isoenzyme do not develop acne, male pattern baldness, or enlarged prostates.[274] The last fact was some of the impetus for development of the steroid 5α-reductase inhibitor **finasteride,** which is widely used to treat benign prostate enlargement.[274,277,278] It is an enzyme-activated inhibitor in which the NADH reduces the C=C bond in the A ring, which is not in the same position as in the substrate. The resulting anion cannot become protonated but instead adds to the $NAD^+$ as shown in Eq. 22-15.

A number of other androgens are present in the body. The adrenal glands make **dehydroepiandrosterone** (DHEA; Fig. 22–11), which circulates in human blood as its sulfate ester in higher concentration than that of any other androgen.[279–283] However, this steroid is absent from most species. DHEA can be taken up by tissues and converted to testosterone, estrogens, or other steroids (Fig. 22–11).[280] Recent attention has been focused on this hormone because it reaches a peak plasma concentration at age 20–25 years and by age 70 has fallen to 1/5 this value or less.[283] Should older men supplement their circulating DHEA by oral ingestion of 25–50 mg per day of DHEA sulfate? The hormone depresses blood cholesterol and lowers blood glucose in diabetic individuals.[280,282] It seems to




(22-15)

promote increased energy metabolism.[281] It may fight obesity and atherosclerosis,[282] increase levels of estrogen and other steroid hormones in the brain,[284] and enhance memory and immune function.[285–285c] However, the hormone may be metabolized differently in different tissues, and its pathway of biosynthesis in the brain is uncertain.[279,286] Will men synthesize more testosterone from DHEA or make more estrogens in their adipose tissues? Young women should not take DHEA. It may increase the testosterone and dihydrotestosterone levels in the blood manyfold, and the women may become hirsute and masculinized.[280] However, most DHEA is converted to estrogen which may be of value to older women.[285a]

In addition to their role in sexual development androgens have a generalized "anabolic" effect causing increased protein synthesis, especially in muscles.[287–289] They promote bone growth, and the adolescent growth spurt in both males and females is believed to result from androgens. The greater height attained by men results in part from the higher concentration of androgen than is present in women. Many synthetic steroids have been made in an attempt to find "anabolic hormones" with little or no androgenic activity. The effort has been at least partially successful, and the use of anabolic hormones by athletes has become both widespread and controversial.

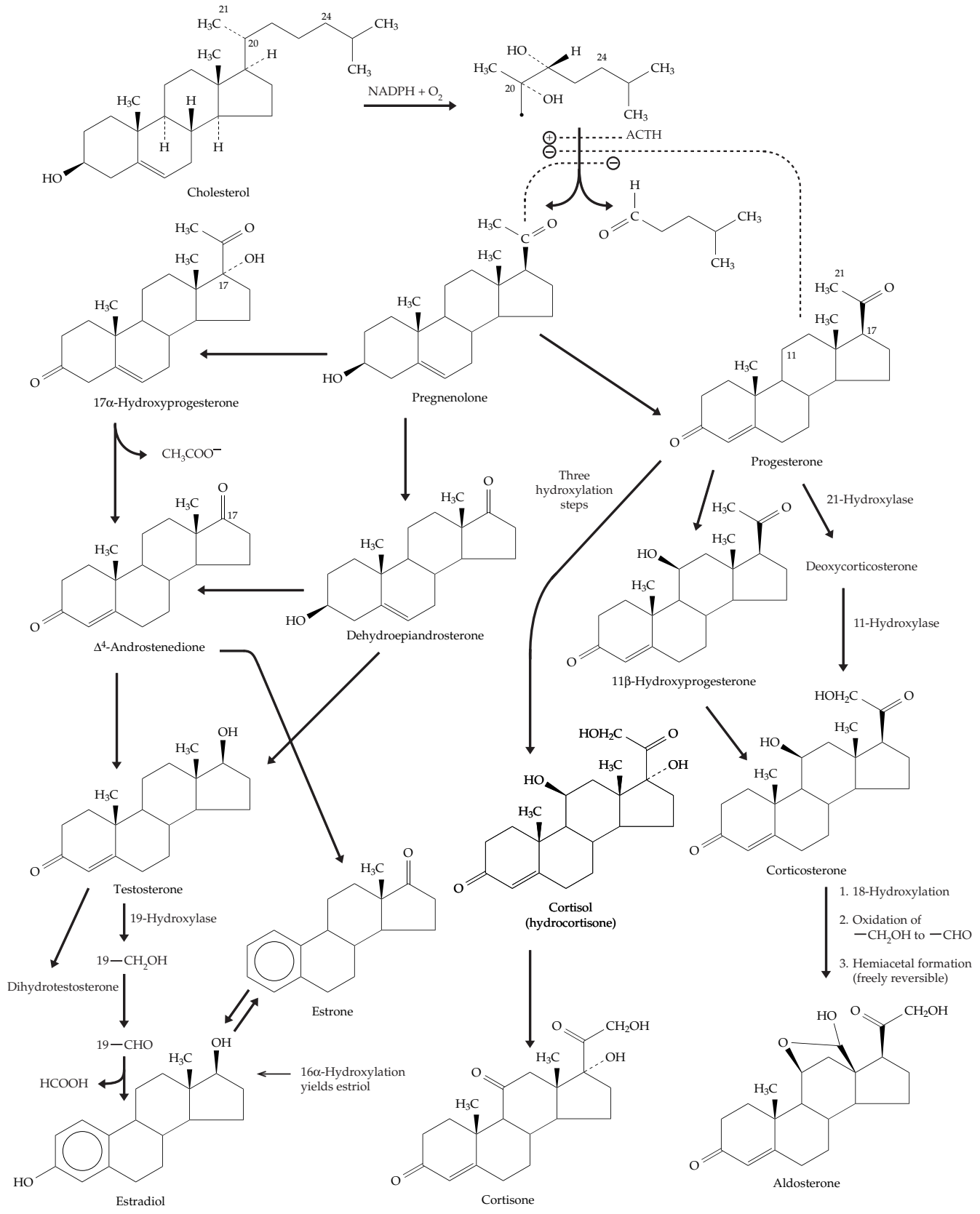
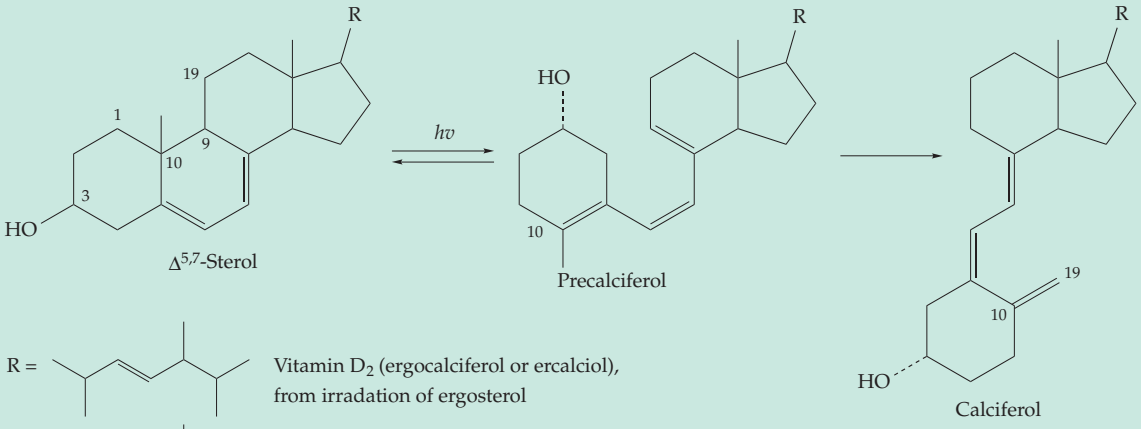



**Figure 22-11** Biosynthesis of some steroid hormones.

## BOX 22-C VITAMIN D

A lack of vitamin D causes **rickets**, a disease of humans and other animals in which the bones are soft, deformed, and poorly calcified. Rickets was recognized by some persons to result from a dietary deficiency well over a hundred years ago, and the use of cod liver oil to prevent the disease was introduced in about 1870. By 1890 an association of rickets with a lack of sunlight had been made.

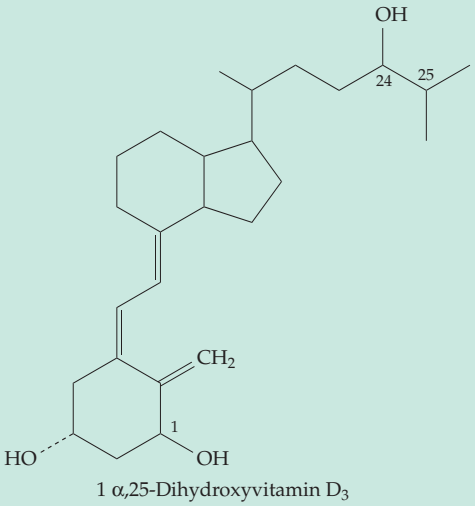



However, it was not until 1924, when Steenbock and Hess showed that irradiation of certain foods generated protective activity against the disease, that vitamin D (**calciferol**) was recognized as a second lipid-soluble vitamin. Vitamin D is a family of compounds formed by the irradiation of $\Delta^{5,7}$-unsaturated sterols such as ergosterol and 7-dehydrocholesterol.[a–e] The former yields **ergocalciferol** (vitamin $D_2$) and the latter **cholecalciferol** (vitamin $D_3$).

At low temperature the intermediate **precalciferol** can be isolated. Irradiation sets up a photochemical steady-state equilibrium between the $\Delta^{5,7}$-sterol and the precalciferol. At higher temperatures the latter is converted to calciferol.[f] Other products, including toxic ones, are produced in slower photochemical side reactions. Therefore, the irradiation of ergosterol for food fortification must be done with care.

With normal exposure to sunlight enough 7-dehydrocholesterol is converted to cholecalciferol in the skin that it was concluded that no dietary vitamin D is required by most adults except during pregnancy. However, recently it has been recognized that old and sick adults probably need 400–600 I.U. per day to maintain calcium absorption and to prevent osteoporesis and fractures.[g,gh] It is usually recommended that children receive ~20 μg (400 I.U.) of ergocalciferol per day in their diet. Larger amounts are undesirable, and at a tenfold higher level vitamin D is seriously toxic.[h]

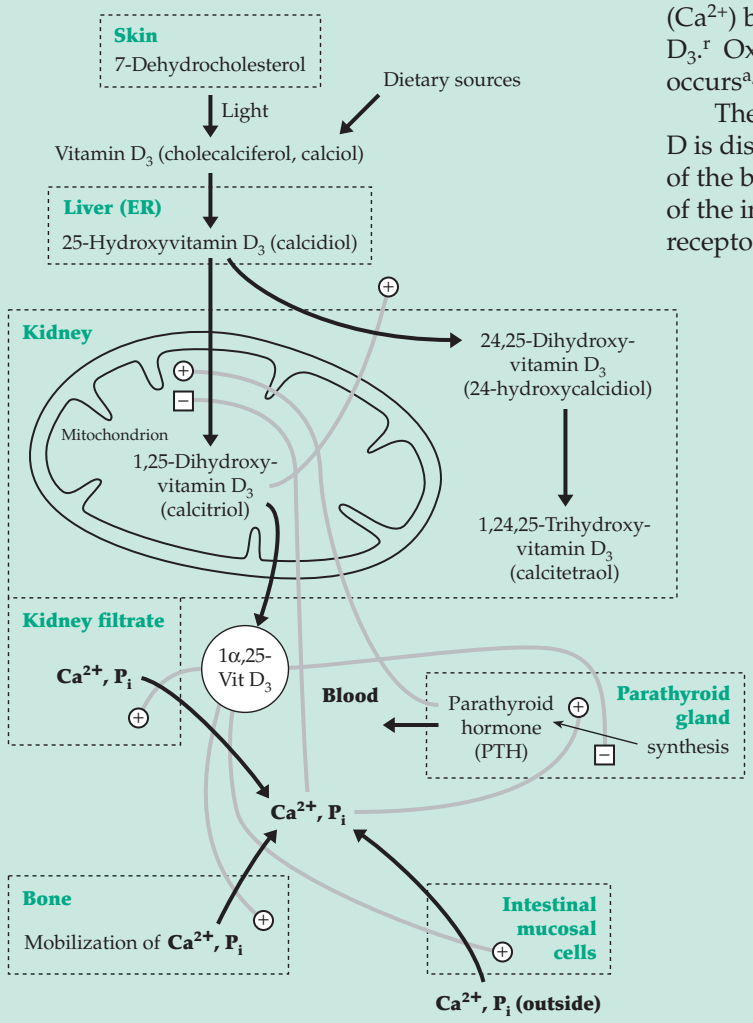
The principal function of vitamin D is in the control of calcium metabolism. This is accomplished through the mediation of polar, hydroxylated metabolites, the most important of which is **1α,25-dihydroxyvitamin D3** (1,25-dihydroxycholecalciferol or calcitriol).[a,i,j] This compound may be properly described as a steroid hormone and vitamin D itself as a hormone precursor formed in the skin. The major hydroxylation reactions of




vitamin D are summarized in the accompanying scheme. The first hydroxylation to 25-hydroxyvitamin $D_3$ occurs largely in the liver,[jk] but the subse-

## BOX 22-C VITAMIN D (continued)

quent cytochrome P450 catalyzed 1α-hydroxylation takes place almost entirely in the kidneys.[k,l] Since it is 1,25-dihydroxy derivative that is essential for control of calcium ion metabolism, human patients




with damaged kidneys often suffer severe demineralization of their bones (renal osteodystrophy). Administration of synthetic 1α,25-dihydroxyvitamin $D_3$ provides an effective treatment for these persons and also for children with an inherited defect in production of this hormone.[a,m] However, large doses, tested as an antileukemia drug, caused a severe hypercalcinemia limiting its use.[n]

A second major vitamin D metabolite is 24*R*,25-dihydroxyvitamin $D_3$, a compound that circulates in the blood at a concentration 10 times higher than that of the 1,25-isomer.[a,b] However, no biological function has been discovered, and like a series of other polar metabolites (>30) it is probably on a pathway of inactivation and degradation of vitamin D. 1α,25-Dihydroxyvitamin D is also hydroxylated at C-24.[o] Additional hydroxylations occur at the 23- and 26-positions.[p,q] The 24-OH is often converted to an oxo group. Oxidation at C-26 together with cyclization yields 26,23 lactol and lactone species. The 25-hydroxy-26,23-lactone suppresses serum ($Ca^{2+}$) by competing with 1,25-dihydroxyvitamin $D_3$.[r] Oxidative cleavage of the side chains also occurs[a,s] as in the metabolism of cholesterol.

The hormonally active 1α,25-dihydroxyvitamin D is distributed through the bloodstream to all parts of the body. It is taken up rapidly by nuclei of cells of the intestinal lining where it binds to a 55-kDa receptor protein. In response, the cells synthesize **calbindins** (Chapter 6), $Ca^{2+}$-binding proteins which facilitate the uptake of calcium ions by the body.[t–v] (see also Fig. 6-7).

Other target organs for the action of 1,25-dihydroxyvitamin D include the kidneys, bone, muscle,[vw] and skin. The hormone promotes reabsorption of both $Ca^{2+}$ and inorganic phosphate by kidney tubules. In bone it binds to a specific receptor where it promotes the mobilization of calcium ions. This effect may result in part from stimulation of calcium-activated ATPase of the outer membrane of bone cells. Dissolution of bone also requires the presence of **parathyroid hormone** (**PTH**), the 83-residue hormone secreted by the parathyroid gland. In women past the age of menopause and in elderly men the production of 1,25-dihydroxyvitamin D decreases.[w] This may be a cause of the serious bone loss (**osteoporosis**) frequently observed. Treatment with 1,25-dihydroxyvitamin $D_3$ or a synthetic analog seems to be helpful to such individuals.[x,xy] See also Chapter 30, Section A,5.

There is another important member of the $Ca^{2+}$ homeostatic system. While vitamin D and PTH act together to increase the calcium level of the blood, **calcitonin**, a hormone of the thyroid gland, lowers the level of $Ca^{2+}$ by promoting deposition of calcium in bone by the osteoblasts. The overall effect is to hold the concentration of $Ca^{2+}$ in the blood at 2.2–2.6 mM in most animals with bones serving as a mobile reserve. Another role for vitamin D is suggested by the observation that 1,25-dihydroxyvitamin $D_3$ inhibits the growth promoting effect of interleukin-2 on mitogen-activated lymphocytes (Chapter 31).

## BOX 22-C (continued)

Like other steroid hormones 1,25-dihydroxyvitamin $D_3$ acts to regulate gene transcription. It binds to a specific receptor, a member of the v-*erb*-A superfamily of ligand-activated transcription factors and a relative of the steroid receptor family.[a,j,y] Like other hormone receptors this vitamin $D_3$ receptor (VDR) has some rapid "nongenomic" actions such as causing an increase in intracellular [$Ca^{2+}$] as well as slower effects on transcription.[y–bb] Like the steroid receptors it is found in both cytoplasm and nucleus.[y] When occupied by 1,25-dihydroxyvitamin D, the receptor binds tightly to specific dihydroxyvitamin $D_3$ response elements in the DNA. These are found in promoter sequences for genes such as that of bone protein **osteocalcin**.[cc] In some promoters the response element binds heterodimers of VDR with another receptor, e.g., the retinoid X receptor (Table 22-1).[cc,dd] One effect of this response element is to activate the gene for the 24-hydroxylase involved in degradation of 1,25-dihydroxyvitamin D.[dd] Heterodimers of VDR with the thyroid hormone receptor activate the transcription of genes for calbindins $D_{28K}$ and $D_{9K}$.[u] VDR also binds to repressor sequences, e.g., in the parathyroid gland.[ee] See also Chapter 28 and Table 22-1.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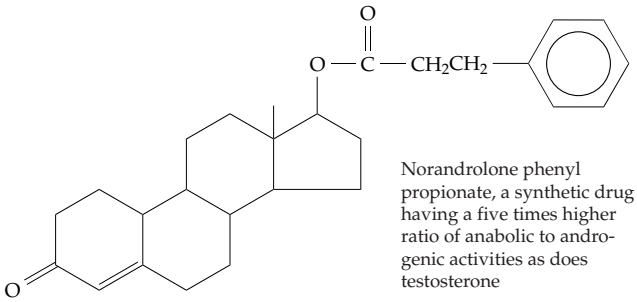
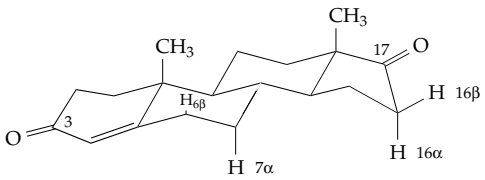



Norandrolone phenyl propionate, a synthetic drug having a five times higher ratio of anabolic to androgenic activities as does testosterone

Several sex-dependent differences have been observed in the action of cytochrome P450 isoenzymes on steroid hormones.[290,290a] Thus, androstenedione is hydroxylated by rat liver enzymes specific for the 6β, 7α, 16α, and 16β positions.[291] Of these the 16 hydroxylase is synthesized only in males, and synthesis of the 6 hydroxylase is also largely suppressed in females.




Δ4-Androstenedione

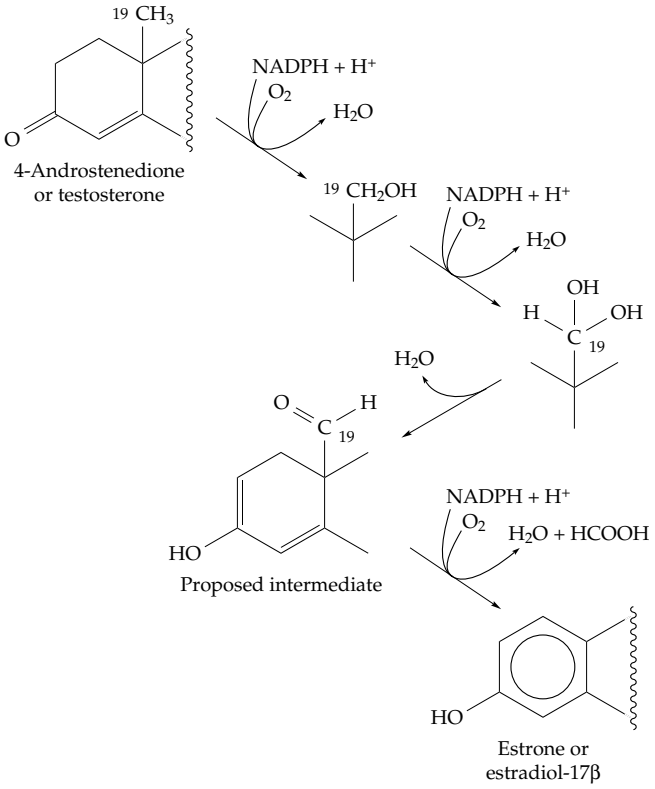
A female-specific 15β hydroxylase acts on steroid sulfates such as corticosterone sulfate and forms the major urinary excretion product of that hormone in female rats.[292] These sex-specific differences in enzymes are thought to be related to secretions of growth hormone that are in turn controlled by the "programming" of the hypothalamus by androgen during the neonatal period in rats[272] or during human fetal development.

## 4. Estrogens

The principal **estrogenic** or female hormone is estradiol-17β. It is formed by oxidative removal of C-19 of testosterone followed by aromatization of the A ring.[268,293–295] All of the estrogenic hormones have this aromatic ring. Its formation involves three steps of hydroxylation followed by elimination of formate (Eq. 22-16). This **aromatase** appears to be a unique cytochrome P450, which catalyzes all of the steps of Eq. 22-16. It accepts electrons from NADPH via the flavoprotein NADH-cytochrome P450 reductase, which serves as the intermediate electron carrier.[296] It probably acts by a mechanism related to that illustrated for lanosterol 14α-demethylase on the right-hand side of Fig. 22-8.[294] This enzyme is the target for synthetic enzyme-activated inhibitors.[293] One of these is an androstenedione derivative with an acetylenic group attached to C-19. Passage through the first two steps of Eq. 22-16 generates a conjugated ketone to which a nucleophilic group of the enzyme can add irreversibly to inactivate the enzyme (Eq. 22-17). The C-17 acetylenic progesterone antagonist (norethyndrone) is also an enzyme activated inhibitor of the aromatase.

Estrogens are formed largely in the ovary and during pregnancy in the placenta. Estrogens are also synthesized in the testes, and the estrogen content of the horse testis is the highest of any endocrine organ. Target tissues for estrogens include the mammary glands, the uterus, and many other tissues throughout the body. Estrogens act on the growing ends of the long bones to stop growth and are therefore responsible, in part, for the shorter stature of females as compared to males. They are responsible for the overall higher fat content of the female body and for the smoother skin of the female. Recent attention has been focused on the effects of estrogens on brain neurons. Estrogens stimulate sprouting of axons and dendrites in neurons in cell cultures, and there is preliminary evidence that the hormone improves memory in healthy women and in those with Alzheimer disease.[285]

The cooperative action of progesterone and estradiol regulate the menstrual cycle. At the beginning of the cycle the levels of both estrogen and progesterone are low. Estrogen synthesis increases as a result of release of **follicle-stimulating hormone** (FSH) from the anterior pituitary. This hormone stimulates growth of the graafian follicles of the ovary which in turn produce estrogen. At about the midpoint of the cycle, as a result of the action of the pituitary **luteinizing hormone** (LH), an ovum is released and progesterone secretion begins. The latter is essential to maintenance of pregnancy. If a blastocyst is not implanted, hormone production decreases and menstruation occurs.

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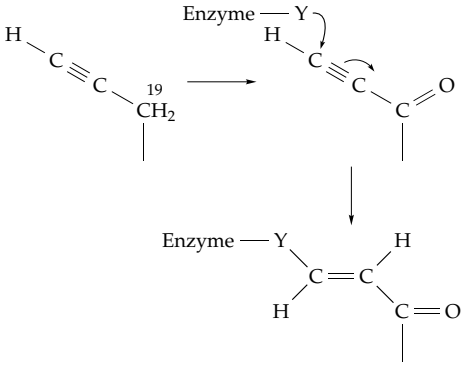
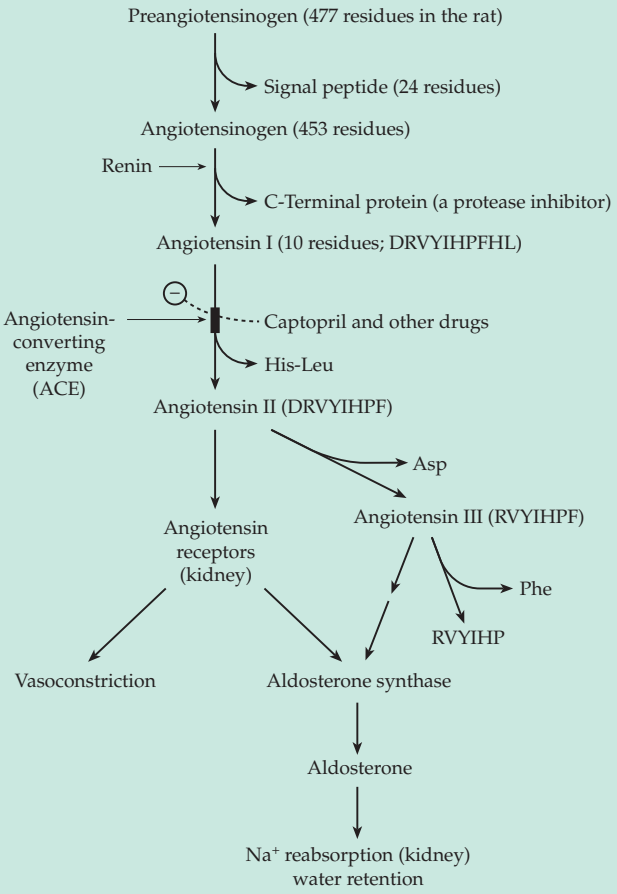



(22-17)

## BOX 22-D THE RENIN-ANGIOTENSIN-ALDOSTERONE SYSTEM AND THE REGULATION OF BLOOD PRESSURE

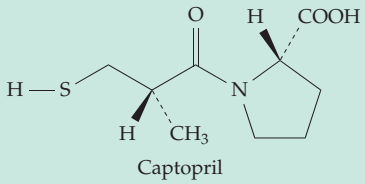
The mineralocorticoid **aldosterone** was isolated and identified in 1953. Although the function of adrenal cortical hormones in regulation of electrolytes had been known for many years, the special role of aldosterone had been overlooked.[a] Aldosterone works in concert with the aspartate protease **renin** and the octapeptide **angiotensin II** to regulate blood pressure. Angiotensin II, which is the most potent pressor substance known, is formed in the liver from the 477-residue (in the rat) **preangiotensinogen** as shown in the following cascade:




This **renin–angiotensin system** is a peptide hormone-generating system that operates in blood plasma rather than within tissues.[b,c]

Angiotensinogen, which is secreted by the liver and circulates in the blood, is converted to the physiologically inactive decapeptide angiotensin I by cleavage of a Leu-Leu peptide bond by the 328-residue renin.[d–f] Its precursor **preprorenin** is produced in the kidneys by the juxtaglomerular cells as well as in some other tissues and undergoes several steps of processing before the active enzyme is formed.[g–j] Active renin is released from the kidney cells into the bloodstream in response to various stimuli which include low arterial pressure resulting from constriction of the renal arteries or loss of blood.[k] Parathyroid hormone, glucagon, other adrenergic agonists, cAMP, some prostaglandins, low levels of aldosterone or $Na^+$, or high $K^+$ all induce secretion of renin. High blood pressure, α-adrenergic agonists, some prostaglandins, angiotensin, vasopressin, high $Na^+$, or low $K^+$ concentration decrease secretion of renin. It has been suggested that these diverse effects may be modified by a rise or fall in the $Ca^{2+}$ concentration, high $Ca^{2+}$ inhibiting secretion of renin, and low $Ca^{2+}$ favoring secretion.

The only known physiological substrate for renin is angiotensinogen,[e] but it may also act on related proteins in the brain and other organs.[g] The inactive angiotensin I is converted to angiotensin II by the metal-containing carboxydipeptidase known as **angiotensin-converting enzyme**.[l,m] This enzyme is a target for drugs such as captopril, which is used to control high blood pressure (hypertension).[i] A zinc-dependent aminopeptidase may cut off the



Captopril

N-terminal aspartate to form angiotension III,[n] and degradation of angiotensions II and III can be initiated by removal of the C-terminal phenylalanine by a prolylcarboxypeptidase.[o]

Angiotensin II has a variety of effects. By constricting blood vessels it raises blood pressure, and by stimulating thirst centers in the brain it increases blood volume. Both angiotensins II and III also act on the adrenal gland to promote the synthesis and release of aldosterone. Most of the effects of angiotension II are mediated by 359-residue seven-helix G-protein linked receptors which activate phospholipase C.[p,q,qr] Like other steroid hormones aldosterone acts,via mineralocorticoid receptors, to control transcription of a certain set of proteins. The end effect is to increase the transport of $Na^+$ across the renal tubules and back into the blood. Thus, aldosterone acts to decrease the loss of $Na^+$ from the body. It promotes retention of water and raises

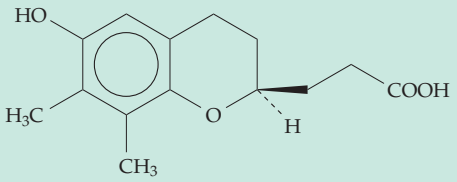
## BOX 22-D THE RENIN–ANGIOTENSIN–ALDOSTERONE SYSTEM AND THE REGULATION OF BLOOD PRESSURE (continued)

blood pressure.[c,r,s] Its primary function is to provide adequate $Na^+$ to cells.[s] Dietary sodium appears to have little or no effect on blood pressure.[t]

The control of blood pressure is considerably more complex than it is described here. Another hormone system involving the peptide **bradykinin** and prostaglandins acts to lower blood pressure. Bradykin is also cleaved by the angiotensin-converting enzyme but is *inactivated* by the cleavage.[u,v] At least ten human genes have been shown to affect blood pressure.[c] One of these is the structural gene for angiotensin-converting enzyme, which has been linked to hypertension in both rats and humans.[w,x]

While several antagonists of angiotensin-converting enzyme are widely used to treat hypertension, they are not free of harmful side effects.[u,y,z] One alternative approach is to inhibit renin.[v,y]

While the angiotensins promote release of aldosterone, the **atrial natriuretic hormone**[r,aa–cc] inhibits release. This group of 21- to 33-residue polypeptides, secreted by cells of the atria (auricles) of the heart, also inhibits release of renin and promotes secretion of both $Na^+$ and water. Thus, they antagonize the action of aldosterone, which promotes $Na^+$ retention. However, there is uncertainty as to the significance of these peptides. The following metabolite of γ-tocopherol (Fig. 15-24) has been isolated from urine and is proposed as a new endogenous natriuretic factor.[dd]




2, 7, 8-trimethyl-2-(β-carboxyethyl)-6-hydroxychroman


[a] Tait, J. F., and Tait, S. A. S. (1978) *Trends Biochem. Sci.* **3**, N273–N275
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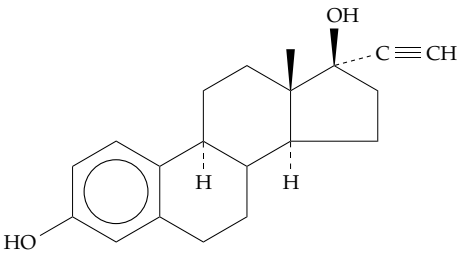
Administration of estrogens and progestins inhibits FSH and LH secretion from the pituitary (feedback inhibition) and hence ovulation. This effect is the action of contraceptive pills. A small amount of the synthetic estrogen 17-ethynylestradiol may be taken daily for 10–15 days followed by a combination of estrogen plus a progestin such as norethindrone for 0–15 days. Alternatively, a progestin alone may be

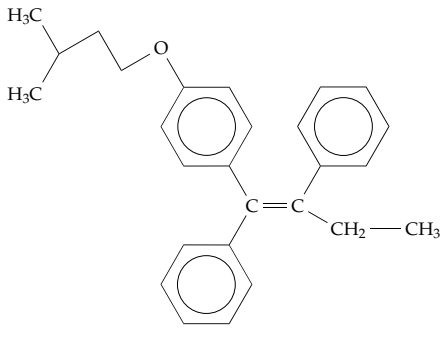


17-Ethynylestradiol

ingested over the entire period. Another synthetic compound with estrogenic activity is diethylstilbestrol. Its once widespread use in promoting growth of cattle and other animals has been discontinued because of carcinogenic action in rats fed large amounts of the compound.

Human cancers of the breast and endometrium are stimulated by estrogen. However, the mechanism is unknown.[297,298] It has usually been assumed that the proliferation of cells induced by estrogens leads to mutations and cancer. However, estrogens can form adducts with DNA after oxidation to 2- and 4-hydroxy derivatives and further oxidation to quinones.[297,298] In a similar manner prostate cancer is promoted by androgens. It has also been observed that in the United States the incidence of cancers of the ovary and endometrium has declined substantially during the past 35 years, perhaps as a result of the anti-estrogenic activity of the progestin in the widely used oral contraceptives.[299] This observation led to the idea of **hormonal chemoprevention**, the deliberate use of hormone antagonists to slow cancer formation.[299a] This may be especially attractive to persons carrying known cancer-susceptibility genes. The synthetic antiestrogen **tamoxifen** is being evaluated as a chemopreventive agent for breast cancer.[299,299b] However, a planned large-scale trial was postponed because of uncertainities about safety.[300]

In addition to steroids there are plant flavonoids (Box 21-E) that have estrogenic activity. These labile compounds are among the **"environmental estrogens."** In addition, there are many much more stable compounds, including the insecticides atrazine and DDT, PCBs, and phenolic softeners for plastics, that have weak estrogenic activity. Alarm has been sounded



*trans*-Tamoxifen

by some who maintain that these **xenoestrogens** are contributing to breast cancer, to reproductive difficulties in animals, and to low sperm counts in men.[301] It seems surprising that such small amounts of weakly estrogenic compounds could have such large effects. Some experiments suggest that two weak xenoestrogens may cooperate to give larger effects,[302,303] but this concept needs further evaluation.

Although estrogens are usually regarded as female hormones, they are present in small amounts in male blood and in high concentration in semen. Male reproductive tissues contain estrogen receptors, and mice deficient in these receptors are sterile and their testes degenerate.[304] Furthermore, as in females estrogen stops growth of long bones in late puberty. A few men lacking estrogen or estrogen receptors have grown very tall (>2.1 m and still growing).[305]

## 5. The Steroid Receptor Family

The principal mode of action of steroid hormones is to stimulate transcription of specific sets of genes. The plasma concentrations of these hormones are low, typically ~$10^{-9}$ M, but they have a high affinity for their protein receptors, some of which are located initially in the cytosol but are found largely in the nucleus. The earliest identification of steroid receptors was accomplished with radioactive $^3$H-labeled progesterone, estrogens, and glucocorticoids.[306,307] Autoradiographs of thin sections of tissue made after the uptake of the hormones revealed that the radioactivity was concentrated in the nuclei. The protein receptors were then isolated and were found to bind both to the hormone and to specific sequences in DNA, the hormone **response elements**.[308] The progesterone, estrogen, and glucocorticoid receptors are multidomain proteins with two of the domains having highly conserved sequences and structures. One of these domains binds to DNA and the other to the steroid hormone. Their amino acid sequences are also related to those of the v-*erb-A* oncogene (Table 11-3).[309] With this knowledge available and through use of methods of "reverse genetics," receptors for the other steroid hormones and also for vitamin D, retinoic acid, and thyroid hormone were identified as members of the family as were several "orphan" receptors of as yet unknown function (Table 22-1). Invertebrates have similar receptors. At least eight are present in *Drosophila*,[307] and the family is present in the nematode *Caenorhabditis*.[310]

Because of their hydrophobic character the steroid hormones or other nonpolar ligands diffuse through membranes into cells. There they may encounter a variety of binding proteins that affect their access to a receptor.[307,311] Some receptors, including glucocorticoid receptors, are found in the cytosol. After a hor-

**TABLE 22-1**
**Known Members of the Steroid Receptor Family[a]**

| | |
|---|---|
| Glucocorticoid[b–d] | Thyroid α, $\beta_1$, $\beta_2$[r] |
| Mineralocorticoid[e,f] | Retinoic acid α, β, γ [s] |
| Progesterone[g,h] | Retinoid-X α,β[t–w] |
| Androgen[i,j,k] | Peroxisome proliferators[x,y] |
| Estrogen[l–o] | Farnesoid X: bile acids[z,aa] |
| Vitamin $D_3$[p,q] | Orphan receptors, 8 or more |

[a] See Fuller (1991) *FASEB J.* **5**, 3092–3099
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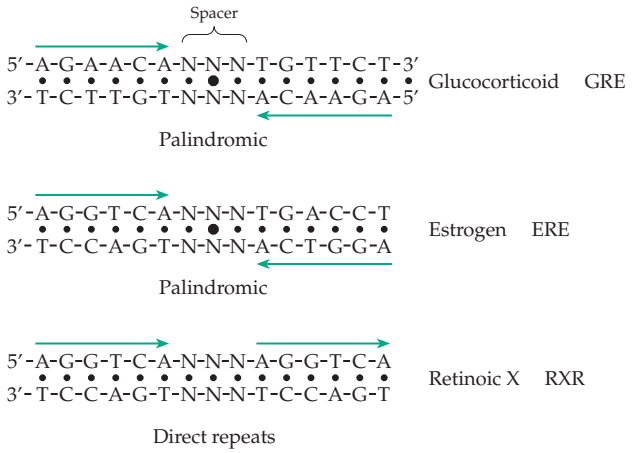
mone binds to a cytosolic receptor, the complex apparently undergoes time and temperature dependent alterations that activate the receptor before it diffuses into the nucleus and binds to its proper response element in the DNA.[312] This process has been observed directly for glucocorticoid receptors labeled by fusion with green fluorescent protein (Box 23-A).[313]

The conserved 68-residue DNA binding domain of the glucocorticoid receptor contains two $Zn^{2+}$ ions, each coordinated by four cysteine $-CH_2-S^-$ groups with tetrahedral geometry. These two consecutive motifs form structures somewhat similar to those of the "zinc fingers" shown in Fig. 5-38.[314–316] However, the overall folding pattern is different from those considered in Chapter 5. The two zinc-binding sites lie at the N termini of a pair of helices that cross at right angles near their centers. One of these is a DNA-recognition helix that fits into the major groove of DNA thereby allowing interaction of its amino acid side chains with the bases of the DNA response elements.

The response elements for glucocorticoids and estrogen receptors contain short palindromic sequences with various three-nucleotide "spacer" sequences in the center as follows.[308,314,316–318] Two receptor proteins bind to the palindromic DNA forming a homodimeric receptor pair. For the 9-*cis* retinoic acid receptor RXR-α the response element contains a pair of direct repeats of a 6-base consensus sequence with a two-base pair spacer:




The RXR-α receptor binds differently and tends to form *heterodimeric* pairs with other receptors.[319,320] All of these receptors undergo conformational changes when agonists or antagonists bind.[320,321] Estrogens can also bind to androgen receptors, perhaps in playing their essential role in male reproductive physiology.[322] There are more than one type of receptor for each group of steroid hormones, and these may interact differently with the various response elements in DNA[323] making the effects of hormones complex and hard to analyze. Interactions with additional proteins

also affect the response of a cell to hormones.[324] Furthermore, steroid hormones have **transcription-independent effects**. For example, progesterone binds to oxytocin receptors[325] as well as to other steroid receptors, which affect a broad range of biochemical processes.[326]

## F. Other Steroids

The **saponins** are a series of steroid glycosides with detergent properties that are widespread among higher plants.[327] Some are toxic, and among these toxic materials are compounds of extraordinary medical importance. Best known are the steroid glycosides of *Digitalis*, among them **digitonin** (Fig. 22-12). The particular arrangement of sugar units in this molecule imparts a specificity toward heart muscle. The compound is extremely toxic; in small amounts it acts to increase the tone of heart muscle and is widely used in treatment of congestive heart failure.[328] The maintenance dose is only 0.1 mg / day. Another toxic glycoside and heart stimulant is **ouabain** (Fig. 22-12). Ouabain is a specific inhibitor of the membrane-bound $(Na^+ + K^+)$-ATPase believed to be the ion pump that keeps intracellular $K^+$ concentrations high and $Na^+$ concentrations low (Chapter 8). Similar glycosides account for the extreme toxicity of the leaves of the oleander and the roots of the lily of the valley. A steroid glycoside from red squill is used as a rat poison. A number of alkaloids (nitrogenous bases) are derived from steroids. An example is **solanidine** (Fig. 22-12), which is present in the skins and sprouts of potatoes, making both quite toxic.

Some animals also contain toxic steroids. **Batrachotoxin** of the Columbian poison arrow frog (Fig. 22-12) is present in amounts of only 50 μg per frog.[329,330]

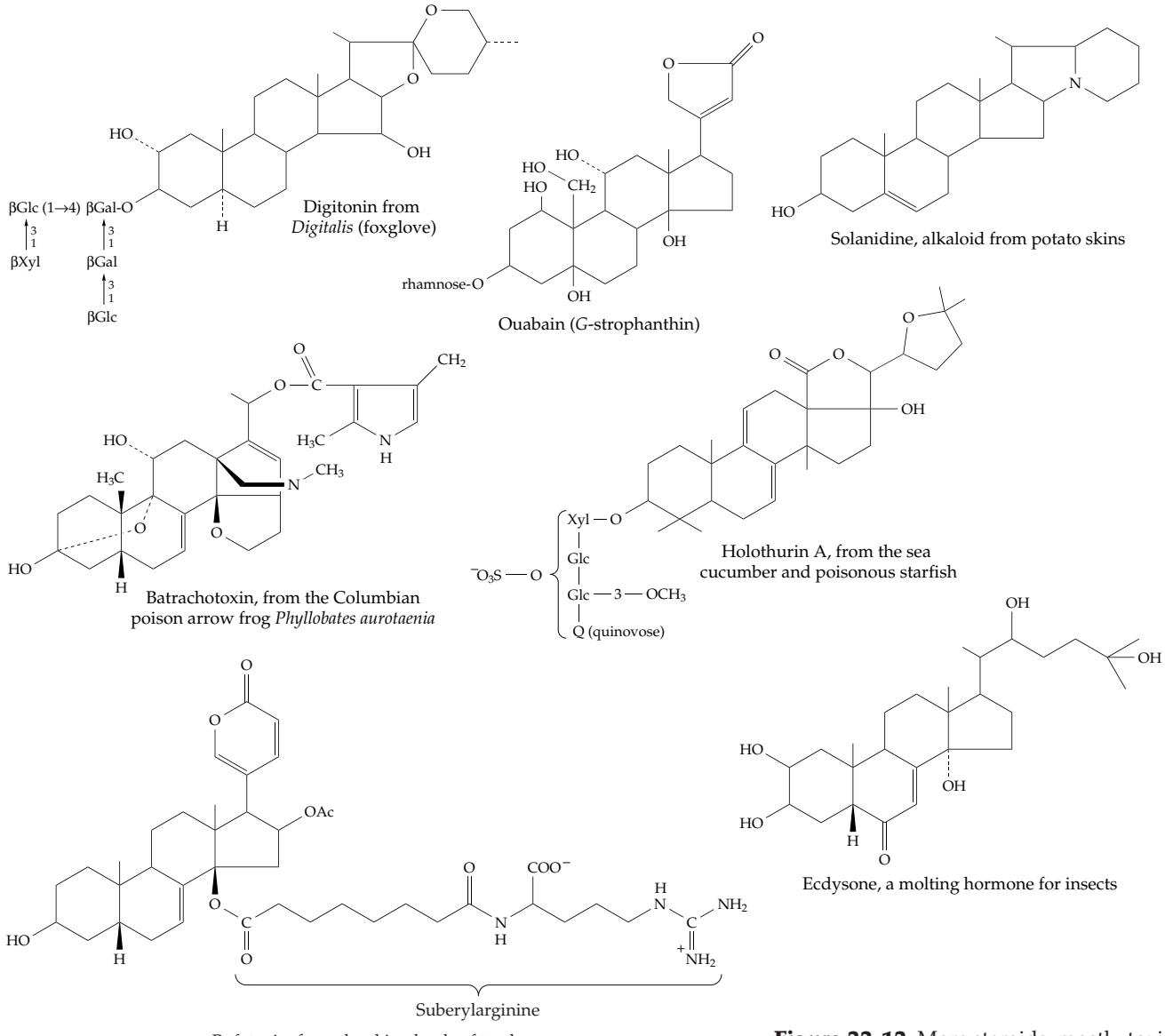
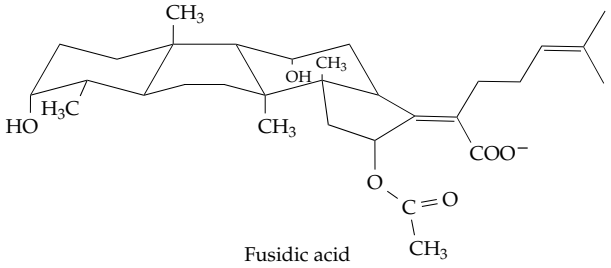



**Figure 22-12** More steroids, mostly toxic.

The toxin acts on nerves to block transmissions to the muscle by increasing the permeability of membranes to sodium ions. It is specifically antagonized by **tetrodotoxin** (Fig. 30-16). Batrachotoxin alkaloids are present also in certain birds.[330a] Some echinoderms make powerful steroid toxins such as **holothurin A** (Fig. 22-12), a surface active agent that causes irreversible destruction of the excitability of neuromuscular tissues. The common toad produces in its skin steroid toxins such as **bufotoxin** (Fig. 22-12), which are sufficiently powerful to teach a dog to leave toads alone.

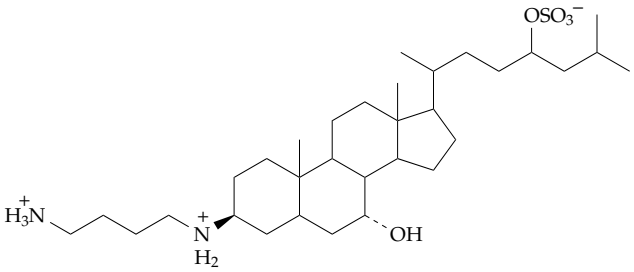
**Ecdysone**, a highly hydroxylated steroid (Fig. 22-12), is a molting hormone for insects.[331,332] Several molecules with ecdysone activity are known, and some of these are produced by certain plants. Although ecdysones are needed by insects for larval molting, they are toxic in excess. Perhaps plants protect themselves from insects by synthesizing these substances.

Among the antibiotics **fusidic acid** is a steroid. An inhibitor of chloramphenicol acetyltransferase[333] it is highly inhibitory to staphyococci but almost noninhibitory to *E. coli*. Note the boat conformation of the B ring.



Fusidic acid

Another steroid antibiotic, **squalamine**,[334] was isolated from the stomachs of sharks. It is effective against gram-positive and gram-negative bacteria, and some fungi as well.[335]



Squalamine

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## Study Questions

1. Outline the sequence and chemical mechanisms of the reactions involved in the conversion of acetyl-CoA into mevalonate.

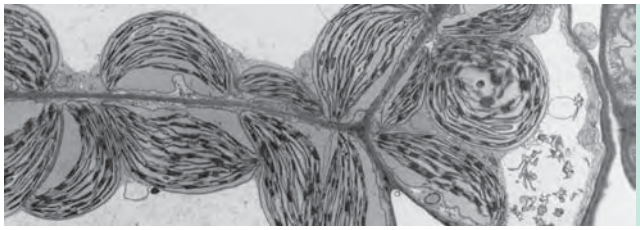
2. a) Show the structures of the reactants for the hydroxymethylglutaryl CoA synthase reaction.
   b) Free coenzyme A is liberated in the above reaction. From which molecule did it come? Explain the metabolic purpose behind the liberation of free CoA.

3. List as many substances as you can that are of polyprenyl origin and are present
   a) in foods
   b) in various commercial products

4. What distinctly different functions do 3-hydroxy-3-methylglutaryl-CoA synthases serve in the cytosol and in mitochondria of the liver?

5. Outline the functions of mitochondrial enzymes in the conversion of fructose into cholesterol in the liver.

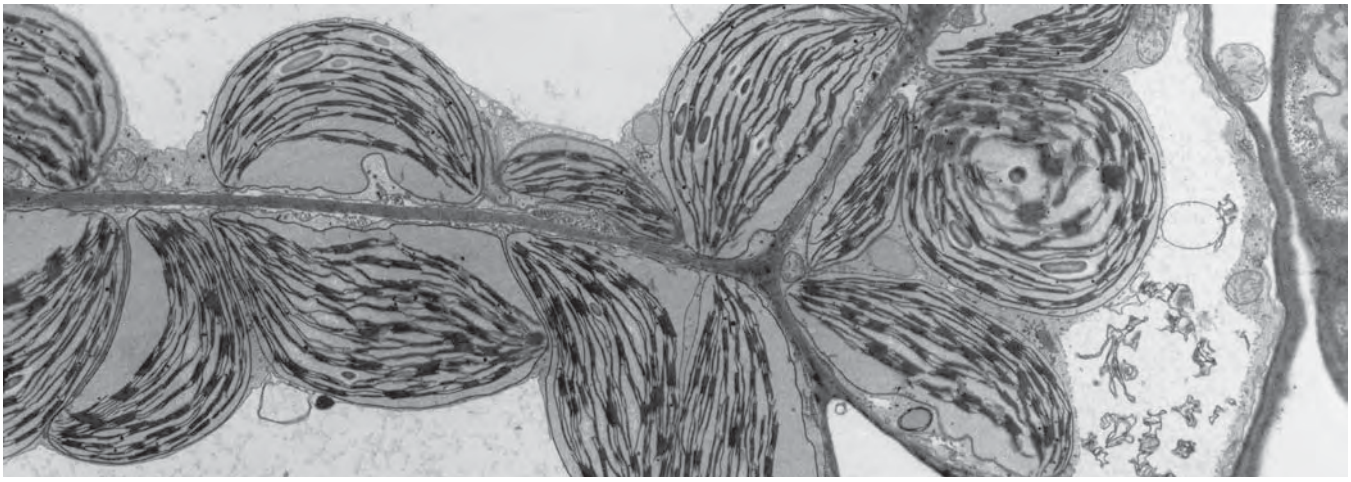
6. How do you think that hydroxycitrate, an inhibitor of ATP citrate lyase, would affect the ability of liver to convert dietary fructose into bile acids?



Chloroplasts fill most of the cytoplasm around the junction of three cells of *Arabidopsis thaliana* seen in this micrograph. Both grana stacks and stroma lamellae (pictured in more detail in Fig. 23-19) can be seen. Also present are several small mitochondria. Portions of the large vacuoles, characteristic of plant cells, are seen at top, right, and bottom. Micrograph courtesy of Kenneth Moore.

# Contents

# 23 Light and Life



Light plays a pervasive role in human life. The earth is bathed in light from the sun, and from this light comes not only warmth but also energy for all living organisms. Of the $3 \times 10^4$ kJ $m^{-2}$ of light energy falling on the earth each day,[1,2] ~30 kJ $m^{-2}$ are captured by photosynthesis.[3] Light penetrating the atmosphere allows us to see and provides color to our environment. It controls the flowering of plants, the germination of seeds and spores, the greening of seedlings, and the daily cycles of many organisms. High in the stratosphere ultraviolet light reacts with oxygen to create a protective blanket of ozone. The ultraviolet light that is not screened out by the ozone layer kills bacteria, tans our skin, and often mutates our DNA, inducing many cancers.[4,5] Organisms, from bacteria to higher plants, display **phototaxis**, the ability to move toward a source of light or to orient themselves with respect to a source of light. In plants the chloroplasts assume an orientation that maximizes efficiency of light absorption. Plants grow toward light (**phototropism**), and some organisms avoid light. Many organisms emit light.

Many of our most important experimental techniques involve the use of light or of other forms of electromagnetic radiation of a wide range of energies. X-rays, ultraviolet light, infrared light, and microwaves all serve in the study of biomolecules.

## A. Properties of Light

Light is a form of electromagnetic radiation and possesses characteristics of both waves and particles (**photons**). The energy of a photon is usually measured by the frequency (or by the wavelength in a vacuum to which it is inversely related, Table 23-1). A portion of the electromagnetic spectrum is shown on a logarithmic scale in Fig. 23-1.[1] At the high-energy end (off the scale of the figure to the right) are cosmic rays and gamma rays, while at the low-energy end radio waves extend to wavelengths of many kilometers. The narrow range of wavelengths from about 100 nm to a few micrometers, which is the subject of this chapter, includes the ultraviolet, visible, and near infrared

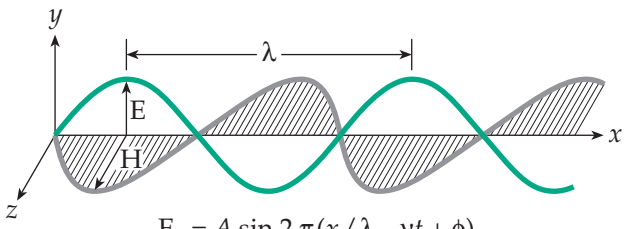
**TABLE 23-1**
**Some Properties of Light**

| | |
|---|---|
| Velocity of light in a vacuum | $c = 2.998 \times 10^8$ m $s^{-1}$ |
| Velocity of light in a medium | $c' = c/n$ where $n$ = refractive index |
| Wave number (in nm) | $\bar{\nu} = 1/\lambda$; $\bar{\nu}$ (in $cm^{-1}$) $= 10^7/\lambda$ |
| Frequency | $\nu = c/\lambda = c\bar{\nu}$<br>$\nu$ (in hertz) $= 2.998 \times 10^{10}\,\bar{\nu}$ ($cm^{-1}$) in a vacuum |
| Energy of quantum | $E = h\nu = hc\bar{\nu}$<br>$E$ (joules) $= 1.986 \times 10^{-23}\,\bar{\nu}$ ($cm^{-1}$)<br>$E$ (eV) $= 1.240 \times 10^{-4}\,\bar{\nu}$ ($cm^{-1}$) |
| Energy of einstein | $E = Nh\nu = Nhc\bar{\nu}$<br>$= 6.023 \times 10^{23}\,hc\bar{\nu}$<br>$E$ (joules) $= 11.961\,\bar{\nu}$ ($cm^{-1}$)<br>$E$ (kcal) $= 2.859 \times 10^{-3}\,\bar{\nu}$ ($cm^{-1}$) |

ranges. The second line of Fig. 23-1 shows this region expanded. Note that the range of light reaching the earth's surface is narrow, largely being confined to wavelengths of 320–1100 nm. The human eye responds to an even more limited range of 380–760 nm, in which all of the colors of the rainbow can be found. The aromatic rings of proteins and nucleic acids absorb maximally at 280 and 260 nm, respectively. Even though these wavelengths are largely screened out by the ozone layer of the stratosphere, enough light penetrates to cause mutations and to damage the skin of the unwary sunbather.

The energy of a quantum of light is proportional to the **wave number** or **frequency**. The wave number $\bar{\nu}$ is the reciprocal of wavelength and is customarily given in units of $cm^{-1}$ (**reciprocal centimeters**). Most of the absorption spectra in this book are plotted against wave number in $cm^{-1}$. The frequency $\nu$ in **hertz** is equal to $c'\nu$, where $c'$ is the velocity of light in a medium. (The velocity of light in a vacuum is designated $c$ and is equal to $3.00 \times 10^8$ m $s^{-1}$.) The energy of a quantum of light $E$ is equal to $h\nu$, where $h$ is Planck's constant, $6.626 \times 10^{-34}$ J $s^{-1}$. From a chemical viewpoint, we are more interested in the energy of one **einstein**, i.e., one "mole" of light ($6.023 \times 10^{23}$ quanta). The energy in kJ per einstein is 11,960 $\bar{\nu}$ (in $cm^{-1}$, vacuum). Energy relationships are summarized in Table 23-1. The lower three scales of Fig. 23-1 also show the relationships of $\bar{\nu}$, $\nu$, and $E$ to wavelength.

The light wave is characterized by oscillating electrical and magnetic fields.[2,3,6] For propagation of light in the $x$ direction the electric field vector **E**, which is customarily plotted in the $y$ direction, is a function of the wavelength $\lambda$ and the time (Eq. 23-1).




$$E_y = A \sin 2\pi(x/\lambda - \nu t + \phi) \qquad (23\text{-}1)$$

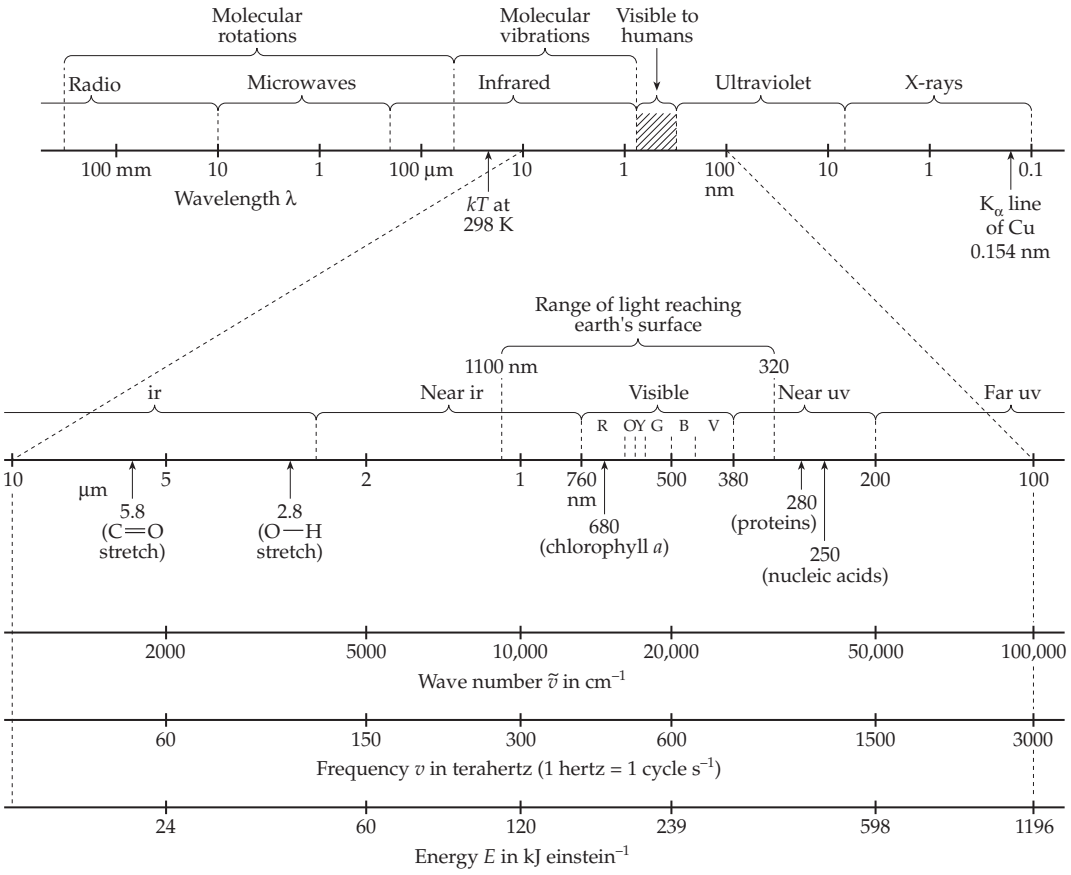



**Figure 23-1** A part of the electromagnetic spectrum. The letters V, B, G, Y, O, R over the visible part of the spectrum refer to the colors of the light. The position marked "$K_a$ line of Cu" is the wavelength of X-rays and most widely employed in X-ray diffraction studies of proteins and other organic materials.

The magnetic vector H is at right angles to the electric vector and is given by Eq. 23-2.

$$H_z = (\varepsilon / \mu)^{-} A \sin 2\pi(x/\lambda - \upsilon t + \phi) \qquad (23\text{-}2)$$

The velocity of propagation of light in a medium, $c'$ (Eq. 23-3), depends upon both ε, the **dielectric constant** of the medium, and μ, the **magnetic permeability**.

$$\text{Velocity in a medium: } c' = c/(\varepsilon/\mu)^{-} = c/n \qquad (23\text{-}3)$$

The **refractive index** of a medium relative to a vacuum is given the symbol $n$. It is the factor by which the velocity of light in a vacuum is diminished in a medium. It is a function of wavelength. For the 589 nm sodium line $n$ is 1.00029 for air and 1.33 for water at 25°C.

The term ϕ in Eqs. 23-1 and 23-2 is a **phase factor**. Most light is called **incoherent** because ϕ varies for the many photons making up the beam. **Coherent light** produced by **lasers** contains photons all with the same phase relationship. If the electric vectors of all the photons in a beam of light are in the same plane (as will be the case for light emerging after passage through certain kinds of crystals), the light is called **plane polarized**. The direction of polarization is that of the electric vector E. **Circularly polarized light**, in which the electric vector rotates and traces out either a left-handed or right-handed helix, can also be generated. A beam of left-handed circularly polarized light, together with a comparable beam polarized in the right-handed direction, is equivalent to a beam of plane polarized light. Conversely, plane polarized light can be resolved mathematically into right- and left-handed circularly polarized components.

## B. Absorption of Light

Absorption of light is fundamental to all aspects of photochemistry and provides the basis for absorption spectroscopy.[3,5–11] Light absorption is always **quantized**. It can take place only when the energy $h\nu$ of a quantum is equal to the difference in energy between two energy levels of the absorbing molecule (Eq. 23-4).

$$E_2 - E_1 = h\nu \qquad (23\text{-}4)$$

Not only must the difference $E_2 - E_1$ be correct for absorption but also there must always be a change in the dipole moment of the molecule in going from one energy level to another. Only when this is true can the electric field of the light wave interact with the molecule. A further limitation comes from the symmetry properties of the wave functions associated with each energy level. Quantum mechanical considerations show that transitions between certain energy levels are allowed, while others are forbidden. Consideration of such matters is beyond the scope of this book, but the student should be aware that the quantum mechanical selection rules that express this fact are an important determinant of light absorption.

Many types of light source are used in chemical measurements. Of great importance is the recent development of lasers that deliver very short pulses of light. Pulses as short as five femtoseconds (5 fs)[11a,11b] and even less[11c] are being utilized for very rapid spectroscopy and excitation of fluorescence. Structures are being determined by ultrafast electron diffraction[11d] or X-ray diffraction.[11e,11f] It takes 200 fs or more for a chemical bond to stretch and break during a reaction. The cleavage and formation of bonds during this time can be observed using 5-fs pulses. Lasers with pulses in the attosecond range may soon be used to observe movements of electrons.[11g]

### 1. Quantitative Measurement of Light Absorption, Spectroscopy

An absorption spectrum is a plot of some measure of the intensity of absorption as a function of wavelength or wave number. The **transmittance** of a sample held in a **cell** (or **cuvette**) is the fraction of incident light that is transmitted, i.e., transmittance = $I/I_o$ where $I_o$ is the intensity of light entering the sample and $I$ is that of the emerging light. The transmittance is usually defined for a single wavelength, i.e., for **monochromatic** light. The absorbance (or optical density) is defined by Eq. 23-5, which also states the **Beer–Lambert law**. The length (in centimeters) of the

$$\text{Absorbance} = A = \log_{10}(I_o/I) = \varepsilon c l \qquad (23\text{-}5)$$

light path through the sample is $l$, $c$ is the concentration in moles per liter, and ε is the **molar extinction coefficient** (molar absorptivity or molar absorption coefficient), whose units are liter $mol^{-1}$ $cm^{-1}$ (or $\mathbf{M^{-1}\,cm^{-1}}$). The reader can derive Eq. 23-5 by assuming that in a thin layer of thickness $dx$ the number of light quanta absorbed is proportional to the number of absorbing molecules in the layer. Integration from $x$ = 0 to $l$ gives the Beer–Lambert law. Equation 23-5 generally holds very well for solutions containing single ionic or molecular forms. However, it is usually valid only for monochromatic light. Furthermore, making precise measurements of absorbance is not easy. At $A$=1 only 10% of the incident light is transmitted, and the utmost care is required to obtain a value of $A$ good to within ±0.05. At $A$=2 only 1% of incident light is transmitted, and the value of $A$ will be much less reliable. Very low absorbances are also difficult to measure. In view of the importance that

spectrometry has played in biochemistry, it may seem surprising that the first reliable commercial laboratory ultraviolet–visible spectrophotometers became available in 1940 and the first commercial infrared spectrometer in 1942.[12]

## 2. The Energy Levels of Molecules

The energy of molecules consists of **kinetic** (translational), **rotational**, **vibrational**, and **electronic** components. The corresponding rotational, vibrational, and electronic energy levels are always quantized. Light quanta of wavelengths 0.2–20 mm (50–0.5 $cm^{-1}$; frequencies of 1.5 x $10^{12}$ to 1.5 x $10^{10}$ $s^{-1}$) with energies of 0.6–0.006 kJ/einstein are sufficient to excite molecules from a given rotational energy level to a higher one. Spectra in this "far infrared" or "microwave" region often consist of a closely spaced series of lines. For example, the rotational spectrum of gaseous HCl is a series of lines at 20.7 $cm^{-1}$ intervals beginning at that wave number and reaching a maximum at about 186 $cm^{-1}$ (54 μm). The energies involved in absorption of such light are far lower than energies of activation for common chemical reactions and lower than the average translational energy of molecules in solution at ordinary temperatures (3/2 $k_BT$ or 3.7 kJ/mol at 25°C). However, they are still much higher than energies involved in the nuclear transitions of NMR spectra (Chapter 3). Compare 500 Hz for a proton resonating at 1 ppm in a 500 MHz NMR spectrometer with the $10^{10}$–$10^{12}$ $s^{-1}$ frequencies of microwave spectra.

Vibrational energies range from about 6 to as much as 100 kJ $mol^{-1}$ with corresponding wave numbers of ~500–8000 $cm^{-1}$. The resulting absorption spectra are in the infrared region. Excited electronic energy levels are ~120 – 200 kJ $mol^{-1}$, and the spectral transitions are at 10,000–100,000 $cm^{-1}$ (1000–100 nm wavelengths) in the visible and ultraviolet region.

## 3. Infrared (IR) and Raman Spectra

Absorption in the near infrared region is dominated by changes in vibrational energy levels. A typical wave number is that of the "amide A" band at 3300 $cm^{-1}$ (3.0 μm wavelength), approximately $10^{14}$ $s^{-1}$. First let us consider the stretching vibrations of a diatomic molecule. The two nuclei of the molecule can be thought of as connected with a spring. The energy of oscillation is approximately that of a harmonic oscillator. Application of quantum theory shows that the discrete energy levels that can be assumed by the oscillator are equally spaced. The difference between each pair of successive energy levels is $h\nu$, where ν is the frequency of light that must be absorbed to raise the energy from one level to the next. In the ground state (unexcited state) the molecule still possesses a **zero-point energy,** $E = 1/2\ h\nu_o$, equal to half the energy needed to induce a transition.

While the harmonic oscillator is a good approximation to the behavior of a molecule in the lower vibrational energy states, marked deviations occur at higher energies. At the lower energy levels the change in the distance between the atomic centers during the course of the vibration amounts to only ±10% or less, but as the energy becomes greater the bond stretches more and the motion becomes **anharmonic**. The energy states of molecules are often in the form of **Morse curves** in which energy is plotted against internuclear distance (Fig. 23-2). As the internuclear distance becomes very short, the energy rises steeply. As the bond is stretched, there comes a point at which addition of more energy ruptures the bond. A diatomic molecule will dissociate into atoms and more complex molecules into fragments. Vibrational energy levels can be portrayed as horizontal lines at appropriate heights on the Morse curve (Fig. 23-2).

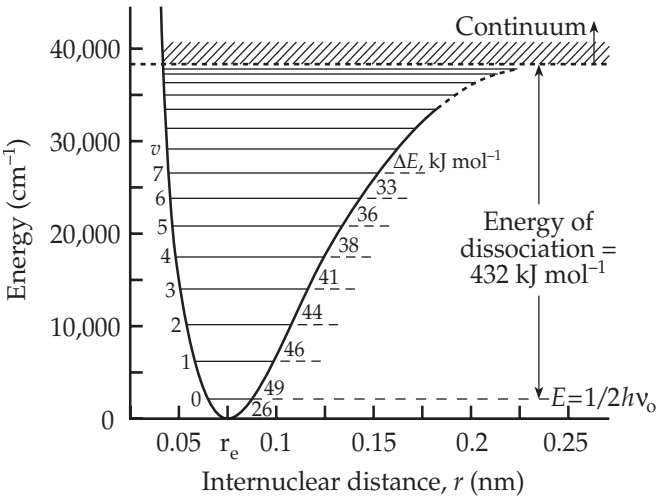



**Figure 23-2** The potential energy of the hydrogen molecule as a function of internuclear distance, and the position of its vibrational energy levels. ΔE values are energy differences between successive levels; $v$ designates vibrational quantum numbers. Adapted from Calvert and Pitts,[2] p. 135.

Because there are many rotational energy levels corresponding to each vibrational level, IR spectra contain absorption bands resulting from simultaneous changes in both the vibrational and rotational energy levels of molecules. Instead of single peaks corresponding to single transitions in vibrational energy, progressions of sharp bands at closely spaced intervals are observed. An example is provided by the band corresponding to the stretching frequency of the H–Cl

bond in gaseous HCl at 2886 $cm^{-1}$ (3.46 μm). There is actually no band at this wave number but a series of almost equally spaced bands on either side of the fundamental frequency from ~2600 to ~3100 $cm^{-1}$ at intervals of ~21 $cm^{-1}$, i.e., the wave number of the rotational frequency seen in the microwave spectrum (Herzberg,[8] p. 55). The effect is to broaden the band as seen in a low-resolution spectrum. This is only one cause of the broadening of IR bands in solution. Another cause is interaction with solvent to provide a heterogeneity in the environments of the absorbing molecules.

The IR spectra of diatomic molecules are relatively easy to interpret, but for more complex substances the infrared absorption bands often cannot be associated with individual chemical bonds. Instead, they correspond to the **fundamental vibrations** (normal vibrations) of the *molecule*. Fundamental vibrations are those in which the center of gravity does not change. For a molecule containing $n$ atoms, there are $3n - 6$ such vibrations. They are sometimes dominated by a vibration of a single bond, but often involve synchronous motion of many atoms. The fundamental vibrations of a molecule are described by such words as *stretching*, *bending* (in-plane and out-of-plane), *twisting*, and *deformation*. Rarely are all $3n - 6$ bands seen in an infrared spectrum. Some of the vibrations, e.g., the symmetric stretching of the linear $CO_2$ molecule, are not accompanied by any change in dipole moment, while other bands may simply be too weak to be observed clearly.

Vibrations involving many atoms in a molecule, i.e., **skeletal vibrations**, are often found in the region of 700–1400 $cm^{-1}$ (14–7 μm). Vibrational frequencies that are dominantly those of individual functional groups can often be identified in the range 1000–5000 $cm^{-1}$ (10–2 μm). Examples of the latter are the stretching frequencies of C–H, N–H, and O–H bonds, which have wave numbers of ~2900, 3300, and 3600 $cm^{-1}$, respectively. The energy (and frequency) of the vibrations increases as the difference in electronegativity between the two atoms increases. When a bond connects two heavier atoms, the frequency is lower, e.g., the wave number for C–O in a primary alcohol is ~1053 $cm^{-1}$. For a double bond it increases; for C=O it is ~1700 $cm^{-1}$. This C=O stretching frequency usually gives rise to one of the strongest bands observed in IR spectra. Hydrogen bonding has a strong and characteristic effect. Thus, the O–H frequency at ~3600 $cm^{-1}$ is decreased to ~3500 $cm^{-1}$ by hydrogen bonding.

Theory predicts that for a harmonic oscillator only a change from one vibrational energy level to the next higher is allowed, but for anharmonic oscillators weaker transitions to higher vibrational energy levels can occur. The resulting "overtones" are found at approximate multiples of the frequency of the fundamental. Combination frequencies representing sums and differences of frequencies of individual IR bands may also be seen. The intensities of these bands are low, but their presence at relatively high energies in the near IR region (4000–12,500 $cm^{-1}$) means that they may be easier to observe than the fundamental frequencies in the more crowded IR region.[12a] Development of the very sensitive **Fourier-transform infrared spectroscopy** (FTIR) has made it possible to record the complex IR spectra of macromolecules in dilute aqueous solutions rapidly. The water spectrum is substracted digitally.[13–16] FTIR has been utilized to study amide groups in peptides, carboxyl groups in proteins,[17] conformations of sugar rings in DNA,[18] and the ionization state of phosphate groups.[19] New computational methods involving use of two-dimensional representations provide simplifications in interpretation of IR spectra.[19a] Another variant is **total reflection FTIR**, a technique that records spectra of thin films and has permitted the recording of transient changes in protein spectra with microsecond time resolution.[20,21]

***Vibrational frequencies of amide groups.*** The IR absorption bands of amide groups, which are present in both proteins and in the purine and pyrimidine bases, have attracted a great deal of attention.[13,22–23a] The **amide I** band at ~1680 $cm^{-1}$ is associated with an in-plane normal mode of vibration that involves primarily C=O stretching. (The band is designated I′ if the N–H has been exchanged to form N–$^2$H.)[16] The **amide II** band at ~1500 $^{-1}$ and the **amide III** band at ~1250 $cm^{-1}$ both arise from in-plane modes that involve N–H bending, while the higher frequency **amide A** band at ~3450 $cm^{-1}$ involves N–H stretching. It is shifted to ~3300 $cm^{-1}$ when the N–H is hydrogen bonded. Examples of IR spectra of proteins, including the amide bands A, I, and II, are shown in Fig. 23-3. The band shapes are complex. Those of the amide bands I and III depend upon the conformation of the peptide chain. For example, (Fig 23-3B) amide groups in α helices have an amide I band about 20 $cm^{-1}$ higher than do those in β structures.

If peptide chains can be oriented in a regular fashion, it may be useful to measure **infrared linear dichroism**.[24,25] Absorption spectra are recorded by passing plane polarized light through the protein in two mutually perpendicular directions, with the electric vector either parallel to the peptide chains or perpendicular to the chains. Such a pair of spectra is shown in Fig. 23-3A for oriented fibrils of insulin. In this instance, the insulin molecules are thought to assume a β conformation and to be stacked in such a way that they extend transverse to the fibril axis (a cross-β structure). When the electric vector is parallel to the fibril axis, it is perpendicular to the peptide chains. Since the amide I band is dominated by a carbonyl stretching motion that is perpendicular to the

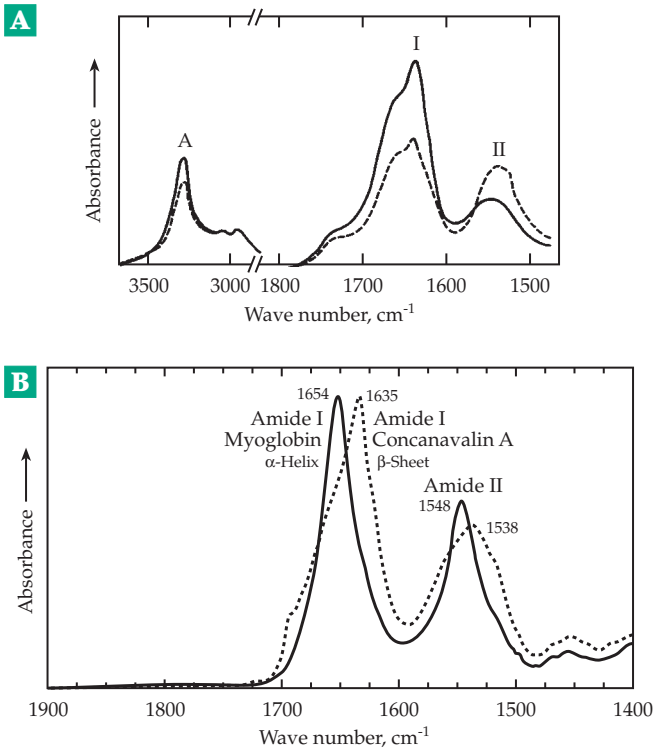



**Figure 23-3** Infrared absorbance spectra of the amide regions of proteins. (A) Spectra of insulin fibrils illustrating dichroism. Solid line, electric vector parallel to fibril axis; broken line, electric vector perpendicular to fibril axis. From Burke and Rougvie.[24] Courtesy of Malcolm Rougvie. See also Box 29-E. (B) Fourier transform infrared (FTIR) spectra of two soluble proteins in aqueous solution obtained after subtraction of the background $H_2O$ absorption. The spectrum of myoglobin, a predominantly α-helical protein, is shown as a continuous line. That of concanavalin A, a predominantly β-sheet containing protein, is shown as a broken line. From Haris and Chapman.[14] Courtesy of Dennis Chapman.

peptide chains in the β structure, this band is enhanced when the electric vector is also perpendicular to the peptide chains and is diminished when the electric vector is parallel to the peptide chains (perpendicular to the fibril axis, Fig. 23-3A). The same is true of the amide A band which is dominated by an N–H stretch. On the other hand, the dichroism of the amide II band is the opposite because it tends to be dominated by an N–H bending, which is in the plane of the peptide group but is longitudinal in direction. In **isotope-edited FTIR**, heavy atoms such as $^{13}C$ are introduced to shift IR bands and assist in their identification. The method can be combined with measurement of linear dichroism of oriented peptides.[25]

The loss of the amide II band in $D_2O$ is one of the major tools for studying protein dynamics.[13,26] (see Chapter 3, Section I,5). In some cases the four main secondary structures, α helix, β sheet, β turn, and random coil, can be distinguished.[27] The amide bands of pyrimidines can also be observed in IR spectra of pyrimidines. Figure 23-4A shows the spectrum of 1-methyluracil in $H_2O$ and also in $D_2O$. Notice that the amide II band is totally lacking in $D_2O$. The same figure also shows the IR spectrum of 1-methyluracil contaning $^{18}O$ in the 4 position. The shift of 9 $cm^{-1}$ in the amide II position is part of the evidence that the NH bending vibration is extensively coupled to C=O and C=C stretching modes.

***Raman spectra.*** In a collision between a photon and a molecule, the photon may undergo **elastic collision** in which the photon loses no energy but changes its direction of travel. Such scattering is known as **Rayleigh scattering** and forms the basis for a method of molecular mass determination. Sometimes **inelastic** collisions occur in which both the

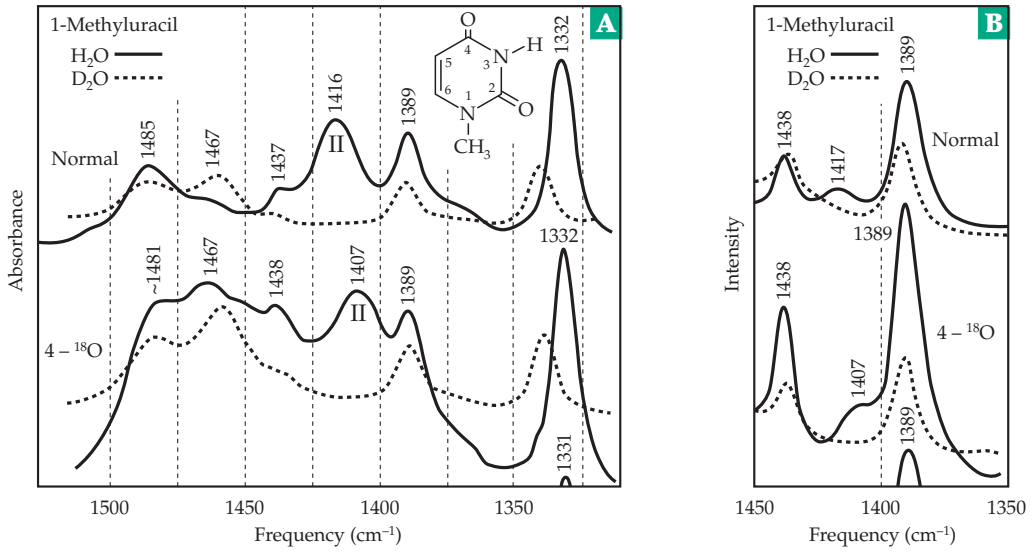



**Figure 23-4** (A) Infrared and (B) Raman spectra of 1-methyluracil in $H_2O$ and $D_2O$. Spectra for normal 1-methyluracil and for the specific isotopic derivatives with $^{18}O$ in the 4 position are shown. From Miles *et al.*[28]

molecule and the photon undergo changes in energy. Since such changes must be quantized and involve vibrational and rotational levels of the molecule, the spectrum of the scattered light (Raman spectrum) contains much of the same information as an ordinary IR spectrum. However, the selection rules are not the same. Some transitions are "infrared active" and others are "Raman active." IR-active transitions can occur only when the dipole moment varies with time as a molecule vibrates, whereas Raman-active transitions require that the polarizability change with time. For this reason, it, s useful to measure both IR and Raman spectra on the same sample. Until recently, Raman spectroscopy was not used much in biochemistry because of the low intensity of the scattered light. However, with laser excitation the technique is practical[7,13,23,28,29] and can be applied to aqueous or nonaqueous solutions, and to solid or dispersed forms of macromolecules.

Both amide I and amide III bands are seen in Raman spectra of proteins.[30] Lippert *et al.* devised the following method for estimating the fractions of α-helix, β sheet, and random coil conformations in proteins.[31] The amide I Raman bands are recorded at 1632 and 1660 $cm^{-1}$ in $D_2O$ (amide I'). The amide III band, which is weak in $D_2O$, is measured at 1240 $cm^{-1}$ in $H_2O$. The intensities of the three bands relative to the intensity of an internal standard (the 1448 $cm^{-1}$ $CH_2$ deformation) are related to those of standard poly-L-lysine in known conformations. See also Craig and Gaber.[32] The Raman spectrum of 2-methyluracil is shown in Fig. 23-4B. Note the low intensity of the amide II band relative to that of the amide I band, a characteristic of Raman spectra. Linear dichroism observed by polarized Raman microspectrophotometry has provided information about orientation of indole rings of tryptophan in filamentous virus particles.[33,33a]

In **resonance Raman spectroscopy**[34–37] a laser beam of a wavelength that is absorbed in an electronic transition is used. The scattered light is often strongly enhanced at frequencies differing from that of the laser by Raman frequencies of groups within the chromophore or of groups in another molecule adjacent to the chromophore. The resonance effect not only increases the sensitivity of Raman spectroscopy but also allows a person to study specifically the vibrational spectrum of a selected aromatic group or other structure within a macromolecule. Problems associated with the technique are fluorescence, which may be $10^6$ times as strong as the Raman emission, and photochemical damage from the intense laser beam. Fluorescence is often quenched with KI (see Section C,1).

If the exciting laser has a frequency $\nu_0$ and the frequency of a vibrationally excitation in a molecule is $\nu_1$ the Raman spectrum will contain a pair of bands,

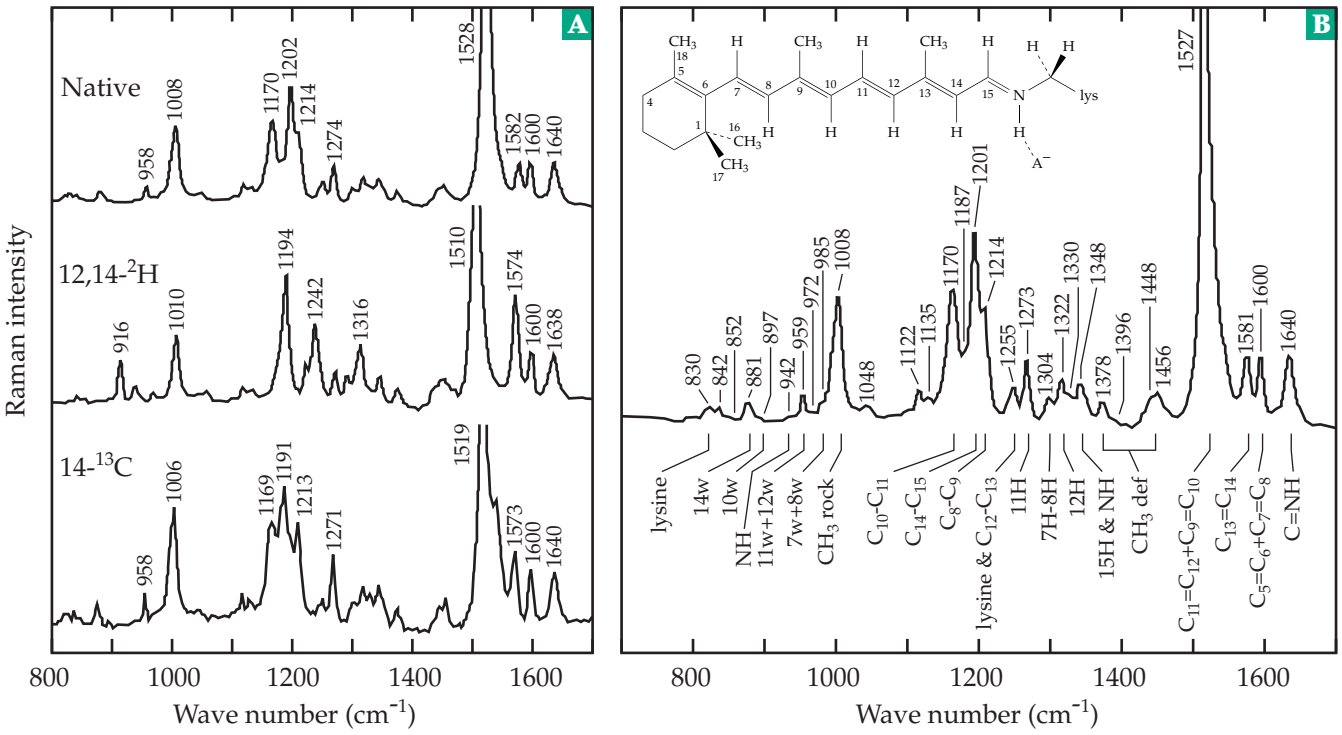



**Figure 23-5** Resonance Raman spectra. (A) Of the retinaldehyde-containing bacteriorhodopsin $bR_{568}$ (see Fig. 23-45) and its 12,14-$^2$H and 14-$^{13}$C isotopic derivatives. (B) Of $bR_{568}$ labeled with the dominant internal coordinates that contribute to the normal modes. From Lugtenburg *et al.*[37]

the stronger one or "stokes" band of energy $h(\nu_0 - \nu_1)$ and a weaker one or "anti-stokes" band of $h(\nu_0 + \nu_1)$. Special techniques such as **coherent anti-stokes** Raman scattering provide a means of getting around the fluorescence problem.[13,36,38] Raman spectroscopy is also possible with excitation of ultraviolet absorption bands. It can be applied to peptides,[39–41] tryptophan or tyrosine residues of proteins,[42] nucleic acid bases,[43–45] heme proteins,[46–48] other metalloproteins,[49] flavin coenzymes,[50–52] pyridoxal phosphate,[53,54] flavoproteins,[54a] carotenoid-containing proteins,[55] and to substrates undergoing cleavage in the active site of papain.[56] Resonance Raman spectroscopy is very useful in the study of adducts of CO, NO, and $O_2$ with heme proteins because coupled vibrational modes of both the porphyrin rings and axial ligands can be observed.[56a,b] Resonance Raman spectra are strongly amplified for samples adsorbed to specially prepared colloidal silver particles.[57] Examples of resonance Raman spectra are shown in Fig. 23-5.

## 4. Electronic Spectra

Biochemists make extensive use of spectroscopy in the ultraviolet (UV) and visible range. Visible light begins at the red end at ~12,000 $cm^{-1}$ (800 nm) and extends to 25,000 $cm^{-1}$ (400 nm). The ultraviolet range begins at this point and extends upward, the upper limit accessible to laboratory spectrophotometers being ~55,000 $cm^{-1}$ (180 nm). The energies covered in the visible–UV range are from ~140 to ~660 kJ/mol. The latter is greater than the bond energy of all but the strongest double and triple bonds (Table 6-7). It is understandable that UV light is effective in inducing photochemical reactions. Even the lower energy red light, which is used by plants in photosynthesis, contains enough energy per einstein to make it feasible to generate ATP, to reduce $NADP^+$, and to carry out other photochemical processes. Although the energies of light absorbed in electronic transitions are large, the geometry of molecules in the excited states is often only slightly altered from that in the ground state. The amount of vibration is increased, and the molecule usually expands moderately in one or more dimensions.

The significance of light absorption in biochemical studies lies in the great sensitivity of electronic energy levels of molecules to their immediate environment and to the fact that spectrophotometers are precise and sensitive. The related measurements of circular dichroism and fluorescence also have widespread utility for study of proteins, nucleic acids, coenzymes, and many other biochemical substances that contain intensely absorbing groups or **chromophores**.[58]

***Shapes of absorption bands.*** Electronic absorption bands are usually quite broad, the width of the band at half-height often being 3000–4000 $cm^{-1}$. The breadth arises largely from the coupling of electronic excitation to changes in the vibrational and rotational energy levels. Inhomogeneity of environments in the solvent also contributes. Shapes of absorption bands are to a large extent determined by the **Franck–Condon principle**, which states that no significant change in the positions of the atomic nuclei of the molecule occurs during the time of the electronic transition. Since the frequency of light absorbed during these transitions is ~$10^{15}$ to $10^{16}$ $s^{-1}$, the absorption of light energy occurs within $10^{-15}$ to $10^{-16}$ s, the time equivalent to the passage of one wavelength of light. During this period the vibrational motions of the nuclei are almost insignificant because of the much lower frequencies of vibration. Two types of potential energy curves for excited states of molecules are shown in Fig. 23-6.[2] In the first the geometry of the molecule is little changed between ground state and excited state. At room temperature most molecules are in the lowest energy states of at least the most energetic of the various vibrational modes of the molecule ($3/2\ k_BT$ ~300 $cm^{-1}$). Therefore, the most probable transitions occur from the lowest vibrational states of the ground electronic states. The most probable internuclear distance for a molecule in the ground state is the equilibrium distance $r_e$ (Fig. 23-2). Since that distance is the same in all of the vibrational levels of the electronically excited state, transitions to any of these states may occur. The transition to the first vibrational level of the excited state is most likely. The result is an absorption spectrum in which the sharp band representing the "0–0 transition" is most intense and in which there are progressively weaker bands corresponding to the 0–1, 0–2, 0–3, etc., transitions (Fig. 23-6A). Many organic dyes with long series of conjugated double bonds have spectra of this type.[59,60]

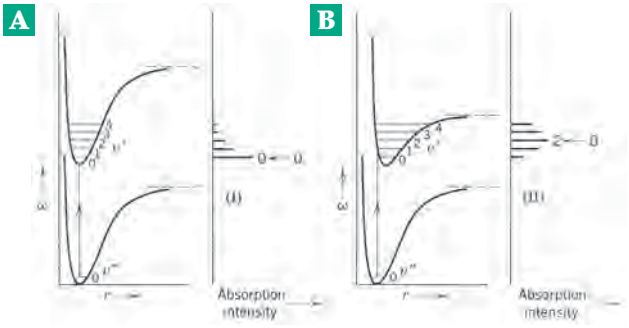



**Figure 23-6** Typical potential energy curves for two types of band spectra: (A) For a transition in which the equilibrium internuclear distances $r_e$ are about equal in the ground and excited states. (B) For a transition in which $r_e'$ (excited state) > $r_e$ (ground state). From Calvert and Pitts,[2] p. 179.

A second type of spectrum is illustrated in Fig. 23-6B. In this instance, the molecule has expanded in the excited state, and $r_e$ is greater than in the ground state. The Franck–Condon principle suggests that a transition is likely only to those vibrational levels of the excited state in which the internuclear distance is compressed for a significant fraction of the time, approximately to that of $r_e$ in the ground state. Examination of Fig. 23-6B explains why the resulting absorption spectra tend to have weak 0–0 bands and stronger bands corresponding to transitions to higher levels.

For real spectra of polyatomic molecules the situation is more complex. Some molecules in the ground state do occupy higher vibrational levels of the less energetic modes. Therefore, there will be weaker lines, some of which lie on the low-energy side of the 0–0 transition. Since in polyatomic molecules there are several normal modes of vibration, there will be other progressions of absorption bands paralleling those shown in Fig. 23-6 and filling in the valleys between them. All of the bands are broadened by rotational coupling and by interactions with solvent.

An example of a molecule giving a spectrum of the type shown in Fig. 23-6B is toluene. The vapor phase spectrum contains a large number of sharp lines, some of which can be seen in the low-resolution spectrum of Fig. 23-7. Several progressions can be identified.[61] One begins with the intense 0–0 line at $37.48 \times 10^3$ cm$^{-1}$ and in which spacing of ~930 cm$^{-1}$ between lines corresponds to a vibration causing symmetric expansion of the ring (ring breathing frequency), a frequency that can also be observed in the infrared spectrum. Other progressions beginning at the 0–0 line involve additional modes of vibration with frequencies (in the excited electronic state) of 460, 520, and 1190 cm$^{-1}$. Additional weaker bands are "buried" in the valleys in Fig. 23-6. When the spectrum of toluene is measured in solution, the sharp lines are broadened, but there are still indications of vibrational structure.

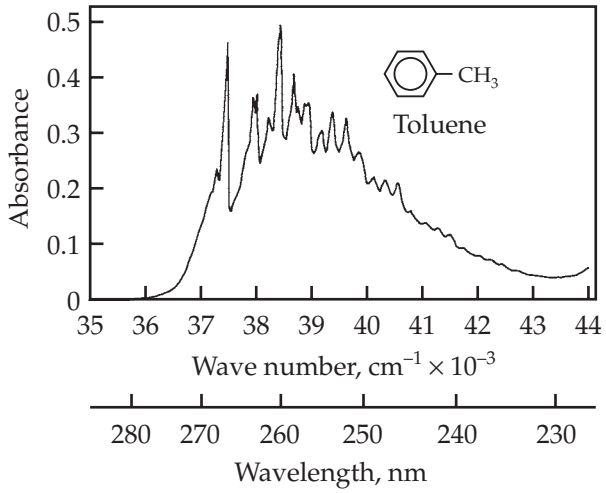



**Figure 23-7** The spectrum of the first electronic transition of toluene vapor at low resolution. Cary 1501 spectrophotometer.

Gaussian curves (normal distribution functions) can sometimes be used to describe the shape of the overall envelope of the many vibrationally induced subbands that make up one electronic absorption band, e.g., for the absorption spectrum of the copper-containing blue protein of *Pseudomonas* (Fig. 23-8) Gaussian bands are appropriate. They permit resolution of the spectrum into components representing individual electronic transitions. Each transition is described by a **peak position**, **height** (molar extinction coefficient), and **width** (as measured at the half-height, in cm$^{-1}$). However, most absorption bands of organic compounds are not symmetric but are skewed

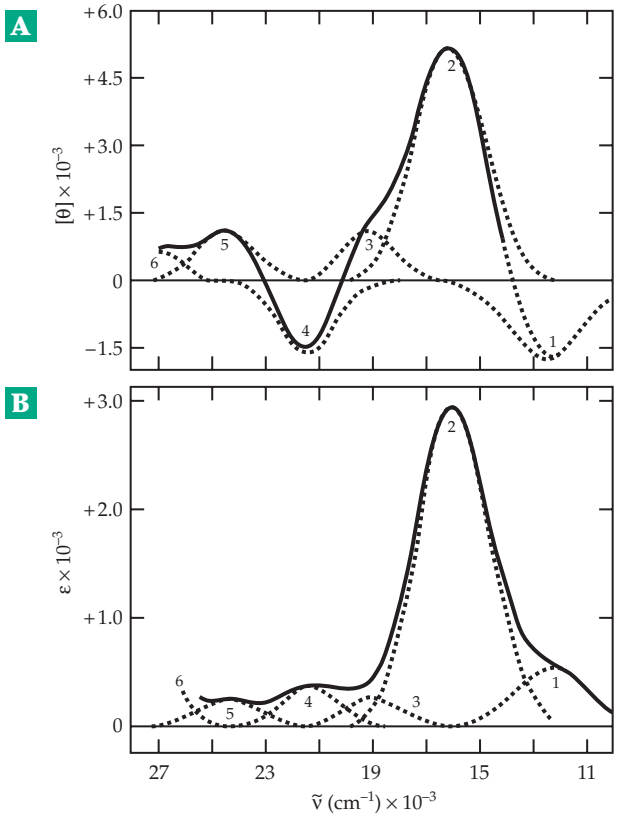



**Figure 23-8** Resolution of the visible circular dichroism (ellipticity) spectrum (A) and absorption spectrum (B) of the *Pseudomonas* blue protein into series of overlapping Gaussian bands (—). The numbers 1 to 6 refer to bands of identical position and width in both spectra. Absorption envelopes resulting from the sum of the set of overlapping Gaussian bands (—) correspond within the error of the measurement to the experimental spectra. The *dashed part* of the CD envelope above 700 nm was completed by a curve fitter with the use of a band in the position of *band 1* of the absorption spectrum. From Tang *et al.*[68]

toward the high-energy side. It is best to fit such bands with a skewed function such as the **log normal** distribution curve.[62–64] In addition to position, height, and width, a fourth parameter provides a measure of **skewness**. Computer-assisted fitting with log normal curves gives precise values for the positions, widths, and intensities. In general, the peak position is somewhat to the high-energy side of the 0–0 transition.

Absorption spectra plotted as a linear function of wavelength are sometimes fitted with Gaussian curves. However, Gaussian curves only occasionally give a good fit for such spectra, and it is undesirable to measure bandwidths in nanometers. It is wave number that is proportional to energy. Spectral bands tend to have similar widths across the visible–ultraviolet range when plotted against wave number but not when ploted against wavelength. Another approach to the quantitative description of spectra is to fit the major progressions of vibrational subbands with series of narrow Gaussian curves.[65–67]

***Classification of electronic transitions.*** The intense 600-nm absorption band of the copper blue protein in Fig. 23-8 is attributed to a $d$–$d$ transition of an electron in the metal ion from one $d$ orbital to another of higher energy.[68] The intensity is thought to arise from transfer of an electron from a cysteine thiolate to the copper (p. 883). The electronic transitions in most organic molecules are of a different type. Transitions lying at energies <55,000 $cm^{-1}$ are classified as either $\pi$–$\pi^*$ or $n$–$\pi^*$. In the $\pi$–$\pi^*$ transitions an electron is moved from a bonding $\pi$ molecular orbital to an antibonding ($\pi^*$) orbital. Such a transition is present in ethylene at 61,540 $cm^{-1}$ (162.5 nm) with a maximum molar extinction coefficient $\varepsilon_{max}$ of ~15,000 $M^{-1}$ $cm^{-1}$. An $n$–$\pi^*$ transition results from the raising of an electron in an unshared pair of an oxygen or nitrogen atom into a $\pi^*$ antibonding orbital. These transitions are invariably weak. For example, acetone in $H_2O$ shows an $n$–$\pi^*$ transition at 37,740 $cm^{-1}$ (265 nm). The value of $\varepsilon_{max}$ is ~240 and the width is about 6400 $cm^{-1}$. A characteristic of $n$–$\pi^*$ transitions is a strong shift to lower energies as the compound is moved from water into less polar solvents. For example, the peak of the acetone band lies at 36,920 $cm^{-1}$ in methanol and at 35,970 $cm^{-1}$ (278 nm) in hexane. Such a solvent shift is often taken as diagnostic of an $n$–$\pi^*$ transition, and it is often stated that the $\pi$–$\pi^*$ bands shift in the opposite direction upon change of solvent character. However, the latter is not true for many of the polar chromophores found in biochemical substances. Thus, the $\pi$–$\pi^*$ bands of tyrosine also shift to lower energies when the molecule is moved from water into hexane. However, the magnitude of the shift is much less than for the $n$–$\pi^*$ band of acetone.

A molecule can have several different excited states of increasing energies. In benzene and its derivatives there are three easily detectable $\pi$–$\pi^*$ transitions (see Fig. 3-13). The first is a weak band centered at ~260 nm in toluene (Fig. 23-7) and ~275 nm in tyrosine (Fig. 3-13) with $\varepsilon = 10^2$ to $10^3$. The second is a band at a higher frequency (at 1.35  0.10 times the frequency of the first band) with $\varepsilon_{max}$ often as high as $10^4$. The third band is found at still higher energies with $\varepsilon_{max}$ reaching $5 \times 10^4$. The excited-state energy levels represented by these transitions were labeled $^1L_b$, $^1L_a$, and $^1B_a$ by Platt. Other authors described the levels in terms of the symmetries of the molecular orbitals, the ground state being $^1A_{1g}$, and the three excited states $^1B_{2u}$, $^1B_{1u}$, and $^1E_{1u}$. In these symbols the superscript 1 indicates that the excited states are **singlet** in nature; that is, the electrons remain paired in the excited states. Absorption of visible and ultraviolet light almost always leads to singlet excited states initially. For more complex ring systems the number of possible electronic transitions increases, but attempts are often made to relate these transitions back to those of benzene.

The intensities of electronic transitions vary greatly. The area ($\mathcal{A}$) under the absorption band, when $\varepsilon$ is plotted against wave number $\bar{\nu}$, is directly proportional (Eq. 23-6) to a dimensionless quantity called the **oscillator strength** $f$.

$$f = \frac{2.303\, m_e c^2}{\pi N e^2} F\mathcal{A} = 4.32 \times 10^{-9}\, F\mathcal{A} \qquad (23\text{-}6)$$

In this equation $m_e$ and $e$ are the mass and charge of the electron, $c$ is the velocity of light, $N$ is Avogadro's number, and $\mathcal{A}$ is the area in a plot of $\varepsilon$ vs $\nu$ in $cm^{-1}$; $F$ is a dimensionless correction factor that is related to the refractive index of the medium and is near unity for aqueous solutions. If the area is approximated as that of a triangle of height $\varepsilon_{max}$ and width (at half-height) $W$, we find that for a typical absorption band of $\varepsilon_{max} = 10^4$ and $W = 3000$ $cm^{-1}$, $f = 0.13$.

The oscillator strength is related to the probability of a transition and can become approximately 1 only for the strongest electronic transitions. However, it is rarely this high. For example, the oscillator strength is $\sim 10^{-4}$ for $Cu^{2+}$ and $\sim 2 \times 10^{-3}$ for the toluene absorption band shown in Fig. 23-7. The low intensity of absorption bands of benzene derivatives is related to the fact that these transitions are quantum mechanically forbidden for a completely symmetric molecule. It is only because of coupling with asymmetric vibrations of the ring that the $^1L_b$ transition of benzene becomes weakly allowed. In the benzene spectrum the 0–0 transition is completely absent, and only those progressions involving uptake of an additional 520 $cm^{-1}$ of a nonsymmetric vibrational energy are observed. In the case of toluene and phenylalanine, the asymmetry of the ring introduced by the substituents permits the 0–0 transition to occur and leads to a higher oscillator

strength than that observed with benzene. The $^1L_a$ transition of benzenoid derivatives is also partially forbidden by selection rules, and only the third band begins to approach an oscillator strength of one.

***Use of plane polarized light.*** The intensity of a spectral transition is directly related to the **transition dipole moment** (or simply the transition moment), a vector quantity that depends upon the dipole moments of the ground and excited states. For aromatic ring systems, the transition dipole moments of the π–π* transitions lie in the plane of the ring. However, both the directions and intensities for different π–π* transitions within a molecule vary.

The transition moment has a dimension of length (usually given in angstroms) and can be thought of as a measure of the extent of the charge migration during the transition. Light is absorbed best when the directions of polarization (i.e., of the electric vector of the light) and of the transition moment coincide. This fact can easily be verified by light absorption measurements on crystals. As with infrared spectra of oriented peptide chains (Fig. 23-3), the electronic spectra of crystals display a distinct dichroism. Crystals of coenzyme-containing proteins (Fig. 23-9) are very appropriate for spectroscopy with polarized light because the chromophores are spaced far enough apart to avoid electronic interaction and have absorbances low enough to record with crystals of the order of 0.1 mm thickness.[69,70]

In contrast to π–π* transitions, the *n*–π* transitions of heterocyclic compounds and carbonyl-containing rings are often polarized in a direction perpendicular to the plane of the ring. Linear dichroism of cytosine, adenine, and other nucleic acid bases has been measured on single crystals and in partially oriented polymer films.[71] Magnetically induced linear dichroism provides a new tool for study of metalloproteins.[72]

***Relationship of absorption positions and intensity to structures.*** While quantum mechanical calculations permit prediction of the correct number and approximate positions of absorption bands, they are imprecise. For this reason, electronic spectroscopy also relies upon a combination of empirical rules and atlases of spectra that can be used for comparison purposes.[74–76] The following may help to orient the student. The position of an absorption band shifts **bathochromically** (to longer wavelength, lower energy) when the number of conjugated double bonds increases. Thus, **butadiene** absorbs at 46,100 $cm^{-1}$ (217 nm) vs the 61,500 $cm^{-1}$ of ethylene. As the number of double bonds increases further, the bathochromic shifts become progressively smaller (but remain more nearly constant in terms of wavelength than wave number). For **lycopene** (Fig. 23-10) with 11

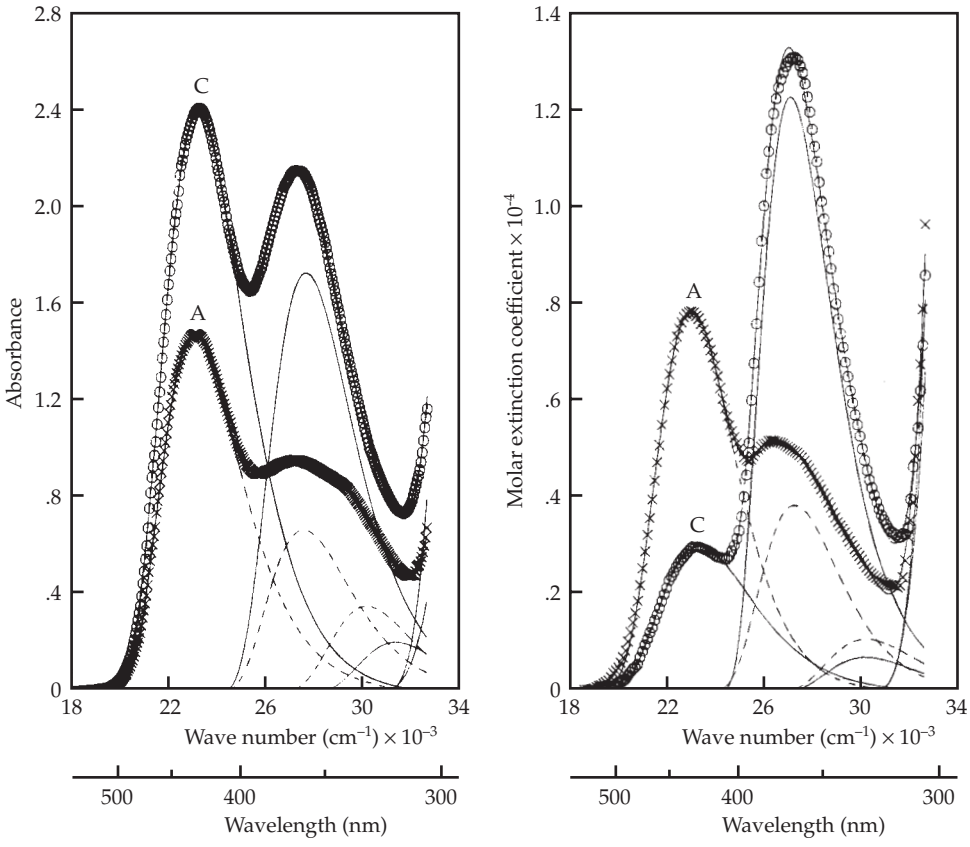



**Figure 23-9** Polarized absorption spectra of orthorhombic crystals of cytosolic aspartate aminotransferase. The light beam passed through the crystals along the *b* axis with the plane of polarization parallel to the *a* axis (A) or the *c* axis (C). Left, native enzyme at pH 5.4; right, enzyme soaked with 300 mM 2-methylaspartate at pH 5.9. The band at ~430 nm represents the low pH protonated Schiff base form of the enzyme. Upon soaking with 2-methylaspartate the coenzyme rotates ~30° to form a Schiff base with this quasisubstrate. The result is a large change in the *c*/*a* polarization ratio. The ~364 nm band in the complex represents the free enzyme active site in the second subunit of the dimeric enzyme.[70,73] Courtesy of C. M. Metzler.

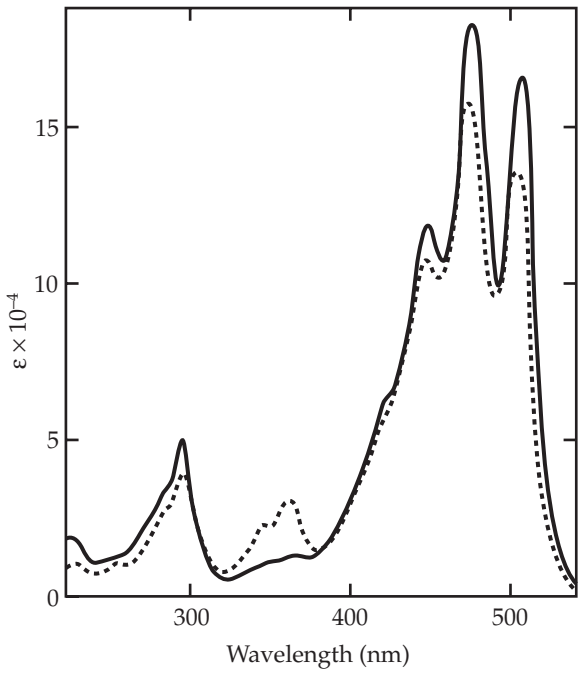



**Figure 23-10** The absorption spectrum of lycopene (plotted vs wavelength). Note the vibrational structure, which has a spacing of ~1200–1500 $cm^{-1}$. The solid line is for all-translycopene while the dashed line is that of the sample after refluxing 45 min in the dark. The new peak at ~360 nm arises from isomers containing some cis double bonds.

conjugated double bonds the absorption band is located at 21,300 $cm^{-1}$ and displays distinct vibrational structure (Fig. 23-10). Certain ring molecules such as the porphyrins and chlorophylls have spectra that can be related back to those of the linear polyenes. Note (Fig. 16-7) that the porphyrin α and β bands represent vibrational structure of a single electronic transition, whereas the intense Soret band results from a different transition.

Substituted benzenes almost invariably absorb at lower energies than the parent hydrocarbon. The stronger the electron withdrawing or donating ability of the substituent, the larger the bathochromic shift. The magnitude of the shift has been correlated with the Hammett σ constants. Thus, the first absorption band of tyrosine in water is shifted 2600 $cm^{-1}$ toward the red from that of benzene, while that of the dissociated tyrosine anion is shifted 4700 $cm^{-1}$, very roughly in proportion to the $\sigma_p$ values of Box 6-C. Especially large shifts are observed when functional groups of opposite types (that is, an electron donating group vs an electron accepting group) are both present in the same ring. The effects of ortho and meta substituent pairs are closely similar (in contrast to the differing electronic effects of ortho and meta pairs in chemical reactivity). Substituent pairs in para positions yield somewhat different spectral shifts. When there are more than two substituents, the two strongest groups often dominate in determining the character of the spectrum. Useful empirical rules have been developed.[77,78]

***Spectra of proteins and nucleic acids.*** Most proteins have a strong light absorption band at 280 nm (35,700 $cm^{-1}$) which arises from the aromatic amino acids tryptophan, tyrosine, and phenylalanine (Fig. 3-14). The spectrum of phenylalanine resembles that of toluene (Fig. 23-7),[61] whose 0–0 band comes at 37.32 x $10^3$ $cm^{-1}$. The vibrational structure of phenylalanine can be seen readily in the spectra of many proteins (e.g., see Fig. 23-11A). The spectrum of tyrosine is also similar (Fig. 3-13), but the 0–0 peak is shifted to a lower energy of ~35,500 $cm^{-1}$ (in water). Progressions with spacings of 1200 and 800 $cm^{-1}$ are prominent.[79] The low-energy band of tryptophan consists of two overlapping transitions $^1L_a$ and $^1L_b$.[65] The $^1L_b$ transition has well-resolved vibrational subbands, whereas those of the $^1L_a$ transition are more diffuse. Tryptophan derivatives in hydrocarbon solvents show 0–0 bands for both of these transitions at approximately 289.5 nm (34,540 $cm^{-1}$). However, within proteins the $^1L_a$ band may be shifted 3–10 nm (up to 1100 $cm^{-1}$) toward lower energies, probably as a result of hydrogen bonding to other groups in the protein. The largest shifts can occur when the NH group of the indole ring is hydrogen bonded to $COO^-$, a ring nitrogen of histidine, or a carbonyl group of amides.[80] In an aqueous medium the $^1L_b$ band of tryptophan is shifted to higher energies and the $^1L_a$ band to lower energies than in a hydrocarbon solvent.

In addition to the three aromatic amino acids, disulfide bonds absorb in the near ultraviolet region as indicated in Fig. 3-14. Since the absorption characteristics depend upon the dihedral angles in the disulfide bridges, it is difficult to accurately evaluate the contribution of this chromophore to the 280-nm band.

Tyrosine, tryptophan, and phenylalanine all have additional transitions in the high-energy UV region of the spectrum (Fig. 3-13). Even more intense are the absorption bands of the amide groups, which become significant above 45,000 $cm^{-1}$.[81] These include a weak $n-\pi^*$ transition at ~45,500 $cm^{-1}$ (210 nm) overlapped by a strong $\pi-\pi^*$ transition at ~52,000 $cm^{-1}$ (192 nm).[82] Histidine also has absorption bands in this region.

As with polypeptides, the light absorption properties of polynucleotides reflect those of the individual components. The spectra of the purine and pyrimidine bases as ribonucleosides are shown in Fig. 5-5. The number of individual electronic transitions and their origins are not immediately obvious, but many measurements in solutions and in crystals, as well as theoretical computations,[7,83,84] have been made. Cytosine has $\pi-\pi^*$ transitions at ~275, 230, 200, and

185 nm,[83] the two highest energy bands being overlapped. Adenine derivatives have seven $\pi-\pi^*$ transitions.[71] Spectra of flavins contain at least four intense transitions (Fig. 15-8).[85]

Whereas proteins have their low energy absorption band at ~280 nm, polynucleotides typically have maxima at ~260 nm (38,500 $cm^{-1}$). A phenomenon of particular importance in the study of nucleic acids is the **hypochromic effect**. In a denatured polynucleotide the absorption is approximately the sum of that of the individual components. However, when a double helical structure is formed and the bases are stacked together, there is as much as a 34% depression in the absorbance at 260 nm. This provides the basis for optical measurement of DNA melting curves (Fig. 5-45).[45,86] The physical basis for the hypochromic effect is found in dipole–dipole interactions between the closely stacked base pairs.[7,86,87]

***Difference spectra and derivative spectra.*** Changes in light-absorbing properties of proteins and nucleic acids are often measured as a function of some quantity such as pH, temperature, ionic environment, or the presence or absence of another interacting molecule. The induced changes in the spectrum are small but can be seen if the *difference* between the two spectra, one "unperturbed" and the other in the presence of some "perturbant," is recorded. The perturbant might be an additional reagent, an altered solvent (e.g., with added glycerol, $D_2O$), a change in pH, or temperature. The difference spectrum shown in Fig. 23-11B arises from the binding of an inhibitor succinate together with a substrate carbamoyl phosphate to the catalytic subunit of aspartate carbamoyltransferase (Fig. 7-20).[88] The difference spectrum appears as a pair of peaks and a valley in the aromatic amino acid region. With proper interpretation (caution!) difference spectra can be used to infer something about the change in environment of aromatic amino acids in a protein.[89]

Difference spectra are usually recorded by placing the unperturbed spectrum in the *reference* light beam of a spectrophotometer and the perturbed solution in the *sample* beam in carefully matched cuvettes. However, the spectrum shown in Fig. 23-11B was obtained by recording the two spectra independently and subtracting them with the aid of a computer. The same data have been treated in another way by fitting two log normal curves (p. 1283) to the absorption bands and plotting the differences between the mathematically

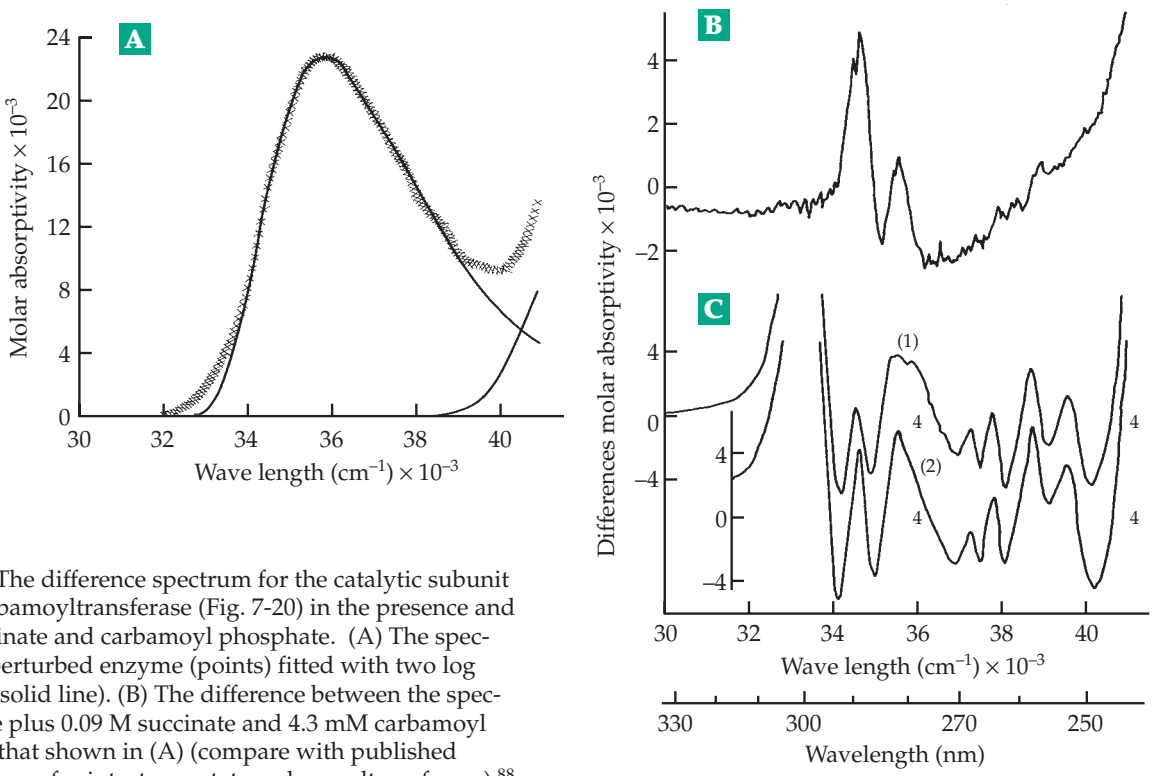



**Figure 23-11** The difference spectrum for the catalytic subunit of aspartate carbamoyltransferase (Fig. 7-20) in the presence and absence of succinate and carbamoyl phosphate. (A) The spectrum of the unperturbed enzyme (points) fitted with two log normal curves (solid line). (B) The difference between the spectrum of enzyme plus 0.09 M succinate and 4.3 mM carbamoyl phosphate and that shown in (A) (compare with published difference spectrum for intact aspartate carbamoyltransferase).[88] (C) Curve 1, "fine structure plot" obtained by subtracting the spectrum in A from the smooth curve obtained by summing the two log normal curves. Curve 2, a similar plot for enzyme plus succinate and carbamoyl phosphate. The enzyme was supplied by G. Nagel and H. K. Schachman and the spectra were recorded by I.-Y. Yang.

smooth fitted curve and the experimental points taken at close intervals[90,91] as shown in Fig. 23-11C. The two "fine structure plots" obtained in this way are an alternative way of representing the same data that gave rise to the difference spectrum. The method has the advantage that information about the overall band shape is obtained from the computer-assisted curve fitting process. Thus, the binding of succinate and carbamoyl phosphate caused an almost insignificant shift (of 20 $cm^{-1}$) in the overall band position and a very slight broadening. The principal effect is an enhancement in the vibrational structure at 34,600 $cm^{-1}$ in the 0–0 band of the two tryptophan residues present in the subunit. The cause of this change is not entirely obvious, a weakness of difference spectroscopy. Another way of examining a spectrum such as that in Fig. 23-11A is to plot a mathematical derivative of the curve. Both second[92] and fourth derivatives[91,93,94] yield curves similar to the difference plots of Fig. 23-11C.

## 5. Circular Dichroism and Optical Rotatory Dispersion

The circular dichroism of a sample is the difference between the molar extinction coefficients for left-handed and right-handed polarized light (Eq. 23-7) and is observed only for chiral molecules.[7,95–97]

$$\Delta\varepsilon = \varepsilon_L - \varepsilon_R \quad (\text{units are } M^{-1}\ cm^{-1}) \tag{23-7}$$

The **dichrograph** gives a direct measure of $\Delta\varepsilon$. A circular dichroism (CD) spectrum often resembles an absorption spectrum, the peaks coming at the same positions as the peaks in the absorption spectrum of the same sample. However, the CD can be either positive or negative and may be positive for one transition and negative for another (Fig. 23-8). It is most convenient to plot $\Delta\varepsilon$ directly as a function of wavelength or wave number. However, much of the literature makes use of the **molar ellipticity** (Eq. 23-8):

$$\text{Molar ellipticity} = [\theta] = 3299\ \Delta\varepsilon \quad (\text{units are degrees } cm^2\ \text{decimole}^{-1}) \tag{23-8}$$

The **rotational strength** may also be evaluated (Eq. 23-9):

$$\text{Rotational strength} = \int [(\Delta\epsilon)/\lambda] d\lambda \tag{23-9}$$

The integration is carried out over the entire absorption band for a given transition.

Circular dichroism is closely related to **optical rotatory dispersion**, the variation of optical rotation with wavelength. Optical rotation depends upon the difference in refractive index ($\eta_L - \eta_R$) between left-handed and right-handed polarized light. Rotation $\alpha$ is measured as an angle in degrees or radians. Data are customarily reported in terms of **specific rotation**, that of a hypothetical solution containing 1 g / ml in a 1 dm (decimeter) tube. Specific rotation is calculated (Eq. 23-10) from the observed rotation, the concentration $c'$ in g $ml^{-1}$, and the length of the tube $l'$ in decimeters.

$$\text{Specific rotation} = [\alpha] = \alpha_{obs} / c'l' \tag{23-10}$$

The **molecular rotation** is defined by Eq. 23-11 in which $M_r$ is the molecular mass and $c$ and $l$ are in moles per liter and cm, respectively.

$$\begin{aligned}\text{Molecular rotation} = [\phi] &= 100\ \alpha_{obs} / cl \\ &= [\alpha]\ M_r / 100\end{aligned} \tag{23-11}$$

It is often multiplied by a factor of $3/(\eta^2 + 2)$ to correct for a minor effect of the polarizability of the field acting on the molecules. The rotation in the radians per centimeter of light path can be related (Eq. 23-12) directly to the wavelength of the light and the refractive indices $\eta_L$ and $\eta_R$.

$$\alpha\ (\text{radians / cm}) = [\alpha]\ c' / 1800 = \pi / \lambda [\eta_L - \eta_R] \tag{23-12}$$

In contrast to circular dichroism, optical rotary dispersion (ORD) extends far from absorption bands into spectral regions in which the compound is transparent. As an absorption band is approached, the optical rotation increases in either the positive or negative direction. Then, within the absorption band it drops abruptly through zero and assumes the opposite sign on the other side of the band (the Cotton effect). Although the occurrence of optical rotation in nonabsorbing regions of the spectrum provides an advantage to ORD measurements, the interpretation of ORD spectra is more complex than that of CD spectra. In principle, the two can be related mathematically and both are able to give the same kind of chemical information.[7]

The CD in the $d$–$d$ bands of the blue copper protein (Fig. 23-8) arises in part from the fact that within the protein the copper ion is in an asymmetric environment. For a similar reason, the aromatic amino acids of proteins often give rise to circular dichroism. In the case of tyrosine, the sign of the CD bands can be either positive or negative but is the same throughout a given transition. The CD bands are similar in shape to the absorption bands.[36,98] The behavior of phenylalanine is more complex. The progression of vibrational subbands at 930 $cm^{-1}$ intervals above the 0–0 band all have the same sign, and the intensities relative to that of the 0–0 band are similar to those in absorption. However, the vibrations of wave numbers equal to

that of the 0–0 transition plus 180 and 520 $cm^{-1}$ sometimes give rise to CD bands of the opposite sign, and the relative intensity relationships are variable.[61,98]

The binding of a symmetric chromophore to a protein or nucleic acid often induces CD in that chromophore. For example, the bands of enzyme-bound pyridoxal and pyridoxamine phosphates shown in Fig. 14-9 are positively dichroic in CD, but the band of the quinonoid intermediate at 20,400 $cm^{-1}$ (490 nm) displays negative CD. When "transimination" occurs to form a substrate Schiff base (Eq. 14-26), the CD is greatly diminished. While the coenzyme ring is known to change its orientation (Eq. 14-39; Fig. 14-10), it is not obvious how the change in environment is related to the change in CD.

A series of octant rules make it possible to predict the sign and magnitude of CD to be expected for $n-\pi^*$ transitions of simple carbonyl compounds.[99] Theoretical approaches to the CD and ultraviolet absorption of proteins in the high-energy ultraviolet region have also been developed. In a regular β structure, in an α helix or in a crystalline array, the transitions of adjacent amide groups may be **coupled**, the excitation energy being delocalized. This **exciton** delocalization leads to a splitting (Davydov splitting) into two transitions of somewhat different energies and polarized in different directions.[7,9] The amide absorption band at 52,600 $cm^{-1}$ is split in an α helix into components at ~48,500 and 52,600 $cm^{-1}$. Furthermore, low-energy $\pi-\pi^*$ and $n-\pi^*$ states are close together in energy, a fact that allows mixing of the two states and appearance of rotational strength in the $\pi-\pi^*$ band with a sign opposite to that in the $n-\pi^*$ band.[100]

Both the sign and intensity of the CD bands of peptides also depend upon conformation. Well-defined differences are observed among α helices, β structure, and random-coil conformations. Measurements may be extended into the "vacuum ultraviolet" region—up to 60,000 $cm^{-1}$ in aqueous solutions.[101] A useful empirical approach is to deduce spectra of helices, β structures, and unordered peptide chains from measured spectra together with an examination of actual structures obtained by X-ray crystallography[7,95,97,102,103] (Fig. 23-12). Note that the CD curve for the α helix has a deep minimum at 222 nm, whereas the β form has a shallower minimum. The random structure has almost no CD at the same wavelength. The approximate helix content of a protein is often estimated from the depth of the trough at 222 nm in the CD spectrum. Better predictions can be made by using a computer-assisted comparison of an experimental CD spectrum with those of a series of proteins of known 3-D structure.[7]

The circular dichroism of polynucleotides at 275 nm is a linear function of both the helix winding angle and the base pair twist.[103] Measurement of CD spectra on large polynucleotides or large molecular aggregates

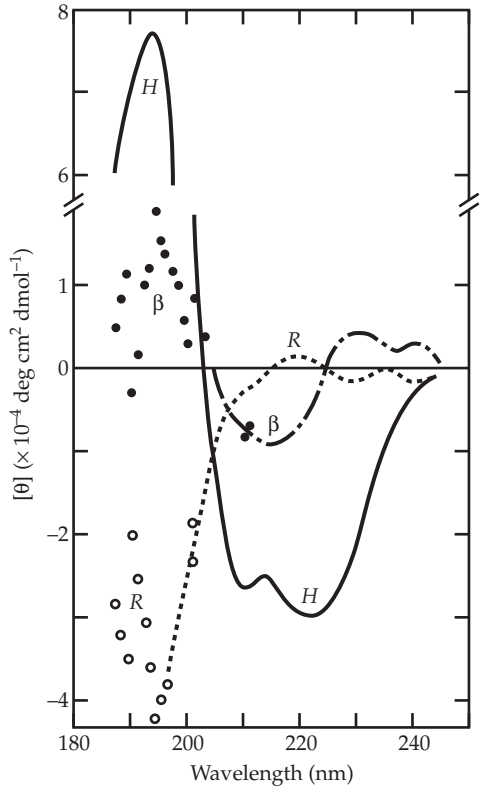



**Figure 23-12** Circular dichroism of the helix (H), β, and unordered (R) form computed from the CD of five proteins. Points are plotted when a smooth curve could not be drawn. From Y.-H. Chen *et al.*[102]

is complicated by differential light scattering of right and left circularly polarized light. However, the phenomenon can also provide new structural information.[104] Progress has been made in attempts to predict the optical rotation of molecules from quantitative values for the polarizabilities of individual atoms.[105–109]

**Vibrational circular dichroism** involves IR absorption bands. The technique has been applied to sugars,[110] oligosaccharides,[111] proteins,[112] and nucleic acids.[113] The related **vibrational Raman optical activity** has also been applied to polyribonucleotides.[114]

## 6. Photoacoustic Spectroscopy

Photoacoustic spectra are recordings of the energy emitted as heat after absorption of monochromatic light. The sample is placed in a closed photoacoustic cell. The light beam, which is chopped at an audiofrequency, induces a periodic heating and cooling of the gas in contact with the sample in the cell. This is sensed as sound by a sensitive microphone. The

resulting electrical signals are sent to a computer for analysis. The output is an absorption spectrum resembling that measured optically. The samples do not have to be transparent.[5] A related technique is photoacoustic calorimetry.[115,116]

### 7. X-Ray Absorption and Mössbauer Spectroscopies

The importance of X-ray diffraction (discussed in Chapter 3) to biochemistry is obvious, but techniques related to absorption of X-rays and γ-rays have also come into widespread use.[117] Abbreviations such as **XANES** and **EXAFS** are common in the metalloprotein literature. The names arise from the sharp increase in the absorption coefficient for X-rays as their energy is increased to what is called the **K absorption edge**. At slightly lower energies absorption of an X-ray by an atom leads to expulsion of an electron or the raising of an electron to an excited state. Absorption of X-rays will expel all except the inner 1*s* electrons. As the energy is increased further, the stepwise increase in absorption that constitutes the edge is observed. At higher energies the absorption decreases. However, with a high-resolution instrument distinct oscillations are observed on the high-energy side of the edge, extending for ~20 eV. This is **X-ray absorption near-edge structure** (XANES). When an X-ray absorbing atom in a molecule is surrounded by other atoms, a fine structure that depends upon the nature of these atoms and their distances from the absorbing atom is observed over a range of several hundred electron volts above the edge. This is **extended X-ray absorption fine structure** (EXAFS).[117]

The EXAFS technique has been especially useful for metalloproteins. It has often provided the first clues as to the identity of atoms (O, N, S) surrounding a metal atom and either covalently bonded to it or coordinated with it (Chapter 16). Interpretations are often difficult, and a common approach is to try to simulate the observed spectrum by calculation from a proposed structure.[118] Tautomerism in crystalline Schiff bases (see Eq. 23-24) has been studied by near-edge X-ray absorption fine structure (NEXAFS) employing soft X-rays.[119]

**Mössbauer spectroscopy**, also called recoil-free nuclear resonance absorption, depends upon resonant absorption of γ-rays emitted by a radioactive source by atomic nuclei.[120] The phenomenon was initially difficult to observe, but the German physicist Mössbauer devised a way in which to record the absorption of a quantum of energy equal to the difference in two energy states of the atomic nucleus. The method depends upon a Doppler effect observed when the sample or source moves. Consequently, Mössbauer spectra, such as that in Fig. 16-18, are plots of absorption versus velocity. Mössbauer spectroscopy has been applied to numerous metalloproteins, especially those containing iron centers. It is a major tool in investigation of Fe–S proteins.[121,122] Since $^{56}Fe$ is "silent" in Mössbauer spectroscopy, proteins are often enriched with $^{57}Fe$ for observation.

## C. Fluorescence and Phosphorescence

An electronically excited molecule is able to lose its excitation energy and return to the ground state in several ways. One of these is to reemit a quantum of light as fluorescence.[7,123–127] The intensity and spectral properties of fluorescent emission can be measured by illuminating a sample in a cuvette with four clear faces with the measuring photomultiplier set at right angles to the exciting light beam. In absorption spectrophotometry we measure a difference between the light intensity of the beam entering the sample and that emerging from the sample. In fluorescence spectroscopy we measure the absolute intensity of the light emitted. Although this intensity is small, the measurement can be made extremely sensitive, far more so than can light absorption. For this reason, fluorescence is widely used for detection and analysis, e.g., in DNA sequencers. Enzyme kinetics can be studied with fluorescent substrates at very low concentrations.[127a] Fluorescent antibodies, DNA chips, and numerous bioassay and imaging methods are dependent upon measurement of fluorescence. Fluorescence can also yield a wealth of information about the chemical and physical properties of electronically excited states of molecules.

### 1. Excitation and Emission Spectra

Measurements of the intensity of fluorescence at any wavelength vs the wavelength of monochromatic light used to excite the fluorescence give a fluorescence **excitation spectrum**. The excitation spectrum is an example of an **action spectrum**, which is a measure of any response to absorbed light. At very low concentrations of pure substances, action spectra tend to be identical to absorption spectra. However, since the observed response (fluorescence in this case) is proportional to light absorbed, action spectra should be compared to plots of 1–T (where T = transmittance, Section B,1) vs wavelength rather than to plots of ε vs λ. The two plots are proportional at low concentrations. For a discussion of action spectra see Clayton.[123]

A fluorescence **emission spectrum** is a record of fluorescence intensity vs wavelength for a constant intensity of exciting light. Excitation and emission spectra for a flavin and for the indole ring of tryptophan are both given in Fig. 23-13. The heights of the

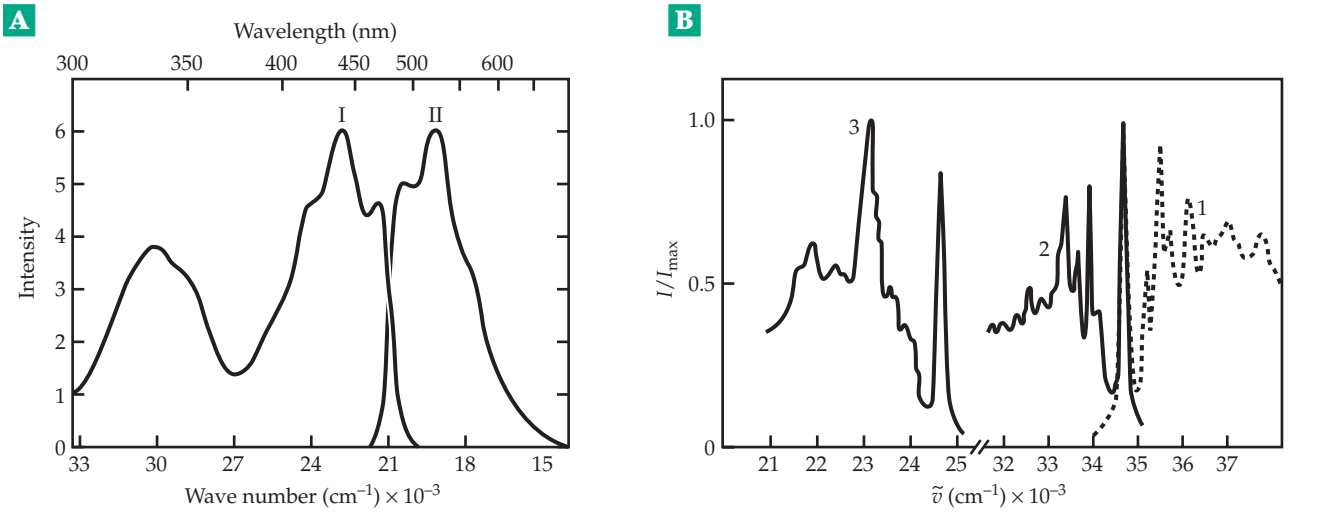



**Figure 23-13** (A) Corrected emission and excitation spectra of riboflavin tetrabutyrate in *n*-heptane. Concentration, about 0.4 mg $I^{-1}$. Curve 1: excitation spectrum; emission at 525 nm. Curve 2: emission spectrum; excitation at 345 nm. From Kotaki and Yagi.[128] (B) Indole in cyclohexane, T = 196 K. 1, Fluorescence excitation spectrum; 2, fluorescence spectrum; and 3, phosphorescence spectrum. From Konev.[125]

emission spectra have been adjusted to the same scale as that of absorption. The fluorescent emission is always at a lower energy than that of the absorbed light. The excitation and emission spectra overlap only slightly, and the emission spectrum is an approximate mirror image of excitation spectrum. To understand this, refer to the diagram in Fig. 23-14. Absorption usually leads to a higher vibrational energy state after light absorption than before. However, most of the excess vibrational energy is dissipated before much fluorescent emission occurs. The excited molecule finds itself in the lowest vibrational state of the upper electronic state, and it is from this state that the bulk of the fluorescent emission takes place. Furthermore, whereas absorption usually occurs from the lowest vibrational state of the ground electronic level, fluorescence can populate many excited vibrational states of the ground electronic state (Fig. 23-14). Consequently, as indicated in the figure, the fluorescent emission spectrum consists of a series of subbands at lower energies than those observed in the absorption spectrum. The two spectra have only the 0–0 transition in common. As can be seen from Fig. 23-14, even the two 0–0 transitions do not coincide exactly. The peak of emission is shifted toward slightly lower energies than that of absorption because during or immediately following absorption of a photon there is some rearrangement of solvent molecules around the absorbing molecule to an energetically more stable arrangement. Just as excess vibrational energy is dissipated in the excited state, so relaxation of these solvent molecules around the excited chromophore leads to a small shift in energy. A similar relaxation

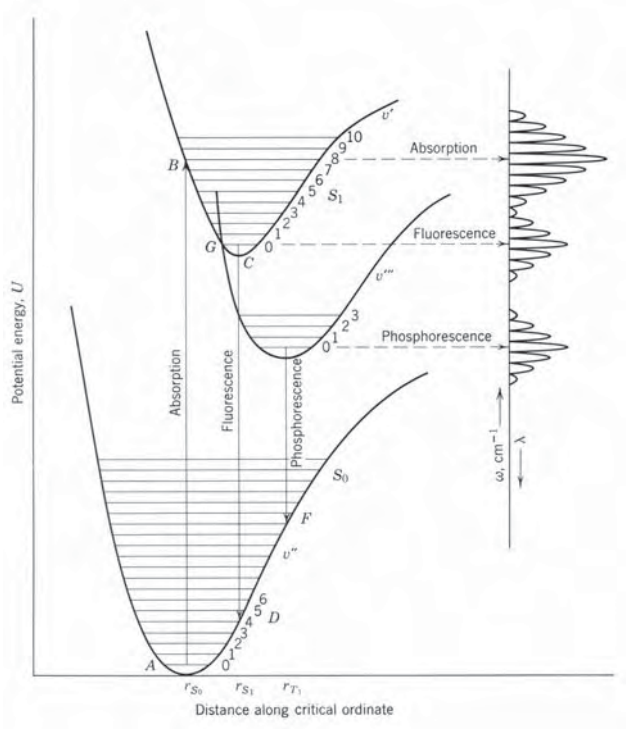



**Figure 23-14** Potential energy diagram for the ground state $S_0$ and the first excited singlet $S_1$ and triplet $T_1$ states of a representative organic molecule in solution. G is a point of intersystem crossing $S_1 \rightarrow T_1$. For convenience in representation, the distances *r* were chosen $r_{S_0} < r_{S_1} < r_{T_1}$; thus, the spectra are spread out. Actually, in complex, fairly symmetric molecules, $r_{S_0} \sim r_{S_1} < r_{T_1}$ and the 0–0 absorption and fluorescence bands almost coincide, but phosphorescence bands are significantly displaced to the lower wavelengths. From Calvert and Pitts,[2] p. 274.

occurs in the ground state of a molecule that has just emitted a photon as fluorescence. This also contributes to the shift in position of the 0–0 band in fluorescence (see Parker,[124] p. 13).

Several molecular properties can be measured using emission and excitation spectra. These include fluorescence lifetime, efficiency, anisotropy of the emitted light, mobility of chromophores, rates of quenching, and energy transfer to other chromophores.

***Fluorescence lifetimes.*** Why are some molecules fluorescent, while others are not? The possibility for fluorescent emission is limited by the radiative lifetime $\tau_r$, which is related by Eq. 23-13 to the first-order rate constant $k_f$ for exponential decay of the excited state by fluorescence.

$$\tau_r = 1/k_f \qquad (23\text{-}13)$$

The radiative lifetime is a function of the wavelength of the light and of the oscillator strength of the transition. For molecules absorbing in the near UV, the approximation of Eq. 23-14 is often made.

$$1/\tau_r \sim 10^4 \varepsilon_{max}) \qquad (23\text{-}14)$$

Thus, if $\varepsilon$ = 10,000, the radiative lifetime (the time in which the fluorescence decays to $1/e$, its initial value) is $\sim 10^{-8}$ s (10 ns). If the absorption is more intense, the lifetime is shorter, and if it is less intense, it is longer. Other modes of deexcitation compete with fluorescence; therefore, the shorter the radiative lifetime the more likely that fluorescence will be observed.

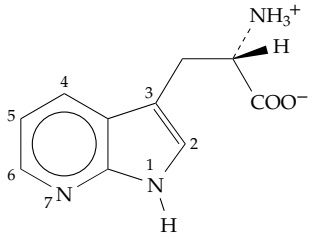
The actual lifetime $\tau$ of an excited molecule is usually less than $\tau_r$ because of the competing nonradiative processes. The sum of their rate constants can be designated $k_{nr}$. The **fluorescence efficiency** (or **quantum yield**) $\phi_F$ is given by Eq. 23-15.

$$\phi_F = k_f/k_f + k_{nr}) = k_r\tau \quad \text{where } \tau^{-1} = k_f + k_{nr} \qquad (23\text{-}15)$$

For a highly fluorescent molecule such as riboflavin, $\phi_f$ may be 0.25 or more.[129] For tryptophan in water it is about 0.14, and in proteins it varies from near zero to 0.35.[130]

***Time-resolved fluorescence spectroscopy.*** The fluorescence lifetime $\tau$ can be measured with either of two different types of fluorometer.[7,127,131–133] **Pulse fluorometers** use pulsed lasers that can deliver pulses of light lasting as little as one picosecond or less. This permits rapid excitation and permits the direct observation of emitted light, using photon countings, over the entire range of time from a few picoseconds to milliseconds required for decay of the fluorescence.[131] The observed value of $\tau$ for riboflavin 5'-phosphate ($\varepsilon_{max}$ = 12,200 at 450 nm) at 25°C is ~5 ns.[134] That for tryptophan is 3 ns.

**Phase fluorometers** utilize continuous irradiation by a beam of light that is sinusoidally modulated. If the frequency of the modulation is set correctly, there will be a phase difference in the modulation of the fluorescent emission that will depend upon $\tau$. Phase fluorometry can yield the same information as does pulse fluorometry.[127,132,133] By using two or more modulation frequencies the decay rates and fluorescence lifetimes for various chromophores in a protein can be observed. For example, the protein **colicin A** (Box 8-D) contains three tryptophans W86, W130, and W140. Their fluorescence decays with lifetimes $\tau_1$, $\tau_2$, $\tau_3$ of ~0.6–0.9 ns, 2.0–2.2 ns, and 4.2–4.9 ns at pH 7. While $\tau_3$ originates mainly from W140, both of the other tryptophans contribute to $\tau_1$ and $\tau_2$. Changes in fluorescence intensity with pH reflect a p$K_a$ value of 5.2.[135] Tryptophan, which often occurs at only one or a few places in a protein, is a useful fluorescent probe for study of protein dynamics. The optical properties of 7-azatryptophan, 2-azotryptophan, and 5-hydroxytryptophan are even better because their absorption maxima occur at longer wavelengths. These amino acids can be biosynthetically introduced in place of tryptophan in proteins.[136–138] The maximum fluorescence of tryptophan in one protein is at 350 nm, but for 7-azatryptophan in the same protein it was shifted to 380 nm.[136]



7-Azatryptophan

***Triplet states, phosphoresence, and quenching.*** In addition to emitting fluorescent radiation, molecules can often pass from the excited singlet state to a lower energy **triplet state**, in which two electrons are now unpaired and the molecule assumes something of the character of a diradical (see Fig. 23-14). This process, known as **intersystem crossing**, competes directly with fluorescence and shortens the fluorescence lifetime. The triplet state is long-lived (e.g., for tryptophan in water at 20°C it is 1.2 ms[139]) and is responsible for much of the photochemical behavior of molecules. It also gives rise to the delayed light emission known as **phosphorescence**, as is illustrated in Figure 23-14. Other processes that compete with fluorescence are **photochemical reactions** of the singlet excited state and **internal conversion**. The

latter is the process by which a molecule moves from the lowest vibrational state of the upper electronic level to some high vibrational state of the unexcited electronic level. This is the principal means of depopulating the electronic state and competes directly with fluorescence.

The rate of relaxation by nonradiative pathways can be increased by addition of **quenchers**. Quenching of fluorescence occurs by several mechanisms, many of which involve collision of the excited chromophore with the quenching molecule. Some substances such as iodide ion are especially effective quenchers. The fluorescence efficiency of a substance in the absence of a quencher can be expressed (Eq. 23-16) in terms of the rate constants for fluorescence ($k_f$), for nonradiative decay ($k_{nr}$), and for phosphorescence ($k_p$):

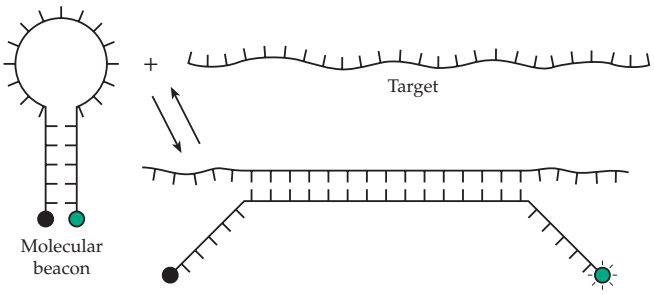
$$\phi_f = k_f / (k_f + k_{nr} + k_p) \qquad (23\text{-}16)$$

In the presence of a quencher, Q, there is an additional rate process for relaxation. The ratio of the fluorescence efficiency in the absence of ($\phi_f^0$) and the presence of a quencher is given by the **Stern–Volmer equation**.[7,140]

$$\phi_f^0 / \phi_f = 1 + K[Q] = 1 + k_Q \tau_0 [Q] \qquad (23\text{-}17)$$

The constant $K$ is known as the Stern–Volmer quenching constant; $k_Q$ is the rate constant for the quenching reaction, and $\tau_0$ the lifetime in the absence of quencher. Fluorescence quenching of tryptophan in proteins by acrylamide or $O_2$ has been used to determine whether tryptophan side chains are accessible to solvent or are "buried" in the protein.[141,142] The long-lived phosphorescence of tryptophan can be studied in a similar way.[143]

A recent application of fluorescence quenching is the development of "**molecular beacons**" for detection of viruses such as the AIDS viruses HIV-1 and HIV-2.[144] A single-stranded oligonucleotide is synthesized with a 25- or 33-nucleotide sequence complementary to a sequence in the target viral RNA. At the ends of this sequence are two 6-nucleotide arms with complementary sequences that will form a stable double-helical stem at the annealing temperature used for PCR amplification of the viral nucleic acid. The end of one arm carries a covalently bonded fluorescent dye, e.g., a fluorescein or rhodamine derivative. The other arm carries a potent covalently linked fluorescence quencher such as 4-(4'-dimethylaminophenylazo)benzoic acid. When the arms form a duplex, the quencher will be next to the fluorophore and no fluorescence will be seen upon irradiation with light of a suitable exciting wavelength. However, if viral DNA is present it will hydridize with the central polynucleotide, keeping the fluorophore and quenchers far apart




(23-18)

and allowing the beacon to signal the presence of a virus (Eq. 23-18). As few as ten retroviral genomes could be detected. By using a series of molecular beacons with different colored fluorescence and specific for different viruses, it is possible to test for more than one virus simultaneously.

***Anisotropy.*** Light emitted from excited molecules immediately after absorption is always partially polarized, whether or not the exciting beam consists of plane polarized light. When light polarized in a vertical plane is used for excitation, part of the emitted light (of intensity $I_v$) will have its electric vector parallel to that of the exciting light. The remainder of intensity $I_h$, will be polarized in a horizontal plane. The **polarization *P*** of the emitted radiation is defined by Eq. 23-19 and the **anisotropy *R*** by Eq. 23-20. After excitation by a laser pulse both the fluorescence and its anisotropy decay with time and can be measured. The decay of $R$ (but not of $P$) can usually be described as the sum

$$P = (I_v - I_h) / (I_v + I_h) \qquad (23\text{-}19)$$

$$R = (I_v - I_h) / (I_v + 2\, I_h) \qquad (23\text{-}20)$$

of simple exponential curves, which are readily obtained by phase fluorometry. These can in turn be related to specific types of motion, such as rotation of the emitting molecule or group.[7,145,146] Rotation of tryptophan rings, both free and restricted, has been studied in a variety of proteins.[145,147] However, interpretation is difficult.[130,148] The rotational rates obtained from anisotropy measurements are strongly affected by the viscosity of the medium (see Eq. 9-35).

## 2. Fluorescence Resonance Energy Transfer (FRET)

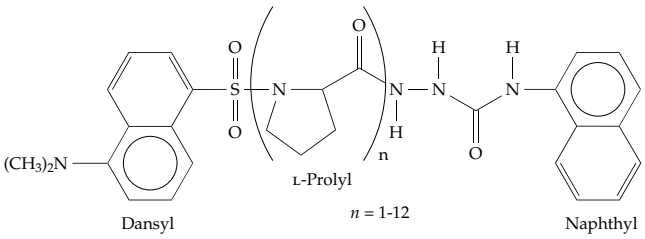
Electronic excitation of one chromophore sometimes elicits fluorescence from a different chromophore that is located nearby. For example, excitation of a monomolecular layer of dye can induce fluorescence in a layer of another dye spaced 5 nm

away. Excitation of tyrosine residues in proteins can lead to fluorescence from tryptophan, and excitation of tryptophan can cause fluorescence in dyes attached to the surface of a protein or in an embedded coenzyme.[134] Such fluorescence resonance energy transfer (**FRET**) is expected for molecules, when the fluorescence spectrum of one overlaps the absorption spectrum of the other. The mechanism is not one of fluorescence emission and absorption but of nonradiative resonant transfer of energy. Resonant transfer of energy is of major biological significance in photosynthesis (Section E). Most of the chlorophyll molecules, which absorb light in a chloroplast, transfer the absorbed energy in a stepwise fashion to a **reaction center**.

Förster[149] calculated that the rate of energy transfer $k_t$ should be proportional to the rate of fluorescence $k_f$, to an orientation factor $K^2$, to the spectral overlap interval $J$, to the inverse fourth power of the refractive index $n$, and to the inverse sixth power of the distance $r$ separating the two chromophores.

$$k_t \propto k_f K^2 J n^{-4} r^{-6} \qquad (23\text{-}21)$$

Besides predicting the inverse sixth power dependence of energy transfer, Förster provided a formula for calculating $R_0$, the distance between chromophores at which 50% efficient singlet-singlet energy transfer takes place. $R_0$ is commonly of the order of 2.0 nm. Making use of these relationships, Stryer proposed a method of measuring distances between chromophores. He calibrated the method by constructing a series of molecules containing various lengths of the rigid threefold polyproline helix to which dansyl groups were attached at one end and naphthyl groups




at the other.[150] By exciting the naphthyl group, which has the higher energy absorption band and is strongly fluorescent, the characteristic lower energy emission of the dansyl group could be observed if energy transfer took place. Since the fluorescent emission band of the naphthyl group overlaps the absorption band of the dansyl group, efficient transfer was expected. The results of a plot of transfer efficiency against distance is shown in Fig. 23-15. The inverse sixth power dependence was followed quite accurately with a value of $R_0$ ~3.4 nm. Having calibrated his "spectroscopic ruler," Stryer turned his attention to biochemical macromolecules. Attaching the same kinds of fluorescent probe to the visual light receptor rhodopsin, Wu and Stryer were able to estimate distances between specific parts of the molecule and to draw some conclusions about the overall shape.[151]

More recently the FRET technique has been widely applied to a broad range of biochemical problems. Sensitivity has been improved to the extent that fluorescence of single molecules can be detected.[152–154] Use of **terbium** ($Tb^{3+}$) or **europium** ($Eu^{3+}$) ions, which can provide luminous labels for metal-binding sites, has provided another advance. These ions absorb light poorly and are therefore only weakly fluorescent. However, they can be excited by resonance energy transfer and become brilliantly luminous. This **luminescence resonance energy transfer** (**LRET**) is a variant of FRET, which allows distances up to ~10 nm to be measured.[155–157] Another advance is the ability to graft into specific proteins fluorescent tags such as the intact **green fluorescent protein** (Section J)[158,159] or an amino acid sequence such as CCXXCC in which the four –SH groups of the cysteines serve to trap an arsenic derivative of fluorescein (see Box 12-B).[159] **Confocal laser scanning microscopy** (Chapter 3) is basic to many applications.[160]

Specific applications of FRET and LRET include observation of myosin movement (Fig. 19-14),[157] measurement of distances between binding sites on tubulin,[161] determining stoichiometry of subunit assembly in a γ-aminobutyrate receptor of brain,[162] association of proteins in peroxisomes,[160] study of hydridization of deoxyribonucleotides,[163] verifying the handedness of various forms of DNA,[164] and other studies of DNA and RNA.[164a,b]

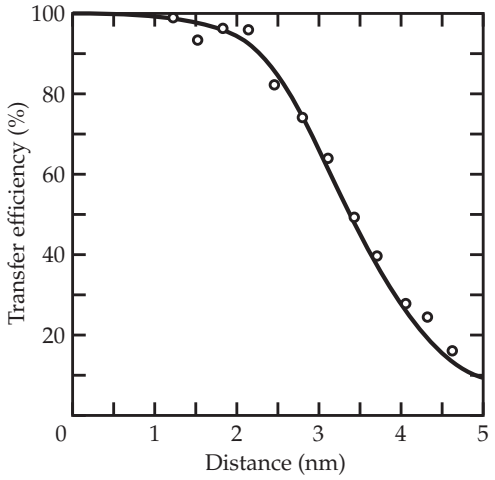



**Figure 23-15** Efficiency of energy transfer as a function of distance between α-naphthyl and dansyl groups at the ends of a polyprolyl "rod" (L-prolyl)$_n$. The observed efficiencies of transfer for $n$ = 1 to 12 are shown as points. The solid line corresponds to an $r^{-6}$ distance dependence. From L. Stryer.[150]

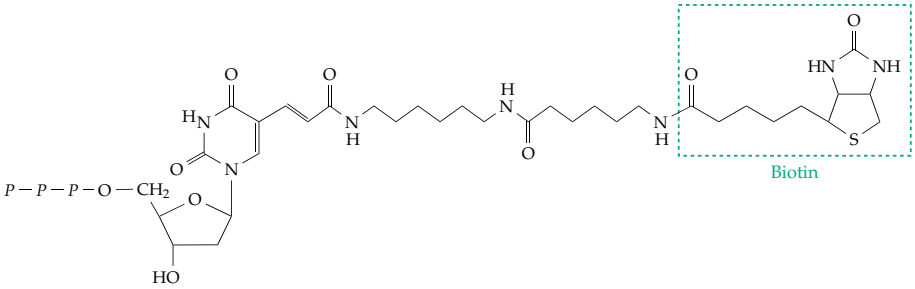
Using the Förster equation the distance between the two calcium-binding sites in parvalbumin (Fig. 6-7) has been estimated by energy transfer from Eu(III) in one site to Tb(III) in the other[165] to within 10–15% of the distance of 1.18 nm based on X-ray crystallography.

## 3. Energy-Selective Spectroscopic Methods

At low enough temperatures vibrational fine structure of aromatic chromophores may be well resolved, especially if they are embedded in a suitable matrix such as argon or $N_2$, which is deposited on a transparent surface at 15 K. This **matrix isolation spectroscopy**[77,166] may reveal differences in spectra of conformers or, as in Fig. 23-16, of tautomers. In the latter example the IR spectra of the well-known amino-oxo and amino-hydroxy tautomers of cytosine can both be seen in the matrix isolation IR spectrum. Figure 23-16 is an IR spectrum, but at low temperatures electronic absorption spectra may display sharp vibrational structure. For example, aromatic hydrocarbons dissolved in *n*-heptane or *n*-octane and frozen often have absorption spectra, and therefore fluorescence excitation spectra, which often consist of very narrow lines. A laser can be tuned to excite only one line in the absorption spectrum. For example, in the spectrum of the carcinogen 11-methylbenz(*a*)anthrene in frozen octane three major transitions arise because there are three different environments for the molecule. Excitation of these lines separately yields distinctly different emission spectra.[77] Likewise, in complex mixtures of different hydrocarbons emission can be excited from each one at will and can be used for estimation of amounts. Other related methods of energy-selective laser spectroscopy include **fluorescence line narrowing**[167] and **spectral hole burning**.[167,168]

## 4. Analytical Applications of Fluorescence

Because of the high sensitivity with which fluorescence can be detected, its measurement is important as an analytical tool. As a result of improved techniques **fluorescence microscopy** has become one of the most important of all tools in biological studies.[168a] New types of microscopes (see also pp. 129–131) have increased resolution beyond what was thought possible.[168b–d] Studies such as those of lipid metabolism in the transparent zebrafish are possible using substrates that carry fluorescent labels.[168e] As mentioned in the preceding paragraph, many aromatic compounds can be detected by their fluorescence. The relatively weak fluorescence of proteins and nucleic acids can be greatly enhanced by the binding of a highly fluorescent dye to the macromolecules. Fluorescent antibodies are widely employed for this purpose. Fluorescent labels are rapidly replacing radioisotopes in analysis of nucleic acids. For example, biotin may be attached to a pyrimidine base of a nucleoside triphosphate by a long spacer arm. The modified base can then be incorporated enzymatically into polynucleotides, e.g., in the synthesis of probes used for hybridization. The attached biotin can be detected by binding to avidin or streptavidin (Box 14-B) and use of fluorescent antibodies to this



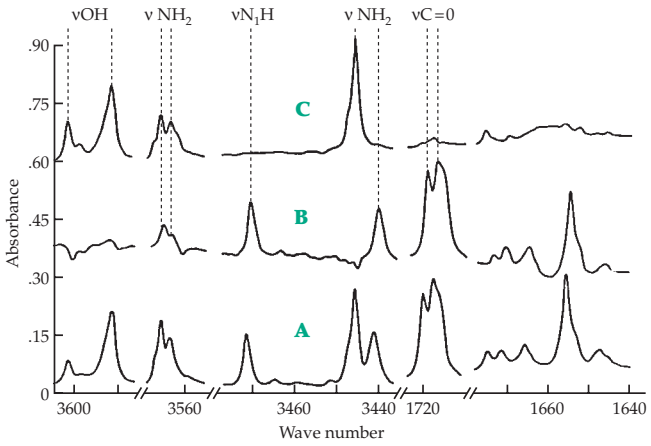
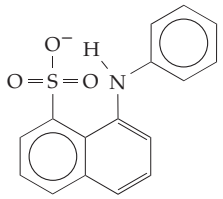



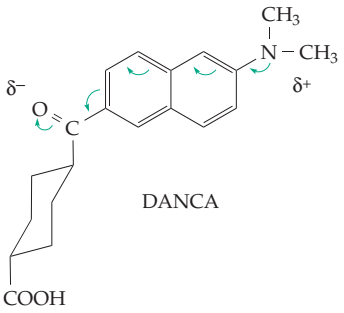

**Fig. 23-16** Infrared spectrum of cytosine in the –OH, NH, and C=O stretching region in an argon matrix in 15 K. (A) Initial spectrum of a sample containing both tautomers. (B) Spectrum of the amino-oxo tautomer calculated by substraction of the spectrum of the amino-hydroxy tautomer. (C) Spectrum of the aminohydroxy tautomer obtained by irradiating the initial sample in order to photochemically convert it into the aminohydroxy tautomer. Spectra (B) and (C) were also corrected for residual amounts of the second tautomer. From Szczesniak *et al.*[166] Courtesy of W. B. Person.

protein.[169,170] Fluorescent dyes can also be covalently attached to nucleotides. Fluorescent dideoxynucleoside triphosphates are used as chain terminators in DNA sequencing (Chapter 5). Using a different dye that fluoresces at a different wavelength for each of the four dideoxynucleosides, polynucleotides can be sequenced automatically using a single column rather than four parallel lanes as in Fig. 5-49.

Fluorescent "probes" such as **1-anilinonaphthalene-8-sulfonate** or 1,6-diphenyl-1,3,5-hexatriene embedded in membranes, contractile fibers, etc., can reveal changes in mobility that accompany alterations in physiological conditions. For example, molecular changes occurring in membranes during nerve conduction and in mitochondria during electron transport



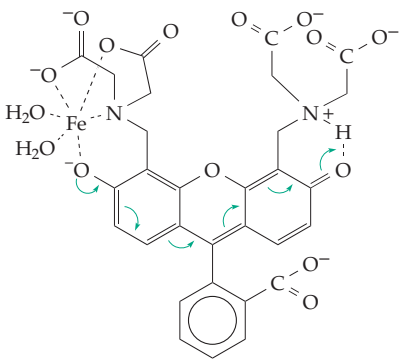
1-Anilinonaphthalene-8-sulfonate



DANCA

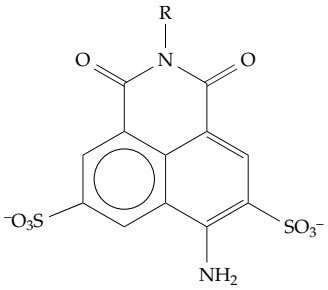
can be observed.[146,171] Another type of probe is exemplified by 2′-(*N*,*N*-dimethylamino)-6-4-*trans*-cyclohexamoic (DANCA). Its emission maximum shifts from 390 nm in cyclohexane to 520 nm in water, presumably as a consequence of increased polarization of the molecule in the excited state, as indicated by the green arrows in the accompanying structural formula.[172] DANCA can be used to obtain some idea of the polarity of sites within macromolecules to which it binds.

Study of calcium ions in living cells has been immensely aided by calcium fluorophores[173] (Box 6-D), which are often derivatives of EDTA (Table 6-9). One of these, **calcein**,[174] is also very specific toward $Fe^{3+}$. The natural calcium-dependent luminous protein, aequorin (Section J), is also widely used.



$Fe^{III}$ - calcein • $H^+$

Nontoxic but highly fluorescent dyes are used to study diffusion within and between cells. The so called Lucifer dyes have been employed to trace shapes and branching patterns in neurons.[175]



Structural formula for Lucifer dyes
For Lucifer yellow
R = —NH—CO—NH—$NH_2$

The recently developed **fluorescence correlation spectroscopy** permits studies of molecular association in one femtoliter of solution using a confocal or two-photon microscope. Two lasers are used to excite two fluorophores of different colors, each one on a different type of molecule. Fluorescence of single molecules can be detected, and molecular associations can be detected by changes in the distribution of the fluctuations in fluorescence intensity caused by Brownian motion.[176–178] A different type of advance is development of computer programs that analyze chromosomes stained with a mixture of dyes with overlapping spectra and display the result as if each chromosome were painted with a specific color.[179–180a] Yet another advance is development of **semiconductor nanocrystals** (or "quantum dots") with narrow absorption bands and intense fluorescence. The wavelength of absorption and fluorescence depends upon the size of the crystals. For example, CdSe crystals of diameter 2–5 nm coated with silica or with a surfactant fluoresce across the visible range. They have a variety of uses in biological staining.[181,182]

## D. Photochemistry

Because of their high energy, molecules in either the singlet or triplet photoexcited state undergo a greater variety of chemical reactions than do molecules in the ground state.[5,183,184] Many of these photochemical reactions arise from the triplet state that is formed from the singlet by intersystem crossing. Selection rules forbid transitions between excited triplet state and ground state; therefore, the radiative lifetime of the triplet state is long. The diradical character of the triplet state also makes it unusually reactive. Despite its forbidden character, nonradiative deexcitation of the triplet state is possible, and phosphorescence is observed for most molecules at low temperatures if the solvent is immobilized as a glass. The intense light

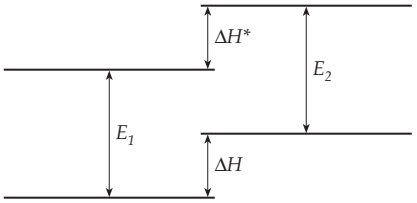
from lasers can also induce a variety of photochemical processes that arise from absorption of two or more photons.

One of the simplest of photochemical processes is the dissociation or uptake of a proton by an excited molecule as a result of a change in the p$K_a$ value of a functional group. Various other bond cleavages may lead to dissociation into ions or radicals. Photoelimination and photoaddition reactions both occur. Molecules may be isomerized, a process of importance in visual receptors. Excited molecules may become strong oxidizing agents able to accept hydrogen atoms or electrons from other molecules. An example is the **photooxidation** of EDTA by riboflavin (which undergoes photoreduction as shown in Fig. 15-8). A biologically more important example is in photosynthesis, during which excited chlorophyll molecules carry out **photoreduction** of another molecule and are themselves transiently oxidized. A frustrating aspect of investigation of photochemical reactions is that the variety of reactions possible often leads to a superabundance of photochemical products, e.g., see the thin layer chromatogram of cleavage products of riboflavin in Fig. 3-5. However, biological photoprocesses are usually much more specific.

## 1. Chemical Equilibria in the Excited State

When pyridoxamine with a dipolar ionic ring structure (Fig. 14-9) and an absorption peak at 30,700 $cm^{-1}$ (326 nm) is irradiated, fluorescence emission is observed at 25,000 $cm^{-1}$ (400 nm). When basic pyridoxamine with an anionic ring structure and an absorption peak at 32,500 $cm^{-1}$ (308 nm) is irradiated, fluorescence is observed at 27,000 $cm^{-1}$ (370 nm), again shifted ~5500 $cm^{-1}$ from the absorption peak. However, when the same molecule is irradiated in acidic solution, where the absorption peak is at 34,000 $cm^{-1}$ (294 nm), the luminescent emission at 25,000 $cm^{-1}$ is the same as from the neutral dipolar ionic form and abnormally far shifted (9000 $cm^{-1}$) from the 34,000 $cm^{-1}$ absorption peak.[185,186] The phenomenon, which is observed for most phenols, results from rapid dissociation of a proton from the phenolic group in the photoexcited state. A phenolic group is more acidic in the excited state than in the ground state, and the excited pyridoxamine cation in acid solution is rapidly converted to a dipolar ion.

The variation of fluorescence intensity with pH can provide direct information about the p$K_a$ in the excited state. Förster suggested the following indirect procedure for estimating excited-state p$K_a$ values for phenols. Let $E_1$ represent the energy of the 0–0 transition (preferably measured as the mean of the observed 0–0 transition energies in absorption and fluorescent emission spectra); let $E_2$ represent the energy of the 0–0 transition in the dissociated (anionic in the case of a phenol) form, while $\Delta H$ and $\Delta H^*$ represent the enthalpies of dissociation in the ground and excited states, respectively. It is evident from the diagram that Eq. 23-22 holds.




$$E_1 - E_2 = \Delta H - \Delta H^* \qquad (23\text{-}22)$$

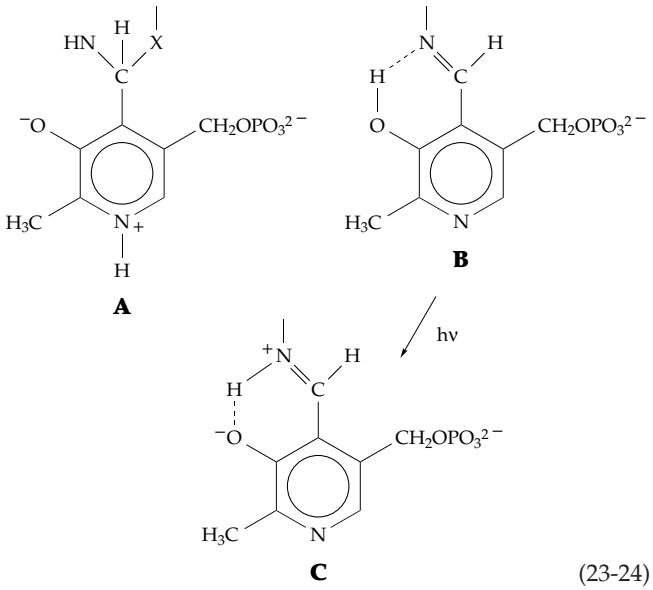
If we assume that the changes in entropy for the reaction are the same in the ground and excited state, Eq. 23-23 follows.

$$\log_{10}(K^*/K) = Nh\,(\Delta\,\bar{\nu})/(2.3RT)$$
$$\text{or}$$
$$pK^* = pK - (2.1 \times 10^3)\Delta\nu\ (cm^{-1}) \text{ at } 25°C \qquad (23\text{-}23)$$

For example, a shift in the spectrum of the basic form by 1000 $cm^{-1}$ to a lower wave number compared with the acid form corresponds to a decrease of 2.1 units in p$K_a$ for dissociation of the acid form. Whereas it is desirable to use both absorption and fluorescent measurements to locate the approximate positions of 0–0 bands, absorption measurements alone are often used, and the positions of the band maxima are taken. Thus, for pyridoxamine the shift in absorption maximum from 34,000 $cm^{-1}$ in the protonated form to 30,700 $cm^{-1}$ in the dissociated form suggests that the p$K_a$ of pyridoxamine of 3.4 in the ground state is shifted by 6.9 units to –3.5 in the excited state. Bridges *et al.*[186] evaluated this same p$K_a$ from the pH dependence of fluorescence as p$K^*$ ~ –4.1.

While phenols and amines are usually more acidic in the singlet excited state than in the ground state, some substances, e.g., aromatic ketones, may become more *basic* in the photoexcited state.

Observation of an abnormally large shift in the position of fluorescent emission of pyridoxal phosphate (PLP) in glycogen phosphorylase answered an interesting chemical question.[187,188] A 330 nm (30,300 $cm^{-1}$) absorption band could be interpreted either as arising from an adduct of some enzyme functional group with the Schiff base of PLP and a lysine side chain (structure A) or as a nonionic tautomer of a Schiff base in a hydrophobic environment (structure B, Eq. 23-24). For structure A, the fluorescent emission would be expected at a position similar to that of pyridoxamine. On the other hand, Schiff bases of the



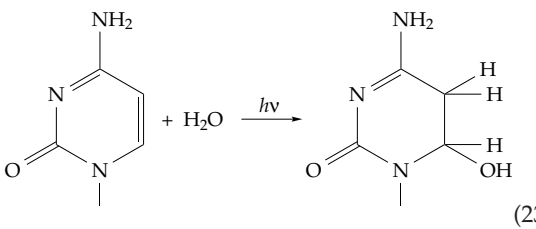

type indicated by structure B would be expected to undergo a photoinduced proton shift (phototautomerization) to form structure C[124,187] with an absorption band at 430 nm (23,300 $cm^{-1}$) and fluorescent emission at a still lower energy. Since the observed fluorescence was at 530 nm, it was judged that the chromophore does have structure B.

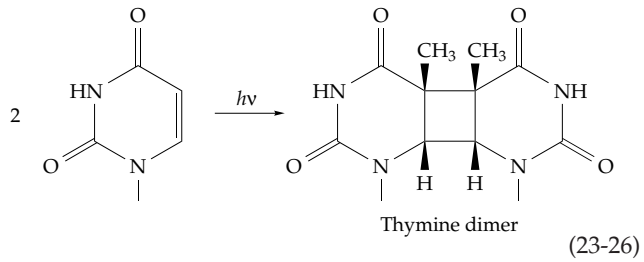
The rate of proton dissociations from the excited states of molecules can be measured directly by nanosecond fluorimetry.[189]

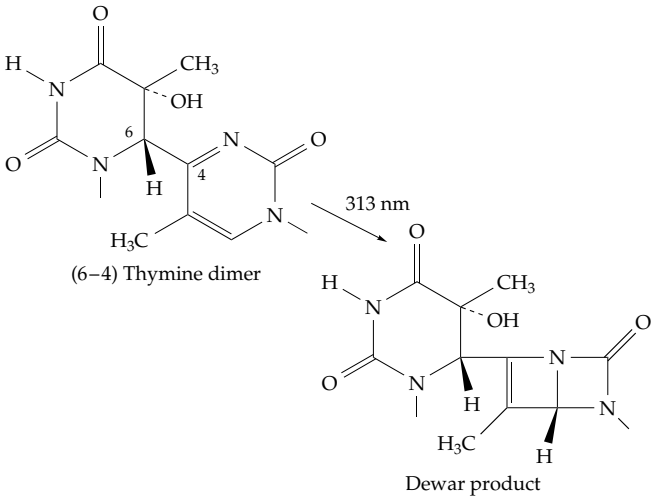
## 2. Photoreactions of Nucleic Acid Bases

Photochemical reactions of the purines and pyrimidines assume special significance because of the high molar extinction coefficients of the nucleic acids present in cells. Light is likely to be absorbed by nucleic acids and to induce photoreactions that lead to mutations.[190] Both pyrimidines and purines undergo photochemical alterations, but purines are only about one-tenth as sensitive as pyrimidines. **Photohydration** of cytidine (Eq. 23-25) is observed readily. The reaction is the photochemical analog of the hydration of α,β-unsaturated carboxylic acids. Uracil derivatives also undergo photohydration.

A more important reaction is the photodimerization of thymine (Eq. 23-26), a reaction also observed with uracil. A variety of stereoisomers of the resulting

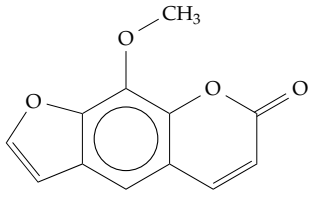


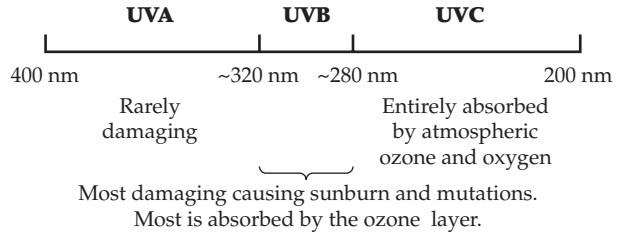

cyclobutane-linked structure are formed. The one shown in the equation predominates after irradiation of frozen thymine. Another important isomer is the 6–4 thymine dimer.[191,191a] Both of these types of **cyclobutane dimers** block DNA replication. This accounts for much of the lethal and mutagenic effect of ultraviolet radiation on organisms. The matter is sufficiently important that a special "excision repair" process is used by cells to cut out the thymine dimers (Chapter 27, Section E). In addition, light-dependent **photolyases**, discussed in Section I, act to reverse the dimerization reactions.

Light can also cause addition and other crosslinking reactions between DNA and proteins or other cell constituents.[192,193] One use of such reactions in the laboratory is DNA "photo footprinting" (Fig. 5-50), a technique which reveals contact regions between DNA and associated proteins.[190] Another type of cellular damage is caused by photosensitization of DNA by a light-absorbing intercalating agent such as **8-methoxypsoralen**.[194,195] DNA as well as adjacent proteins can be damaged.



### 3. Sunburn, Cancer, and Phototherapy

Ultraviolet light is sometimes classified according to its energy and capacity for damaging cells as follows:[195a]



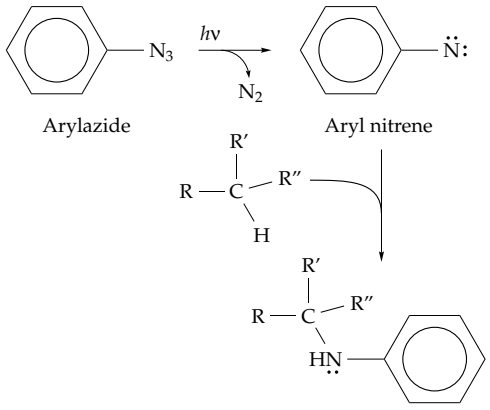

The UVB radiation causes most damage to skin. UVA radiation is at least an order of magnitude less damaging and is usually harmless to human skin. The UVC solar radiation is all absorbed by ozone and dioxygen of the atmosphere. UVC radiation produced by ultraviolet lamps is usually all absorbed in the epidermis.[5]

Ultraviolet light damages proteins as well as DNA. Residues of Trp, Tyr, His, Cys, and Met are especially susceptible to photolysis, or photooxidation by $O_2$, or by singlet oxygen. Also damaged are unsaturated lipids, porphyrins, flavins, etc. Kynurenic acid (Fig. 25-11) and urocanic acid (Eq. 14-44), an important ultraviolet filter in skin,[196] are also decomposed by light.

Repeated sunburn ages skin and may induce cancer.[4] However, light also has beneficial effects. It allows us to see, provides a source of vitamin D (Box 22-C), induces enzymatic repair of some DNA damage, and provides all of our food energy, directly or indirectly, by photosynthesis.

Light is used in **phototherapy**. This happens most frequently in the irradiation of newborn babies with white light to isomerize bilirubin (Fig. 24-24) from the 4*Z*, 15*Z* form to more readily excreted forms such as the 4*E*, 15*Z* isomer. About half of newborns have some **jaundice** (elevated bilirubin), and if it is severe it must be treated promptly to avoid neurological damage (see Chapter 24). There are sometimes complications, but the treatment is usually effective.[5] **Psoriasis** is frequently treated by irradiation with UVB light, which is thought to inhibit growth of the abnormal skin cells. Ten to 35 treatments are usually required, and the condition may return after some years. An alternative treatment is irradiation with UVA light after ingestion of 8-methoxypsoralen or other psoralen derivative.[5] Another skin condition that may respond to phototherapy is **vitiligo** (Box 25-A). Treatment with UVA and psoralen derivatives may stimulate repigmentation. If depigmentation is extreme, the remaining pigmentation may be reduced by bleaching with the monobenzyl ester of hydroquinone.

**Photodynamic therapy** is a cancer treatment that involves intravenous injection of a light-absorbing molecule such as a porphyrin, which may be taken up preferentially by cancer cells. Laser irradiation by deeply penetrating red light (650–800 nm wavelength) causes oxygen-dependent photosensitization.[5,197,198] Improvements in lasers, in fiber optics, and in photosensitizers may lead to widespread use of this type of therapy both for cancer and for some other conditions.[5,199]




(23-27)

### 4. Photoaffinity Labels

Photochemically reactive molecules have often been used as labels for specific sites in proteins and nucleic acids. Psoralen derivatives serve as relatively nonspecific photochemically activated crosslinking agents for DNA and double-stranded RNA.[195] **Aryl azides** are converted by light to aryl nitrenes, which react in a variety of ways including insertion into C–H bonds (Eq. 23-27).[200,201] In some cases UV irradiation can be used to join natural substrates to enzymes or hormones to receptors. For example, progesterone, testosterone, and other steroids have been used for direct photoaffinity labeling of their receptors.[202] Synthetic **benzophenones** have also been used widely as photoactivated probes.[203]

### 5. Microphotolysis and Ultrafast Light-Induced Reactions

Fluorescence microphotolysis, or photobleaching, has been widely used to study translational mobility of lipids and proteins in membranes. An attenuated laser beam may be focused down to the diameter of a cell or less. Then the intensity can be suddenly increased by several orders of magnitude, bleaching any fluorescent material present. The return of fluorescent material by free diffusion from a neighboring region (**fluorescence recovery after photobleaching**) or by diffusion through a membrane into a cell can then be

observed.[204,205] Diffusion coefficients of labeled biopolymers or of components of cells can be evaluated, and translation and metabolism of lipids and other components can be followed.[206]

Laser-based techniques are being used for ultrafast observation of the results of a photochemical process, e.g., the light-induced dissociation of CO from the hemoglobin • CO complex. A dissociating laser pulse can be as short as 100 fs (0.1 ps) or less. This is shorter than the time of vibrational motion of nuclei in an electronically excited state (~0.3 ps). Using IR spectroscopy, events that follow can be observed at intervals as short as 0.1 ns.[207] X-ray diffraction measurements using 150 ps pulses have allowed direct observation of the CO dissociated from hemoglobin or myoglobin and its recombination with the same protein.[208–210] Femtosecond dynamics of electron transfer along a DNA helix is also being studied.[211,212]

## 6. Optical Tweezers, Light-Directed Synthesis, and Imaging

The radiation pressure exerted by light is very weak. A bright laser beam of several milliwatts of power can exert only a few piconewtons (pN) of force. However, a force of 10 pN is enough to pull a cell of *E. coli* through water ten times faster than it can swim.[213] In about 1986, it was found that a laser beam focused down to a spot of ~ one λ (~1 μm for an infrared laser) can trap and hold in its focus a refractile bead of ~1 μm diameter. This "optical tweezers" has become an important experimental tool with many uses.[213,214] For example, see Fig. 19-19. Not only are optical tweezers of utility in studying biological motors but also mechanical properties of all sorts of macromolecules can be examined. For example, DNA can be stretched and its extensibility measured.[215] Actin filaments have even been tied into knots![216]

Light-directed solid state synthesis of peptides[217] and oligonucleotides is another new tool.[218] Development of this technology may provide new advances in preparation of DNA chips (Chapter 26) with a higher density of components than are now available.

X-rays and more recently NMR (MRI)-imaging have become well known to us. It might seem impossible to use visible light for a similar type of imaging. However, a laser beam can pass through a person's head. Is it possible, using computer-based techniques, to create an image from the emergent light? Efforts are being made to do exactly this.[219,220] **Optical coherence tomography** using backscattered infrared light and related fast techniques have higher resolution than MRI, computerized tomography (CT), or ultrasound.[220–221a] Ultrabright synchrotron radiation is also being used in **infrared microspectrophotometry**.[222,223]

## E. Photosynthesis

The photochemical reduction of $CO_2$ to organic materials[224–228] is the basic source of energy for the biosphere. Nevertheless, the process is limited to a few genera of photosynthetic bacteria (Table 1-1), eukaryotic algae, and higher green plants. Photosynthetic bacteria include the distinctly different purple, green, and bluegreen (cyanobacteria) groups, each of which has a different array of photosynthetic pigments. However, the basic mechanism of transduction of solar energy into chemical energy is the same in all of the bacteria and in green plants.

As discussed in Chapter 17, photosynthesis involves the incorporation of $CO_2$ into organic compounds by reduction with NADPH with coupled hydrolysis of ATP. This is most often via the Calvin–Benson cycle of Fig. 17-14. In a few organisms a reductive tricarboxylic acid cycle is employed. The idea that the chloroplasts of plants or the pigmented granules of photosynthetic bacteria generate NADPH or reduced ferredoxin plus ATP (Chapter 17) is now thoroughly accepted. However, it was not always obvious. Consider the overall equation (Eq. 23-28) for formation of glucose by photosynthesis in higher plants:

$$6\,CO_2 + 6\,H_2O \rightarrow 6\,O_2 + C_6H_{12}O_6 \qquad (23\text{-}28)$$

$$6\,CO_2 + 12\,H_2O^* \rightarrow 6\,O_2^* + C_6H_{12}O_6 + 6\,H_2O \qquad (23\text{-}29)$$

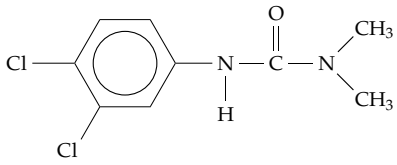
The stoichiometry of the reaction suggests that all 12 of the oxygen atoms of the evolved $O_2$ might come from $CO_2$ or that some might come from $CO_2$ and some from $H_2O$. In fact, water supplies both of the oxygen atoms needed for formation of $O_2$, as is indicated by the asterisks in Eq. 23-29. This possibility was suggested by van Niel[229,230] in 1931. He pointed out that in bacterial photosynthesis no $O_2$ is produced, and that bacteria must have access to a reducing agent to provide hydrogen for the reduction of $CO_2$ (Eq. 23-30).

$$H_2A \xrightarrow{h\nu} A + 2[H] \qquad (23\text{-}30)$$

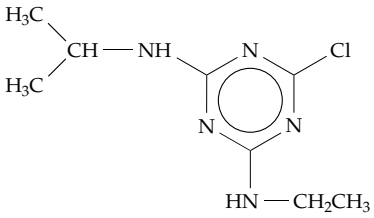
In this equation, $H_2A$ might be $H_2S$ (in the purple sulfur bacteria), elemental $H_2$, isopropanol, etc. From a consideration of these various reactions, van Niel concluded that in the $O_2$-producing cyanobacteria and eukaryotic plants water serves as the oxidizable substrate in Eq. 23-30 and is cleaved to form $O_2$ and to provide hydrogen atoms for reduction. This photochemical cleavage is the only known biological oxidation reaction of $H_2O$. No oxidizing agents present in living things are powerful enough to dehydrogenate water except for the photochemical **reaction centers** of photosynthetic organisms.

## 1. Two Photosystems, the Z Scheme, and Reaction Centers

It had long been known that for green plants light of wavelength 650 nm was much more efficient than that of 680 nm. However, Emerson and associates[34] in 1956 showed that a combination of light of 650 nm *plus* that of 680 nm gave a higher rate of photosynthesis than either kind of light alone. This result suggested that there might be two separate photosystems. What is now known as **photosystem I** (PSI) is excited by far red light (~700 nm), while **photosystem II** (PSII) depends upon the higher energy red light of 650 nm. Additional evidence supported the idea. Hill had shown many years before[231] that mild oxidizing agents such as ferricyanide and benzoquinone can serve as substrates for photoproduction of $O_2$, while Gaffron[232] found that some green algae could be adapted to photooxidize $H_2$ to protons (Eq. 23-30) and to use the electrons to reduce NADP. Thus, photosystem I could be disconnected from photosystem II. The powerful herbicide **dichlorophenyldimethylurea** (DCMU) was found to block electron transport between the two photosystems. In the presence of DCMU electrons from such artificial donors as ascorbic acid or an indophenol dye could be passed through photosystem I.




3-(3,4-Dichlorophenyl)-1,1-dimethylurea




Atrazine, another PSII inhibitor

***The Z scheme.*** The result of these and other experiments was the development of the series formulation or zigzag scheme of photosynthesis[233] which is shown in Fig. 23-17. Passage of an electron through the system requires two quanta of light. Thus, four quanta are required for each NADPH formed and eight quanta for each $CO_2$ incorporated into carbohydrate.

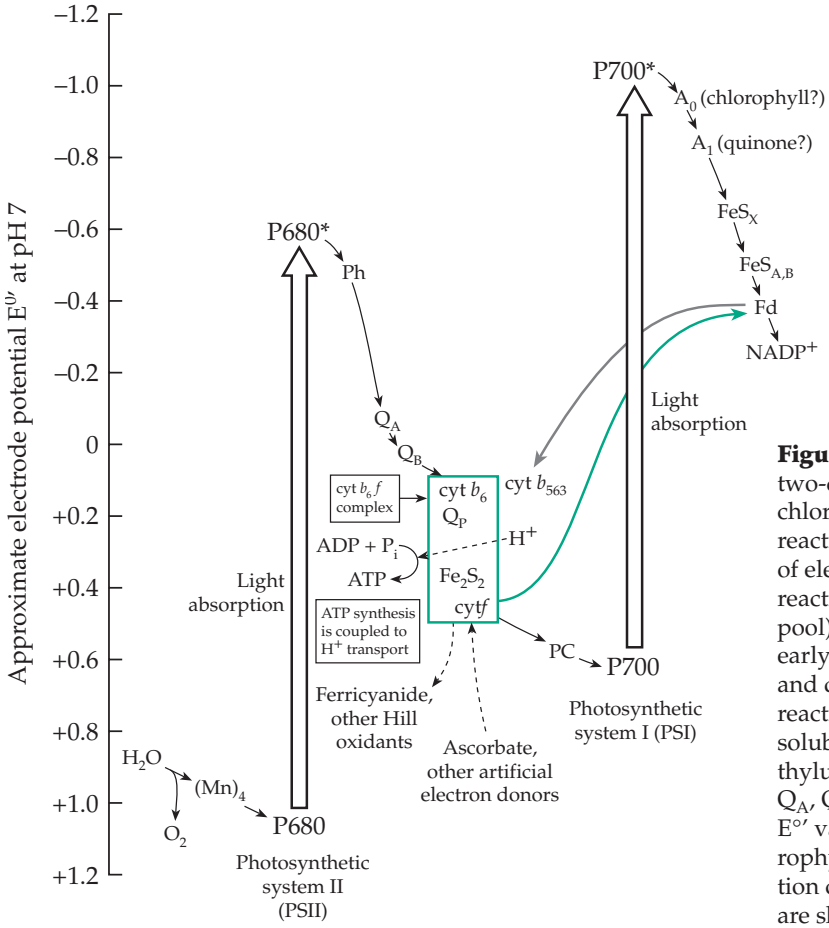



**Figure 23-17** The zigzag scheme (Z scheme) for a two-quantum per electron photoreduction system of chloroplasts. Abbreviations are P680 and P700, reaction center chlorophylls; Ph, pheophytin acceptor of electrons from PSII; $Q_A$, $Q_B$, quinones bound to reaction center proteins; PQ, plastoquinone (mobile pool); Cyt, cytochromes; PC, plastocyanin; $A_0$ and $A_1$, early electron acceptors for PSI, possibly chlorophyll and quinone, respectively; $F_x$, $Fe_2S_2$ center bound to reaction center proteins; $F_A$, $F_B$, $Fe_4S_4$ centers; Fd, soluble ferredoxin; and DCMU, dichlorophenyldimethylurea. Note that the positions of P682, P700, Ph, $Q_A$, $Q_B$, $A_0$, and $A_1$ on the E°′ scale are uncertain. The E°′ values for P682 and P700 should be for the (chlorophyll / chlorophyll cation radical) pair in the reaction center environment. These may be lower than are shown.

An important experiment of Emerson and Arnold[35] employed very short flashes of light and measurement of the quantum efficiency of photosynthesis during those flashes. A striking fact was observed. At most, during a single turnover of the photosynthetic apparatus of the leaf, one molecule of $O_2$ would be released for each 3000 chlorophyll molecules. However, it could be calculated that for each $O_2$ released only about eight quanta of light had been absorbed. It followed that about 400 chlorophyll molecules were involved in the uptake of one quantum of light. This finding suggested that a large number of chlorophyll molecules act as a single light receiving unit (usually called a light-harvesting or antenna complex) able to feed energy to one **reaction center**. The concept is now fully accepted.

***Electron transport and photophosphorylation.*** Two molecules of NADPH are required to reduce one molecule of $CO_2$ via the Calvin–Benson cycle (Fig. 17-14), and three molecules of ATP are also needed. How are these formed? The Z scheme provides part of the answer. There is enough drop in potential between the upper end of PSI and the lower end of PSII to permit synthesis of ATP by electron transport. It is likely that only one molecule of ATP is formed for each pair of electrons passing through this chain. Since, according to Fig. 17-14, one and a half molecules of ATP are needed per NADPH, some other mechanism must exist for the synthesis of additional ATP. Furthermore, many other processes in chloroplasts depend upon ATP so that the actual need for photogenerated ATP may be larger than this.

Arnon[234,235] demonstrated that additional ATP can be formed in chloroplasts by means of **cyclic photophosphorylation**: Electrons from the top of PSI can be recycled according to the dashed lines in Fig. 23-17. An electron transport system, probably that of the Z scheme, is used to synthesize ATP. As isolation of proteins and cloning of their genes progressed, it became clear that a complex of proteins known as **cytochrome $b_6f$** is closely related to the cytochrome $bc_1$ of mitochondria (Fig. 18-8).[236–237a] As in that complex, cytochrome $b_6$ carries two hemes, designated $b_h$ and $b_l$ with $E_m$ of −84 and −158 mv, respectively. $E_m$ values are for *Chlamydomonas* (see Fig. 1-11). Heme $b_l$ is closer to the positively charged membrane surface (lumen side) and heme $b_h$ is closer to the negative surface (stroma side). In maize cytochrome $b_6$ is a 23-kDa subunit, and the c-type cytochrome *f* is a larger ~ 34-kDa subunit whose heme is close to the lumenal side. Its $E_m$ value is +330 mv. A 20-kDa Rieske Fe–S protein (Chapter 16) and an additional 17-kDa subunit complete the core four-subunit complex, which is found in green plants, green algae,[236] and cyanobacteria.[238,239] Other smaller subunits are also present. It is usually assumed that a Q cycle equivalent to that of mitochondrial complex III (Fig. 18-9) operates in the pumping of protons across the thylakoid membrane (Fig. 23-18).[237b,c] However, at high rates of photosynthesis the electron transfer may bypass cytochrome *f*.[237d] The associated ATP synthase is also subject to complex regulatory mechanisms.[237e] The Rieske protein is encoded by a nuclear gene, but genes for other subunits are chloroplastic. Electrons may be carried from the cytochrome *f* subunit to PSI by plastocyanin[240] or, in many algae and cyanobacteria, by the small **cytochrome $c_6$**.[241–242b] It is often synthesized when copper is inadequate for synthesis of plastocyanin. Figure 23-18 is a schematic view of PSI, PSII, and the intermediate cyt $b_6f$ complex in a thylakoid membrane.

In spite of the close similarities in structures and function, there are distinct differences between cyto-

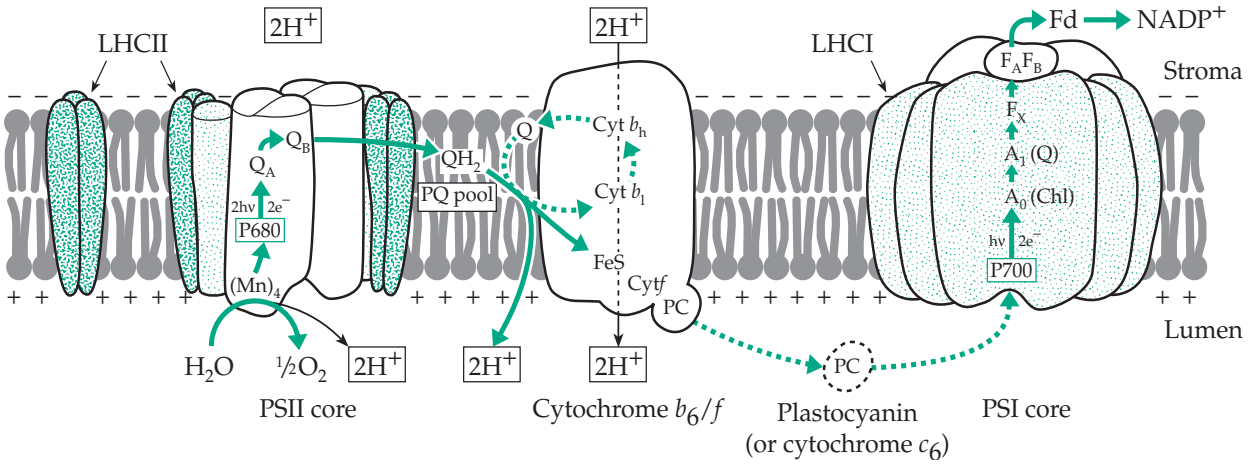



**Figure 23-18** Schematic view of photosynthetic reaction centers and the cytochrome $b_6f$ complex embedded in a thylakoid membrane. Plastocyanin (or cytochrome $c_6$ in some algae and cyanobacteria) carries electrons to the PSI core.

chrome $b_6f$ and the cytochrome $bc_1$ of mitochondria.[243] Among these are the presence of stoichiometrically bound chlorophyll *a* and β-carotene[238,244] in the photosynthetic complex. The function of the chlorophyll is uncertain, but the carotene is probably there to quench the chlorophyll triplet state,[244] which would probably cause photodamage via formation of singlet $O_2$.

***Bacterial photosynthesis.*** What is the relationship of the Z scheme of Fig. 23-17 to bacterial photosyntheses? In photoheterotrophs, such as the purple *Rhodospirillum*, organic compounds, e.g., succinate, serve as electron donors in Eq. 23-30. Because they can utilize organic compounds for growth, these bacteria have a relatively low requirement for NADPH or other photochemically generated reductants and a larger need for ATP. Their photosynthetic reaction centers receive electrons via cytochrome *c* from succinate ($E°' = +0.03$ V). The centers resemble PSII of chloroplasts and have a high midpoint electrode potential $E°'$ of 0.46 V. The initial electron acceptor is the $Mg^{2+}$-free bacteriopheophytin (see Fig. 23-20) whose midpoint potential is −0.7 V. Electrons flow from reduced bacteriopheophytin to menaquinone or ubiquinone or both via a cytochrome $bc_1$ complex, similar to that of mitochondria, then back to the reaction center P870. This is primarily a cyclic process coupled to ATP synthesis. Needed reducing equivalents can be formed by ATP-driven reverse electron transport involving electrons removed from succinate. Similarly, the purple sulfur bacteria can use electrons from $H_2S$.

In contrast, the reaction centers of green sulfur bacteria resemble PSI of chloroplasts. Their reaction centers also receive electrons from a reduced quinone via a cytochrome *bc* complex.[245] However, the reduced form of the reaction center bacteriochlorophyll donates electrons to iron–sulfur proteins as in PSI (Fig. 23-17). The latter can reduce a quinone to provide cyclic photophosphorylation. Cyanobacteria have a photosynthetic apparatus very similar to that of green algae and higher plants.

## 2. Chloroplast Structure

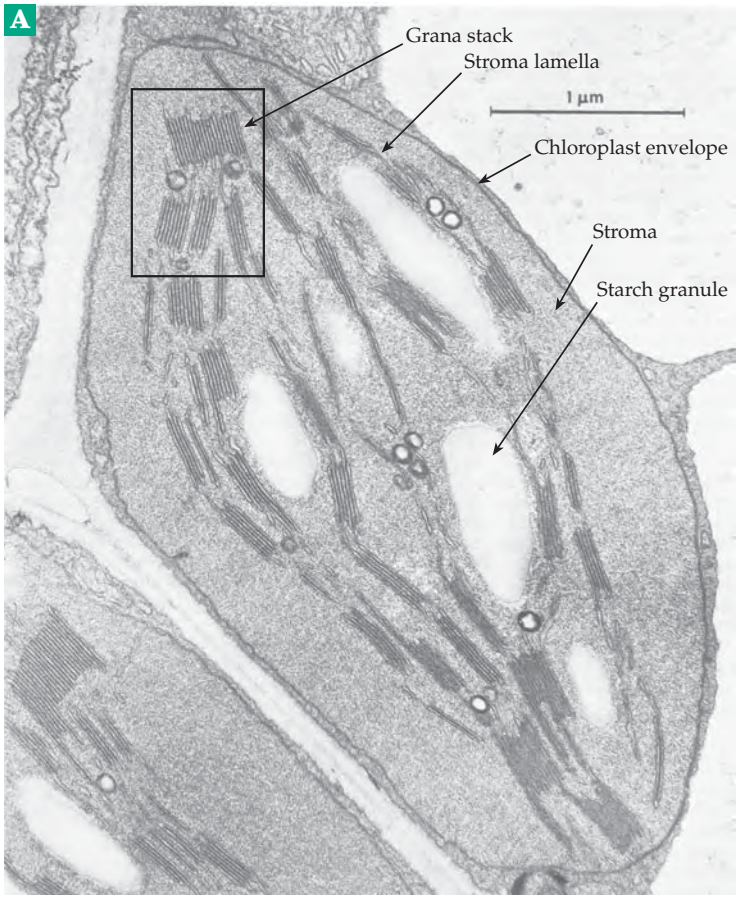
Chloroplasts come in various sizes and shapes, but all contain a small number of DNA molecules ranging in size from 120–160 kb. Complete sequences are known for DNA from chloroplasts of a liverwort (121,025 bp),[246] tobacco (155,844 bp),[247] maize (*Zea mays*),[248] and other plants. The 140,387 bp DNA from maize chloroplasts is a circular molecule containing the genes for 23S, 16S, 5S, and 4.5S RNA, for 30 species of tRNA, and for 70 different proteins. Among them are subunits of RNA polymerase, NADH dehydrogenase, subunits of both PSI and PSII, rubisco (large subunit), cytochromes *b* and *f*, six subunits of ATP synthase, and others. As with mitochondria, some subunits of the enzyme complexes that provide the cell with energy (e.g., ATP synthase) are encoded in the nucleus.

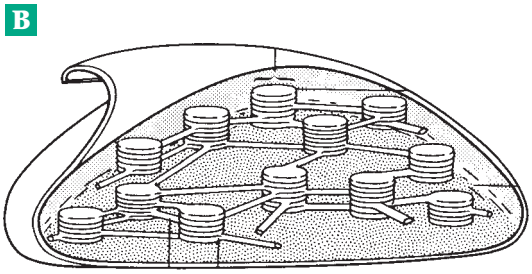
A characteristic of chloroplast genomes is the presence of a pair of large (in maize 22,348 bp) inverted repeat sequences. Since they can form a large hairpin structure with a very large loop, they may stabilize the gene sequence. The mutation rate within the repeat sequence is lower than in the single-stranded regions. The same genes are found in corresponding positions in both maize and rice. Both genomes have a number of sites of departure from the standard genetic code. These "editing sites" give rise to C→U transitions in the RNA transcripts.

Most land plants have similar chloroplast DNA sequences, but considerable divergence is observed among algae.[249] For example, the red alga *Porphyra purpura* has 70 genes not found in chloroplasts of land plants. Each gene of the chloroplasts of the dinoflagellate *Heterocapsa triquetra* is carried on its own DNA minicircle.[250] However, ~2000 chloroplast proteins are encoded by nuclear DNA. The corresponding proteins are synthesized on cytoplasmic ribosomes and are transported into the chloroplasts.[249a] Some of these proteins must pass through both the double membrane of the envelope and the thylakoid membrane. As in mitochondria (Fig. 18-4) an array of different transport proteins are required. They are distinctly different from the mitochondrial transport proteins and involve their own unique targeting mechanisms.[251–253a]

***Chloroplast membranes.*** Like the other energy-producing organelles, the mitochondria chloroplasts are surrounded by an outer double membrane or **envelope** and also contain an internal membrane system.[225–227,254–255a] Within the colorless **stroma** are stacks of flattened discs known as **grana** (Fig. 23-19). The discs themselves (the **thylakoids**) consist of pairs of closely spaced membranes 9 nm thick, each pair being separated by a thin internal space or loculus (Fig. 23-19). At least 75 different proteins are present in the isolated membranes. There is also a high content of **galactosyl diacylglycerol**, **digalactosyl diacylglycerol**, and **sulfolipid** (Chapter 8, Section A,4). Lipids account for half of the mass of thylakoid membranes.

Through the use of freeze-fracture and freeze-etching techniques of electron microscopy, it is possible to see, embeddded in the thylakoid membranes, particles which may represent individual **photosynthetic units** (also called quantosomes).[227,256–258] They are about 20 nm in diameter, and at least many of them presumably contain a reaction center surrounded by light-collecting chlorophyll–protein complexes. Others may represent the cytochrome $b_6f$ complex and



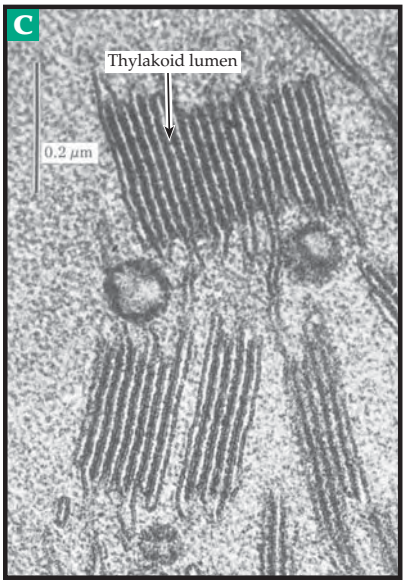




**Figure 23-19** (A) Electron micrograph of alfalfa leaf chloroplast. Courtesy of Harry T. Horner, Jr., Iowa State University. (B) Schematic drawing of a chloroplast. From Hall and Rao[227] (C) Enlargement of a portion of (A) to show grana stacks more clearly.

ATP synthase, whose knobs also protrude into the stroma. A photosynthetic unit can also be defined chemically by the number of various types of molecules present in a chloroplast membrane for each four manganese atoms (Table 23-2). Separate units contain PSI and PSII. These reaction centers appear to have a different distribution within the thylakoids, the PSI units being located principally in the unstacked membranes and the PSII units in the grana stacks.[255,259]

***Photosynthetic pigments and their environments.*** The chlorophylls (Fig. 23-20) are related in structure to the hemes (Figs. 16-5, 16-6), but ring IV (D) is not fully dehydrogenated as in the porphyrins. The **chlorin** ring system is further modified in chlorophyll by the addition of a fifth ring (V) containing an oxo group and a methyl ester. Ring V has been formed by crosslinking between the propionic acid side chain of ring III and a methine bridge carbon to give the parent compound **pheoporphyrin**. Chlorophylls contain constituents around the periphery that indicate a common origin with the porphyrins (Fig. 24-23). However, one of the carboxyethyl groups is esterified with the long-chain phytyl group in most of the chlorophylls. Chlorophyll *a* is the major pigment of chloroplasts and is a centrally important chromophore for photosynthesis in green plants. Most of the other chlorophylls, as well as the carotenoids and certain other pigments, are referred to as **accessory pigments**. Many of them have a light-receiving antenna function. Carotenoids are also photoprotectants. The relative numbers of pigment molecules in the photosynthetic units (average of PSI and PSII) of spinach chloroplasts are given in Table 23-2.

While the structure of chlorophyll *a* shown in Fig. 23-20 is the predominant one, other forms exist, e.g., with

$$-CH_2CH_3 \text{ or } -\underset{\displaystyle OH}{\underset{|}{CH}}-CH_3$$

replacing the vinyl group on ring I or with vinyl or hydroxyethyl replacing the ethyl group on ring II. The same kind of variation occurs for chlorophyll *b*.[260] In 80% acetone chlorophyll *a* has a sharp absorption band at 663 nm (15,100 $cm^{-1}$), but within chloroplasts the absorption maximum is shifted toward the red, the majority of the chlorophyll absorbing at 678 nm. Chlorophyll *b* (Fig. 23-20) is also nearly always present in green leaves.

The absorption peak in acetone is at 635 nm (15,800 $cm^{-1}$). Chlorophyll *c* found in diatoms, brown algae (Phaeophyta), and dinoflagellates (Fig. 1-9) lacks the phytyl group. Chlorophyll *d* contains a formyl group on ring I.[261]

Photosynthetic bacteria contain **bacteriochlorophylls** in which ring II is reduced (Fig. 23-20). The absorption band is shifted to the red from that of chlorophyll *a* to ~770 nm. The most abundant chlorophylls of green sulfur bacteria, **bacteriochlorophylls *c*, *d***, and ***e*** (or *Chlorobium* chlorophylls), contain a hydroxyethyl group on ring I; ethyl, *n*-propyl, or isobutyl groups on ring II; often an ethyl group instead of methyl on ring III; and a methyl group on the methine carbon linking rings I and IV. A variety of polyprenyl side chains can replace the phytyl group of the chlorophylls of higher plants.[262,263] The **pheophytins**, which are identical to the chlorophylls but lack the central magnesium ion, play an essential role in photosynthetic reaction centers. They can be formed in the laboratory by splitting the $Mg^{2+}$ out from chlorophyll with a weak acid. Other derivatives are the **chlorophyllides** formed by hydrolysis of the methyl ester group and **chlorophyllins** formed by removal of both the methyl and phytyl groups.

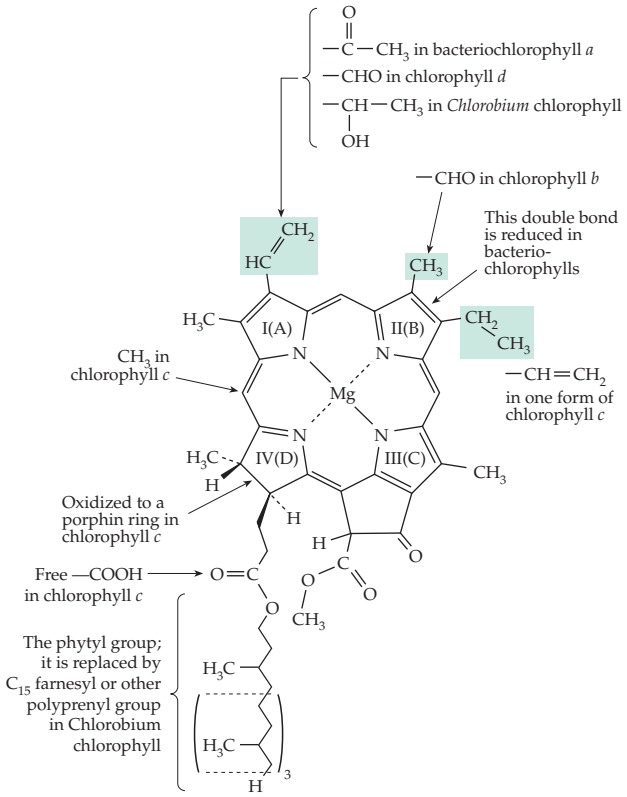
Since chlorophyll can be removed readily from chloroplasts by mild solvent extraction, it might appear that it is simply dissolved in the lipid portion of the membranes. However, from measurements of dichroism (Gregory,[226] p. 111) it was concluded that the chlorophyll molecules within the membranes have a definite orientation with respect to the planes of the thylakoids and are probably bound to fixed structures. The absorption spectrum of chlorophyll in leaves has bands that are shifted to the red by up to 900 $cm^{-1}$ from the position of chlorophyll *a* in acetone. Most green plants contain at least four major chlorophyll bands at ~662, 670, 677, and 683 nm as well as other minor bands[264] (Fig. 23-21). This fact suggested that

**TABLE 23-2**
**Approximate Composition of Photosynthetic Units in a Spinach Chloroplast[a]**

| Component | Number of molecules[b] |
|---|---|
| Chlorophyll *a* | 160 |
| Chlorophyll *b* | 70 |
| Carotenoids | 48 |
| Plastoquinone A | 16 |
| Plastoquinone B | 8 |
| Plastoquinone C | 4 |
| α-Tocopherol | 10 |
| α-Tocopherylquinone | 4 |
| Vitamin $K_2$ | 4 |
| Phospholipids | 116 |
| Sulfolipids | 48 |
| Galactosylglycerides | 490 |
| Iron | 12 atoms |
| Ferredoxin | 5 |
| Cytochrome $b_{563}$ | 1 |
| Cytochrome $b_{559}$ | |
| Cytochrome *f* | 1 |
| Copper | 6 atoms |
| Plastocyanin | 1 |
| Manganese | 2 atoms |
| Protein | 928 kDa |

[a] Averaged for PSI and PSII. After Gregory, R. P. F. (1971) *Biochemistry of Photosynthesis*, Wiley, New York [data of Luchtenthaler, H. K., and Park, R. B. (1963) *Nature* (*London*) **198**, 1070] and White, A., Handler, P., and Smith, E. L. (1973) *Principles of Biochemistry*, 5th ed., p. 528, McGraw-Hill, New York.

[b] Numbers of molecules assuming 2 $Mn^{2+}$ ions per unit (4 for PSII and 0 for PSI).




Name of parent ring system:

| | |
|---|---|
| without ring V closed: | chlorin |
| with ring V closed: | pheoporphyrin |
| with free — COOH on ring V: | chlorophyllides |
| with free — COOH on both rings II and V: | chlorophyllins |
| with $Mg_{2+}$ replaced by 2 $H^+$: | pheophytins |

**Figure 23-20** Structures of the chlorophylls.

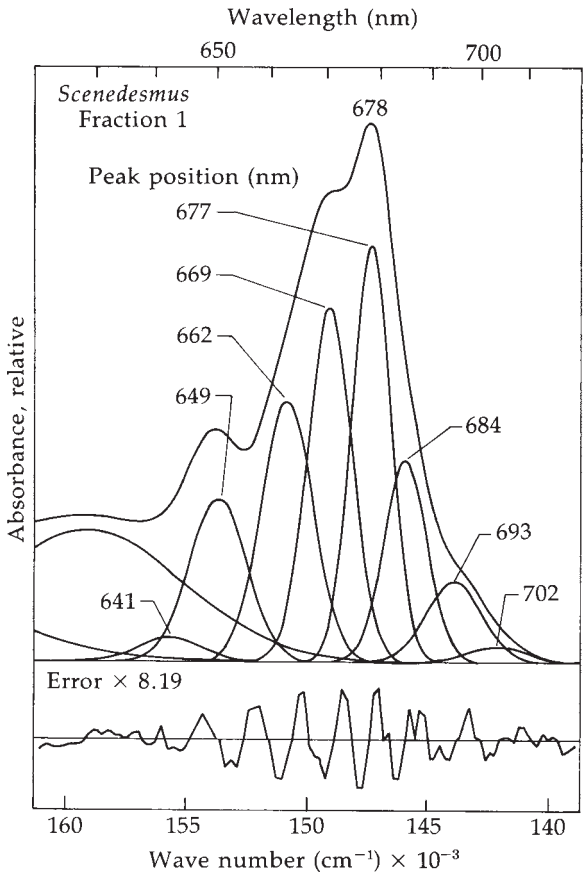



**Figure 23-21** Absorption spectrum of chlorophyll in a suspension of chloroplast fragments from the green alga *Scenedesmus* showing the multicomponent nature of the chlorophyll environments. From French and Brown.[264]

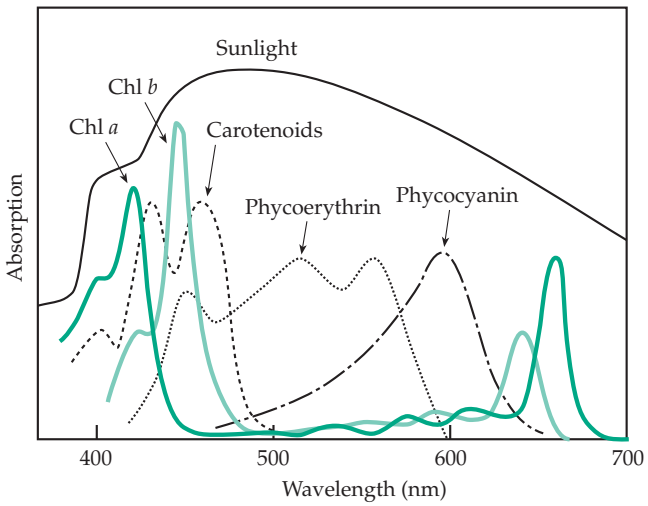



**Figure 23-22** Absorption spectra of chlorophylls and accessory pigments compared. Redrawn from G. and R. Govindjee,[265] and from J. J. Wolken.[266]

the chlorophyll exists in a number of different environments. As a result, the absorption is spread over a broader region leading to more efficient capture of light. Only a small fraction of the total chlorophyll is in the reaction centers; that for PSI absorbs at ~700 nm and that for PSII at ~682 nm.

Bacteriochlorophyll in *Chromatium* has three absorption bands with peak positions at 800, 850, and 890 nm. The last includes the reaction center bacteriochlorophyll and is the only form that fluoresces. Recent studies have established that most if not all chlorophyll is bound to specific proteins, a fact that can account for the various overlapping absorption bands.

The **carotenes** and **carotenoids** are very important accessory pigments (Fig. 23-22). The major component in most green plants is β-carotene. Green sulfur bacteria contain γ-carotene in which one end of the molecule has not undergone cyclization and resembles lycopene (Fig. 22-5). Chloroplasts also contain a large variety of oxygenated carotenoids (xanthophylls). Of these, neoxanthin, violaxanthin (Eq. 22-12), and lutein (p. 1240) predominate in higher plants and green algae. *Euglena* and related microorganisms contain much antheraxanthin (Eq. 22-10). A light-collecting protein from marine dinoflagellates contains both chlorophyll *a* and several molecules of the carotenoid peridinin. See Fig. 23-29.[267,268] Brown algae and diatoms contain mostly fucoxanthin and zeaxanthin (Fig. 22-5), while the bacterium *Rhodospirillum rubrum* synthesizes spirilloxanthin (p. 1240).

It is a striking fact that there are no naturally occurring green plants that lack carotenoid pigments.[269] Carotenoidless mutants are used in photosynthesis research, but they apparently cannot survive under natural conditions. Carotenoids not only participate as members of the light-receiving complex but also confer protection to chlorophyll against light-induced destruction by singlet oxygen. This accounts for the fact that carotenoids are usually intimately associated with chlorophyll in the pigment complexes. For example, see Figs. 23-29 and 23-30 and discussion on pp. 1308–1310.

A third class of accessory pigment of more limited distribution are the **open tetrapyrroles**, sometimes called "plant bile pigments" because of their relationship to the pigments of animal bile (Fig. 24-24). Among these are the **phycocyanins**, which provide the characteristic color to cyanobacteria. They are conjugated proteins (biliproteins) containing covalently bound phycocyanobilin (Fig. 23-24).[270] The red **phycoerythrins** of the Rhodophyta contain bound phycoerythrobilin (Fig. 23-23), an isomer of phycoerythrobilin. There are four common isomeric **bilins**, each having a different number of conjugated double bonds.[272,273] Together, they provide for a broad range

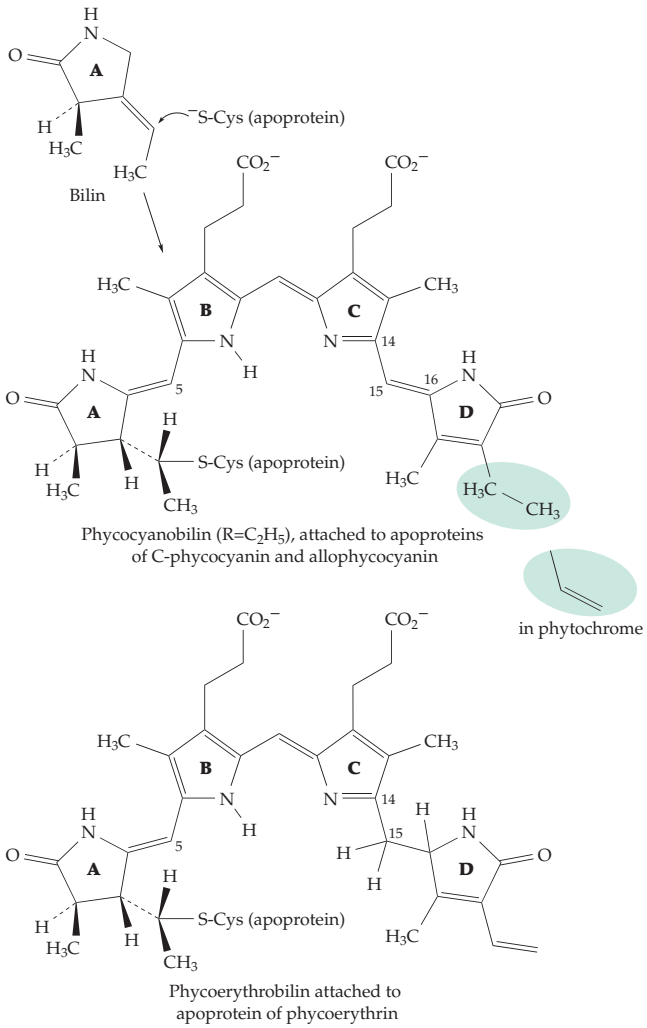



**Figure 23-23** Structures of the open tetrapyrroles of plants. See also Fig. 24-24. After Szalontai *et al.*[271]

of colors from blue to red (Table 23-3). The bilins are attached to proteins via addition of a cysteine –SH group to the vinyl group of ring A of the bilin (Fig. 23-23). A double attachment may be formed by addition of –SH groups to both vinyl groups.[273–276] Isolated tetrapyrrole pigments tend to have a helical structure and to absorb light at lower wavelengths than do the protein-bound pigments which assume elongated conformations.

There are three major classes of conjugated phycobiliproteins,[273,277] all of which are αβ heterodimers often associated as $(\alpha\beta)_6$ (Fig. 23-24). The **allophycocyanins** carry one bilin per subunit, the phycocyanins carry one on the α and two on the β subunit, and the phycoerythrins carry two or three on the α subunit and three on the β (Fig. 23-24). Cysteine α-84 is one of the frequent attachment sites.[273] Three-dimensional structures are known for several of these proteins[278–281]

**TABLE 23-3**
**The Common Bilin Pigments Present in Phycobiliniproteins[a]**

| Isomer | Number of conjugated double bonds | Absorption maximum (nm) when conjugated to proteins |
|---|---|---|
| Phycocyanobilin | 8 | ~640 |
| Phycobiliviolin | 7 | ~590 |
| Phycoerythrobilin | 6 | ~550 |
| Phycourobilin | 5 | ~490 |

[a] Wedemayer, G. J., Kidd, D. G., Wemmer, D. E., and Glazer, A. N. (1992) *J. Biol. Chem.* **267**, 7315–7331.

The bilins are derived biosynthetically rather directly[282] from biliverdin IXα, whose formation is described in Fig. 24-24. The addition of an apoprotein –SH group to a carbon–carbon double bond of the bilin is catalyzed by a specific lyase.[283] **Phytochrome** (Section H) arises in a similar way[284] as does the blue biliprotein **insecticyanin** (Box 21-A).[285]

## 3. The Light-Receiving Complexes

Irradiation of chloroplasts leads to easily measurable fluorescence from chlorophyll *a*, but no fluorescence is observed from chlorophyll *b* or from other forms of chlorophyll, carotenoids, or other pigments. It appears that the latter all serve as light-collecting or antenna pigments that efficiently transfer their energy to chlorophyll *a* at the reaction centers.[286] As is evident from Fig. 23-22, the light-collecting pigments generally have higher energy absorption bands than do the reaction centers. Thus, a broad range of wavelengths of light are absorbed by an organism, and energy from all of them is funneled into the reaction centers. The light-collecting pigments are bound to specific proteins, which are located close to the reaction centers and are arranged to provide efficient energy-transfer. Distances between adjacent pigment molecules vary from 1 to 7 nm.[287]

***Phycobilosomes.*** Algal and cyanobacterial phycocyanins and phycoerythrins are aggregated in special granules that are on the outsides of the photosynthetic membranes. The granules in the cyanobacteria are known as phycobilosomes (Fig. 23-24).[272,286,286a,288] The $(\alpha\beta)_6$ hexamers form the disks of the phycobilosomes. These are held together by linker proteins,[281,289] which fit asymmetrically into the central cavities. As is indicated in Fig. 23-24C, the disks and linker proteins are assembled into rods which are joined to form the phycobilosomes. The

latter are organized into closely packed parallel arrays on the surface of the photosynthetic membranes.

***Purple photosynthetic bacteria.*** The reaction centers of *Rhodobacter spheroides*, *Rhodospirillum rubrum*, and related purple bacteria are embedded in the plasma membrane. Each center is surrounded by a ring of bacteriochlorophyll *a* molecules bound noncovalently to heterodimeric (αβ) protein subunits made up of ~52- to 54-residue chains. Each αβ dimer binds two molecules of BChl *a*, whose central $Mg^{2+}$ ions are coordinated by conserved histidine imidazole groups, as well as a molecule of spirilloxanthin. About 15–17 αβ subunits form the ring, which is designated LH1 (Fig. 23-25A).[291–293] Most of these bacteria also have smaller rings, designated LH2, floating in the membrane near the LH1 complex. The LH2 rings (Figs. 23-25B, C) consist of about nine αβ subunits with associated BChl *a* and carotenoid.[294–298] Under some conditions a third complex LH3 may be formed.[298a] In *Rhodopseudomonas acidophila* nine of the 27 BChl *a* molecules absorb light maximally at ~800 nm and are designated **B800**. The other 18, designated **B850**, absorb maximally at ~860 nm.[294,299] The B850 BChl *a* molecules have direct contact with the chromophores of neighboring molecules, allowing for easy energy transfer. The B800 chromophores are more isolated.

Low-temperature (1.2 K) single-molecule spectroscopic techniques have been used to obtain the fluorescence-excitation spectra shown in Fig. 23-26C. For an ensemble of LH2 complexes (upper trace) the spectral absorption bands are broad, but for individual LH2 complexes structure can be seen clearly for the B800 chromophores but not for the B850 chromophores. This difference has been interpreted to mean that the excitation energy of an electronically excited B850 molecule is delocalized over the whole ring of 18 BChl *a* molecules as an **exciton**. This permits both fast and efficient energy transfers from B800 to B850 and from B850 of one LH2 ring to another or to an LH1 ring and to the reaction center (Fig. 23-27).[299–300a] Energy transfer may occur by the Förster dipole–dipole mechanism (Section C,2).[298] Many of the antenna are supported by binding through their $Mg^{2+}$ ion to an imidazole group of a protein as can also be seen in reaction center chlorophylls (Fig. 23-31C). Hydrogen-bonding to the C13-oxo groups of the chlorophylls may also be possible.[300b] The orientations of the transition dipole moments of the chlorophyll molecules may be arranged to facilitate rapid energy transfer.[300c]

***Green sulfur and nonsulfur bacteria.*** In these organisms chlorophylls are present in rodlike particles

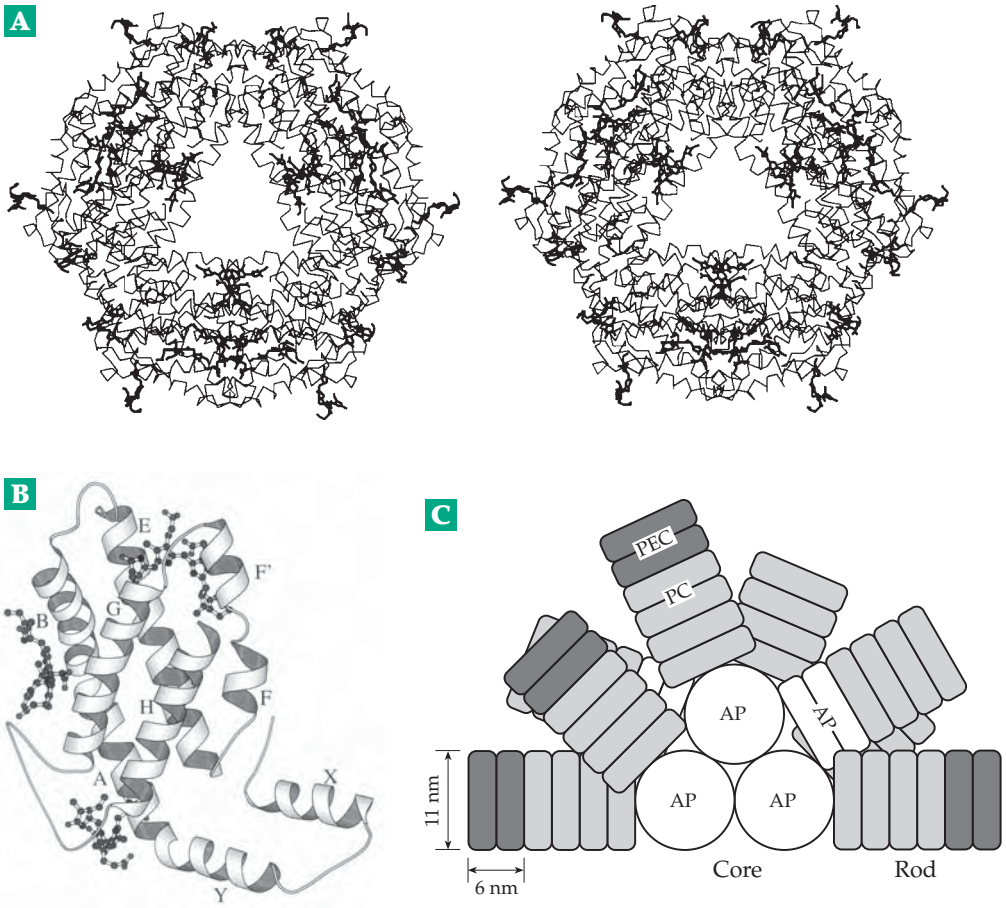



**Figure 23-24** (A) Stereoscopic view of a hexameric $(\alpha\beta)_3$ phycobiliprotein. (B) The β subunit of the complex with two molecules of bound phycoerythrobilin and one of phycourobilin. From Chang *et al.*[279] (C) Schematic representation of a phycobilosome of a strain of the cyanobacterium *Anabaena*. Each disk in the structure contains an $(\alpha\beta)_3$ phycobiliprotein. The circles marked AP are cross-sections of rods, each one composed of about four disks of allophycocyanin (AP). The projecting rods contain C-phycocyanin (PC) and phycoerythrocyanin (PEC). From Lao and Glazer.[290]

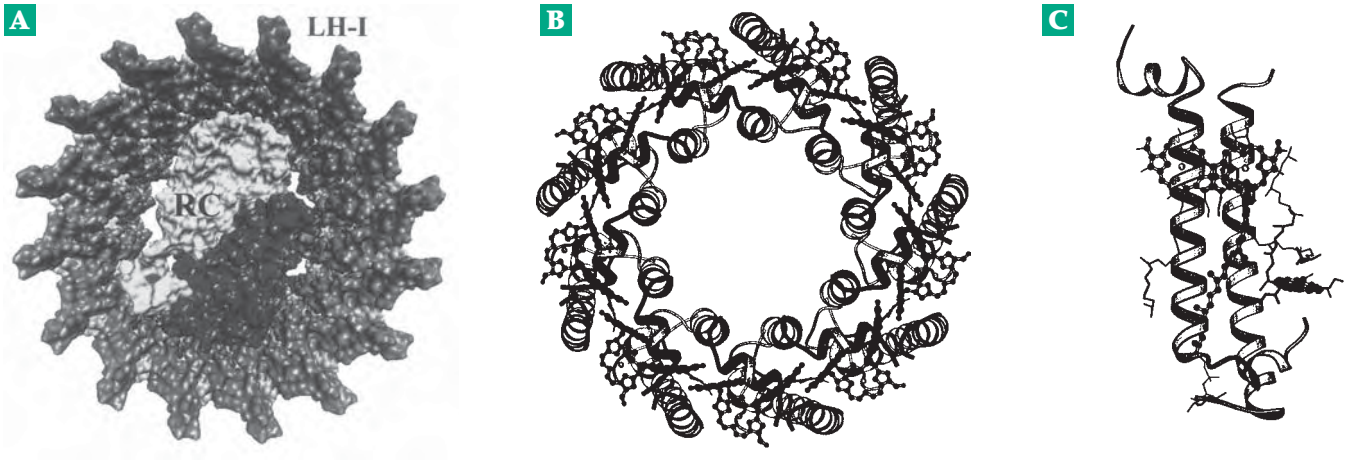



**Figure 23-25** (A) The van der Waals contact surface of the periplasmic face of the reaction center and surrounding light-harvesting complex LH1 of *Rhodobacter sphaeroides*. Made with VMD by Theoretical Biophysics Group, UIUC. See also Hu and Schulten.[291] (B) Ribbon drawing of the structure of the circular light-harvesting complex LH2 of the purple photosynthetic bacterium *Rhodopseudomonas acidophila*. The tetrapyrrole rings of the 18 bacteriochlorophyll molecules are also shown. (C) Structure of one of the nine αβ protomers with three associated bacteriochlorophylls. One of these is near the top of the protein, and the other has its chromophoric group protruding at nearly a right angle on the right side. The complete phytyl side chains are also depicted in a stick representation. (B) and (C) are from Prince *et al.*[295] MolScript drawings courtesy of N. W. Isaacs.

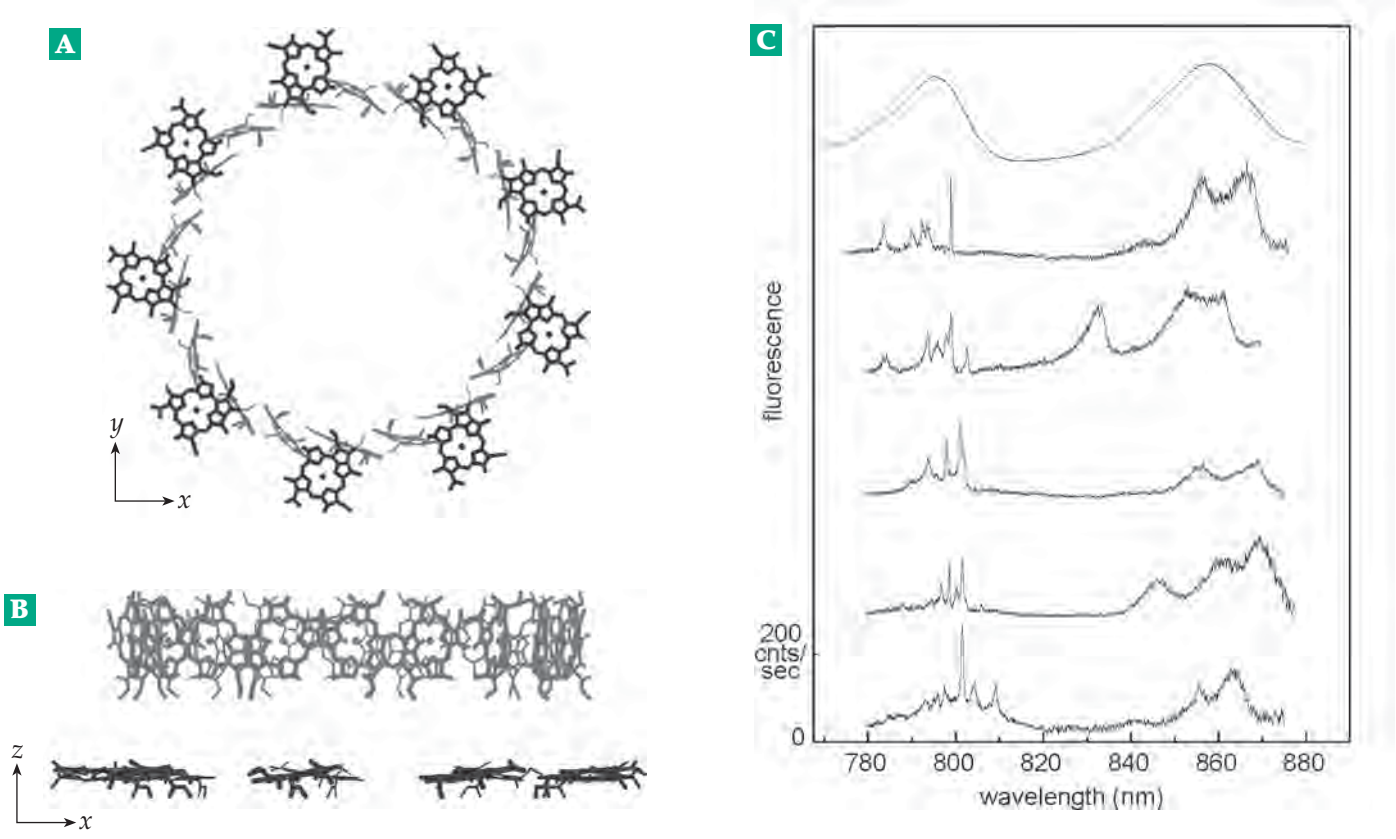



**Figure 23-26** (A), (B) Arrangement of bacteriochlorophyll chromophores in the cyclic LH2 array of *Rhodopseudomonas acidophila*. The B850 subunits are gray while the B800 subunits are black. (C) Fluorescence-excitation spectra. Top trace, for an ensemble of LH2 complexes, other traces, for several individual LH2 complexes at 1.2K. Fine structure is evident for the B800 but not for the B850 chromophores. From van Oijen *et al.*[299] with permission.

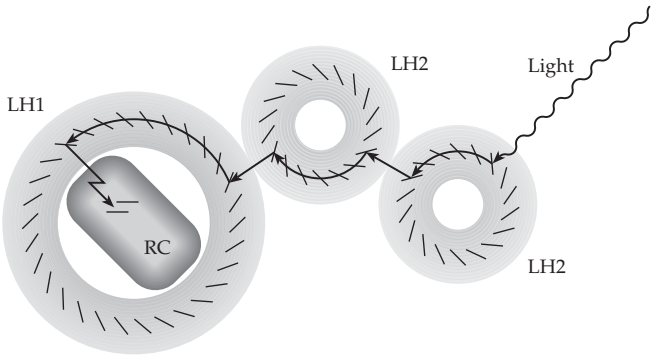



**Figure 23-27** Illustration of proposed exciton transfer of the energy of light absorbed by bacteriochlorophyll *a* of purple bacteria. Energy absorbed by the light harvesting complex LH2 is transferred in steps to another LH2, to LH1 and to the reaction center. The short lines within the circles represent the edges of the BChl*a* chromophores. After Kühlbrandt[300] with permission.

of protein present within **chlorosomes**, baglike structures which may be as large as 100 x 260 nm and are attached to the inside of the cytoplasmic membranes,[301–302a] which contain the reaction centers (Fig. 23-28). The over 10,000 light-collecting chlorophylls *c*, *d*, or *e*, which may be present per reaction center, allow these bacteria to live in extremely weak light. The bacteria also contain a smaller "antenna" of ~5000 BChl *a* present as a complex with a water-soluble protein. The three-dimensional structure of this protein is also shown in Fig. 23-28. Each 45-kDa subunit of the trimeric protein contains seven embedded molecules of bacteriochlorophyll *a*.[302,303] Other light-collecting chlorophyll–protein complexes may contain an even higher ratio of chlorophyll to protein.[286]

***Eukaryotic plants and cyanobacteria.*** Photosynthetic dinoflagellates, which make up much of the marine plankton, use both carotenoids and chlorophyll in light-harvesting complexes. The carotenoid **peridinin** (Fig. 23-29), which absorbs blue-green in the 470- to 550-nm range, predominates. The LH complex of *Amphidinium carterae* consists of a 30.2-kDA protein that forms a cavity into which eight molecules of peridinin but only two of chlorophyll a (Chl *a*) and two molecules of a galactolipid are bound (Fig. 23-29).[268]

The allenic carotenoid **fucoxanthin** (Fig. 22-5), which is absent in higher plants, predominates in brown algae, where it occurs in light-harvesting complexes along with Chl *a* and Chl *c*.[306,307]

A family of Chl *a*/*b* binding proteins are found in green plants.[308] These have apparently evolved inde-

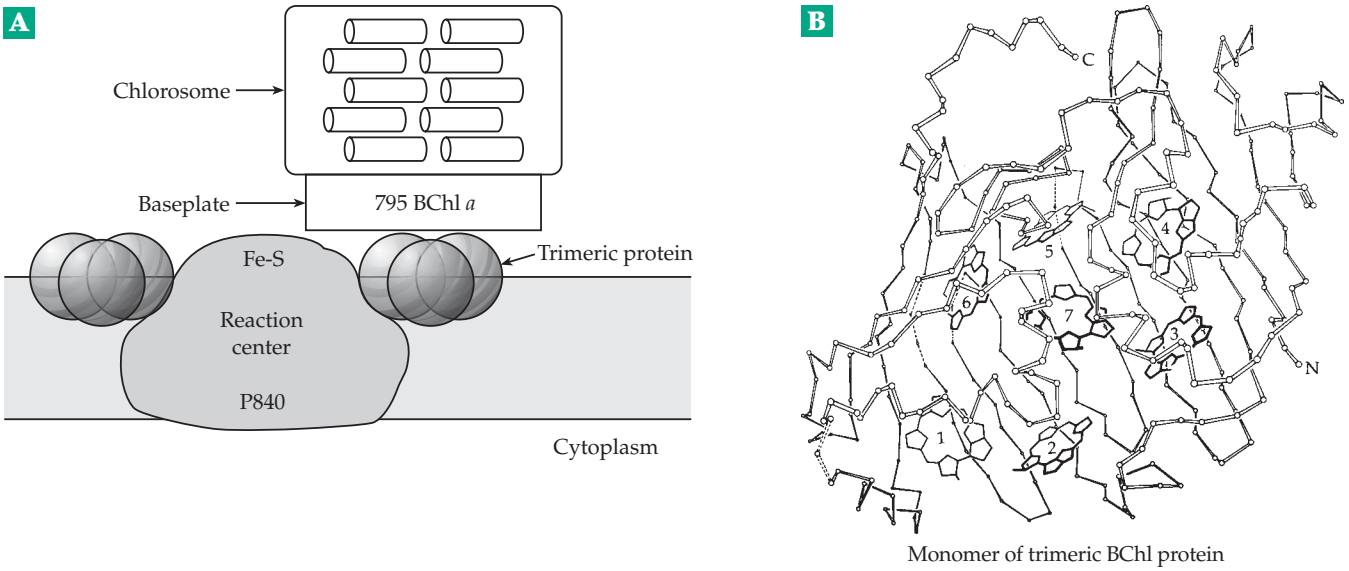



**Figure 23-28** (A) Model of a light-harvesting chlorosome from green photosynthetic sulfur bacteria such as *Chlorobium tepidum* and species of *Prosthecochloris*. The chlorosome is attached to the cytoplasmic membrane via a baseplate, which contains the additional antenna bacteriochlorophylls (795 BChl *a*) and is adjacent to the trimeric BChl protein shown in (B) and near the reaction center. After Li *et al.*[302] and Rémigy *et al.*[304] (B) Alpha carbon diagram of the polypeptide backbone and seven bound BChl *a* molecules in one subunit of the trimeric protein from the green photosynthetic bacterium *Prosthecochloris*. For clarity, the magnesium atoms, the chlorophyll ring substituents, and the phytyl chains, except for the first bond, are omitted. The direction of view is from the three-fold axis, which is horizontal, toward the exterior of the molecule. From Fenna and Matthews.[305] See also Li *et al.*[302]

pendently of chlorophyll-binding proteins of green bacteria.[309] Quantitatively most important is the complex known as LHCII, the major Chl *a* / *b* protein associated with PSII and which may also provide energy to PSI. This one protein, whose structure is shown in Fig. 23-30,[310] is thought to bind half of all of the chlorophyll in green plants. The protein is organized as trimers.[311] Each 232-residue monomer binds 5–6 Chl *b*, 7–8 Chl *a*, ~ two molecules of lutein, and one of neoxanthin.[310,311] LHCII also carries all four characteristic thylakoid lipids: mono- and digalactosyl diacylglycerols, phosphatidylglycerol, and sulfoquinovosyl diacylglycerol.[312] The Chl *a* and Chl *b* molecules are in close contact (Fig. 23-30). Subpicosecond transient absorption spectroscopy[311] indicates that half of the Chl *b* to Chl *a* energy transfers occur in < 0.2 ps. Notice the close association of the two luteins in Fig. 23-30 with the chlorophyll rings. The carotenoids are thought to quench chlorophyll triplet states to prevent formation of singlet oxygen.

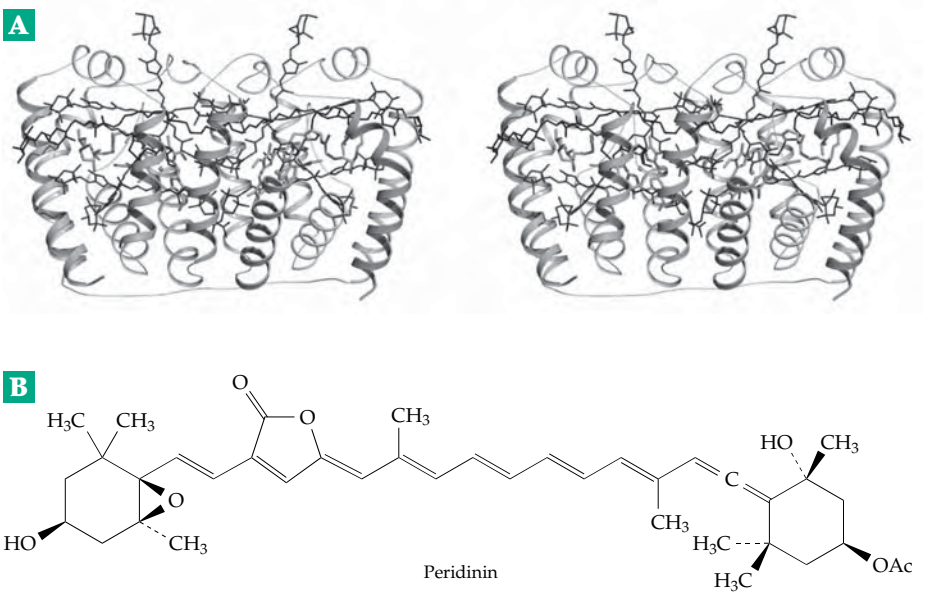



**Figure 23-29** (A) Stereoscopic drawing of light-harvesting complex from the dinoflagellate protozoan *Amphidinium carterae*. The central cavity contains eight molecules of peridinin, two of which can be seen protruding from the top. Deeply buried toward the bottom are two molecules of Chl *a*. Also present are two molecules of digalactosyl diacylglycerol. From Hofmann *et al.*[268] Courtesy of Wolfram Welte. (B) Structure of peridinin.

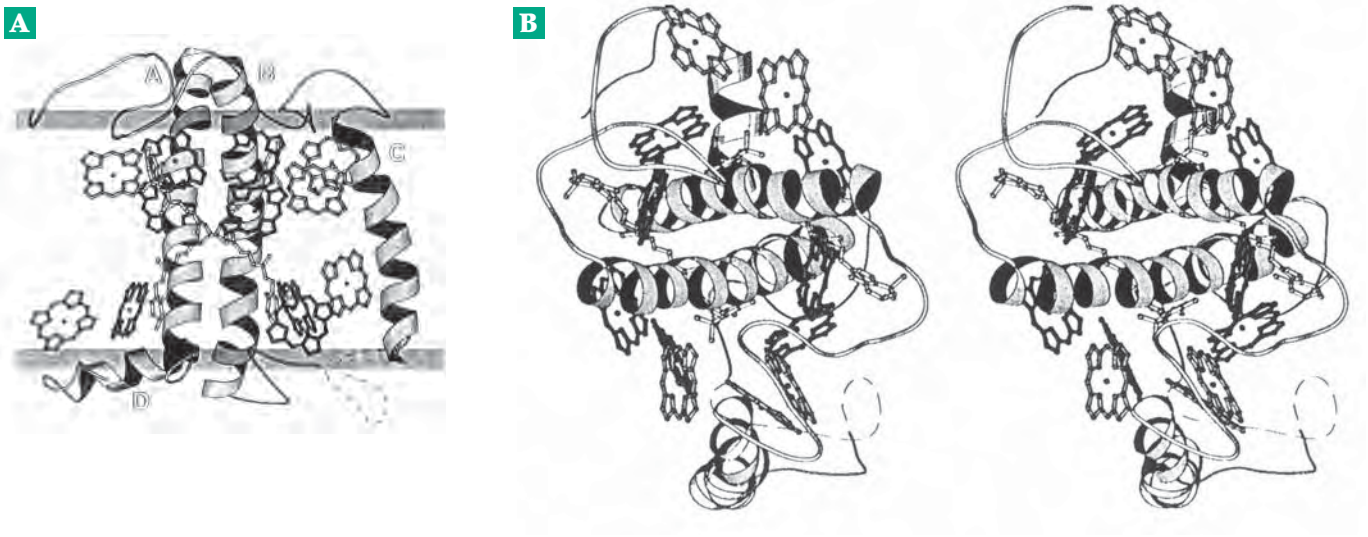



**Figure 23-30** Views of light-harvesting protein LHCII of green plants. (A) Side view indicating the approximate position in the lipid bilayer of the thylakoid membrane. Helices are labeled A–D. (B) Stereoscopic top view from the stromal side of the membrane. The structure, at 0.34 nm resolution, was determined by electron crystallography on highly ordered two-dimensional crystals. MolScript drawings from Kühlbrandt *et al.*[310] Courtesy of Werner Kühlbrandt.

PSII also contains several additional chlorophyll-binding proteins, designated CP24, CP26, CP29, CP43, CP47, etc. These lie on both sides of a pair of reaction-center cores.[313–315a] A large fraction of the LHCII complexes are separate from the reaction centers and are mobile, while a smaller fraction are bound to the outer ends of the core complex.[308,314,315] The light-harvesting chlorophylls of PSI are arranged around the core. Some are bound in the N-terminal part of the major core subunits, the products of genes *PsaA* and *PsaB*.[316] They bind ~90 Chl *a* and 14 β-carotene molecules per reaction center. (See Fig. 23-33.) In addition, the peripheral LHCI, composed of four different proteins arranged around the core, binds ~110 Chl *a* and Chl *b* and ~70 molecules of xanthophyll.[317] Some species of cyanobacteria use antenna rings around their PSI trimers instead of phycobilinosomes.[317a]

In every case the light-harvesting complexes are arranged to allow rapid and very efficient transfer of electronic excitation energy from one chromophore to another and finally to the chromophores of the reaction center.[317b] The speed and efficiency appear to depend upon very rigid structures of the proteins and precise orientations of the bound chromophores to allow direct excitonic transfer of energy at distances of less than 2 nm or transfer by the Förster mechanism at distances not exceeding 10 nm.[302] An example of the precision of protein structures was observed when a posttranslationally modified asparagine $N^5$-methylasparagine at position 72 of the β subunit of many phycobiliniproteins was substituted by aspartate or glutamine. The fluorescence lifetime of the nearby bilin was reduced 7–10% in the mutants, an effect that could cut the >95% efficiency of energy transfer from the phycobilinisomes to the PSII reaction center.[318]

## 4. The Reaction Centers and Their Photochemistry

The initial or primary processes of photosynthesis occur in the reaction centers in which chlorophyll or bacteriochlorophyll absorbs a photon.[318a] Then, the chlorophyll, in its singlet excited state (Chl*), donates an electron to some acceptor A to form a radical $A^-$ and to leave an oxidized chlorophyll $Chl^+$ radical (Eq. 23-31).

$$\mathrm{Chl} \xrightarrow{h\nu} \mathrm{Chl}^* \xrightarrow{\mathrm{A}} \mathrm{Chl}^+ + \mathrm{A}^- \qquad (23\text{-}31)$$

In the scheme of Fig. 23-18, acceptor A is $Q_A$ for PSII and $A_0$ for PSI. The oxidized chlorophyll ($Chl^+$) quickly reacts further by receiving an electron from some donor.

The photooxidation of chlorophyll indicated in Eq. 23-31 is accompanied by bleaching in the principal light absorption band. However, since there is so much light-gathering chlorophyll for each reaction center, the effect is small. The study of the process has been aided greatly by preparation of isolated bacterial photochemical reaction centers.

***Reaction centers of purple bacteria.*** The exact composition varies, but the properties of reaction centers from several genera of purple bacteria are similar. In *Rhodopseudomonas viridis* there are three peptide chains designated H, M, and L (for heavy, medium and light) with molecular masses of 33, 28, and 24 kDa, respectively. Together with a 38-kDa tetraheme cytochrome (which is absent from isolated reaction centers of other species) they form a 1:1:1:1 complex. This constitutes reaction center P870. The three-dimensional structure of this entire complex has been determined to 0.23-nm resolution[288,319–323] (Fig. 23-31). In addition to the 1182 amino acid residues there are four molecules of bacteriochlorophyll (BChl), two of bacteriopheophytin (BPh), a molecule of menaquinone-9, an atom of nonheme iron, and four molecules of heme in the *c* type cytochrome. In 1984, when the structure was determined by Deisenhofer and Michel, this was the largest and most complex object whose atomic structure had been described. It was also one of the first known structures for a membrane protein. The accomplishment spurred an enormous rush of new photosynthesis research, only a tiny fraction of which can be mentioned here.

The reaction centers are embedded in the cytoplasmic membranes of the bacteria, with the bottom of the structure, as shown in Fig. 23-31, protruding into the cytoplasm and the heme protein at the top projecting out into the periplasm which lies within infoldings of the plasma membrane. Subunits L and M each contain five ~4.0 nm long roughly parallel helices, which span the cytoplasmic membrane. Another membrane-spanning helix is contributed by subunit H, which is located mainly on the cytoplasmic side. An approximate twofold axis of symmetry relates subunits L and M and the molecules of bound chlorophyll and pheophytin.

Spectral measurements suggesting exciton splitting were among early observations that led to the conclusion that the bacteriochlorophyll involved in the initial photochemical process exists as a dimer or **special pair** (Fig. 23-31),[319,324] a conclusion verified by the structure determination. The special pair of BChl *b* lies in the center of the helical bundle that is embedded in the membrane. Nearly perpendicular to the rings of the special pair are two more molecules of BChl *b*. The central magnesium atoms of all four bacteriochlorophylls are held by imidazole groups of histidine side chains.[319,325] Below the chlorophylls are

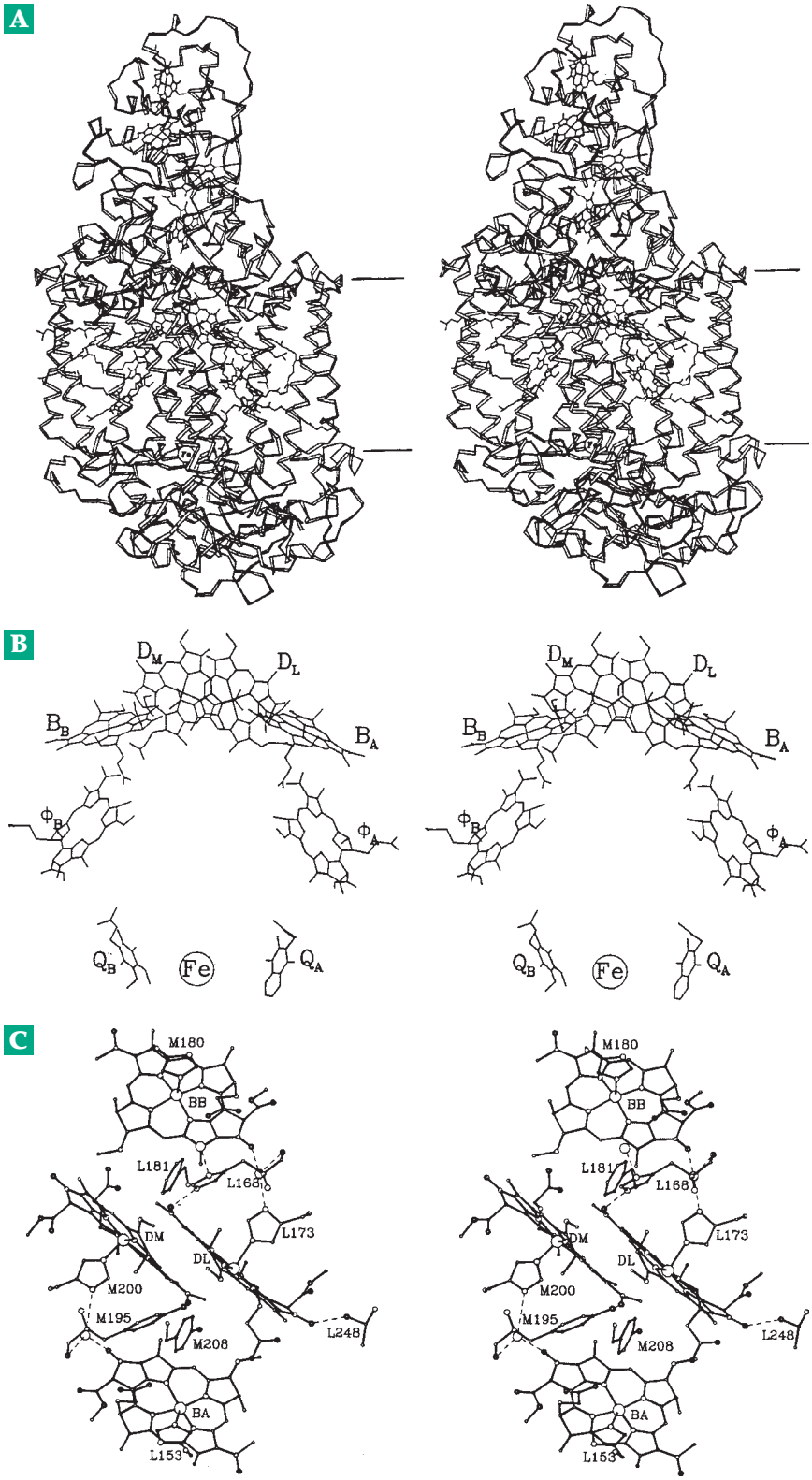



**Figure 23-31** (A) Stereoscopic ribbon drawing of the photosynthetic reaction center proteins of *Rhodopseudomonas viridis*. Bound chromophores are drawn as wire models. The H subunit is at the bottom; the L and M subunits are in the center. The upper globule is the cytochrome *c*. The view is toward the flat side of the L, M module with the L subunit toward the observer. (B) Stereo view of only the bound chromophores. The four heme groups He1–He4, the bacteriochlorophylls (Bchl) and bacteriopheophytins (BPh), the quinones $Q_A$ and $Q_B$, and iron (Fe) are shown. The four hemes of the cytochrome are not shown in (B). From Deisenhofer and Michel.[320] (C) Stereoscopic view of the Bchl *b* molecules along the local twofold axis. The special pair ($D_M$, $D_L$) is in the center with its tetrapyrrole rings almost perpendicular to the plane of the paper; the monomeric chlorophylls are labeled $B_B$ and $B_A$. The four histidine ligands to the magnesium ions of the bacteriochlorophylls as well as two tyrosines (M195 and M208) and three water molecules (large circles) are also shown. From Deisenhofer *et al.*[321] with permission.

the two molecules of bacteriopheophytin and below them the nonheme iron and the menaquinone, the first quinone acceptor $Q_A$. It corresponds to $Q_A$ of PSII shown in Fig. 23-18.

Isolated reaction centers usually contain or will bind a second quinone, which may be ubiquinone-10 ($Q_{10}$)[325a] and which is usually designated $Q_B$. Its binding site is to the left of the nonheme iron in Fig. 23-31 in a position symmetrically related to that of $Q_A$. The reaction centers also contain a carotenoid 1,2-dihydro-

neurosporene.[323] The reaction centers of purple bacterium, *Rhodospirillum rubrum*, each contain one molecule of spirilloxanthin; a variety of carotenoids are present in other species.[326] The reaction centers of a third purple bacterium, *Rhodobacter spheroides*, are closely similar in structure to that in Fig. 23-31 but lack the tetraheme.[327] Reaction centers of these bacteria accept electrons directly from a soluble cytochrome *c*.

***Reaction center kinetics.*** After an 0.8-ps or shorter flash of light the decay of the singlet excited state of the bacteriochlorophyll dimer in isolated reaction centers can be followed by loss of its characteristic fluorescence.[328,329] The lifetime of this excited state in *R. sphaeroides* is only 4 ps indicating a rapid occurrence of the initial electron transfer of Eq. 23-31. A rise in absorbance at 1250 nm is interpreted as formation of the bacteriochlorophyll cation radical $BChl^+$ in the special pair. Other spectral changes support the formation of $BPh^-$ as the first reduction product ($A^-$ in Eq. 23-31). However, this is thought to occur in two steps[323] with the monomeric BChl ($B_A$ in Fig. 23-31B) receiving the electron in ~2.8 ps and passing it to the pheophytin (Phe; $\phi_A$ in Fig. 23-31B) in ~0.7 ps (Eq. 23-32; corresponding steps for PSII of green plants are also shown).

The quinone $Q_A$ (the secondary acceptor) is next reduced by the $BPh^-$ radical in ~200 ps with development of a characteristic EPR signal[321,330] at $g = 1.82$. Over a much longer period of time (~320 ns) an electron passes from the tetraheme cytochrome subunit to the $Chl^+$ radical in the special pair.[323,323a] The relatively slow rate of this reaction may be related to the fact that the bacteriochlorophyll of the special pair is 2.1 nm (center-to-center) from the nearest heme in the cytochrome while BPh and $Q_A$ are only 1.4 nm apart (see Fig. 23-31).[288,331] Over a period of ~24 μs after formation of the radical anion $Q_A^-$ an electron from the $Q_A^-$ radical is passed to $Q_B$, a weakly bound ubiquinone-9, to form the $Q_B^-$ radical. Upon absorption of a second photon by the special BChl pair another electron is passed through the chain to form $Q_A^-Q_B^-$. Uptake of two protons with transfer of the second electron from $Q_A^-$ to $Q_B^-$ yields the ubiquinol $QH_2$, which dissociates from its binding site ($Q_B$) into the ubiquinone pool dissolved in the lipids of the membrane bilayer.[331a–g]

Why is this multistep sequence of electron transfers necessary? A variety of techniques such as femtosecond IR[332,333] and electronic[334–335a] spectroscopy, resonance Raman spectroscopy at low temperatures,[336] and study of many mutants[337–338c] have been directed toward an answer to this question. It has been generally accepted that light energy absorbed by any one of the "monomeric" Chl or pheophytins in the reaction centers is funneled "downhill" to the special pair within 0.1–0.2 ps to generate P*.[334,336] The ultrafast ~3 ps electron transfer from P* to the adjacent monomeric BChl or Chl is necessary to prevent loss of energy by fluorescence from P*. The subsequent energetically downhill transfer to a pheophytin and on to $Q_A$ prevents reverse electron transfer, which could also lead to fluorescence. Both the efficiency and the quantum yield are very high.[339]

The rate of the ultrafast proton transfer becomes even higher at cryogenic temperature, suggesting quantum mechanical tunneling.[331,335,340–340b] The transfer is generally treated using Marcus theory (Chapter 16), which indicates a very small reorganization energy for the process. Another aspect of the process is a possible coupling of a vibrational mode of the protein matrix to the electron transfer. Femtosecond near-IR spectra show low-frequency vibrational modes of the excited-state reaction center chromophores which may facilitate electron transfer.[332,336] The transfer of an electron from the tetraheme cytochrome *c* of *R. viridis*[341] or from the small cytochrome $c_2$ of *Rhodobacter spheroides*[342] to $Chl^+$ of the special pair has similar characteristics but is slower than the initial electron transfer from P*. On the other hand, electron transfers from $Q_A^-$ to $Q_B^-$ involve two distinct steps and coupled uptake of two protons.[343] An unexplained fact is that photochemical electron transport through the reaction centers always occurs through the L-side to $Q_A$ rather than the M-side.[343a–344] However, rapid electron transfer to the pheophytin on the B-side (M-side) has been observed following excitation with blue light. This may

| PSII (Green plants) | *Rhodopseudomonas viridis* |
|---|---|
| $H_2O$ | |
| ↓ | |
| $(Mn)_4$ Manganese cluster | Cytochrome *c* |
| ↓ | ↓ |
| $Y_Z$ (Tyr) | Fe (Tetraheme cytochrome *c*) |
| ↓ | ↓ |
| $Chl_2$ (P680) | $BChl_2$ Special pair cation radical (P870) |
| ↓ | ↓ |
| $Chl_2$* | $BChl_2$* *hν* Electronic excitation of $BChl_2$ |
| ~10 ps ↓ | 2.8 ns ↓ |
| $Chl_A(A_0)$ | $BChl_A$ |
| 35-200 ps ↓ | 0.7 ns ↓ |
| Phe ($A_1$) | $BPhe_A$ |
| ↓ | 200 ps ↓ |
| $Q_A$ (Plastoquinone) | $Q_A$ (Menaquinone) |
| ↓ | 24 μs ↓ 2 $e^-$ in two steps |
| $Q_B$ (Plastoquinone) | $Q_B$ (Ubiquinone) |

(23-32)

## TABLE 23-4
## Properties of Various Reaction Centers

| | *Rhodopseudomonas viridis* (now *Blasatochloris*) | Green plants and Cyanobacteria | | Green sulfur bacteria, *heliobacteria*[a] |
|---|---|---|---|---|
| | | PSII | PSI | |
| Subunits | L / M, H | D1 / D2, CP43, CP47 | PsaA / PsaB nine others | $(PscA)_2$ |
| Masses, kDa | 24 / 28, 33 | 38 / 39.4 | 83 / 83 | 65 / 65 |
| Input | Cyt *c* (4 Fe) | $H_2O$, $(Mn)_4$, $Y_Z$, $Y_D$ | Cyt *f*, plastocyanin or cyt *c* | Cyt $c_1$, $c_2$ |
| Special pair | P870 | P680 $(BChl\ b)_2$ | P700 $(Chl\ a)_2$ | P840 $(Chl\ a)_2$ |
| Monomeric chlorophyll | $BChl_L$, $BChl_M$ | | $A_0$ Both Chl *a* | $A_0$ Both Chl *a*-like |
| Pheophytin | $BPhe_L$, $BPhe_M$ | | $A_0$ (Phe, Phe) | |
| Quinone | $Q_B$, $Q_A$ (Ubiquinone, menaquinone-9) | $Q_B$, $Q_A$ (Plastoquinone) | $Q_B$, $Q_A$ $(A_1)$ (Both phylloquinone in cyanobacteria, plastoquinone in chloroplasts) | $Q_B$, $Q_A$ (Both menaquinone-7)[b] |
| Iron | $Fe^{3+}$ | | $F_X$ $(Fe_4S_4)$ | $F_X$ |
| Output | Ubiquinone Cyt $bc_1$, Cyt $c_2$ | Ubiquinone Cyt $b_6f$ | $F_A$, $F_B$, Fd $NADP^+$ | $F_A$, $F_B$, Fd $NAD^+$ |

[a] Nitschke, W., and Rutherford, A. W. (1991) *Trends Biochem. Sci.* **16**, 241–245
[b] Kjear, B., Frigaard, N.-U., Yang, F., Zybailov, B., Miller, M., Golbeck, J. H., and Scheller, H. V. (1998) *Biochemistry* **37**, 3237–3242

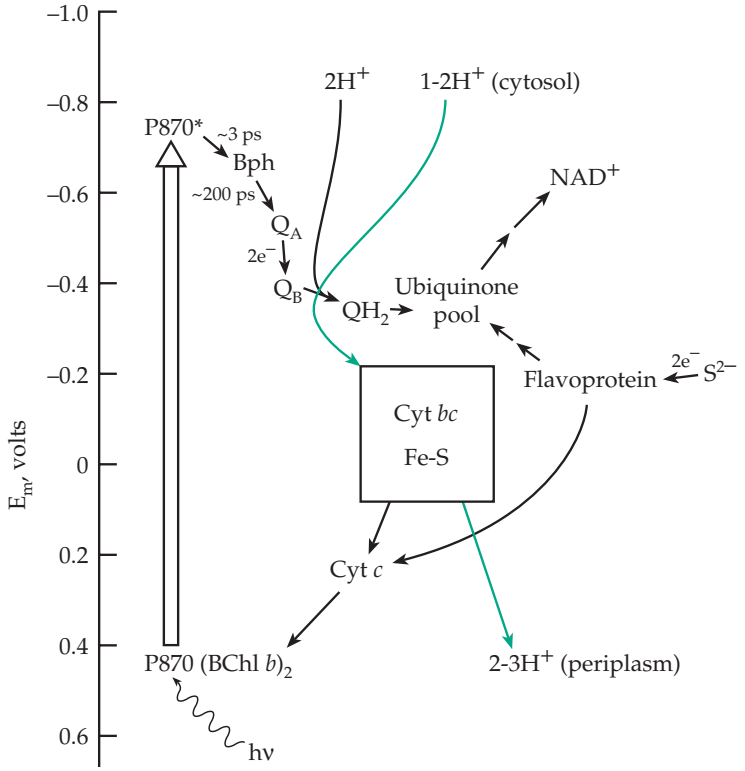



**Figure 23-32** Simplified diagram of cyclic electron flow in purple bacteria. Two protons from the cytoplasm bind to $Q_B^{2-}$ in the reaction center to form $QH_2$ (ubiquinol), which diffuses into the ubiquinone pool. From there it is dehydrogenated by the cytochrome $bc_1$ complex with expulsion of two protons into the periplasm. A third and possibly a fourth proton may be pumped (green arrows) across the membrane, e.g., via the Q cycle (Fig. 18-9). The protons are returned to the cytoplasm through ATP synthase with formation of ATP. Some electrons may flow to the reaction centers from such reduced substrates as $S^{2-}$ and some electrons may be removed to generate NADPH using reverse electron transport.[345]

represent a photoprotective mechanism.[344a] In the PSI system, in which the two phylloquinones are tightly bound,[344b] both the A-side and the B-side seem to function in electron transfer.[344c]

***Cyclic photophosphorylation in purple bacteria.*** $QH_2$ is eventually dehydrogenated in the cytochrome $bc_1$ complex, and the electrons can be returned to the reaction center by the small soluble cytochrome $c_2$, where it reduces the bound tetraheme cytochrome or reacts directly with the special pair in *Rhodobacter spheroides*. The overall reaction provides for a cyclic photophosphorylation (Fig. 23-32) that pumps 3–4 $H^+$ across the membrane into the periplasmic space utilizing the energy of the two photoexcited electrons. These protons can pass back into the cytoplasm via ATP synthases located in the same membrane with their catalytic centers in the cytosol (see Figs. 18-5 and 18-14).

***Comparison with other reaction centers.*** Subunit L of the *R. viridis* reaction center was found to have a 25% sequence homology with a quinone-binding protein now known as D1, a component of the reaction center core of PSII of chloroplasts. This protein was identified as the specific target protein for inhibition by herbicides such as DCMU and atrazine (see Section 1 for structures). These compounds act as competitive inhibitors of quinone binding[346] and bind in the $Q_B$ site in the *R. viridis* reaction center. This fact, together with the discovery that the core of PSII of green plants consists of a heterodimer of the related polypeptides D1 and D2, suggested that PSII is very similar to the bacterial center of Fig. 23-31. Both use a quinone as primary acceptor. However, P680 operates at a more positive potential ($E_m$ ~1 V), consistent with the fact that it must provide an oxidizing agent able to oxidize $H_2O$ to $O_2$ ($E°' = +0.82$ V). Plastoquinone rather than menaquinone is the primary acceptor in PSII. A chlorophyll *a* dimer is apparently the initial electron donor. The methyl ester carbonyl groups on the edges of rings I (Fig. 23-20) of BChl may coordinate to other groups of the proteins.[288,346a] These ester groups are absent in Chl *a*.

The PSI reaction center (P700) of maize chloroplast was also found to contain a pair of homologous polypeptides with appropriately placed histidyl residues for chlorophyll binding.[347] FTIR spectra also indicate the presence of two Chl *a* molecules. Small shifts in IR frequencies upon electronic excitation suggest that one chlorophyll ($P_1$) is hydrogen-bonded through its 109-ester and 9-oxo groups (Fig. 23-20) while the chlorophyll ($P_2$) is free. This may account for the low value of $E_m$ (Fig. 23-17). The charge on $P700^+$ appears to be carried in part on both chlorophylls. However, in the triplet state $^3P700^*$, which may be observed at low temperature, excitation is localized on $P_1$.[348] Although P700 operates at the low $E_m$ of +0.49 V it produces a powerful reducing agent able to reduce ferredoxin. The first identified acceptors are other Fe–S centers present in integral membrane proteins.[345] The reaction centers of green bacteria[349] and PSI of cyanobacteria[245] have similar characteristics. As more genes have been sequenced and X-ray diffraction, electron crystallography, and electron microscopy with single-particle averaging have advanced, the fundamental similarity of all of the photosynthetic centers has been confirmed.[228,300,350–353] Gene sequences are often not highly conserved, but structures are more conserved. An evolutionary relationship of all of the reaction centers can be seen.[316,354]

***PSI of cyanobacteria and green plants.*** The major reaction-center subunits **PsaA** and **PsaB** each have a C-terminal domain, resembling those of the L and M chains of purple bacteria, and a large N-terminal antenna-chlorophyll-binding domain. Cyanobacterial PSI contains ten other subunits, PsaC to PsaF, PsaI to PsaM, and PsaX. Thirty-one transmembrane helices have been assigned to the various subunits, several of which are in positions corresponding closely to those in the reaction centers of the purple bacteria (Fig. 23-33).[355–357] The PSI of higher plants is somewhat larger than that of cyanobacteria and contains somewhat different subunits.[356a,357,357a]

The electron donor to $Chl^+$ in PSI of chloroplasts is the copper protein plastocyanin (Fig. 2-16). However, in some algae either plastocyanin or a cytochrome *c* can serve, depending upon the availability of copper or iron.[345] Both $Q_A$ and $Q_B$ of PSI are phylloquinone in cyanobacteria but are plastoquinone-9 in chloroplasts. Mutant cyanobacteria, in which the pathway of phylloquinone synthesis is blocked, incorporate plastoquinone-9 into the A-site.[345a] Plastoquinone has the structure shown in Fig. 15-24 with nine isoprenoid units in the side chain. Spinach chloroplasts also contain at least six other plastoquinones. Plastoquinones C, which are hydroxylated in side-chain positions, are widely distributed. In plastoquinones B these hydroxyl groups are acylated. Many other modifications exist including variations in the number of isoprene units in the side chains.[358,359] There are about five molecules of plastoquinone for each reaction center, and plastoquinones may serve as a kind of electron buffer between the two photosynthetic systems.

Look at the Z scheme of Fig. 23-17. The lower end of each vertical arrow is located at an electrode midpoint potential $E_m$ (or $E°'$) for the couple $P^+/P$, i.e., for a one-electron reduction of the $Chl^+$ or $BChl^+$ radical.[360] The top of the arrow is at the estimated value of $E_m$ for the excited state P*. It is more negative than the ground-state value of $E_m$ by the energy (in electron volts) of the light absorbed. This is a little misleading

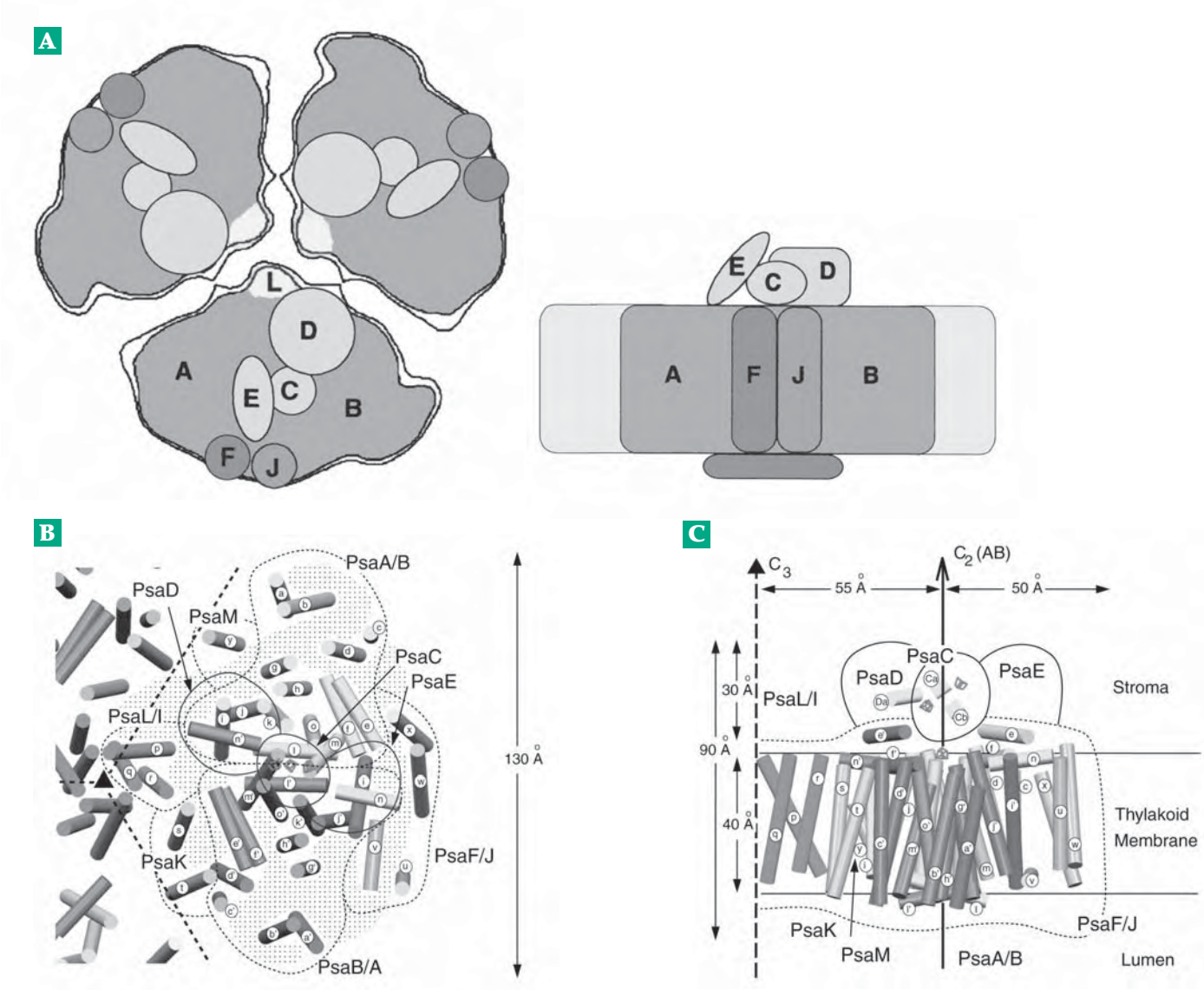
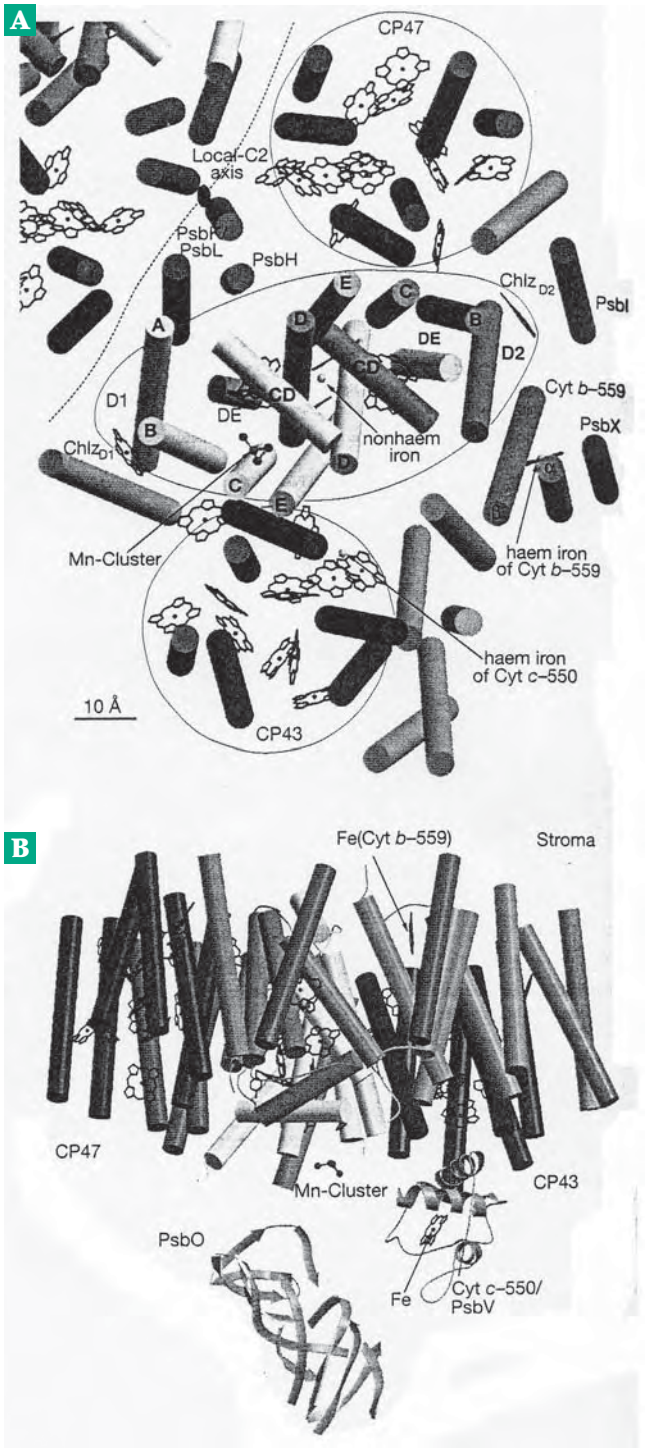



**Figure 23-33** Models of the PSI reaction center of the cyanobacterium *Synechocystis* and of higher green plants. (A) Subunit arrangement in trimeric cyanobacterial PSI as gained from EM and crosslinking experiments with wild type and directed mutant forms. From Kruip *et al.*[355] Courtesy of Jochen Kruip: Left, top view; Right, side view. (B) Schematic representation (top view) of the structure of one monomer based on X-ray data at 0.6 nm resolution. The triangle at the left marks the threefold axis of a trimer and the heavy dashed lines the interfaces between monomers. Thirty-one helices, many of which are transmembrane, are shown as shaded cylinders. Fourteen of these are in the central core (light area) while the other 17 are in peripheral regions (light gray) that bind antenna chlorophyll molecules. (C) Side view, stroma at the top showing stromal subunits PsaC, PsaD, and PsaE. (D) Top view of the trimer showing locations of all α helices and core antenna chlorophylls as well as reaction center cofactors. From Schubert *et al.*[356] Courtesy of Nobert Krauss.

for it is not commonly appreciated that light carries entropy as well as energy. An important consequence of this fact is that not all of the energy of sunlight could be harnessed for chemical work. Knox[361] has calculated that at 700 nm at most 78% of the energy could be captured. See Parson[362] for further discussion. Nevertheless, the photoexcited P700* with $E_m$ = – 1.26 V is able to reduce a series of membrane-bound Fe–S centers of $E°' \sim -0.5$ to $-0.6$ V. There are three of these designated $F_X$, $F_A$, and $F_B$. Center $F_X$ is an $Fe_4S_4$ cluster located at almost the same position as the single $Fe^{3+}$ of the *R. viridis* reaction center (Fig. 23-31B). $F_A$ and $F_B$ are also $Fe_4S_4$ clusters both of which are carried on the small 79-residue PsaC.[363,364] This protein binds to the reaction center on the stromal side as shown in Fig. 23-33. Close to it are two other subunits, PsaD and PsaE, which appear to assist the docking of ferredoxin or flavodoxin to PsaC and cluster $F_X$.[365,366]



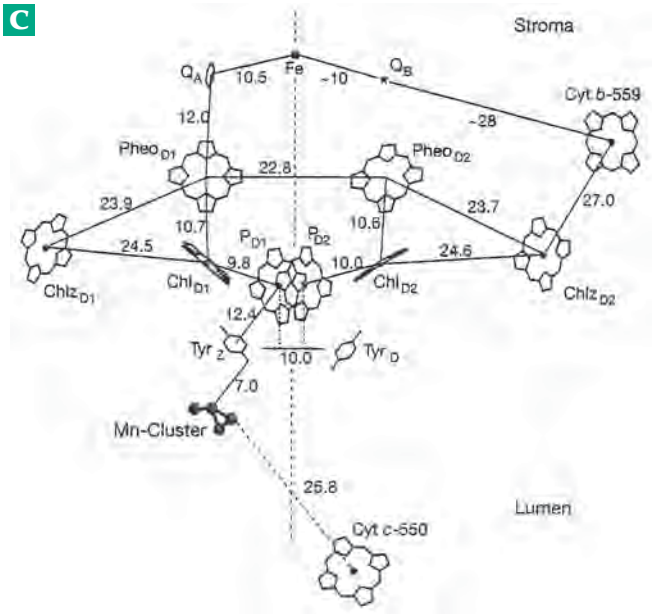




**Figure 23-34** Structure of PSII with assignment of protein subunits and cofactors. (A) Arrangement of transmembrane α-helices and cofactors in PSII. One monomer of the dimer is shown completely, with part of the second monomer related by the local-C2 axis (filled ellipse on the dotted interface). Chlorophyll *a* head groups and hemes are indicated by black wire drawings. The view direction is from the luminal side, perpendicular to the membrane plane. The α-helices of D1, D2, and Cyt *b*-559 are labeled. D1/D2 are highlighted by an ellipse and antennae, and CP43 and CP47 by circles. Seven unassigned α-helices are shown in gray. The four prominent landmarks (three irons and the manganese (Mn) cluster) are indicated by arrows. (B) Side view of PSII monomer looking down the long axis of the D1/D2 subunits from the right side in (A), at slightly tilted membrane plane and rotated 180° so that the luminal side is bottom. PsbO (33K protein) is shown as a β-sheet structure, and Cyt *c*-550 as a helical model. (C) Arrangement of cofactors of the electron transfer chain located in subunits D1 and D2. View direction along the membrane plane. Full lines indicate center-to-center distances (nm) between the cofactors (uncertain to about ± 0.1 nm). The pseudo-C2 axis is shown by the vertical dotted line; it runs throught the non-heme iron Fe and is parallel to the local-C2 axis. The asterisk indicates the putative $Q_8$ binding site. From Zouni *et al.*[371d] Courtesy of Athina Zouni.

The photosynthetic centers of green photosynthetic sulfur bacteria also have centers $F_X$, $F_A$, and $F_B$.

The soluble electron carriers released from the reaction centers into the cytoplasm of bacteria or into the stroma of chloroplasts are reduced single-electron carriers. Bacterial ferredoxin with two $Fe_4S_4$ clusters is formed by bacteria if enough iron is present. In its absence flavodoxin (Chapter 15), which may carry either one or two electrons, is used. In chloroplasts the carrier is the soluble **chloroplast ferredoxin** (Fig. 16-16,C), which contains one $Fe_2S_2$ center. Reduced ferredoxin transfers electrons to $NADP^+$ (Eq. 15-28) via **ferredoxin:NADP$^+$ oxidoreductase**, a flavoprotein of known three-dimensional structure.[367–369]

***PSII and formation of oxygen.*** The structure of PSII has been difficult to determine directly, but its core has been modeled in atomic detail using bacterial reaction centers as a guide.[370,371] More recently electron crystallography provided a three-dimensional image at 0.8 nm resolution.[314,371a] The resolution has been extended down to 4 nm by X-ray crystallography.[371b–e] The structure of the cyanobacterial PSII (Fig. 23-34) is very similar to that of green plants.[371e,f] PSII contains at least 17 protein subunits, all of which are encoded by chloroplast genes. The large structurally similar D1 and D2 form the core. They are encoded by genes *PsbA* and *PsbD* and have molecular masses of 38.0 and 39.4 kDa, respectively.[315] Both $Q_A$ and $Q_B$ are plastoquinone. It is of historical interest that these cofactors were first designated simply as Q, not for quinone but for *quencher*. This is because $Q_A$ apparently quenched the fluorescence of P680, the reaction-center chlorophyll *a*. If chloroplasts are irradiated with 650-nm light, PSII is activated but PSI is not. Under these conditions $Q_A$ becomes reduced, and the fluorescence of $Chl^+$ increases, presumably because the electron acceptor $Q_A$ is absent. If PSI is activated by addition of far-red light, $Q_A$ remains more oxidized, and fluorescence is quenched by a mechanism that appears to depend upon one of the additional bound chlorophylls, as well as the chlorophyll pair $Chl_2$ and the 9-kDa cytochrome $b_{559}$. The latter is an essential PSII subunit (*PsbE* gene),[372,373] which forms a tight complex with the D1/D2 pair.

Other subunits in PII include the 56- and 50-kDa antenna proteins CP43 (*PsbC* gene) and CP47 (*PsbB* gene). Three **extrinsic proteins**, which bind to the lumenal side of the thylakoid membrane, are the 33-kDa **manganese stabilizing protein** (*PsbO* gene),[374,375] cytochrome $c_{550}$, and a 12-kDa subunit (Fig. 23-34). The position of the larger two of these proteins are shown in the model in Fig. 23-34, which is based on 0.8-nm resolution data.[315] These extrinsic proteins seem to function together to facilitate binding not only of Mn ions but also of $Ca^{2+}$ and $Cl^-$, both of which are essential for $O_2$ evolution.[376] Other smaller subunits are also present.[376a] Together with its antenna complexes PSII may form large supercomplexes with as many as 25 subunits.[376b] The distribution of complexes varies in the different regions of the thylakoid, e.g., the stroma lamellae and grana stacks (Fig. 23-19).[376c]

The four-electron dehydrogenation of two water

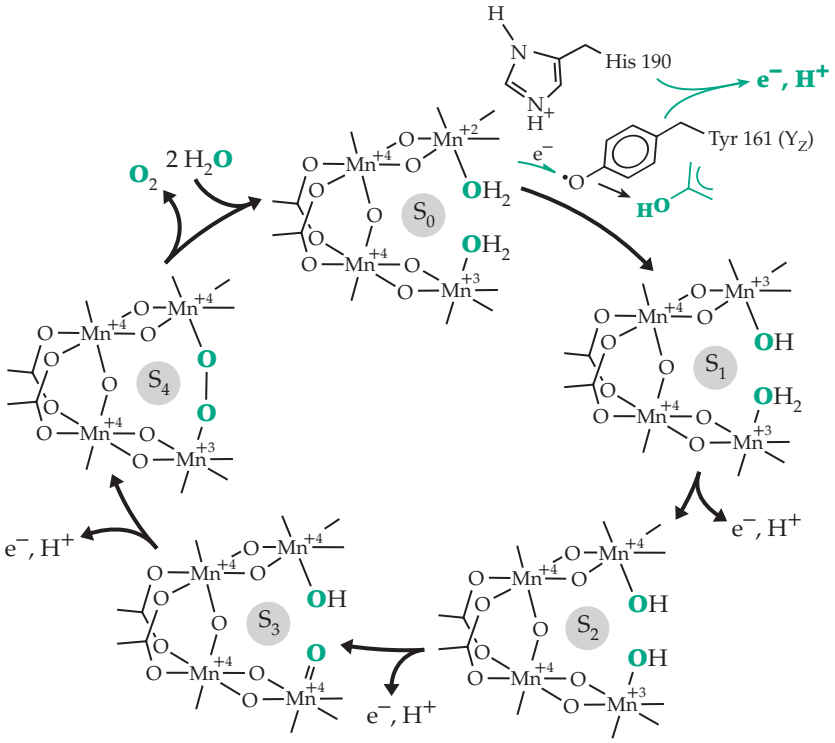



**Figure 23-35** Proposed sequence of S-states of the manganese cluster of photosystem II. The successive states as two molecules of $H_2O$ (green oxygen atoms) are converted to $O_2$ is shown with the successive states $S_0$–$S_4$ labeled. To save space and possible confusion tyrosine 161 ($Y_Z$) and the nearby His 190 are shown only by $S_1$. The $Y_Z$ radical is thought to remove a hydrogen atom or $H^+$ from one bound $H_2O$ and an electron from one Mn ion at each of the four S-states $S_0$–$S_3$ functioning in each case to eject a proton into the thylakoid lumen and to transfer an electron to $P^+$ of the reaction center. However, the exact sequence of $e^-$ transfer and $H^+$ release may not be shown correctly. After Hoganson and Babcock.[392,392a]

molecules to give one molecule of $O_2$ by PSII is still not well understood. From experiments on oxygen evolution in the presence of repeated short flashes of light it was found that a four-quantum process is required.[376–380] There must be some way of storing oxidizing equivalents until enough are present to snap together an oxygen molecule. There is abundant evidence that manganese is required for this process and that the oxidation of $H_2O$ occurs on a cluster of four atoms of manganese.[379]

The 33-kDa protein PsbO, which is present in all oxygen-forming photosynthetic organisms, is closely associated with the $Mn_4$ cluster. Removal of this protein leads to a gradual loss of two of the four Mn ions.[375] The structure of the $Mn_4$ cluster is not yet certain, but on the basis of EXAFS spectroscopy a pair of di-μ-oxo bridged Mn dimers linked by carboxylates and with a fifth μ-oxo bridge as in Fig. 23-35 has been proposed. The distance between Mn ions in the di-μ-oxo-$Mn_2$ groups is ~0.27 nm, and the planes of these groups are roughly parallel to the surface of the thylakoid membranes.[381,382] The protein groups that bind the Mn atoms include carboxylates, as shown in Fig. 23-35, and probably one or more histidine imidazole groups,[383] perhaps of His 332 and His 337 of the D1 chain.

The immediate donor of an electron to the reaction-center cation $P^+$ ($ChlZ^+$) of PSII was identified by EPR spectroscopy as a tyrosine radical.[384] On the basis of directed mutations this tyrosine, which is usually designated $\mathbf{Y_Z}$, is Tyr 161 of the D1 chain and is located ~1.2 nm from one of the chlorophylls of the $(Chl)_2$ pair.[371d,376,380,385–387] The two molecules in the pair are not in close contact, their central Mg atoms being ~1.0 nm apart. One of the two probably forms the $P680^{\bullet+}$ intermediate.[371d] $Y_Z$ is also close to the $Mn_4$ cluster and to the imidazole group of His 190 of subunit D1 as is shown in Fig. 23-34C.[386,388] The $Y_Z^{\bullet}$ radical is able to accept an electron from the $Mn_4$ cluster within 30–1300 μs depending upon the oxidation state of the cluster (see Fig. 23-35).[388] If a proton is transferred synchronously from a bound $H_2O$, a neutral –OH group will be created on $Y_Z$. The proton may then be transferred to His 190, which can eject the proton on its other nitrogen atom into the lumen. Alternatively $Y_Z^{\bullet}$ may accept an electron to become tyrosinate $-O^-$, which then donates an electron to $P^+$, while His 190 accepts a proton directly from a bound $H_2O$.

In a mechanism proposed by Hoganson and Babcock (Fig. 23-35) four successive transfers, each of one $H^+$ + one $e^-$, leads to a three-electron oxidation of Mn ions, e.g., from the $2^+$ and $3^+$ oxidation states to all $Mn^{4+}$, and to joining of the two water oxygens to form a manganese peroxide linkage. Oxidation of the peroxide dianion to $O_2$ by the adjoining $Mn^{4+}$ and $Mn^{3+}$ ions completes the cycle. This mechanism is hypothetical, and various alternatives have been presented.[387–389b] Most assume a structure similar to that shown in Fig. 23-35. Some are based on nonenzymatic model reactions.[390,391] Chloride ions are essential to $O_2$ formation[393,394] especially in going from state $S_2$ to $S_3$ and $S_3$ to $S_0$. This suggests that $Cl^-$ may function in passing electrons between Mn ions.[393,395] Calcium ions are also necessary, but it has been difficult to establish an exact function.[396–397a] A bicarbonate ion may also be an essential ligand in the $Mn_4$ cluster.[398,398a]

***ATP synthesis in chloroplasts.*** The flow of electrons between PSII and PSI (Fig. 23-18) is of great importance for ATP formation. As previously mentioned, plastocyanin is usually the immediate donor to P700 and serves as a mobile carrier to bring electrons to this reaction center. In this function it is analogous to cytochrome *c* of mitochondrial membranes. The essentiality of plastocyanin was shown by study of copper-deficient *Scenedesmus* (Fig. 1-11). The photoreduction of $CO_2$ by $H_2$ is impaired in these cells, but the Hill reaction occurs at a normal rate.

Like mitochondria, chloroplasts (when illuminated) pump protons across their membranes (Fig. 23-18). However, while mitochondria pump protons to the outside, the protons accumulate on the inside of the thylakoids. The ATP synthase heads of coupling factor $CF_1$ are found on the outside of the thylakoids, facing the stromal matrix, while those of $F_1$ lie on the insides of mitochondrial membranes. However, the same mechanism of ATP formation is used in both chloroplasts and mitochondria (Chapter 18).

The cleavage of water at PSII also occurs on the inside of the thylakoids. The splitting of one water molecule leaves two protons (one per electron) inside the thylakoids, while the electrons are "photoejected" through the lipid bilayer to acceptor $Q_A$ on the outside. The chlorophyll in PSI is likewise in contact with the inside of the bilayer with acceptor $A_0$ (Fig. 23-18) on the outside. Since the conversion of $NAD^+$ to NADH on the outside generates a proton, the overall reaction would be the pumping of one and a half protons per electron passing through the Z scheme.

The pathways involved in cyclic photophosphorylation in chloroplasts are not yet established. Electrons probably flow from the Fe–S centers $Fd_x$, $Fd_a$, or $Fd_b$ back to cytochrome $b_{563}$ or to the PQ pool as is indicated by the dashed line in Fig. 23-18. Cyclic flow around PSII is also possible. The photophosphorylation of inorganic phosphate to pyrophosphate ($PP_i$) occurs in the **chromatophores** (vesicles derived from fragments of infolded photosynthetic membranes) from *Rhodospirillum rubrum*. The $PP_i$ formed in this way may be used in a variety of energy-requiring reactions in these bacteria.[399] An example is formation of NADH by reverse electron transport.

***Protection of chloroplasts against radiation and oxygen.*** Carotenoids often act as accessory light-receiving pigments, but an additional function is protecting photosynthetic organisms against toxic effects of light.[400–403] Carotenoid photoprotection has been demonstrated in photosynthetic bacteria and in the reaction centers and light-harvesting complexes (LHC) of green plants. Excited chlorophyll molecules can pass from the singlet ($^1$Chl*) to the triplet ($^3$Chl*) state by intersystem crossing (Fig. 23-14). The triplet chlorophyll can then react with ordinary oxygen ($^3O_2$) to form singlet oxygen ($^1O_2$). The formation of $^3$Chl* is favored when the intensity of sunlight is high and energy is absorbed in the LHCs faster than it can be utilized in the reaction centers. Carotenoids are able to quench excitation of both $^3$Chl* and $^1O_2$. Strains of *Rhodobacter sphaeroides* that lack carotenoids rapidly form both $^3$BChl* and BChl$^+$ cation radicals in their LH1 and LH2 complexes (Fig. 23-26). However, the presence of carotenoids quenches the triplet bacteriochlorophyll effectively and no formation of BChl$^+$ radicals is seen.[403] Another effect of excessive light energy is the reaction of $O_2$ with the highly reducing P700* of photoexcited PSI. This interaction can form triplet oxygen, which can react to generate superoxide anion radicals, $H_2O_2$, and hydroxyl radicals.[224,227,402]

In higher plants the quenching of both $^3$Chl* and $^1O_2$ depends upon carotenoids[404] and also upon the large transthylakoid membrane potential that is generated by high light intensities.[405,406] High light intensity also induces a rapid reductive deoxygenation of epoxycarotenoids via the **xanthophyll** or **violaxanthin cycle** (Eq. 22-10).[401,407,407a,b] Epoxycarotenoids are found only in photosynthetic $O_2$ evolving organisms. Although occurring in response to light, the cyclic photodeoxygenation and reoxygenation is not a non-photochemical process. Violaxanthin contains the epoxy structure at both ends of the molecule. Reduction of one end produces **antheroxanthin** and of both ends **zeaxanthin**. These three carotenoids are found in almost all higher green plants and algae. The de-epoxidation is mediated by ascorbic acid, occurs in the lumen of the thylakoids, and is favored by the low pH developed during illumination. Epoxidation (Eq. 22-10) is catalyzed by a monooxygenase located on the stromal side. The significance of the xanthophyll cycle is puzzling. There may be specific binding sites, perhaps in the inner antenna complex on CP29 (Fig. 23-34), that bind zeaxanthin or antheroxanthin. This could alter the antenna structure to form an "exciton trap" in which the fluorescence lifetime would be decreased and excitation energy would be dissipated rapidly as heat.[408] Zeaxanthin is also found in the macular area of the primate retina.[401]

Recent studies, using an *Arabidopsis* mutant defective in the xanthophyll cycle, point to a chlorophyll-binding protein PsbS, which participates in nonphotochemical quenching at high light intensity.[401a,b] Another *Arabidopsis* protein, which is probably a blue light receptor, participates in an avoidance response by which chloroplasts move to the side wall to avoid strong light.[401c]

Yet another carotenoid function in PSII has been proposed. Under some conditions, when electron flow from $Tyr_z$ is blocked, the bound monomeric chlorophyll Z (Chlz) acts as a secondary electron donor to P680$^+$. The cytochrome $b_{559}$ subunits may have a similar function. Both cyt $b_{559}$ and the carotenoid are essential for assembly of PSII, and both may participate in a protective cycle.[409,410] Chloroplasts generate both $O_2$ and powerful reducing materials such as the membrane-bound FeS centers of PSI, which may form superoxide ions by single-electron donation to $O_2$. Probably for this reason, chloroplasts are rich in superoxide dismutase which converts superoxide to $O_2$ and $H_2O_2$. The latter can diffuse into peroxisomes and react with catalase and peroxidases. It can also be reduced to $H_2O$ within the chloroplasts by ascorbic acid and ascorbate peroxidase.[224,227] The resulting dehydroascorbate (Box 18-D) can be reduced back to ascorbate by glutathione (Box 11-B) and dehydroascorbate reductase, in the following electron transfer sequence:

$$\text{NADPH} \rightarrow \text{G–S–S–G} \rightarrow \text{dehydroascorbate} \rightarrow H_2O_2 \qquad (23\text{-}33)$$

Under extreme conditions of excess light energy **photoinhibition** is observed as a result of damage to the PSII structure.[411–415] The D1 polypeptide is cleaved, probably as a result of oxidation by $^1O_2$ and proteolysis. Damaged proteins are replaced and the PSII structure rebuilt, but the effect is a long-lasting decrease in photosynthetic efficiency. The cyanobacterium *Synechococcus* has three *PsbA* genes and resists UV-B radiation by exchanging a delicate D1 polypeptide with more resistant ones as necessary.[415] Other adaptations to varying light-intensity involve movement of light-harvesting complexes from the thylakoid stacks, which contain much PSII, to the stroma lamellae (Fig. 23-19), which contain more PSI. Some herbicides act by binding into the $Q_B$ site in PSII. They may cause light-induced oxidative stress that kills the plant.[415a]

## 5. Control of Photosynthesis

The key reaction of the Calvin–Benson cycle of $CO_2$ reduction is the carboxylation of ribulose bisphosphate to form two molecules of 3-phosphoglycerate (Eq. 13-48). The properties of ribulose bisphosphate carboxylase (**rubisco**, Figs. 13-10 to 13-12), which catalyzes this reaction, are discussed in Chapter 13. It

is controlled in part by $CO_2$ and by natural inhibitors,[416] but regulation of rubisco starts at the transcriptional level.

***Light-induced transcription.*** Plants depend upon light both as a source of energy and also for control of development. Many genes are activated by light in response to at least three groups of photoreceptors. These are **phytochromes** (Section H) and the blue light responsive **cryptochromes** (Section I) and the ultraviolet light **UV-B photoreceptors**.[417] The synthesis of chlorophyll, of reaction center proteins, and of many enzymes are controlled by light-induced transcription.[418–420] Among these processes are synthesis of both the large and small subunits of rubisco (Fig. 13-10). The small subunits are synthesized in the cytoplasm in a precursor form. After illumination the concentration of the rubisco mRNA may be increased 100-fold.[421,422] On the other hand, the large subunit of the carboxylase is encoded in chloroplast DNA, and stimulation of its synthesis by light appears to be at the translational level.[423]

***Light-induced control via the ferredoxin/thioredoxin system.*** Rubisco is activated by $CO_2$ (Chapter 13) and by fructose 6-*P* and is inhibited by fructose 1,6-$P_2$ (Fig. 23-36),[424] whose accumulation is a signal to turn off the carboxylase. Conversely, fructose 6-*P* in high concentrations turns on the Calvin–Benson cycle. Like the reactions of gluconeogenesis (Chapter 17), photosynthetic $CO_2$ incorporation is dependent on the highly regulated fructose-1,6-bisphosphatase. In chloroplasts it is activated by light through the mediation of reduced ferredoxin and thioredoxin.[424–427] The small mobile thioredoxin (Box 15-C) is reduced to its dithiol form by reduced ferredoxin[428–429a] and then reduces one or more disulfide linkages in the fructose 1,6-bisphosphatase to activate that enzyme (Fig. 23-36). Other light-activated enzymes of the Calvin–Benson cycle include sedoheptulose-1,7-bisphosphatase, the phosphoribulokinase that forms ribulose 1,5-bisphosphate and the $NADP^+$-dependent glyceraldehyde-3-phosphate dehydrogenase. $NADP^+$-dependent malate dehydrogenase, which has a major function in $C_4$ plants (see Fig. 23-38), is totally inactive in the dark but is activated by the ferredoxin–thioredoxin system in the light.[427] The activity of the LHCII complex is also affected.[429b]

Another aspect of chloroplast metabolism is synthesis of starch. Formation of ADP-glucose from glucose 1-phosphate is induced by 3-phosphoglycerate, a "feed-ahead" type of regulation (Fig. 23-36). Although fructose 2,6-bisphosphate is absent from chloroplasts, it has an important regulatory function in the cytoplasm of plants as it does in animals.[425,430] In the plant cytosol triose phosphates from the chloroplasts are converted to fructose 6-*P*, glucose 6-*P*, UDP-glucose, and sucrose. Inorganic phosphate $P_i$, which accumulates in plant vacuoles, also has a regulatory function.[431] It activates the kinase that converts fructose 6-*P* to fructose 2,6-$P_2$ and inhibits the phosphatase that converts the bisphosphate back to fructose 6-*P*. The accumulated fructose 2,6-$P_2$ inhibits fructose-1,6-bisphosphatase and slows the conversion of triose phosphates to sucrose (Eq. 23-34). Accumulation of fructose 6-*P* due to decreased utilization for sucrose formation will have a similar effect. However, both 3-phosphoglycerate and dihydroxyacetone phosphate have opposite effects and will act to remove the inhibition by lowering the fructose 2,6-$P_2$ level and to promote rapid sucrose formation (Eq. 23-34).[430]

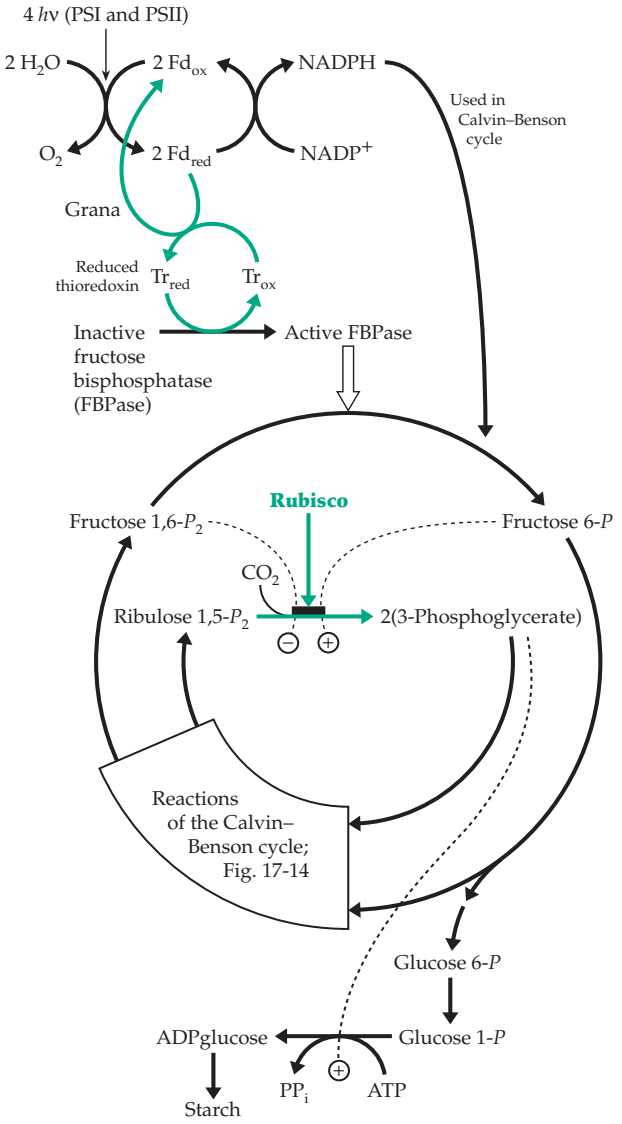
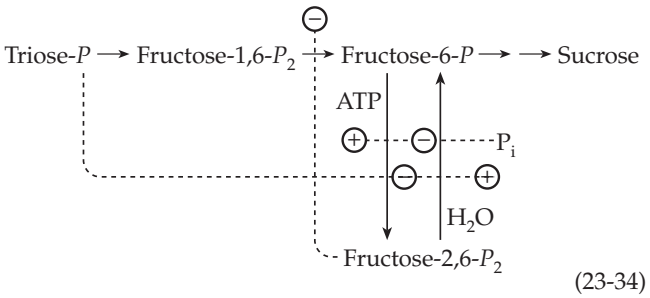



**Figure 23-36** Some control mechanisms for photosynthetic assimilation of carbon dioxide. After Buchanan and Schurmann[424] with modifications.




(23-34)

## 6. Photorespiration; $C_3$ and $C_4$ Plants

The first product of incorporation of $CO_2$ via the Calvin–Benson cycle is 3-phosphoglyceric acid (Box 17-E). It was the rapid appearance of radioactivity from $^{14}CO_2$ in phosphoglycerate and other three-carbon ($C_3$) compounds that permitted Calvin and associates, using green algae, to work out the complex cycle as it is shown in Fig. 17-14. Green algae, spinach, and many common crop plants are often known as **$C_3$ plants**. Another group of plants, mostly of tropical origin and capable of extremely fast growth, behave differently.[224,227,432] In these plants, which include sugar cane, maize, and crabgrass, radioactivity from $^{14}C$-containing $CO_2$ is found first in the $C_4$ compounds oxaloacetate, malate, and aspartate. These **$C_4$ plants** are characterized by high efficiencies in photosynthesis, a fact that explains the rapid growth of crabgrass and the high yield of corn. Maximum rates of $CO_2$ incorporation may attain 40–60 mg of $CO_2$ per square decimeter of leaf surface per hour (~0.3 mmol $CO_2$ $m^{-1}s^{-1}$ or ~0.10 mol $CO_2$ per mol of total chlorophyll per second), more than twice that for common crop plants.

Like all other organisms plants respire in the dark, but illumination of $C_3$ plants markedly increases the rate of oxygen utilization. This light-enhanced respiration (**photorespiration**) may attain 50% of the net rate of photosynthesis. Photorespiration prevents plants from achieving a maximum yield in photosynthesis. For this reason, its understanding and control assume great importance in agriculture. It is difficult to measure the rate of photorespiration, and the literature on the subject often refers instead to the **$CO_2$ compensation point**. This is the $CO_2$ concentration (at a given constant light intensity) at which photosynthetic assimilation and respiration balance. (Similarly, the **light compensation point** is the light intensity at which the rate of photosynthetic $CO_2$ incorporation and that of respiration exactly balance.) Normal air has a $CO_2$ content of ~0.03% or 300 ppm. For common $C_3$ crop plants the $CO_2$ compensation point is ~40–60 ppm at 25°C. The $C_4$ plants are characterized by a much lower $CO_2$ compensation point, often less than 10 ppm. In strong sunlight the $CO_2$ level of air in a field of growing plants drops. Furthermore, as the temperature rises on a hot day, the $CO_2$ compensation point rises. The result is a serious decrease in efficiency of photosynthesis for the $C_3$ plants but not for the $C_4$ plants.

***Metabolism of glycolic acid.*** The 2-carbon glycolic acid is formed in large quantities in the chloroplasts of $C_3$ plants and moves out into the cytosol.[433,434] The major source of this acid is phosphoglycolate whose formation is catalyzed by rubisco in the chloroplasts through competition of $O_2$ for the $CO_2$ binding site of the enzyme (Eq. 13-50). It is easy to understand why an increase in the $O_2$ pressure in air increases the $CO_2$ compensation point for a plant. Another less important source of glycolate is transketolase, which may yield glycolaldehyde as a side product (Eq. 17-15). Glycolaldehyde can be oxidized readily to glycolate. Glycolate is metabolized rapidly, some in the chloroplasts,[435] but most in the peroxisomes. There the flavoprotein glycolate oxidase converts it to glyoxylate with formation of $H_2O_2$ (Fig. 23-37).[436] Some of the hydrogen peroxide formed may react nonenzymatically, decarboxylating glyoxylate to formate and $CO_2$, but most is probably destroyed by peroxidases or catalase. The latter enzyme is lacking in chloroplasts, one reason why oxidation of glycolate must occur in the peroxisomes.

Glyoxylate undergoes transamination to glycine, which can be oxidatively decarboxylated (Fig. 15-20) in the mitochondria. It can also be converted to serine,[437] some of which returns to the peroxisomes to be oxidized to hydroxypyruvic acid and glyceric acid (Fig. 23-37). The latter can be synthesized into glucose. The net result is the stimulation of a large amount of metabolism that ultimately produces $CO_2$ and apparently accounts for the light-induced respirations of plants. Because much of the glycine formed in the peroxisomes is oxidatively decarboxylated in the mitochondria, photorespiration also generates large amounts of $NH_3$. This is recycled into amino acids within the photosynthetic cells (green lines in Fig. 23-37), an energy-requiring process.[438]

Although much metabolism occurs as a result of photorespiration, it appears to waste energy rather than to provide energy to the plant. Why then don't plants avoid this process? Wouldn't a small change in the structure of ribulose bisphosphate carboxylase allow plants to avoid photorespiration and to grow more efficiently? The answer is not clear. It has been difficult to create such modified plants, and there is a possibility that they would not grow well. One theory is that photorespiration protects plants when the $CO_2$ pressure is low and the absorbed light would damage the chloroplasts if there were not a way to utilize the accumulating reduced Fe–S proteins generated by PSI. Photorespiration provides a mechanism.[434] Most

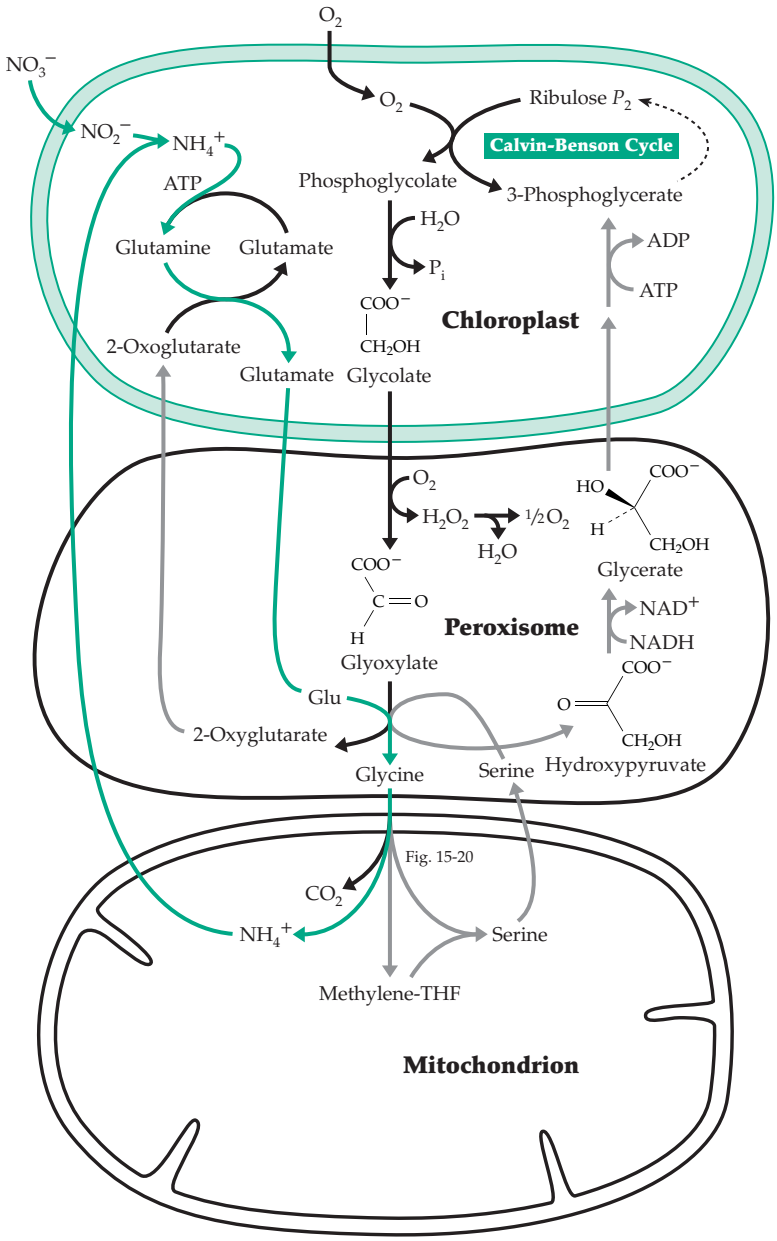
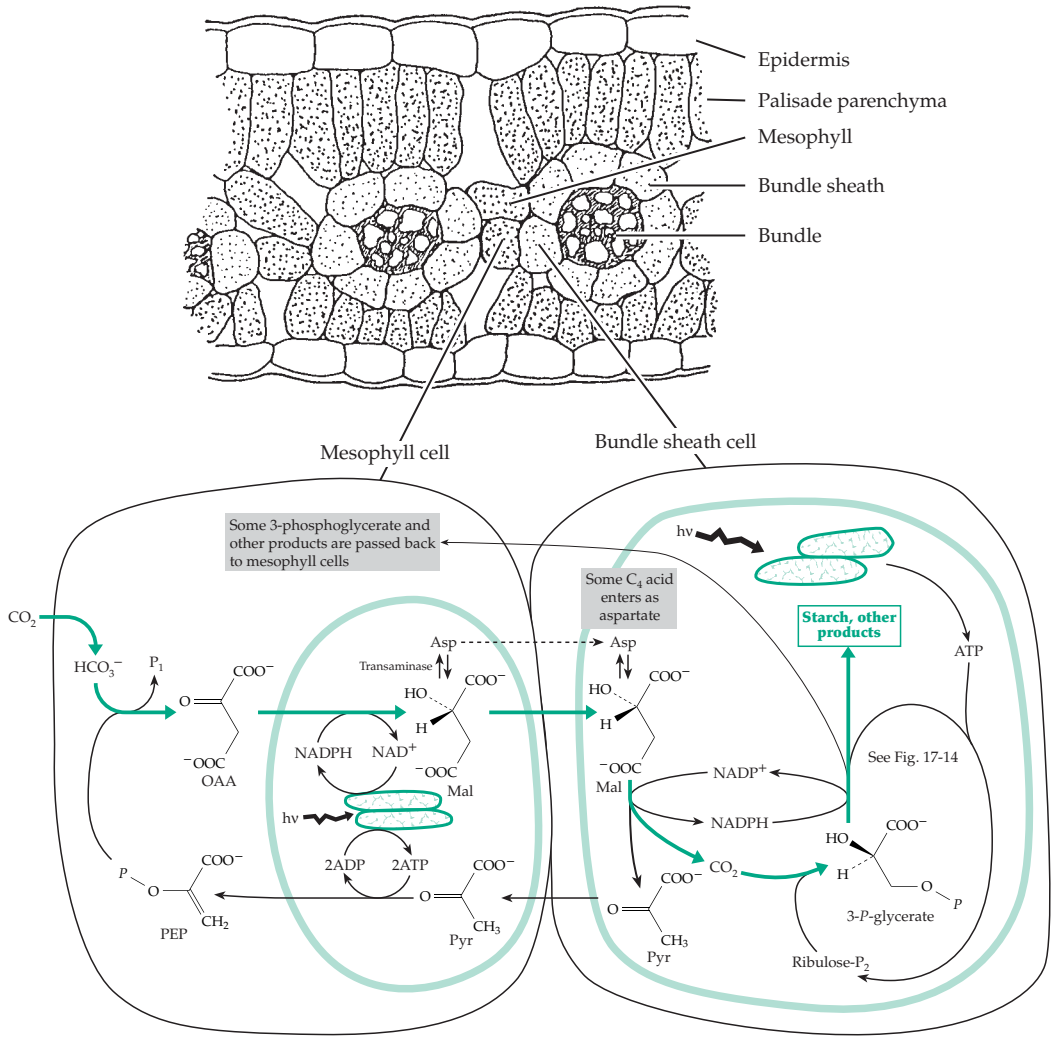



**Figure 23-37** Production of glycolate by chloroplasts and some pathways of its metabolism in peroxisomes and in mitochondria. After Tolbert[436] and Givan *et al.*[438]

efforts to breed plants with lower photorespiration rates or to inhibit it chemically have failed.[433,439]

***The $C_4$ cycle for concentration of carbon dioxide.*** The $C_4$ plants reduce their rate of photorespiration by using a $CO_2$ concentrating mechanism that enables them to avoid the competition from $O_2$. All species of $C_4$ plants have a characteristic internal leaf anatomy in which a single dense layer of dark green cells surrounds the vascular bundles in the leaves. This **bundle sheath** is surrounded by a loosely packed layer of cells, the **mesophyll**, an arrangement that is sometimes called the "Kranz anatomy." In $C_4$ plants there is a separation of the chemical reactions between the mesophyll and the bundle sheath cells. The incorporation of $CO_2$, as bicarbonate ion into oxaloacetate, occurs in the mesophyll cells, principally through the action of PEP carboxylase (Fig. 23-38). Oxaloacetate is reduced to malate by light-generated NADPH. Alternatively, it undergoes transamination to aspartate. Both malate and aspartate then diffuse out of the mesophyll cells and into bundle sheath cells where the malate undergoes oxidative decarboxylation via the malic enzyme (Eq. 14-42) to pyruvate (Fig. 23-38). Aspartate also can be converted to oxaloacetate, malate, and pyruvate in the same cells. The overall effect is to transport $CO_2$ from the mesophyll cells into the bundle sheath cells along with two reducing equivalents, which appear as NADPH following the action of the malic enzyme. The $CO_2$, the NADPH, and additional NADPH generated in the chloroplasts of the bundle sheath cells are then used in the Calvin–Benson cycle reactions to synthesize 3-phosphoglycerate and other materials. Of the $CO_2$ used in the bundle sheath cells, it is estimated that 85% comes via the $C_4$ cycle and only 15% enters by direct diffusion. The advantage to the cell is a higher $CO_2$ tension, less competition with $O_2$, and a marked reduction in photorespiration.

The pyruvate produced in the bundle sheath cell is largely returned to the mesophyll cells where it is acted upon by **pyruvate-phosphate dikinase**.[441] This unusual enzyme (Eq. 17-55) phosphorylates pyruvate to PEP while splitting ATP to AMP and $PP_i$. The latter is in turn degraded to $P_i$. In effect, two high-energy linkages are cleaved for each molecule of pyruvate phosphorylated. Because of this extra energy need, it is thought that cyclic photophosphorylation is probably more important in the chloroplasts of the mesophyll cells than in the bundle sheath cells. It also accounts for the fact that $C_4$ plants are less efficient than $C_3$ plants under cool or shaded conditions.[442] Other $CO_2$-concentrating mechanisms exist in plants.[442a] For example, cyanobacteria accumulate $HCO_3^-$ ions in carboxysomes, polyhedral bodies to which rubisco adheres. An ABC type ATP-dependent transporter powers the bicarbonate accumulation.[442b]




(23-35)

**Figure 23-38** The $C_4$ cycle for concentrating $CO_2$ in the $C_4$ plants. From Haag and Renger with alterations.[440]

***Metabolism in the family Crassulaceae.*** The crassulacean plants are a large group that includes many ornamental succulents such as *Sedum*. They have a remarkable metabolism by which large amounts of malic and isocitric acids are synthesized at night. During the day when photosynthesis occurs these acids disappear. The stomata in the leaves (Chapter 1) stay closed during the day and open only at night, an adaptation that permits the plants to live with little water. However, this requires that the plant accumulates carbon dioxide by night and incorporates it photosynthetically into organic compounds by day.[443] A possible mechanism is shown in Fig. 23-39. On the left side of the figure are reactions by which starch can be broken down at night to PEP. While it would also be possible to produce that compound by the glycolysis reactions, labeling studies have indicated that the pentose phosphate pathway is more important.[330] The PEP acts as the $CO_2$ acceptor to create oxaloacetate, which is then reduced to malic acid. A balanced fermentation reaction (Fig. 23-39; Eq. 23-35) can be written by using the NADPH formed in the conversion of glucose 6-*P* to ribulose 5-*P*. During the day when ATP and NADPH are available in abundance from photoreactions, the conversions on the right side of the figure can take place. The initial step, the release of $CO_2$ from malic acid by the malic enzyme, is the same as that employed by $C_4$ plants. In this case, it is used to release the $CO_2$ stored by night, making it available for incorporation via the Calvin–Benson cycle. The remaining pyruvate is reconverted to starch.

$$C_6H_{10}O_5\ (\text{starch}) + 2\ CO_2\ (g) \rightarrow 2\ C_4H_4O_5^{2-}\ (\text{malate}) + 4\ H^+$$
$$\Delta G'\ (\text{pH } 7) = -159\ \text{kJ mol}^{-1} \qquad (23\text{-}35)$$

Many plants store substantial amounts of malate in their cytoplasm and in vacuoles. It apparently

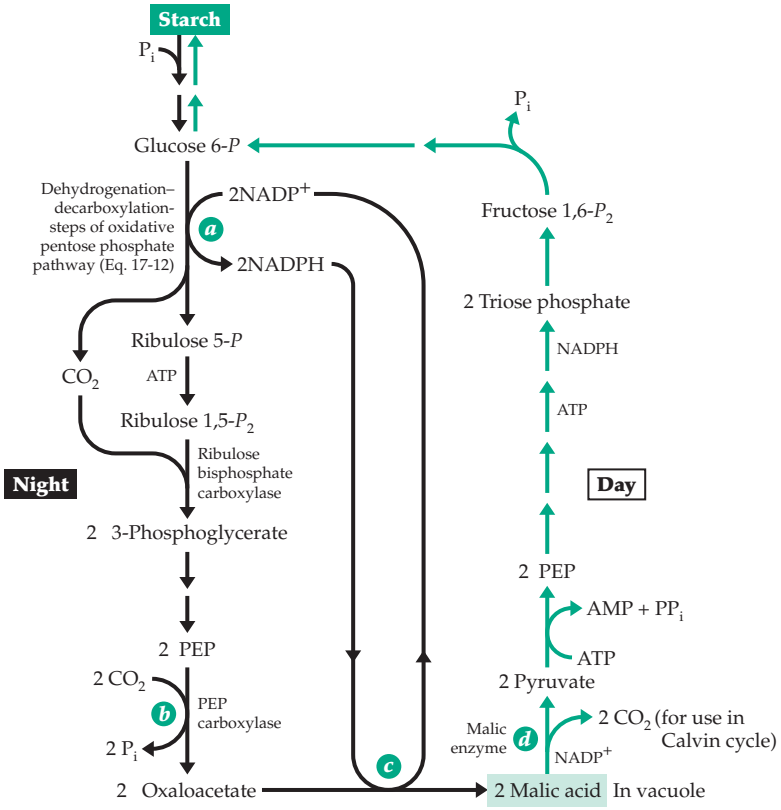



**Figure 23-39** A proposed night–day cycle of crassulacean acid metabolism.

serves as a ready reserve for carbohydrate synthesis.

## 7. Photosynthetic Formation of Hydrogen

A system consisting of chloroplasts, ferredoxin, and hydrogenase has been used to generate $H_2$ photosynthetically.[444] This may be a prototype of a method of solar energy generation for human use. Another photochemical hydrogen-generating system makes use of both the nitrogen-fixing heterocysts and photosynthetic vegative cells of the cyanobacterium *Anabaena cylindrica*.[445] In this instance hydrogen production is accomplished by nitrogenase (Eq. 24-6). Photogeneration of $H_2$ by bacteria is just one of many kinds of photometabolism observed among photosynthetic microorganisms.

# F. Vision

The light receptors of the eye perform a very different function from those of chloroplasts. Visual receptors initiate nerve impulses, and their primary requirement is a high sensitivity. By the use of stacked membranes containing a high concentration of an intensely absorbing molecule[446,447] the most sensitive visual receptors are able to trap nearly every photon that strikes them. The retina of the human eye contains more than $10^8$ tightly packed receptor cells of two types. The **rod cells** are extremely sensitive. Used for night-time vision, they give a "black and white picture" and are concentrated around the periphery of the retina. The retina works as a coincidence detector. An ensemble of ~500 rods must register 5–7 isomerizations within a few tenths of a second in order to trigger a nerve impulse.[448] The less sensitive **cone cells**, which are most abundant in the center of the retina, are of three types with different spectral sensitivities. They provide color vision.

The retinal receptors have a very active metabolism. Human rod cells (Fig. 23-40) may live and function for a hundred years.[190] A self-renewal process leads to a casting off of the older membranous discs from the end of the rod[194] and replacement by new discs at the end nearest to the nucleus. The rod outer segment is surrounded by a plasma membrane. Within the membrane but apparently not attached to it are ~500 stacked discs of ~2 μm diameter and with a repeat distance between centers of ~32 nm. Each disc is enclosed by a pair of membranes each ~7 nm thick with a very narrow space between them. From electron micrographs it appears that this space within the discs is sealed off at the edges. Somewhat larger spaces separate adjacent discs.

The membranes of the rod discs are ~60% protein and 40% lipid (Table 8-3). About 80% of the protein is **rhodopsin** (visual purple), a lipoprotein that is insoluble in water but soluble in detergent solutions. Digitonin is widely used to disperse rhodopsin molecules because it causes no change in optical properties. In addition to rhodopsin, in the outer segment discs of frog retinal rods, there are ~65 molecules of phospholipid and smaller amounts of other materials for each molecule of rhodopsin (Table 8-3). The cone cells have a similar architecture but have a different shape and contain different light receptors. The receptors in the cones are present in deep indentations of the plasma membrane rather than in discs within the cytoplasm.

## 1. Visual Pigments

The rod pigment rhodopsin is readily available from cattle retinas and has been studied for many

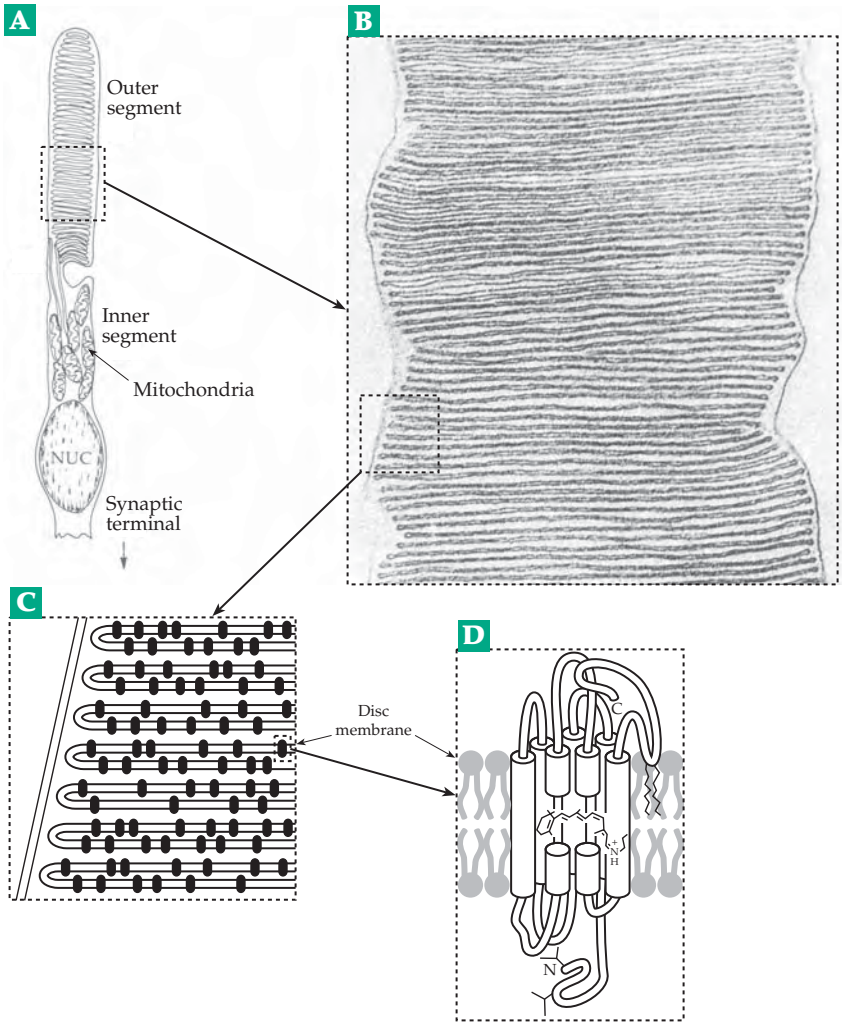
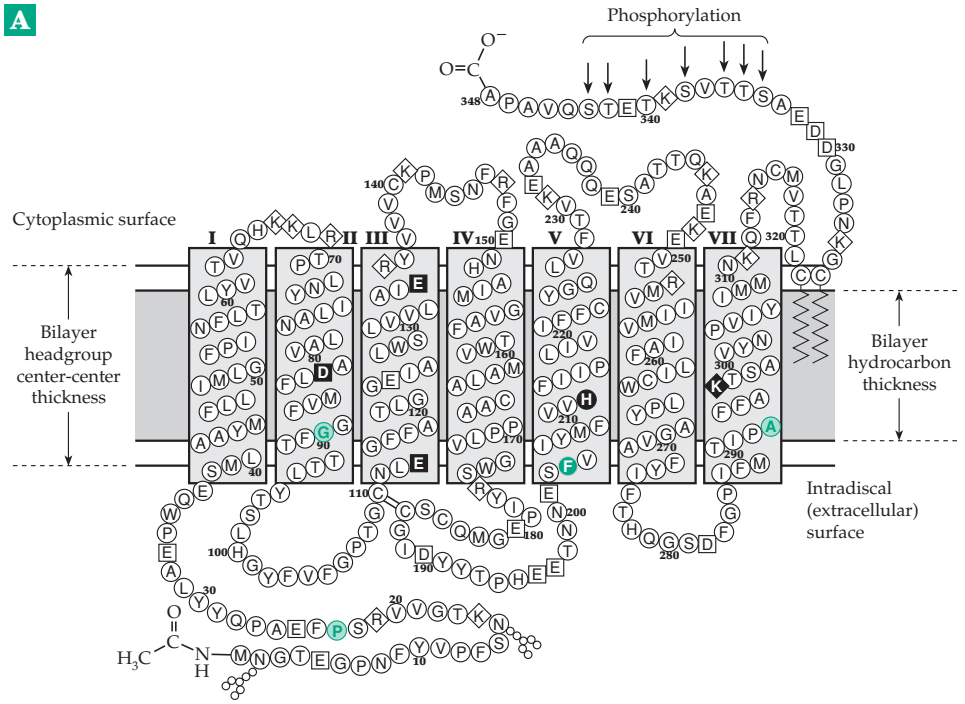



**Figure 23-40** (A) Diagram of a vertebrate rod cell. From Abrahamson and Fager.[446] (B) Electron micrograph of a longitudinal section of the outer segment of a retinal rod of a rat. There are 600–2000 discs per rod and 2 x $10^4$ and 8 x $10^5$ rhodopsin molecules per disc. Courtesy of John E. Dowling. (C) Enlarged section; from Dratz and Hargrave.[451] (D) Schematic drawing of rhodopsin. The two α helices in the front have been partly cut away to reveal the 11-*cis* retinal in protonated Schiff base linkage to lysine 296. From Nathans.[448] Courtesy of Jeremy Nathans.

years. It has a molecular mass of ~41 kDa of which ~2 kDa is contributed by two asparagine linked oligosaccharides.[195] Both bovine and human rhodopsins consist of a 348-residue protein known as **opsin** to which is bound a molecule of vitamin A aldehyde, **retinal**. Human and bovine opsins are 93% homologous. A totally synthetic 1057 base pair gene for bovine rhodopsin was made by Khorana and associates.[449,450] The gene was constructed with 28 unique sites for cleavage by specific restriction endonucleases. These have allowed easy specific mutation of the gene and production of a wide variety of mutant forms of opsin. Similar synthetic genes have been constructed for the three human cone pigments[452] and for the related bacterial protein **bacteriorhodopsin**.[453]

### *Transmembrane structure.*

From its circular dichroism rhodopsin was estimated to be 60% helical, and its amino acid sequence suggested that it contains seven parallel membrane-spanning helices (Fig. 23-41)[451] just as does bacteriorhodopsin (Section G; Fig. 23-45). Rhodopsin and other visual pigments are also members of the large family of **G-protein coupled receptors**, which includes the $\beta_2$ adrenergic receptor pictured in Fig. 11-6. It has been hard to determine the three-dimensional structure of rhodopsin or other receptors of this family. However, their relationship to bacteriorhodopsin, whose structure was obtained in 1975 by electron crystallography[454] and more recently by X-ray crystallography at 0.15 nm resolution,[455] permitted modeling based on similarities among the proteins.[456–458] New results of electron crystallography[459,460] and mass spectrometry[461] have been combined with studies of mutant forms of rhodopsin and other visual pigments to provide the picture given in Fig. 23-41A,B. Recently a higher resolution structure (Fig. 23-41D) has been obtained by X-ray diffraction.[461a–c] Mutations have been introduced in every part of the rhodopsin molecule, and the effects on photoreception, protein stability, and other properties have been observed.[448,450,462–465] Some of the essential residues identified are indicated in Fig. 23-41A, a schematic diagram showing the seven helices and connecting loops. Figure 23-41B shows an end view of the helix bundle with its retinal prosthetic group buried in the interior of the protein. Rhodopsin is roughly cylindrical with a length of 6–6.5 nm and a diameter of ~2.8 nm and is embedded in the phospholipid bilayer with its long axis perpendicular to the membrane surface (Fig. 23-40). The two oligosaccharide chains, which are attached near the N terminus, project into the intradiscal space on the side away from the cytoplasm. Palmitoyl groups on two cysteine side chains help to anchor the protein. Rhodopsin apparently exists in the mem-




● A few residues essential to the mechanism of vision.

○ Point mutations causing serious diseases. P23H is a frequent cause of retinitis pigmentosa. Mutations G90D and A292E cause congenital night blindness.

● The mutation C203R in the green-sensitive pigment causes red-green color blindness.

**Figure 23-41** (A) Model of the topological organization of bovine rhodopsin according to the consensus analysis of Baldwin.[456] Oligosaccharide chains are portrayed on the glycosylation sites Asn 2 and Asn 15. Glutamate 113 provides the counter ion for the *N*-protonated retinal Schiff base. Cysteines 110 and 187 form an essential disulfide linkage. Histidine 211 modulates the interconversion of the metarhodopsin forms I and II. Lysine 296 forms a Schiff base with 11-*cis*-retinal. Cysteines 322 and 323 are sites of palmitoylation, and as many as six serine and threonine hydroxyls (indicated by green arrows) may become phosphorylated during desensitization of rhodopsin. Aspartate 83, glutamate 134, and histidine 211 may be essential for proton movements. The point mutation C203R in the green-sensitive pigment causes red-green color blindness. The mutation

branes as monomers. From the composition it can be calculated[451] that the average center-to-center distance of the cylindrical molecules must be ~5.6 nm.

***The visual chromophores.*** Rhodopsin has been an object of scientific interest for over 100 years.[462] Wald and associates[469,470] established that rhodopsin contains 11-*cis*-retinal bound to the opsin in Schiff base linkage (Eq. 23-36). When native rhodopsin is treated with sodium borohydride, little reduction is observed. However, after the protein is bleached by light, reduction of the Schiff base linkage becomes rapid, and the retinal is incorporated into a secondary amine, which was identified as arising from Lys 296.

In a crystal structure[471] 11-*cis*-retinal has the 12-*s*-*cis* conformation shown at the top in Eq. 23-36 rather than the 12-*s*-*trans* conformation at the center and in which there is severe steric hindrance between the 10–H and 13–$CH_3$. Nevertheless, $^1$H– and $^{13}$C–NMR spectroscopy suggest that the retinal in rhodopsin is in a twisted 12-*s*-*trans* conformation.[472,472a] The Schiff base of 11-*cis*-retinal with *N*-butylamine has an absorption maximum at ~360 nm but *N*-protonation, as in the structure in Eq. 23-36, shifts the maximum to 440 nm with $\varepsilon_{max}$ = 40,600 $M^{-1}$ $cm^{-1}$ (Fig. 23-42). This large shift in the wavelength of the absorption maximum (the *opsin shift*) indicates that binding to opsin stabilizes the light-excited state by almost 33 kJ/mol compared to that in the free *N*-protonated Schiff base. This is evidently the result of a fixed negative charge, that of Glu 113, which is near the polyene chain of the retinal (Fig. 23-41B) and probably separated from it by a hydrogen-bonded water molecule.[473–477]

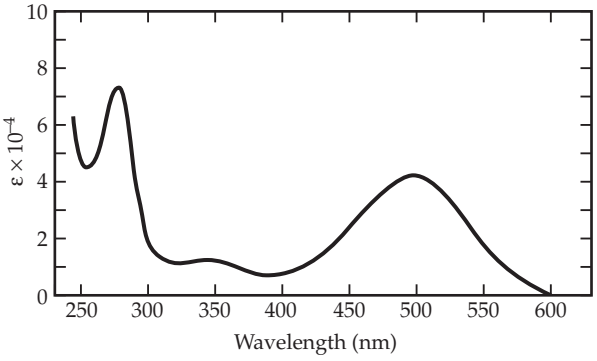
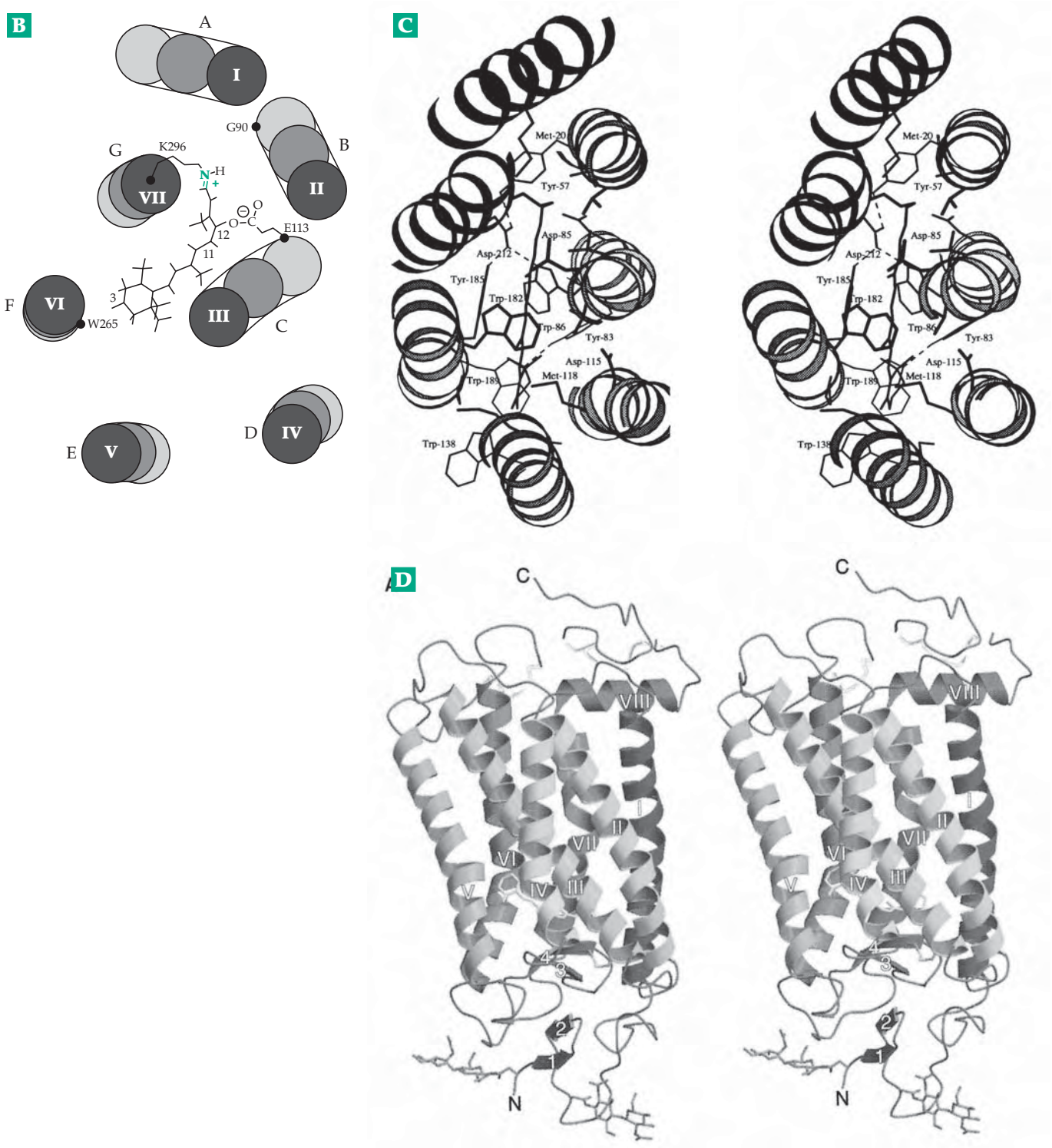
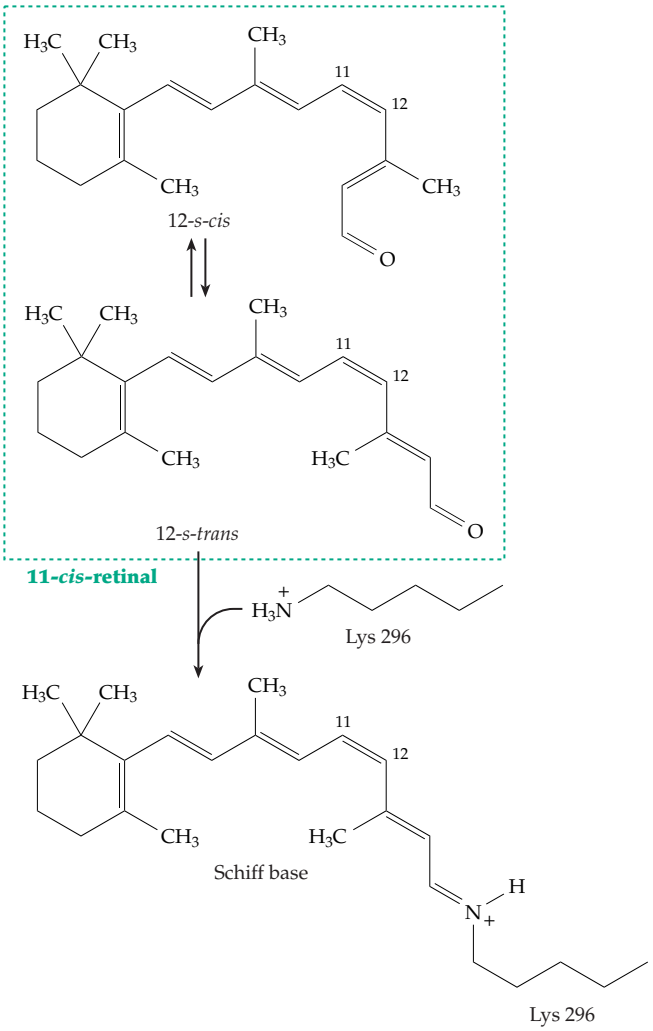



**Figure 23-42** Absorption spectrum of cattle rhodopsin in aqueous dispersion with a nonionic detergent. From H. Shichi *et al.*[486,487]

P23H in rhodopsin is one of the most frequent causes of retinitis pigmentosa, and mutations G90D and A292E cause congenital night blindness. From Barnidge *et al.*[461] with modifications. (B) Structural model of rhodopsin based on the helix arrangement of Baldwin[456] and NMR constraints. The seven transmembrane helices, viewed from the cytoplasmic surface, are shown at three levels indicated by differences in shading. The α-carbons of residues of interest are shown in dots of various sizes, indicating the depth from the cytoplasmic domain. Gly 90, Glu 113, and Lys 296 are on the extracellular half of the transmembrane domain and close to each other in space. The 11-*cis*-retinal chromophore has been incorporated into the model using NMR constraints, which require a close interaction between Glu 113 and $C_{12}$ of the chromophore. The relative position of the β-ionone ring and Trp 265 agrees well with crosslinking data of Zhang *et al.*[466] From Han and Smith.[467] (C) Stereoscopic view of the retinal-binding pocket of **bacteriorhodopsin** viewed from the cytoplasmic surface. The retinal, in Schiff base linkage to Lys 216, runs across the central cavity from top to bottom in this view. From Grigorieff *et al.*[468] (D) Ribbon drawing of bovine rhodopsin (stereoview). From Palczewski *et al.*[461a]






(23-36)

Three types of cone cells in the human retina are needed for color vision. Four genes specify the proteins for rhodopsin and for related cone photoreceptors absorbing blue (~425 nm), green (~530 nm), and red (~560 nm) light.[477–479b] All of the cone opsins also bind 11-*cis*-retinal. The rhodopsin gene is located on human chromosome 3, while that of the blue pigment is found on chromosome 7. However, the green and red sensitive pigment genes, whose sequences are 96% identical,[478] are close together on the q arm of the X-chromosome and near the gene for glucose-6-phosphate dehydrogenase. Examination of cloned DNA from persons with inherited red-green color-blindness shows that loss of a functional form of one of these genes is usually responsible for the problem.[480–482] Among Caucasians 8% of males and 1% of females differ from the normal in their color vision. About 30% of affected males are **dichromats** and lack either the red-sensitive pigment (they are protanopes) or the green-sensitive pigment (deuteranopes). They usually have a partial gene deletion. The other 70% often have hybrid genes created by errors in recombination during cell division.[483] A few deuteranopes have the point mutation C203R in the green pigment. John Dalton, of atomic theory fame, reasoned that his red-green confusion resulted from a blue tint in the vitreous humor of his eyes and ordered that they be dissected after his death (in 1884). There was no blue tint but DNA analysis performed more recently showed that Dalton was a deuteranope.[484] Defects in the blue-sensitive receptor are relatively rare affecting about 1 in 500 persons, while only one person in 100,000 has a total lack of color discrimination.[481,485]

All retinal-dependent visual pigments form Schiff bases with lysine side chains of the photoreceptor proteins. How can the same chromophore be "tuned" to absorb across the wavelength range of 360 to 635 nm? Modern techniques such as resonance Raman[477,479] and FTIR spectroscopies and study of mutant forms[488] have shown that interaction of the conjugated double bond system of the chromophores with immediately adjacent dipoles of side chain groups and peptide linkages is sufficient to account for the great variability in absorption maxima.

Visual pigments of many species have been investigated. Most vertebrate animals have a rhodopsinlike pigment plus a variable number of cone pigments. Mammals typically have only two, a short-wavelength pigment absorbing maximally in the ultraviolet, violet, or blue region[488a-c] and a long-wavelength pigment with maximum absorption in the green or red region.[489] The bottlenose dolphin has only a rod pigment of $\lambda_{max}$ 524 nm.[489] In contrast, the chicken has four cone pigments with maximum absorption for violet, blue, green, and red.[490,490a] The red light receptor, called **iodopsin**, absorbs maximally at 571 nm. However, it binds chloride ions which induce an additional 40-nm red shift. The $Cl^-$-binding site involves His 197 and Lys 200 which are present in an extracellular loop (Fig. 23-41A) and quite far from the bound retinal.[490] Human red and green color vision pigments, and also a green-sensitive pigment of the reptile *Gecko gecko*, undergo spectral shifts upon binding of $Cl^-$ in the same site.[491] However, rhodopsin and most other visual pigments do not share this behavior.

Fishes live in a variety of environments and have a diversity of visual pigments. Goldfish have genes for five opsins, one of which gives rise to an ultraviolet light receptor. They are also sensitive to polarized light.[492] Related visual pigments occur throughout the animal kingdom. Even the eyespot of the alga *Chlamydomonas* (Fig. 1-11) contains rhodopsin with some sequence homology to invertebrate opsins.[493,494] The pineal glands of chickens and probably of reptiles[495–496a] as well as those of fish[497] also contain rhodopsinlike pigments. In a few freshwater marine species the visual pigments (**porphyropsins**) contain **3-dehydroretinal**. The peak positions of light absorption depend both upon the nature of the bound alde-

hyde and on the protein, the latter having the larger effect. Thus, retinal-based pigments absorb in the entire range 467–528 nm (18,900–21,400 $cm^{-1}$). The fruit fly, *Drosophila*, contains 3-hydroxy-11-*cis*-retinal in its rhodopsin and also contains other related photoreceptors.[498]

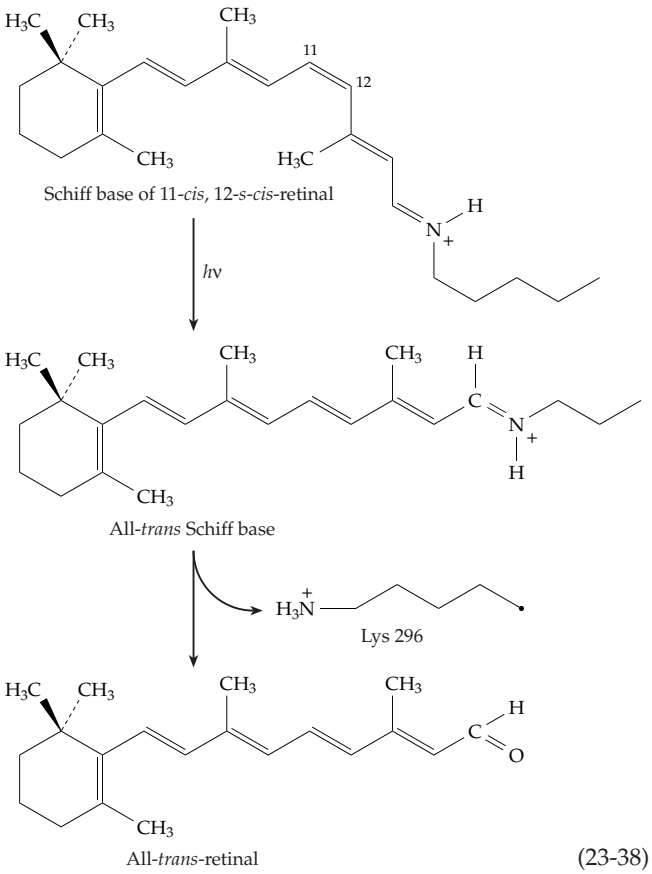
## 2. The Light-Induced Transformation

The retinal Schiff base chromophore is embedded in rhodopsin with its transition dipole moment parallel to the plane of the discs, i.e., perpendicular to the direction of travel of the incoming photons. Absorption of a photon leads to a sequence of readily detectable spectral changes.[37,461b,499,500] The relaxation times indicated in Eq. 23-37 are for 20°C.

Rhodopsin (498 nm, 20.1 x $10^3$ $cm^{-1}$)
$h\nu \downarrow \tau < 6 \times 10^{-12}$s
Bathorhodopsin (prelumirhodopsin; 543 nm, 18.4 x $10^3$ $cm^{-1}$)
$\downarrow \tau$ ~50 ns
Lumirhodopsin (497 nm), 20.2 x $10^3$ $cm^{-1}$)
$\downarrow \tau$ ~ 50 μs
Metarhodopsin I (478 nm, 20.9 x $10^3$ $cm^{-1}$)
$\downarrow\uparrow$ milliseconds
Metarhodopsin II (380 nm, 26.2 x $10^3$ $cm^{-1}$)
$\downarrow$ seconds
All-*trans*-retinal (387 nm, 25.8 x $10^3$ $cm^{-1}$) + opsin (23-37)

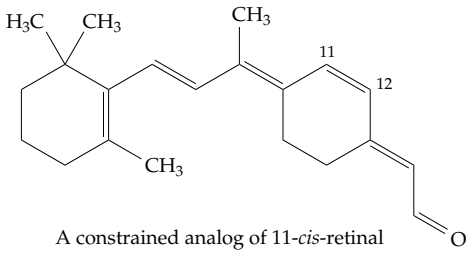
The intermediate chemical species have been named bathorhodopsin, lumirhodopsin, and metarhodopsin I and II. At very low temperatures a transient form **photorhodopsin** with a wavelength maximum at ~580 nm may precede bathorhodopsin.[461b,501–502a] Furthermore, nanosecond photolysis of rhodopsin has revealed a **blue-shifted intermediate** that follows bathorhodopsin within ~40 ns and decays into lumirhodopsin.[500,503,504] The overall result is the light-induced isomerization of the bound 11-*cis*-retinal to all-*trans*-retinal (Eq. 23-38) and free opsin. The free opsin can then combine with a new molecule of 11-*cis*-retinal to complete the photochemical cycle.

What are the chemical structures of the intermediates in Eq. 23-37, and why are there so many of them? The answer to the last question is that the initial photochemical process is very fast. Subsequent conformational rearrangments and movement of protons are slower, occur in distinct steps, and give rise to the observed series of intermediates. To shed light on these processes many experiments have been done with analogs of retinal,[502,505–508] often using very rapid spectroscopic techniques.[37,508] These studies have shown that the isomerization of the Schiff base from 11-*cis* to all-*trans* occurs in the first very rapid step of



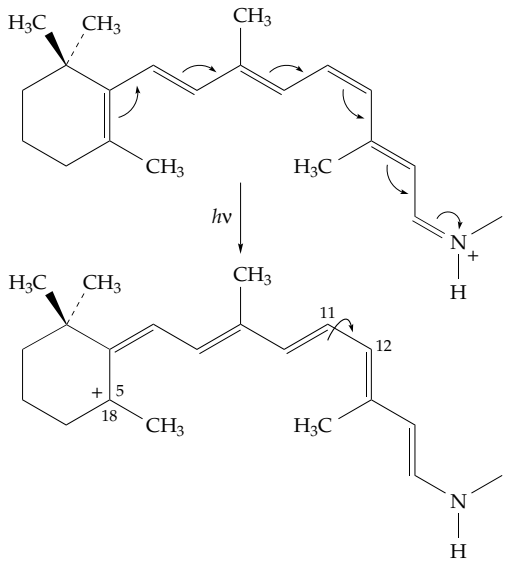

(23-38)

Eq. 23-37.[499,509–510a] Constrained analogs of 11-*cis* retinal also combine with opsin to form rhodopsinlike molecules with absorption maxima near 500 nm.




However, most of these analogs cannot isomerize and illumination does not produce bathorhodopsinlike molecules.[504,509,511]

In the photoexcited state the positive charge on the Schiff base is delocalized. For example, with some of the positive charge located on C5, rotation could occur around the more nearly single C11–C12 bond to give the all-*trans* isomer (Eq. 23-39). However, it seems more likely that simultaneous rotation occurs around two connected single and double bonds.[511a] The mechanism of this photoisomerization, which is among the fastest known chemical reactions, is still being investigated.[511b] In native rhodopsin the conversion to bathorhodopsin occurs with a high quantum




(23-39)

yield of 0.67 within 0.2 ps, a time comparable to the period of torsional vibrations of the retinal. This extreme speed suggests that the isomerization is a concerted process that is **vibrationally coherent**.[511c] Vibrational motion in the electronically excited state is utilized in the isomerization process.[506,512]

The reaction sequence of Eq. 23-37 can be slowed by lowering the temperature. Thus, at 70K illumination of rhodopsin leads to a **photostationary state** in which only rhodopsin, bathorhodopsin, and a third form, **isorhodopsin**, are present in a constant ratio.[510] Isorhodopsin (maximum absorption at 483 nm)[513] contains 9-*cis*-retinal and is not on the pathway of Eq. 23-37. Resonance Raman spectroscopy at low temperature supports a distorted all-*trans* structure for the retinal Schiff base in bathorhodopsin.[510] The same technique suggests the *trans* geometry of the C=N bond shown in Eqs. 23-38 and 23-39. Simple Schiff bases of 11-*cis*-retinal undergo isomerization just as rapidly as does rhodopsin.[514]

Some step in the sequence of Eq. 23-37 must initiate a chemical cascade that sends a nerve impulse out of the rod cell. This is accomplished through a GTP-dependent G protein cascade as outlined in Fig. 23-43. Light-activated rhodopsin initiates the cascade by interacting with the G protein **transducin**. Energy for the activation comes from the quantum of absorbed light. While the primary chemical reaction has long been recognized as the isomerization of the retinal Schiff base,[515] it is not obvious how this generates the signal for transducin to bind and become activated.

The seven helices of rhodopsin form a "box" around the bound retinal. The environment of the retinal is largely hydrophobic. However, there are also buried polar groups, some of which lie in conserved positions in more than 200 G-protein-coupled receptors[458] and internal water molecules whose vibrational spectra can be detected.[516] As in bacteriorhodopsin (Fig. 23-41, C) the buried polar groups and water molecules are doubtless hydrogen-bonded in an internal network. We can anticipate two effects of the isomerization reaction: (1) It will distort the shape of the box in which it occurs. (2) It will break some hydrogen bonds and allow new ones to form and may affect the balance of electrical charges within the protein. This in turn can lead to proton movements and alterations in the internal hydrogen-bonded network. Both of these anticipated effects have been observed.

Conformational changes induced in the rhodopsin protein by the isomerization of the retinal Schiff base include significant movement of the end of helix VI(F) at the cytosolic surface as well as smaller movements of other helices.[517,518] Spectroscopic measurements indicate that the Schiff base nitrogen remains protonated in both the bathorhodopsin and metarhodopsin I forms and in metarhodopsin II, the first long-lived form in the sequence.[518a,b] It seems likely that the proton has jumped via a bridging water molecule and the E113 carboxylate to the external (intradiscal) surface of the molecule. At the same time one or more protons are apparently taken up on the cytoplasmic side.[519] Study of mutant forms suggests that glutamate 134, near the cytosolic surface, and histidine 211 may be involved in proton transport.[520] By analogy with bacteriorhodopsin, aspartate 83 is probably also involved. The combination of conformational change plus altered charge distribution may be needed to create a binding surface with a suitable shape and charge constellation to bind tightly to transducin for the next step.

## 3. The Nerve Impulse

Which of the intermediates in Eq. 23-37 is responsible for initiation of a nerve impulse? Some evidence favored metarhodopsin I,[326] but its lifetime may be too short. On the other hand, the transformation of metarhodopsin I to metarhodopsin II is the slowest step that could trigger a nerve impulse, which must travel the length of the rod to the synapse in about one ms,[521] and metarhodopsin II is generally believed to be the activated signaling form of rhodopsin.[521a–c]

***Transducin, cyclic GMP, and phosphodiesterase.*** The essential consequence of light absorption is an alteration in the membrane potential in the vicinity of the absorbed photon with the resulting propagation of a nerve impulse down the plasma membrane to the synapse by cable conduction (Chapter 30). The type of potential change that is transmitted differs among vertebrates and invertebrates.[522] In the case of mammalian photoreceptors the rod outer segment is permeable to sodium ions so that a large

**dark current** of sodium ions flows in through the plasma membrane and is pumped out by sodium pumps in the inner portion of the cell. Visual stimulation causes this permeability to $Na^+$ to be decreased with an increase in polarization of the membrane. Absorption of a single photon by rhodopsin blocks the outflow of ~$10^6$ sodium ions.

At one time calcium ions seemed to be the logical internal messenger between rhodopsin and the plasma membrane. If light absorption opened channels from the internal space of the rod discs, calcium ions could be released and diffuse quickly to the plasma membrane and block the entrance of sodium ions.[523] However, light *does not* increase the free [$Ca^{2+}$] in the cytoplasm but may decrease it from 500 nM to as low as 50 nM.[524,525] Stryer suggested that the essential messenger is **cyclic GMP** (Chapter 11)[526–528] and that a *decrease* in cGMP concentration initiates the nerve response. Cyclic GMP is apparently responsible for keeping the sodium ion channels open. Absorption of a photon in the rod disc (Fig. 23-43) produces activated rhodopsin R* (metarhodopsin II), which acts as an allosteric effector for the heterotrimeric G protein transducin whose structure and properties have been discussed in Chapter 11. Like proteins $G_s$ and $G_i$ of the adenylate cyclase system, transducin contains three subunits: α, 40 kDa, 350 residues; β, 36 kDa; and γ, ~8 kDa.[529] In the resting state they are associated as $T_{\alpha\beta\gamma}$ with a molecule of GDP bound to the α subunit. When activated rhodopsin R* binds to transducin (step *a*, Fig. 23-43) it catalyzes a rapid exchange of GTP for GDP (step *b*). This is followed by dissociation of $T_{\beta\gamma}$ from $T_\alpha$ GTP. The latter serves as an allosteric effector for a **cGMP phosphodiesterase** bound to the disc surface converting it to an active form (step *c*).[529a,b] The activated phosphodiesterase, an $\alpha\beta\gamma_2$ oligomer,[530] hydrolyzes the cGMP (step *d*, Fig. 23-43), reducing its concentration and thereby inhibiting the $Na^+$ outflow.

Because one molecule of activated cGMP phosphodiesterase can hydrolyze more than $10^5$ molecules of cGMP per second the light response is highly amplified. There is also an earlier stage of amplification. Each molecule of light-activated rhodopsin (R*) is able to catalyze the exchange of GTP for GDP on hundreds of molecules of $T_{\alpha\beta\gamma}$ before R* passes on to other intermediates and releases all-*trans*-retinal from opsin (light green lines, Fig. 23-43).

### *Rhodopsin kinase, recoverin, and arrestin.*

Metarhodopsin II (R*) can become phosphorylated by rhodopsin kinase on as many as seven serine and threonine side chains on its cytoplasmic surface (Fig. 23-41).[531,532] The 45-kDa protein arrestin binds to such phosphorylated R*,[533–535a] which is rapidly deactivated and desensitized so that it is less likely to be immediately reactivated. This is important in the adaptation of the eye to bright light. At the same time $T_\alpha$2GTP is hydrolyzed back to $T_\alpha$2GDP and reforms $T_{\alpha\beta\gamma}$2GDP, and guanylate cyclase regenerates the cGMP.[536] At least four different arrestins are known. Some function in nonvisual tissues. In all cases they seem to serve as "uncouplers" of G protein-coupled receptors.[536a]

Recovery of the inhibited rhodopsin, which occurs most rapidly in dim light, depends upon calcium ions. In dim light both $Ca^{2+}$ and $Na^+$ enter the visual cells through the cGMP-controlled channels. At the same time $Ca^{2+}$ flows out through a $Na^+/Ca^{2+}$ exchanger. When the channels are blocked by cGMP formed in

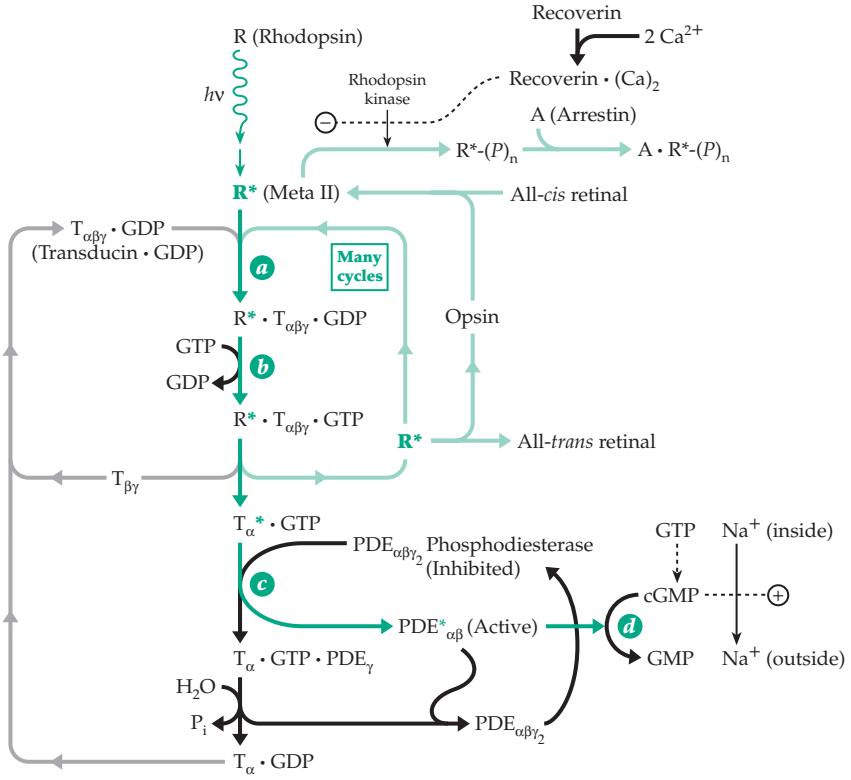



**Figure 23-43** The light-activated transducin cycle. In step *a* photoexcited rhodopsin (R*) binds the GDP complex of the heterotrimeric transducin ($T_{\alpha\beta\gamma}$). After GDP–GTP exchange (step *b*) the activated transducin T*•GTP reacts with the inhibited phosphodiesterase ($PDE_{\alpha\beta\gamma 2}$) to release the activated phosphodiesterase ($PDE_{\alpha\beta}$). Based on scheme by Stryer[528] and other information.

response to light, [$Ca^{2+}$] falls as a result of continuing export by exchange with $Na^+$.[524,537] In the dark the [$Ca^{2+}$] concentration rises again and binds to a 23-kDa calcium sensor molecule called recoverin. Recoverin, like calmodulin (Fig. 6-8), has four EF-hand $Ca^{2+}$-binding motifs and also an unexpected feature. A **myristoyl group** attached at the N terminus is bound into a deep hydrophobic pocket in the protein. However, when two $Ca^{2+}$ ions bind, recoverin undergoes a conformational change that allows the myristoyl group to be extruded and to bind to a membrane surface. This allows recoverin to move out of the cytosol to the surface of the disc membrane where it binds to and inhibits the activity of rhodopsin kinase,[538–541] increasing the sensitivity of photodetection.

***Some details about cone cells and invertebrate vision.*** The biochemistry of retinal cones is less well known but is similar to that of rod cells. Cone pigments are present in the plasma membrane rather than in isolated discs (Fig. 23-40C). Different α, β, and γ subunits of transducin are formed in rods and cones.[522] Many differences are seen among various invertebrate visual systems. Inositol triphosphate ($IP_3$) and $Ca^{2+}$ often serve as signals of photoexcitation. G proteins also play prominent roles.[522]

## 4. Regeneration of Visual Pigments; the Retinal Cycle

How is all-*trans*-retinal released from photobleached pigments and isomerized to 11-*cis*-retinal for the regeneration of the photopigments? Since new 11-*cis*-retinol is continuously brought in from the bloodstream and oxidized to retinal, isomerization can occur in other parts of the body. However, much of it takes place in the **pigment epithelium** of the retina, the layer of cells immediately behind the rod and cone cells. As indicated in Fig. 23-44, all-*trans*-retinal can leave the photoreceptor cells and, after reduction to retinal, be carried to the pigment epithelial cells by an **interphotoreceptor retinoid-binding** protein.[542,543] There it becomes esterified by the action of **lecithin:retinol acyltransferase**, an enzyme that transfers a fatty acyl group from lecithin to the retinol. The resulting retinyl esters are isomerized, and 11-*cis*-retinol is released.[543a] Some is stored as 11-*cis*-retinyl esters but enough is dehydrogenated to 11-*cis*-retinal to meet the needs of the photoreceptor cells and is transported back to them (Fig. 23-44). In the cephalopods the inner segment of the receptor cells contain a second pigment **retinochrome** that carries out a photochemical conversion of all-*trans*-retinal to 11-*cis*-retinal.[544,544a]

## 5. Diseases of the Retina

An important cause of blindness is **retinitis pigmentosa**, an inherited disease affecting about one in 3000 persons. Symptoms include progressive night blindness, degeneration of the rod cells, and gradual loss of cone cells and of nerve function in the retina. An autosomal dominant form of the disease arises from deletions or point mutations in the rhodopsin gene. In the United States 15% of cases arise from the mutation P23H.[545] By 1996 ~70 point mutations that cause retinitis pigmentosa had been discovered.[448,546–548] These mutations are found in all three of the rhodopsin domains: intradiscal, transmembrane, and cytosolic. Other rhodopsin point mutations such as G90D and A292E cause congenital night blindness.[549] Retinitis pigmentosa also arises from defects in **peripherin-RDS**, a structural component of rod cells identified originally from the gene *rds* (retinal degeneration slow) of the mouse.[545,550] Another form of congenital night blindness results from mutations in rhodopsin kinase.[551] A dominant **rod–cone dystrophy** is caused by a defect in the photoreceptor guanylate cyclase.[552] The most frequent cause of combined deafness and blindness in adults (**Usher syndrome**) is a defect in a cell adhesion molecule.[553] In **choroideremia**, another X-linked form of retinitis pigmentosa,

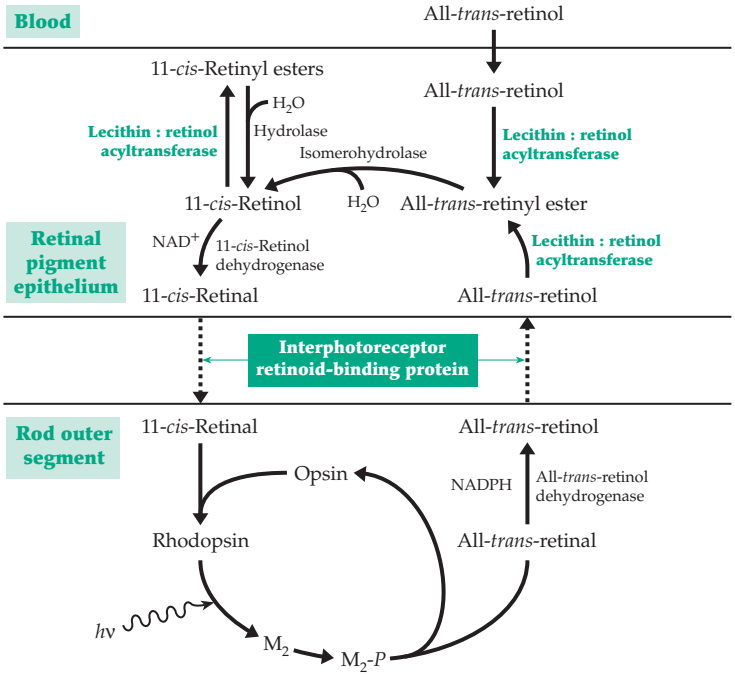



**Figure 23-44** Reactions of retinol and the retinal cycle of mammalian rod cells. After Palczewski *et al.*[543]

the choroid layer behind the pigment epithelium also deteriorates. A geranylgeranyl transferase specific for the Rab family of G proteins is defective.[554]

The most frequent cause of vision loss in the elderly is **macular degeneration**. Mild forms of the disease occur in nearly 30% of those over 75 years of age and more serious forms in 7% of that age group. There are many causes, some hereditary.[555–556a] Excessive accumulation of fluorescent lipofuchsin, perhaps arising in part from Schiff base formation between retinal and phosphatidylethanolamine, is sometimes observed.[557]

### 6. Proteins of the Lens

The lens of the eye encloses cells that cannot be replaced and contains proteins that don't turn over and must last a lifetime.[558] The fiber cells, which make up the bulk of the lens, have no nuclei. They elongate and stretch to cover the central nucleus, the original fetal lens, like the layers of an onion, the edges of the cells interdigitated with the next cell like a piece in a child's construction set.[559] These cells are tightly packed with proteins in aggregates whose size is on the order of the wavelengths of light. The high concentration of proteins is needed to provide transparency and also a high refractive index.[560–562] The membranes of the lens cells acquire increasing amounts of a 28-kDa **major intrinsic protein** as they age.[563] Three classes of soluble lens proteins, called **crystallins**, are found in virtually all lenses. Alpha crystallins, which account for ~40% of the total soluble protein, are heterodimers of ~20-kDa subunits that associate into ~800-kDa complexes.[564] They have a chaperoninlike activity.[565] Beta crystallin, which may account for ~35% of the protein, as well as the γ crystallins are β-sheet proteins with "Greek key" folding motifs.[558]

In addition to the α, β, and γ crystallins many animals have recruited additional "taxon-specific" crystallins δ, ε, λ, etc., that have evolved from prexisting enzymes, chaperonins, or other proteins.[561,566–568] For example, avian and reptilian lenses contain a δ crystallin homologous to argininosuccinate lyase.[569] Many crystallins are derived from dehydrogenases, e.g., for lactate dehydrogenase (duck),[570] hydroxyacyl-CoA dehydrogenase (rabbit),[571] and aldehyde dehydrogenase (squid and octopus).[572] A high concentration of NADH may be present.[568] A crystallin of a diurnal gecko is a retinol-binding protein with bound 3-dehydroretinal (vitamin $A_2$), which probably acts as an ultraviolet filter that improves visual acuity and protects against ultraviolet damage.[567,572a] Human lenses contain small molecules that act as UV filters, e.g., glucosides of **3-hydroxykynurenine** (Fig. 25-11) and 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid. Lenses tend to discolor and become fluorescent with age, in part by irreversible reactions of crystallins with these compounds.[573–574a]

A common problem with lenses is **cataract**, a term that describes any loss of opacity or excessive coloration. There are many kinds of cataract, most of which develop in older persons.[560,562] Since lens proteins are so long-lived deamidation of some asparagine occurs. However, the reactions are slow. One of the asparagines in α crystalline has a half-life of 15–20 years, and some glutamines are undamaged after 60 years.[575]

## G. Bacteriorhodopsin and Related Ion Pumps and Sensors

Under certain conditions, the salt-loving *Halobacterium salinarum* forms a rhodopsinlike protein, which it inserts into patches of **purple membrane** in the surface of the cell. These membranes, which may constitute up to 50% of the cell surface, contain light-operated proton pumps that translocate protons from the inside to the outside of the cells.[454,576–578] In this manner they may provide energy for a variety of cell functions including ion transport and ATP synthesis. The 248-residue retinal-containing **bacteriorhodopsin** makes up 75% of the mass of the membrane. Its molecules aggregate into a two-dimensional crystalline array in the purple patches of the membrane. This allowed determination of the three-dimensional structure to 0.7 nm resolution in 1975 by electron microscopy and neutron diffraction.[454] More recently the structure has been established at progressively higher resolution by electron crystallography[579–581] and X-ray diffraction.[455,582] The most recent studies have been focused on determination of the structural alterations in the protein that accompany the proton pumping.[582a–e] A step-by-step picture is emerging.[582f] Internal changes in the retinal chromophore, movements of protons, and alterations in the shapes of some of the protein helices are involved. The surface loops have been studied both by electron crystallography and by atomic force microscopy.[583] Each bacteriorhodopsin molecule is folded into seven closely packed α-helical segments which extend roughly perpendicular to the membrane. Although 100 residues shorter than rhodopsin, the folding pattern is very similar (Figs. 23-41C; 23-45). The protein molecules form an extremely regular array with phospholipid molecules (mostly of phosphatidylglycerol) filling the spaces between them. The retinal is buried in the interior of the protein and is bound as an *N*-protonated Schiff base with the side chain of lysine 216.

The retinal in bacteriorhodopsin (bR) exists in both all-*trans* and 13-*cis* configurations. The all-*trans* form has an absorption maximum at 568 nm and the 13-*cis*

at 548 nm. The two exist in a slow nonphotochemically mediated equilibrium in the dark.[584,584a] However, in the light the all-*trans* $bR_{568}$ undergoes a rapid photochemical cycle of reactions, which is presented in simplified form in Fig. 23-46. The subscript numbers designate the wavelength of maximum absorption. Many efforts have been made to determine the structures of the intermediates K, L, M, and O and to relate them to a mechanism of proton pumping.[585–585c] Both K and L contain 13-*cis* retinal. Therefore, as with rhodopsin (Eq. 23-39) the very first step is a photochemical isomerization. Intermediate M appears to contain a deprotonated Schiff base, but O is both *N*-protonated and again all-*trans*. It follows logically that the proton bound initially to the Schiff base is pumped out of the cell and is then replaced by a new proton in the O form. However, several questions must be answered if we are to understand this proton pump. Where in the sequence do proton transfers occur? How is the sequence driven by the absorbed light energy? Protons must enter the pump from the cytosol and exit on the exterior (periplasmic) side, flowing out against a concentration gradient. There must be a "gating" or "switch" mechanism that ensures that protons enter and leave the pump in the correct direction.[586]

Some aspects of a possible mechanism for pumping the single proton bound to the retinal Schiff base are included in Fig. 23-46. In $bR_{568}$ the Schiff base bound proton is H-bonded, via a water bridge, to the carboxylate group of Asp 85 (Fig. 23-45B). The charge constellation in the interior of the protein, part of which is shown in this figure, is such that protonation of the Schiff base is stable and the p$K_a$ of the protonated Schiff base is high, with estimates of 16 or above.[587] One cause of the high p$K_a$ is the presence of the nearby negative charges on D85 and D212. Absorption of light and isomerization of the retinal causes a downward movement of the $=NH^+-$ group of the Schiff base[588] and facilitates movement of the Schiff base proton via the water molecule to the D85 carboxylate as indicated by the green arrows in Fig. 23-45B. Loss of the positive charge will instantly substantially raise the p$K_a$ of D85 from a low value, while the loss of the negative charge will lower the p$K_a$ of the Schiff base to closer to 7. The electrostatic interactions of the D212 and E194 carboxylates with the positive charge of R82 may also be altered. At some point in the sequence the interaction of R82 with the E194 carboxylate could cause E204, which is known by spectroscopic measurements to be protonated in the intermediate, to lose its proton to the outside. At some other point, perhaps between $M_{412}$ and another intermediate, $M_{408}$ (Fig. 23-46), a conformational switch must occur to limit flow of a proton back to E204 and to allow a new proton to enter from the cytosol. The D96 carboxylate is thought to accept this proton and to transfer it via a chain of

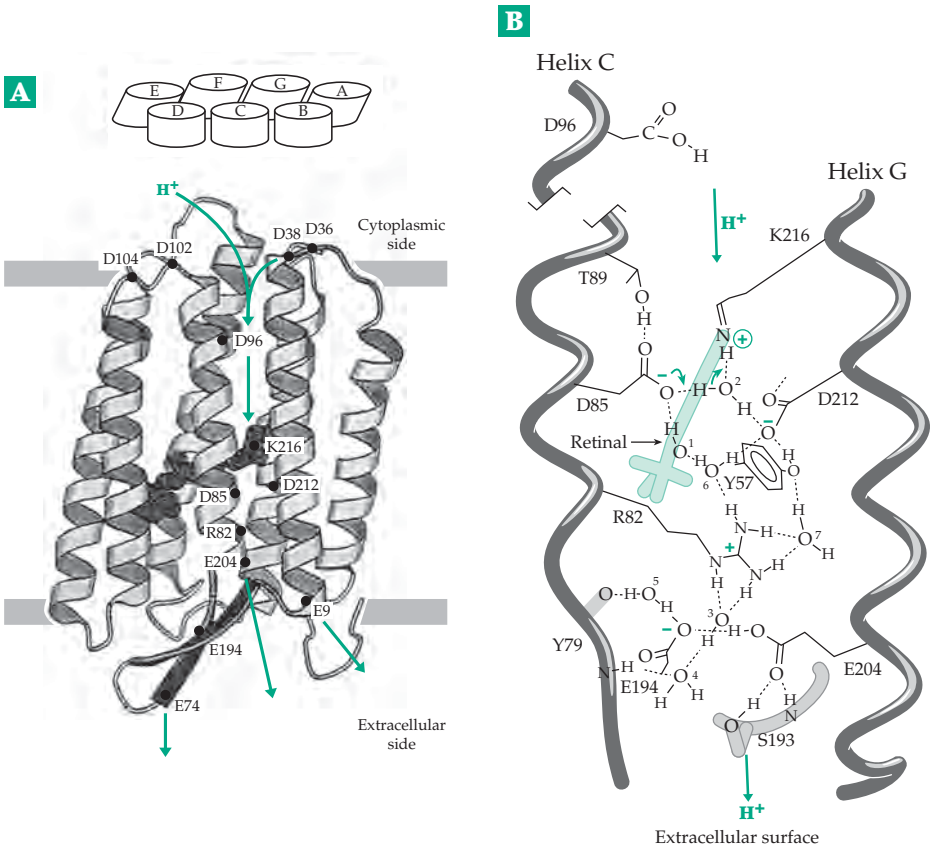



**Figure 23-45** (A) Some aspects of the structure of bacteriorhodopsin. Ribbon diagram with the retinal Schiff base in ball-and-stick representation. At the top the helices are labeled as in Fig. 23-41. The locations of aspartate, glutamate, and arginine residues that might carry protons during the proton pumping action are indicated. Retinal is shown attached to lysine 216. From Kimura *et al.*[580] Courtesy of Yoshiaki Kimura. (B) Schematic drawing illustrating hydrogen-binding observed in the three-dimensional structure at 0.14 nm resolution. From Luecke *et al.*[455] The side chains shown are those thought to be involved in proton transport and in a hydrogen-bonded network with bound water molecules, principally between helices C and G. The positions of many of the hydrogen atoms in this network have not been established. They have been placed in reasonable positions in this figure but may be quite mobile. For another view of the hydrogen-bonded network see Fig. 23-41C.

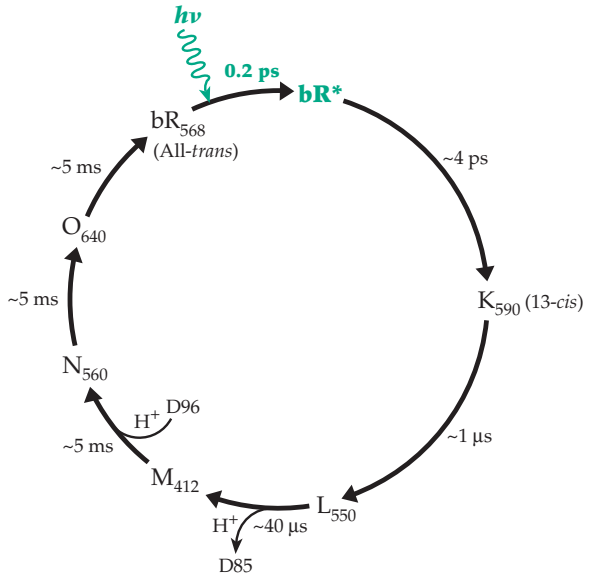
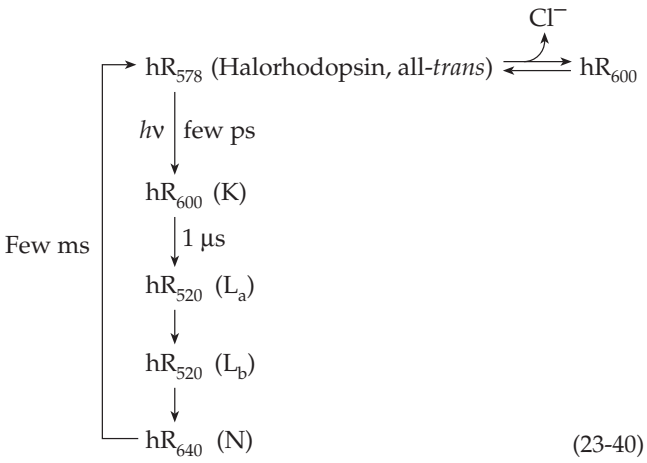



**Figure 23-46** The photoreaction cycle of bacteriorhodopsin. After Bullough and Henderson.[585] The subscript numbers indicate the wavelengths of maximum absorption of each intermediate and the approximate lifetimes are given by the arrows. Resting bacteriorhodopsin as well as intermediates J and O have all-*trans* retinal but K through N are thought to all be 13-*cis*. A proton is transferred from L to aspartate 85 and then to the exterior surface of the membrane. A proton is taken up from the exterior surface via aspartate 96 to form N.

water molecules or water molecules plus protein groups to the Schiff base, which may isomerize back to all *trans* in going from intermediate N to O. Isomerization to all-*trans* O is a slow step. Both $O_{640}$ and $bR_{568}$ are all-*trans* and have a 15-*trans* Schiff base linkage. There may be a difference in protein conformation with the chromophore being more distorted in O than in $bR_{568}$. Many recent studies have provided additional information.[588a–j]

***Halorhodopsin.*** In addition to bacteriorhodopsin there are three other retinal-containing proteins in membranes of halobacteria. From mutant strains lacking bacteriorhodopsin the second protein, **halorhodopsin**, has been isolated. It acts as a light-driven *chloride ion pump*, transporting $Cl^-$ from outside to inside. Potassium ions follow, and the pump provides a means for these bacteria to accumulate KCl to balance the high external osmotic pressure of the environment in which they live.[578] The amino acid sequences of halorhodopsins from several species are very similar to those of bacteriorhodopsin as is the three-dimensional structure.[589] However, the important proton-carrying residues D85 and D96 of bacteriorhodopsin are replaced by threonine and alanine, respectively, in halorhodopsin.[590] Halorhodopsin (hR)




(23-40)

undergoes a light-dependent cycle (Eq. 23-40) that involves an all-*trans* to 13-*cis* photoisomerization with some intermediates resembling those of the bacteriorhodopsin cycle.[590a]

***Sensory rhodopsins.*** The third and fourth light-sensitive proteins of halobacteria are **sensory rhodopsins** (SR)[578,591,591a,b] that are used by the bacteria to control **phototaxis**. These bacteria swim toward long-wavelength light, the maximum in the action spectrum being at ~580 nm. They are repelled by blue or ultraviolet light, the maximum in the action spectrum being at ~370 nm. Evidently the bacteria can detect either a decrease with time in red light intensity or an increase with time in blue light intensity. Either is interpreted as unfavorable and causes the bacteria to tumble and move in a new direction (see Chapter 19). Sensory rhodopsin I (SRI) appears to be able to provide both light responses. Absorption of orange light by $SRI_{587}$, which contains all-*trans*-retinal, yields $SRI_{373}$, in which the retinal Schiff bases have been isomerized to 13-*cis* as in bacteriorhodopsin. The red light response is proportional to the fraction of $SRI_{373}$ present. However, this is converted spontaneously back to $SR_{587}$ within seconds. Nevertheless, photoexcitation of $SRI_{373}$ with blue light causes a faster reconversion and induces swimming reversals, the repellent response.[592,593] SRI exists in the bacterial membranes in a complex with a 57-kDa protein designated **halobacterial transducer I** (HtrI), which resembles bacterial chemotaxis receptors (Figs. 11-8 and 19-5) and is modulated by action of a methyltransferase.[591b,c] Interaction of SRI with HtrI depends upon a histidine residue, H166 of SRI. It may be part of a proton transfer pathway.[593]

**Sensory rhodopsin II** (SRII, also called phoborhodopsin) is specialized for repellant phototaxis.[591a] Blue light converts $SRII_{487}$ in < 1 ms to UV-absorbing $SRII_{360}$. It decays in ~100 ms to $SRII_{540}$ which reverts to the initial $SRII_{487}$ in ~0.5 s. The cycle is accompanied by swimming reversals that result in a repellent

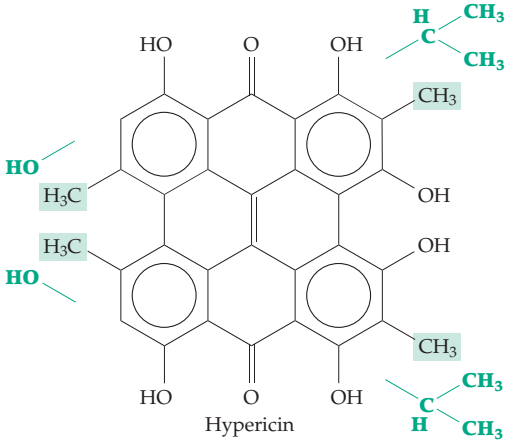
effect of light.[594–596] The three-dimensional structure is known.[593a,b]

Whereas retinal-based proton pumps all have the conserved residues D85 and D96 of bacteriorhodopsin, only the aspartate correspnding to D85 is conserved (as D73) in the sensory rhodopsins. D96 is replaced by tyrosine or phenylalanine.[586] In SRI D73 appears to be protonated and, therefore, does not form a counterion for the Schiff base iminium ion.[597] However, in SRII D73 is apparently unprotonated and available to -serve as a counterion and as a proton acceptor as in bacteriorhodopsin.[597a,b] There is also a corresponding aspartate (Asp 83) in rhodopsin (Fig. 23-41). This suggests a common signaling mechanism for rhodopsin and the sensory rhodopsins. Finally, there are retinal-containing proteins in fungi and in algae. They may serve as blue light receptors.[598,598a]

***The photoactive yellow proteins (xanthopsins).*** A 124-residue, 14-kDa yellow protein isolated from a halophilic phototrophic purple bacterium, *Ectothiorhodospira halophila*, was at first thought to be a rhodopsin-type pigment. However, this photoactive yellow protein (PYP) carries a covalently bonded **coumaroyl (4-hydroxycinnamoyl) group** in thioester linkage,[599–601c] which is completely surrounded by the small protein.[601] The coumaroyl group, which was probably derived from coumaroyl-CoA (Fig. 25-8), is bound as a phenolate anion by hydrogen bonds to tyrosine and glutamate side chains (Eq. 23-41). After a laser flash at room temperature a readily observed intermediate $I_1$ (also called pR) absorbing maximally at 460–465 nm appears within ~3 ns and decays within a few milliseconds to a bleached intermediate $I_2$ (also called pB or pM)[602,603] with maximum absorption at ~355 nm. This returns to the original 446-nm form within a few seconds. Earlier intermediates $I_0$ and $I_0^{\ddagger}$ have been identified by picosecond spectroscopy,[601c,604,605] and others have been identified at low temperatures.[602,606]

The structure of PYP is known to 0.1 nm resolution (Fig. 23-47).[601,607] Structures have also been determined for a very early intermediate by trapping at –100°C[607] and for $I_1$ (pR). The cofactor structures are shown in Eq. 23-41. The light-induced step is apparently the *cis–trans* isomerization,[608] and changes in hydrogen-bonding follow. The hydrogen bond between the phenolate ion of the coumaroyl group and glutamate 46 appears to break, and E46 may donate a proton to the phenolate group to form the 337-nm chromophore of $I_2$.[602,609,609a] The signaling mechanism may be similar to that in sensory rhodopsins.

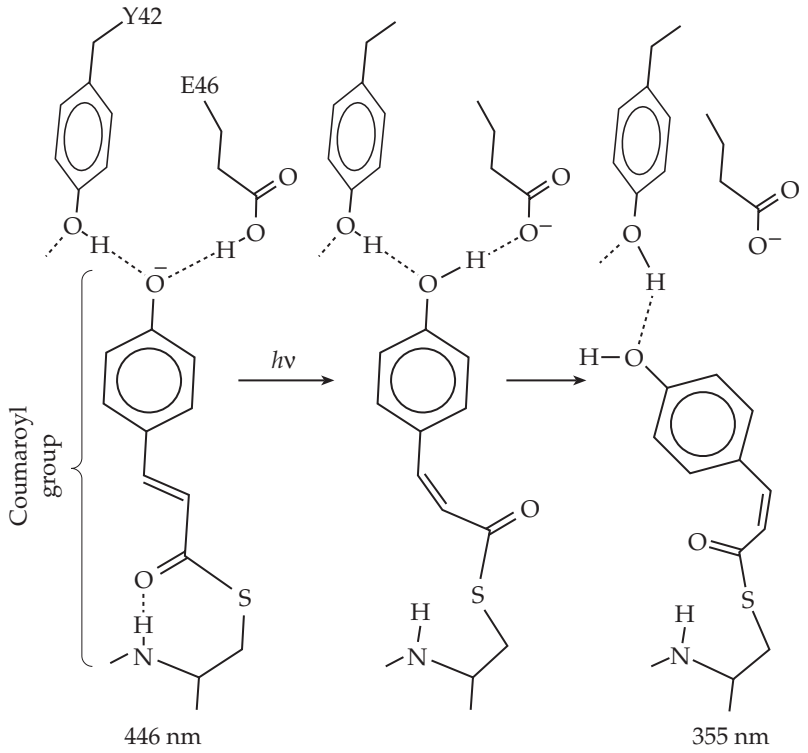
***Stentorin.*** A protein with a bound chromophore called stentorin mediates the light-avoidance response of the protozoan *Stentor*. Stentorin,[610] which is found in pigment granules in the cell surface, is a derivative of **hypericin**, a plant compound with antidepressant activity and the active ingredient in the herb St. John's wort.




Hypericin

Replaced in stentorin by the green groups

Stentorin is covalently bonded to a 16-kDa protein in an acid-labile linkage. Its photocycle is not well investigated, but it is thought to initiate a response via

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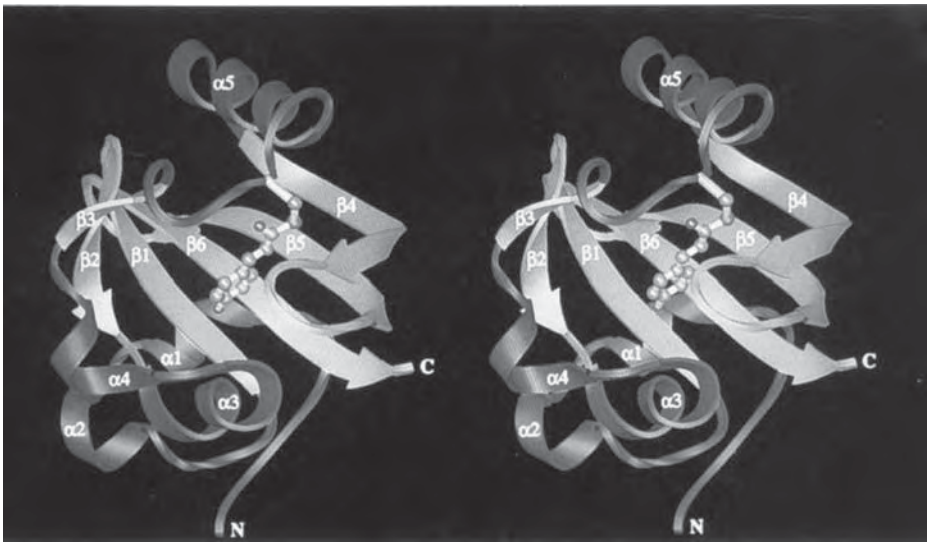



**Figure 23-47** Ribbon drawing of the structure of the 125-residue yellow photoactive protein. The 4-hydroxycinnamoyl chromophore, which is attached to cysteine 69, is represented with balls and sticks. From Borgstahl *et al.*[601] Courtesy of Gloria Borgstahl.

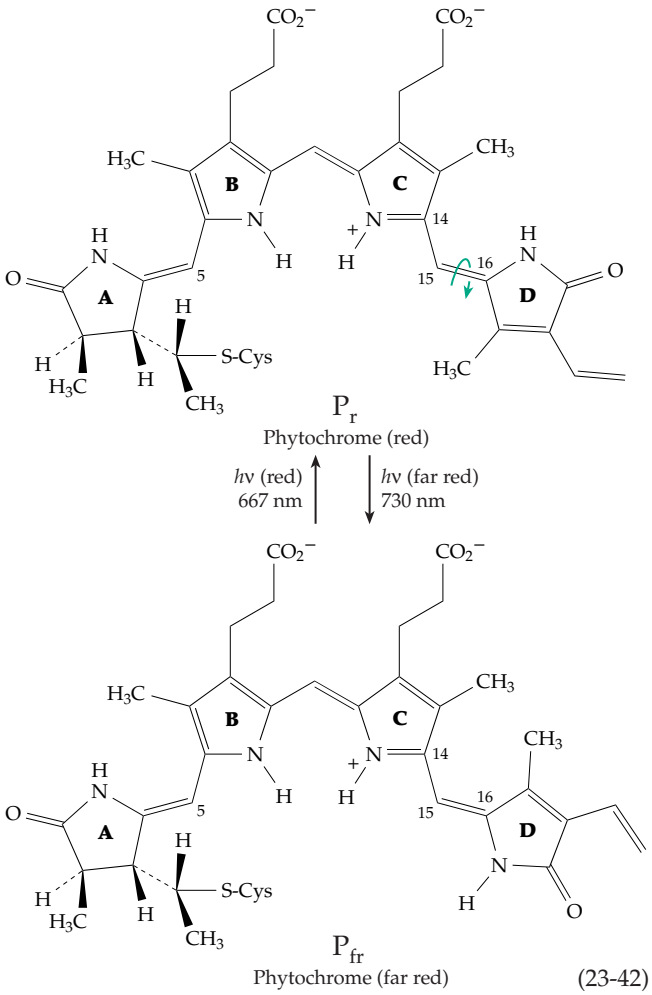
proton transfer.[611] However, stentorin proteins apparently do not belong to the bacteriorhodopsin family.

Hypericin and related compounds have also aroused interest because of their antiviral and antitumor activities.[612,613] Hypericin is a strong photoactivator which produces singlet oxygen with a quantum efficiency of 0.73. However, antiviral activity may involve a radical mechanism.[613] Hypericin is attractive as a possible agent for photodynamic therapy (Section D,3). It can also receive energy from photoexcited firefly luciferin (Section J). A proposed application is to incorporate the gene for the enzyme luciferase of the firefly luminescence system into DNA from the virus HIV. This DNA could be used to promote synthesis of luciferase only in virus-infected cells. Addition of the nontoxic hypericin would lead to photoactivation of hypericin only in virus-infected cells, where the luciferin–luciferase complex would act as a "molecular flashlight" to activate the hypericin and destroy the cell.[613]

## H. Phytochrome

In 1951, it was discovered that a flash of red light (maximum activity at 660 nm) during an otherwise dark period promoted a variety of responses in plants.[614] These included flowering, germination of seeds (e.g., those of lettuce), and the expansion of leaves in dark-grown pea seedlings. Interestingly, the effect of the short flash of red light could be *completely reversed* if followed by a flash of *far-red light* (730 nm). This discovery led to the isolation, in 1959, of the chromoprotein phytochrome, a kind of molecular switch that initiates a whole series of far-reaching effects in plants. The phototransformation[615] is completely reversible (Eq. 23-42; Fig. 23-48), and the switch can be thrown in one direction or the other many times in rapid succession by light flashes.

Green plants have a family of phytochromes. There are five genes for the ~125-kDa chains of about 1100 residues each in *Arabidopsis*,[618–619c] and the corres-

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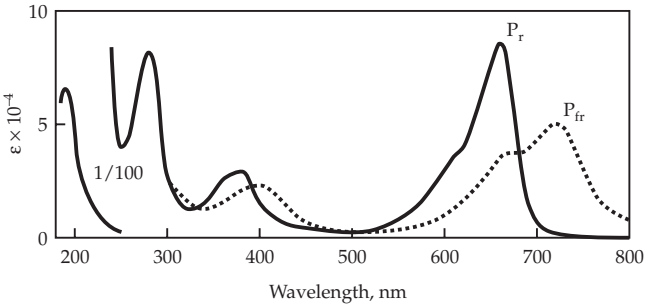



**Figure 23-48** Absorption spectra of red ($P_r$) and far-red ($P_{fr}$) forms of purified oat phytochrome following saturating irradiations with red and far-red light. See Quail[616] and Anderson *et al*.[617]

sponding phytochromes A – E each appear to have distinct functions. The chromophore is an open tetrapyrrole closely related to phycocyanobilin and covalently attached to the peptide backbone near the N terminus through a cysteine side chain (top structure in Eq. 23-42). The initial photochemical reaction is thought to be the $Z \rightarrow E$ isomerizion around the C15 – C16 double bond, but there may also be rotation about the C14 – C15 single bond. The initial step occurs within a few microseconds and up to four intermediate species have been seen in the $P_r \rightarrow P_{fr}$ conversion and at least two other different ones in the $P_{fr} \rightarrow P_r$ conversion.[620–623c]

Phytochromes exist as two distinct domains, the N-terminal domain bearing the chromophore. However, the first 65 residues at the N terminus as well as the C-terminal domain probably interact with other proteins to transmit a signal. The slow responses to phytochrome are thought to involve regulation of transcription.[619b,623d] Thus, the synthesis of mRNA molecules specific for the small subunit of ribulose bisphosphate carboxylase and for the chlorophyll *a*/*b* binding protein increases in response to formation of $P_{fr}$. These responses are quite rapid occurring within 15–30 min.[614] Another response to $P_{fr}$ is a decrease in the amount of the specific mRNA for phytochrome itself. That is, light induces a decrease in the concentration of this light-sensing molecule thereby decreasing the sensitivity of the system.[614]

Phytochrome is found not only in higher plants but also in algae, where it controls the movement of chloroplasts,[611] and also in cyanobacteria.[623e,f] Cyanobacterial phytochromes contain histidine kinase domains, which may function in a two-component system with a response regulator similar to protein CheY of the chemotaxis system in *E. coli* (Fig. 19-5).[624,625] Some nonphotosynthetic bacteria also use bacteriophytochromes for light sensing. In some cases biliverdin (Fig. 24-24) is the chromophore.[625a]

Phytochromes of higher plants also have histidine kinase-like N-terminal domains. Searches for associated signaling proteins have revealed a phytochrome-interacting factor in *Arabidopsis*. A possible partner for phytochrome B, it is a nuclear helix–loop–helix protein that may be a transcription factor.[626–627a] Phytochrome A may signal via a WD-repeat protein to control morphogenesis.[628]

One response under phytochrome control is the closing of leaflets of *Mimosa* at the onset of darkness. The response occurs within 5 min, too short a time to be the result of transcriptional control. This and the finding that some phytochrome is tightly bound to membranes have led to the proposal that one primary effect of phytochrome is to alter membrane properties. It is not certain whether it is $P_r$ or $P_{fr}$ that is active in causing a response, but $P_{fr}$ seems to be the most likely candidate for the "active" form. According to one suggestion, phytochrome in plastid membranes may mediate the release of gibberelins stored within the plastids.[629]

## I. Some Blue Light Responses

Numerous biological responses to light of wavelength 400–500 nm are known. These include phototropism in higher plants, the phototaxis of *Euglena*, and photorepair of DNA. On the basis of action spectra both carotenoids and flavins were long ago proposed as photoreceptors.[630–632] The action spectrum for opening of the stomates in coleoptiles matches the absorption spectrum of zeaxanthin.[631] On the other hand, genetic evidence[633,634] has strengthened the view that a flavin acts as the photoreceptor in the fungus *Phycomyces*. Recently compelling evidence for a flavoprotein receptor for phototropism in *Arabidopsis thaliana* has been obtained. Deficiency of a gene called *nph*1 (nonphototropic hypocotyl 1) is associated with loss of blue light-dependent phosphorylation of a 120-kDa protein. This protein was identified as the product of *nph*1 gene. The **nph1** protein is a soluble autophosphorylating Ser / Thr protein kinase with an N-terminal flavin-binding region. It apparently binds FMN and is a photoreceptor for phototropism in higher plants.[635–636b]

The complexity of the action spectra suggested the existence of more than one receptor.[634] In higher plants there are not only blue light receptors but also violet receptors and phytochrome. In addition to nph1 and a related protein **npl1**, *Arabidopsis* employs two cryptochromes (next section) and **phototropins**.[636c–e] These are also riboflavin 5'-phosphate (FMN)-dependent proteins. The action of light apparently causes addition of a highly conserved cysteinyl –SH group to the C4a position of the flavin.[636c] Phytochrome absorbs blue and ultraviolet light to some extent (Fig. 23-48) as well as red or far-red. This adds

considerable complexity to the interpretation of light responses in plants.[637] The fern *Adiantum* contains a protein with a phytochrome photosensory domain fused to an NPH1 structure. It may mediate both real far-red and blue-light responses.[636] The protist *Euglena* (Fig. 1-9) makes use of a **photoactivated adenylate cyclase**, also a photoactivated enzyme, in a light avoidance response.[637a]

Fungi such as *Neurospora crassa* provide a simpler system for study of blue-light signaling than do green plants.[637] *Neurospora* contains no phytochromes. However, numerous genes including some involved in carotenoid biosynthesis and some that control the circadian cycle are regulated by blue light. Two mutants defective in riboflavin synthesis show a reduced sensitivity to blue light. A deficiency of either of two other genes *wc*-1 and *wc*-2 results in "blind" *Neurospora* unable to respond to light but able to grow. Proteins WC1 and WC2 are probably transcription factors, which act as a heterodimer. WC1, which contains bound FAD, is the photorecetor.[637,637b,c] Recently a rhodopsinlike protein NOP-1 of *Neurospora* has been identified.[637d]

## 1. Cryptochromes

The elusive nature of the principal blue-light receptor in plants gave rise to the name cryptochrome.[632] The gene for a cryptochrome in *Arabidopsis thaliana* was isolated by gene tagging and was cloned. It is surprisingly similar in sequence to the gene for the well-known **DNA photolyase** (Section 2).[638] It was soon recognized that cryptochromes, like photolyases, carry a bound flavin and also an antenna chromophore. The latter is probably a 5,10-methylenetetrahydrofolate, as in plant photolyases. It is a better light absorber than the flavin and passes electronic excitation to the flavin.

Two cryptochrome genes, *cry*1 and *cry*2, are present in *Arabidopsis*. The encoded proteins affect many aspects of plant growth. The cry-1 protein, together with NPH1, has a role in controlling phototropisin[639] while cryptochrome cry-2 affects flowering time, apparently via antagonistic signals from cry-2 and phytochrome B.[640–640b] cry-1 is also involved in controlling the daily rhythm of the plant, the **circadian cycle**. The circadian clock, which is discussed in Chapter 30, provides the organisms an oscillator with a period of about 24 hours. However, the oscillator must be **entrained** by the daylight cycle so that it remains in proper synchrony. The nature of the light signal and the mechanism of the entrainment are being investigated in many different organisms from fungi to human beings. In *Arabidopsis* the cycle is controlled by phytochromes A and B and by the cryptochrome cry-1.[641]

Cryptochrome genes have been found in many organisms. In the fly *Drosophila* cryptochrome appears to interact directly with the clock proteins that control the circadian cycle. Most important are products of two genes *per* (period) and *tim* (timeless). They are helix–loop–helix DNA binding proteins that form heterodimers, are translocated to the nucleus, and repress their own transcription. Morning light leads to a rapid disappearance of the TIM protein. The cryptochrome CRY appears to react directly with TIM to inactivate it. However, details remain to be learned.[642] The circadian clock mechanism appears to be universal and the cryptochrome-2 (*mcry*2 gene) appears to function in the mouse.[643,643a] A human cDNA clone was found to have a 48% identity with a relative of cryptochromes, the **(6–4) photolyase** of *Drosophila*. A second related human gene has also been found. The protein products of these two genes (*hcry*1, *hcry*2) lack photolyase activity. They too may encode cryptochromes.[644]

Where in the body is the light sensed for entraining the circadian cycle? Genes for CRY1 and CRY2 are specifically expressed in ganglion cells of the retina in mice. Severing of the optic nerve destroys both vision and light entrainment of mammals. However, in mice with the retinal degeneration (*rd*) syndrome all rod cells and virtually all cone cells are destroyed but the circadian rhythm is normal.[645] Furthermore, many blind persons with no conscious perception of light have normal light entrainment of their circadian cycle. For these reasons the ganglion cells of the retina, which are close to the location of the master circadian clock in the **suprachiasmatic nucleus** of the brain, are the most probable light sensory cells for the cycle[646,647] (see also Chapter 30). Recent evidence points to a retinal-based photoreceptor, **melanopsin**.[647a,b,c] However, vitamin A-deficient mice still display a normal circadian response.[647d]

## 2. Photolyases

A curious discovery was made many years ago. Bacteria given a lethal dose of ultraviolet radiation can often be saved by irradiating with visible or near ultraviolet light. This **photoreactivation**, which permits many bacteria to survive, results from the action of a **DNA photolyase**,[648,649] which often absorbs light maximally around 380 nm and carries out a photochemical reversal of Eq. 23-26, cutting the pyrimidine–pyrimidine covalent bonds of thymine dimers in DNA. The enzyme is present in cells in such small amounts, only 10–20 molecules per cell, that it was difficult to investigate until the gene had been cloned.[650,651] The significance cannot be doubted, for photoreactivation enzymes appear to be found in most organisms including some mammals. However, there

is some doubt about the presence of a photolyase in the human body.

The *E. coli* DNA photolyase contains a blue flavin radical that arises from **FAD** and absorbs maximally at 580 nm (see also Chapter 15, Section B,6). The enzyme also contains a second chromophore in the form of bound 5,10-methenyltetrahydrofolylpolyglutamate with 3–6 γ-glutamyl residues.[652–653b] as shown in Fig. 23-49. The pterin coenzyme binds near the N terminus in a domain with an α/β folding pattern, while the FAD binds into a larger mostly helical domain. The pterin cofactor is not essential for repair activity, and it is generally agreed that because of its high molar extinction coefficient it acts as an effective **antenna**. It transfers energy in a nonradiative fashion to the $FADH^-$ anion located ~3 nm away.

The enzyme as isolated is in a stable blue radical form (Fig. 23-50; also Fig. 15-13) which must undergo a one-electron light-induced reduction to the anion $FADH^-$ before becoming active. A nearby indole ring of Trp may donate the electron and be reoxidized by a tyrosyl ring.[654] The $FADH^-$ donates an electron to the pyrimidine dimer, initiating the sequence of radical reactions[654a–c] which cleaves both pyrimidine–pyrimidine bonds in the photodimers (Fig. 23-50).

The structures of all of the photolyases are thought to resemble that in Fig. 23-49. However, in one large group, which includes methanogenic bacteria, **8-hydroxy-5-deazariboflavin** acts as the antenna chromophore.[649] Another light-induced defect in DNA is the so-called 6–4 photoproduct, a different pyrimidine dimer. The 6–4 dimers are normally removed in most organisms by efficient **excision repair** (Chapter 27). However, a 6–4 photolyase was discovered in both *Arabidopsis* and *Drosophila* and has also been found in *Xenopus* and the rattlesnake.[655,655a] It has a structure similar to that of the *E. coli* photolyase and presumably acts by a related mechanism[191,656–657a] that uses the light-excited reduced flavin in an electron donation and return cycle as in Fig. 23-50. A homolog of the *Drosophila* 6–4 photolyase gene has been found in human cells, but there is uncertainty about its function.[658] Is it really a photolyase or is it a cryptochrome involved in the circadian cycle?

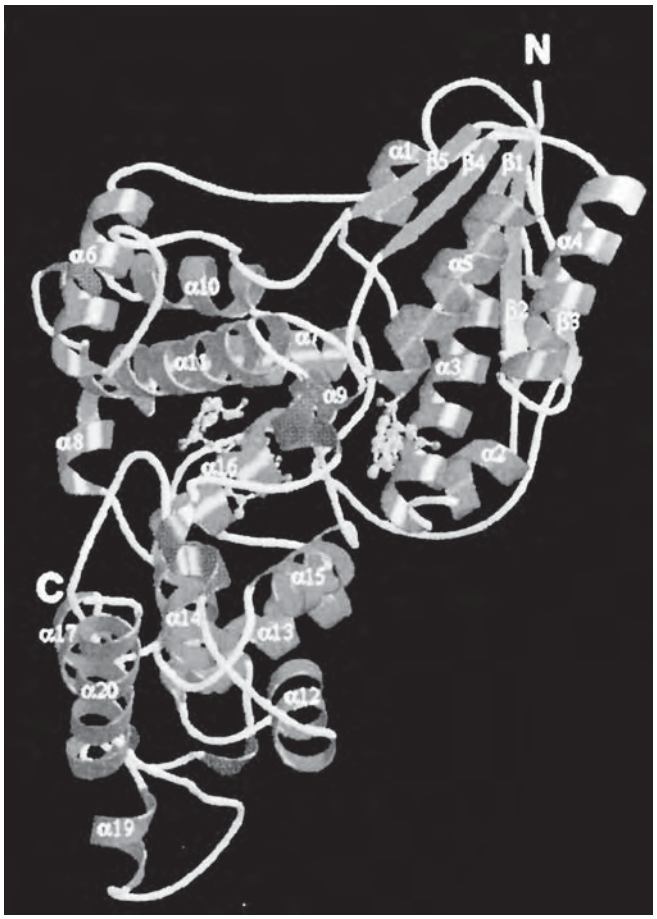
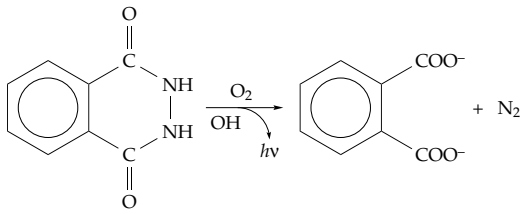



**Figure 23-49** Overall view of the DNA photolyase structure from *E. coli*. The ribbon traces the 471-residue chain. The bound cofactors FAD (left) and 5,10-methenyltetrahydrofolate (right) are shown in ball-and-stick representation. From Park *et al.*[652] Courtesy of Johan Deisenhofer.

## J. Bioluminescence

The emission of visible light by living beings is one of the most fascinating of natural phenomena. Luminescent bacteria, glowing toadstools, protozoa that can light up ocean waves, luminous clams, fantastically illuminated railroad worms,[659] and fireflies[660–661a] have all been the objects of the biochemists' curiosity.[662–664] The chemical problem is an interesting one. The firefly's light with a wavelength of 560 nm (17,900 $cm^{-1}$) has an energy of 214 kJ/einstein. What kind of chemical reaction can lead to an energy yield that high? It is far too great to be provided by the splitting of ATP. Even the oxidation of NADH by oxygen would barely provide the necessary energy.

A clue comes from the fact that chemiluminescence is very common when $O_2$ is used as an oxidant in nonenzymatic processes. The slow oxidation of alcohols, aldehydes, and many nitrogen compounds (Eqs. 23-43, 23-44) is accompanied by emission of light




Luminol, a synthetic luminescent compound

(23-43)

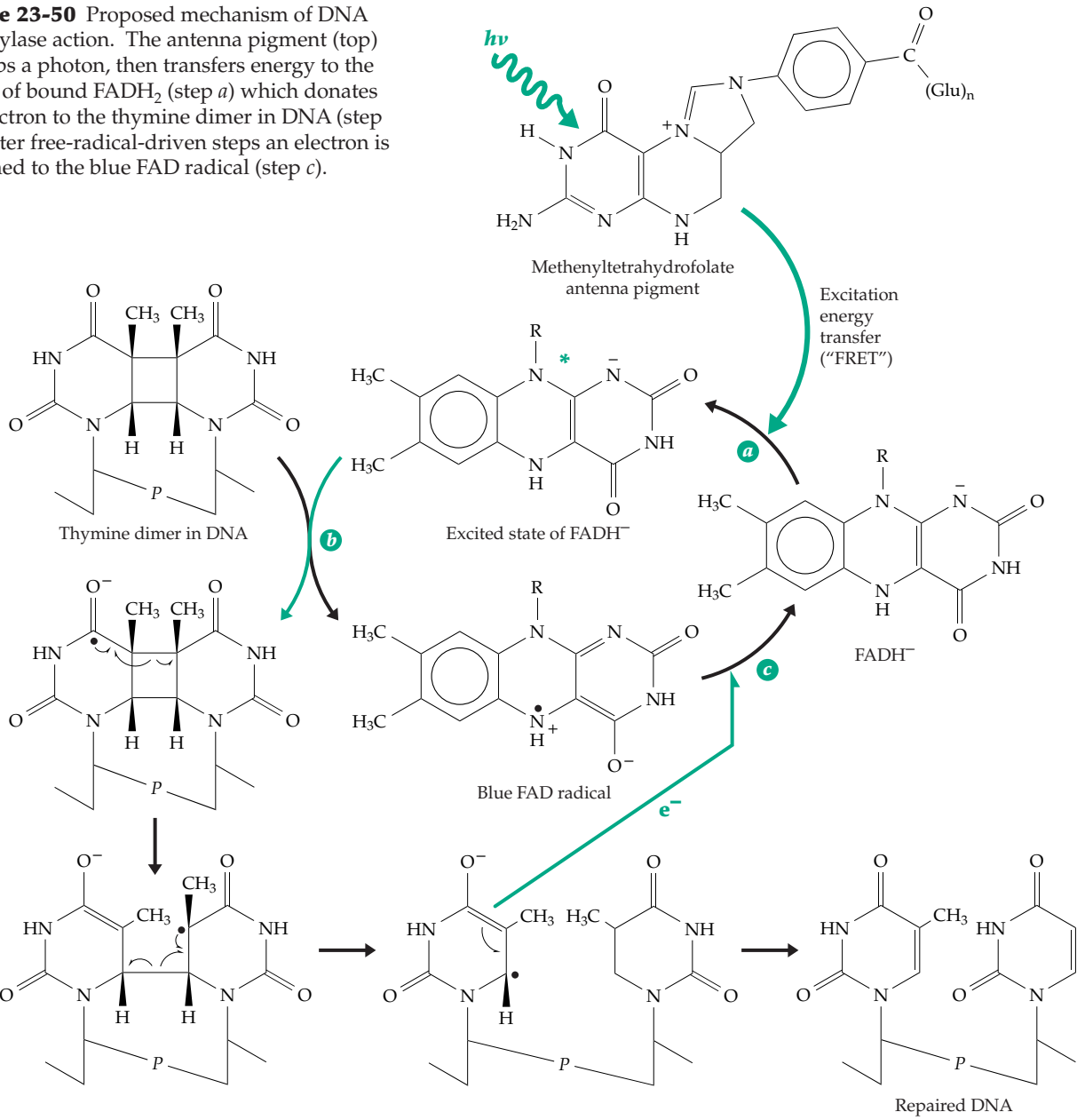
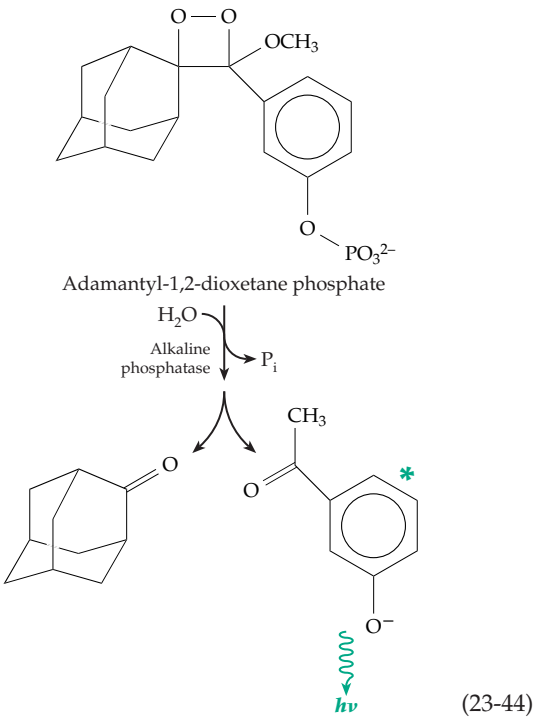



**Figure 23-50** Proposed mechanism of DNA photoylase action. The antenna pigment (top) absorbs a photon, then transfers energy to the anion of bound $FADH_2$ (step *a*) which donates an electron to the thymine dimer in DNA (step *b*). After free-radical-driven steps an electron is returned to the blue FAD radical (step *c*).

visible to the eye. Chemiluminescence is especially pronounced in those reactions that are thought to occur by radical mechanisms. The recombination of free radicals provides enough energy to permit the release of visible light. Cleavage of a peroxide linkage, e.g., in a dioxetane (Eq. 23-44),[665] is often involved.[666] For example, the reaction of Eq. 23-44 is used in a sensitive light-detected assay for alkaline phosphatase.

In view of these facts it is perhaps not so surprising that many organisms have mastered the ability to channel the energy released in an oxygenation reaction into light emission. Attempts to extract luminous materials from organisms date from the last century when the French physiologist, DuBois, in 1887 prepared both a cold-water extract and a hot-water extract of luminous clams.[662] He showed that the material in the cold-water extract, which he named **luciferase**, caused emission of light when a heat-stable material (which he called **luciferin**) present in hot-water extract was added. These names have been retained and are now used in a general way. Thus, the luciferins are a family of compounds whose structures have been determined for a number of species (Fig. 23-51). Firefly luciferin is a carboxylic acid, but it must be activated in an ATP-requiring reaction to give **luciferyl adenylate**, whose structure is shown in the figure. The latter emits light in the presence of $O_2$ and luciferase. It can be seen that the original carboxyl group becomes $CO_2$, while the ring becomes oxidized. In addition, the acyl adenylate linkage is broken. In

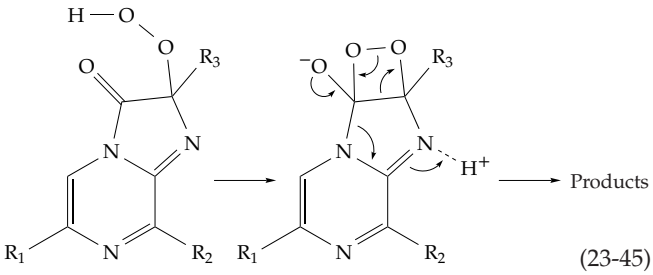



(23-44)

the "sea pansy" *Renilla reniformis* (a coelenterate) the luciferin has quite a different structure.[667,668] However, the reaction with $O_2$ to produce $CO_2$ and an oxidized product causes the light emission, just as in the firefly. The luciferin of *Renilla*, which is called **coelenterazine** (Fig. 23-51), is also found in the jellyfish *Aequorea*, the shrimp *Oplaphorus*,[669] the "firefly squid" *Watasenia scintillans*,[670] and other luminous organisms.

In *Renilla* the coelenterazine is stored as a coelenterazine sulfate, possibly having the structure shown. To convert this storage form to the active luciferin the sulfo group is transferred onto adenosine 3',5'-bisphosphate to form 3'-phosphoadenosine 5'-phosphosulfate, the reverse of step *d* of Eq. 17-38. The luciferin of the ostracod crustacean *Vargula hilgendorfii* has a structure (Fig. 23-51) close to that from *Renilla*. In *Vargula* (formerly *Cypridina*) the luciferin and luciferase are produced in separate glands and are secreted into the surrounding water where they mix and produce a bright cloud of light.[671]

In most mechanisms suggested for luciferase action $O_2$ reacts at the carbon atom that becomes the carbonyl group in the product to form an intermediate peroxide. In the case of *Renilla* luciferin this can easily be visualized as a result of flow of electrons (perhaps one at a time) from the pyrazine nitrogen (at the bottom of the structure in Fig. 23-51) into the $O_2$. According to one proposal, the peroxide group that is formed adds to the carbonyl to form a four-membered dioxetane ring as shown in Eq. 23-45 for coelenterazine peroxide. The latter opens, as indicated by the arrows, to give the products. This theory was tested using



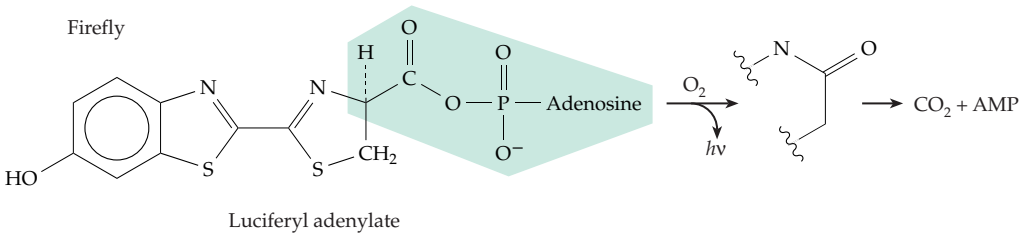

(23-45)

$^{18}O_2$. In the case of *Vargula* luciferin the expected incorporation of one atom of $^{18}O$ into $CO_2$ was observed, but with firefly and *Renilla* luciferins no $^{18}O$ entered the $CO_2$. In these two cases, a somewhat different mechanism may hold.

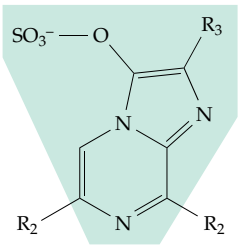
The jellyfish *Aequorea* contains a **photoprotein**, which emits light only when calcium ions are present.[672,673] Since light emission can be measured with great sensitivity (modern photomultipliers can be used to count light quanta) the protein **aequorin** and related photoproteins[674a] are used as a sensitive indicator of calcium ion concentration.[674] (In a similar way the firefly luciferin–luciferase system, which requires ATP for activation, is widely used in an assay for ATP.)

To identify the chromophore in aequorin over 4000 kg of jellyfish were used to obtain 125 mg of electrophoretically pure photoprotein.[674] From this one mg of a chromophoric substance AF-350 (Fig. 23-51) was isolated and characterized as a product. The close relationship to the *Renilla* and *Vargula* luciferins is obvious, and it is thought that coelenterazine is present in aequorin and other photoproteins as a peroxide (as in Eq. 23-45). For this reason no additional oxygen is needed to complete the reaction when $Ca^{2+}$ acts to alter the conformation of the protein.[675–676a] The structure of a photoactive intermediate from the coelenterazine-containing protein obelin (from *Obelia longissima*), however, shows only one oxygen atom attached to C2 (Fig. 23-51)[676a] Although coelenterazine is utilized by many cnidarians they apparently cannot synthesize the compound but must obtain it through their diet. The source of biosynthesis is unknown.[676b] Some dinoflagellates emit light from a 137-kDa luciferin that contains three homologous domains each of which binds a molecule of a tetrapyrrole.[676c] From its structure the latter appears to have arisen from chlorophyll (Fig. 23-20), whose ring has been opened to give a structure somewhat similar to that of phytochrome (Fig. 23-23).[676d]

The first step in the formation of light in the firefly is a reaction with ATP to form luciferyl adenylate (Eq. 23-46, step *a*).[676e] The proton on the carbon may then be removed making use of the electron accepting properties of the adjacent ring system and carbonyl group before addition of the $O_2$. The reactions should be compared to those catalyzed by oxygenases, e.g., Eq. 18-42. The large 62-kDa firefly luciferase has a

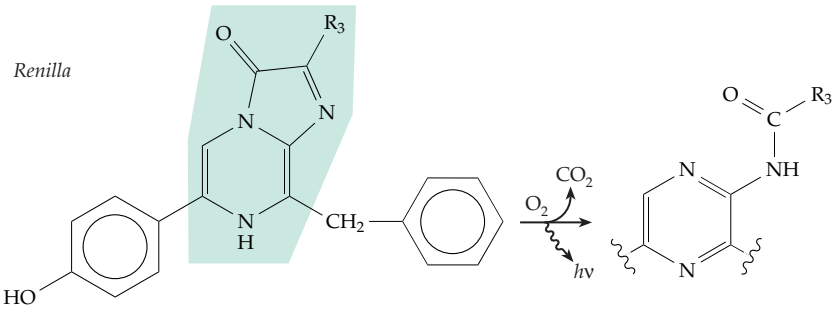



Luciferyl adenylate

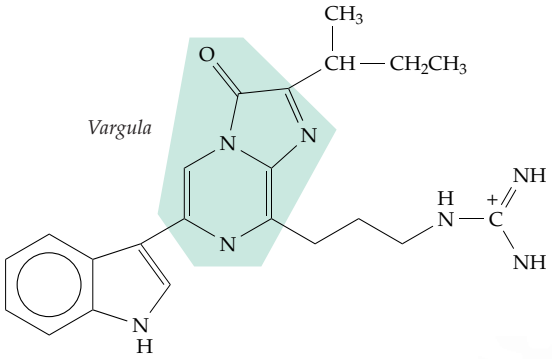


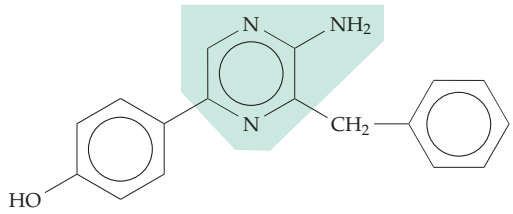

Luciferyl sulfate of *Renilla*

*Renilla*




*Vargula*




Compound AF-350 from *Aequorea*


Photoprotein **obelin** from *obelia* showing an oxy complex of bound coelenterazina. The oxygen atom is green. From Liu *et al.*[676a]

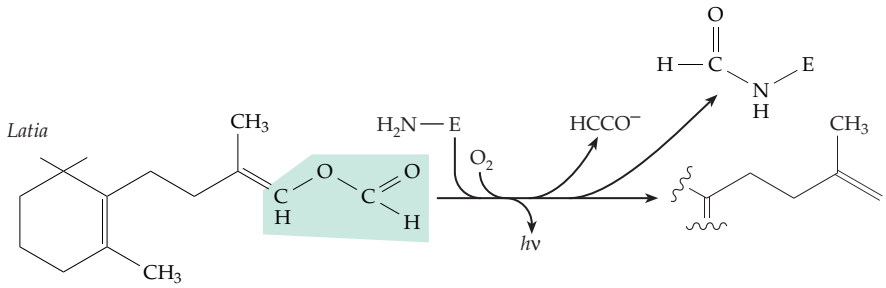
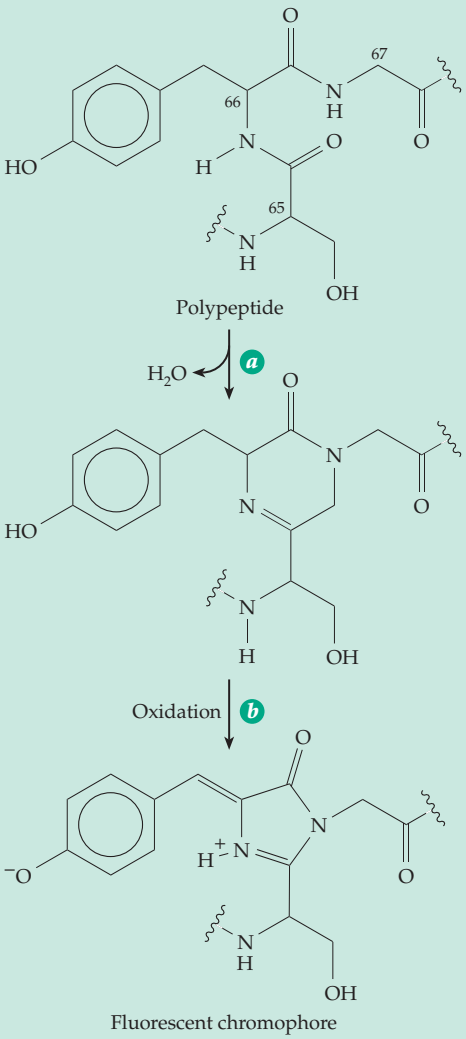


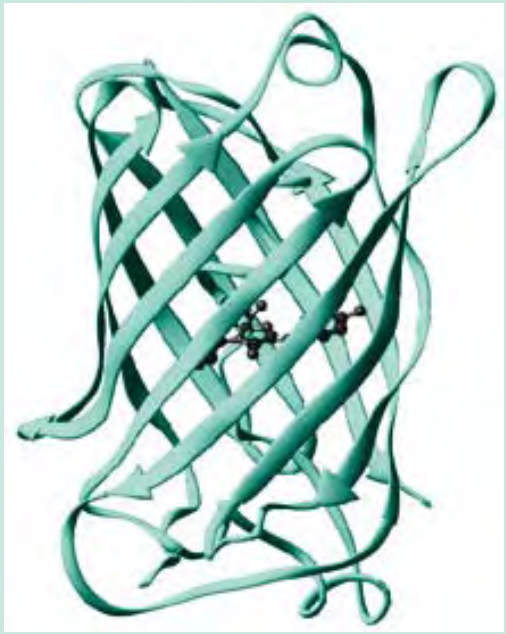

**Figure 23-51** Structures of luciferins from several luminous organisms. The forms shown are the "activated" molecules ready to react with $O_2$. However, compound AF-350 is a breakdown product of the $Ca^{2+}$-activated luminous protein aequorin.

## BOX 23-A THE GREEN FLUORESCENT PROTEIN AND OTHER LIGHT-EMITTING ANTENNAS

The $Ca^{2+}$ -dependent luminescence of the jellyfish *Aquorea*, discussed in the main text, consists of blue light with a maximum intensity at 470 nm. However, the living organism has a more brilliant green luminescence. The excitation energy is transferred in a nonradiationless process to a green fluorescent protein with absorption maxima at 395 and 475 nm and an emission maximum for fluorescence at 508 nm.[a–c] A similar protein is used by *Renilla*.[d] The 238-residue green fluorescent protein (**GFP**) has a compact three-dimensional structure, an 11-stranded antiparallel β barrel with helices at one end and longer loops at the other, a "**β can**." The chromophore lies in the center of the cylinder. Numerous mutant forms have been made but only 15 residues in the terminal regions could be deleted without loss of fluorescence.[e]

The chromophore of GFP is formed spontaneously from -Ser-Tyr-Gly, residues 65–67 of the protein.[a,f,g] The entire protein has been synthesized chemically and forms the fluorescent chromophore just as in the protein produced biologically.[h] The reaction is autocatalytic, requiring only $O_2$ as the oxidant. Although in the living organism it accepts energy from the aqueorin chromophore a near ultraviolet lamp will elicit the fluorescence in the laboratory.



Ribbon drawing of the 238-residue green fluorescent protein showing the embedded chromophore as a ball-and-stick structure.[i] Courtesy of S. James Remington.

The green fluorescent protein is used widely in molecular biology as a fluorescent tag. Its rugged chemical nature, resistance to degradation by proteases and ability to form the chromophore autocatalytically from its own amino acids have permitted many applications. The entire GFP can be attached covalently to numerous cell components. Its gene can be spliced into the genome of an organism to form green-glowing worms, flies, and plants. Put behind a suitable promoter the fluorescent protein may be synthesized or not depending upon the control mechanism of a particular promoter (Chapter 28).[c,j–m]

The phenolic group of the GFP chromophore is apparently dissociated in the form absorbing at 395 nm and is in a tautomeric equilibrium with the other species. However, some histidine-containing replacement mutants have pH-dependent spectral changes in which the dipolar ionic form shown above, and absorbing at a longer wavelength, loses

**BOX 23-A (continued)**

a proton to form the anion. Observation of the excitation spectra for fluorescence of such mutant proteins within cells provides a new method for measuring the internal pH of cells and their organelles.[n–q] Some mutants emit blue or yellow light.[r,s] Two different color mutants have been fused with a molecule of calmodulin in such a way that the $Ca^{2+}$-induced conformational change in calmodulin allows fluorescence resonance energy transfer (FRET) between the two fluorophores. This provides a new type of calcium ion indicator.[s–u] A structurally similar red fluorescent protein, produced by a coral, extends the range of colors available as biological markers and may be useful in applications based on resonance energy transfer.[v,w,x]

Bioluminescent bacteria of the genus *Photobacterium* produce large amounts of a highly fluorescent 189-residue **lumazine protein** which contains bound 6,7-dimethyl-8-ribityllumazine (see Fig. 25-20).[y,z] Like the green fluorescent protein, it serves as a secondary light emitter receiving its energy by transfer from the flavin primary emitter. Its presence shifts the light-emission from the 495 nm of the luciferase to as low as 470 nm. *Vibrio fischeri* synthesizes a **yellow fluorescent protein** with either bound FMN or riboflavin. Its emission is at 542 nm, a longer wavelength than that of the luciferase emission. The value to the bacteria may be the higher quantum yield of fluorescence from the antenna emitter than from the luciferase. The luciferase fluorescence has a lifetime of 10 ns but on addition of the yellow fluorescent protein it is decreased to 0.25 ns with a greatly intensified emission.[u]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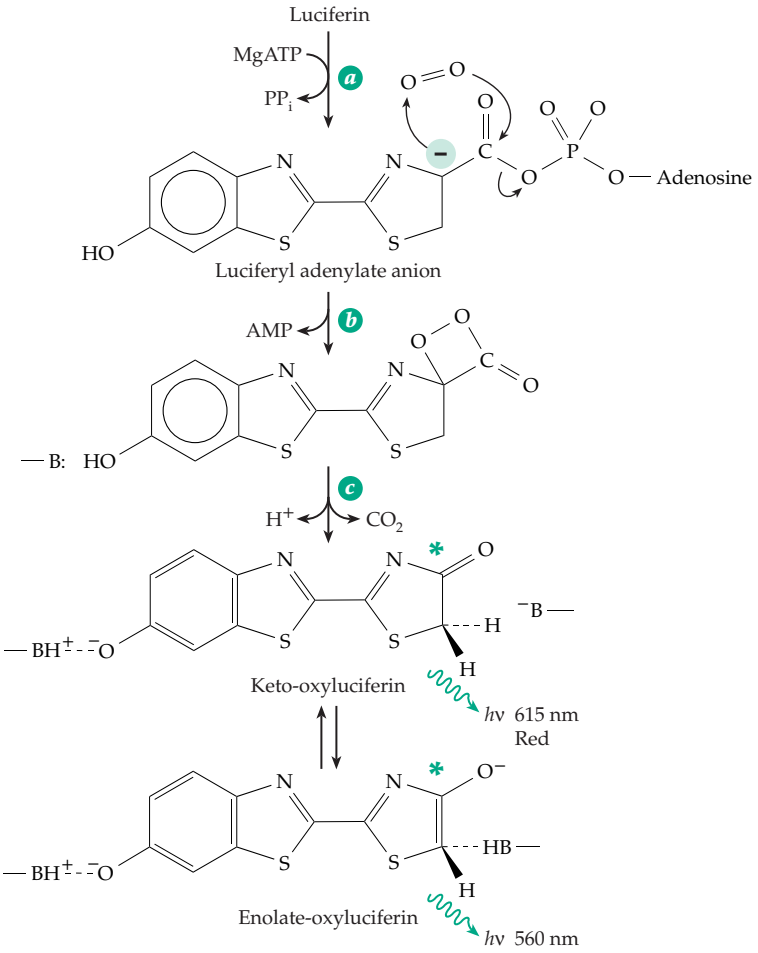
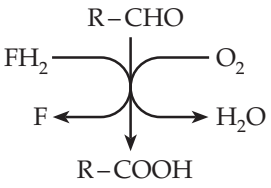
two-domain structure that suggests that domain movement may be essential to bring reactants together.[660] The structure is homologous to those of acyl-CoA ligases and peptide synthetases which share a similarity in step *a*. Formation of the dioxetane intermediate is assisted by the loss of AMP (Eq. 23-46, step *b*). The electronically excited decarboxylation product interacts with groups in the protein. It apparently exists as an anion bound to acidic and basic groups of the protein. An equilibrium between oxo- and enolate forms is thought to regulate the color of the emitted light which can vary from red to yellow and green in various fireflies, other beetles, and larvae.[661,661a,677,677a] Oxyluciferin can be reconverted to luciferin for the next flash.[677b]

A very different light-producing reaction is used by the limpet *Latia*. The luciferin is an unusual terpene derivative (Fig. 23-51) that lacks any chromophore suitable for light emission.[678] Evidently oxidation of this luciferin causes electronic excitation of some other molecule, presumably a "purple protein" which is also needed for luminescence. A complex of luciferin plus the purple protein is believed to react with the luciferase (abbreviated E-$NH_2$ in Fig. 23-51). It is




(23-46)



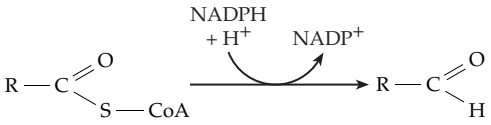

(23-47)

thought that the formyl group is released from its enolic ester linkage in the luciferin. A Schiff base of the resulting aldehyde may form with the enzyme and react with oxygen. Yet another type of luciferin is found in dinoflagellates (Fig. 23-51).[679]

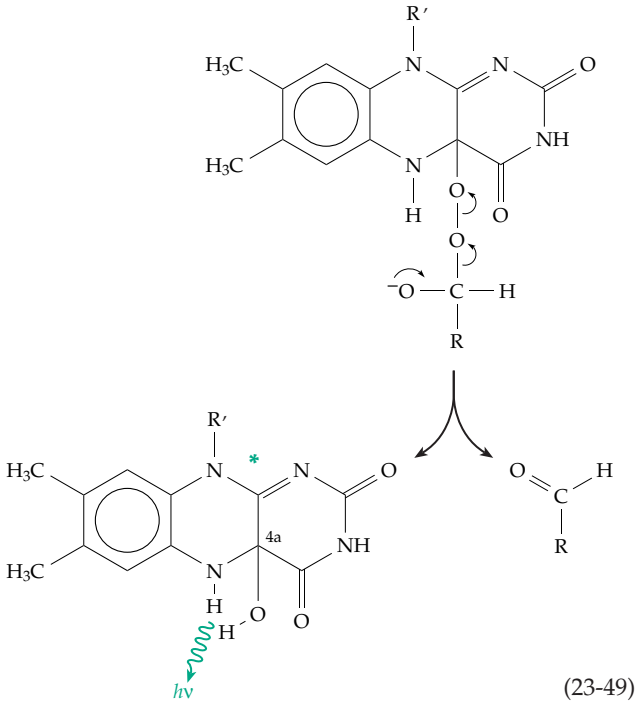
Luminescent bacteria all appear to obtain light from a riboflavin-5'-phosphate dependent oxygenase, which converts a long-chain aldehyde (usually *n*-tetradecanal) to a carboxylic acid (Eq. 23-47). Here $FH_2$ is the riboflavin 5'-*P*, which is thought to be supplied by a flavin reductase.[679a]

Bacterial luciferases are αβ heterodimers with subunit masses of ~40(α) and 35 (β) kDa.[664,680,681] In *Vibrio harveyi* these are encoded by the *lux A* and *lux B* genes. At least five other genes are essential for light production including two regulatory genes.[682,683] The tetradecanal and other long-chain aldehydes are supplied by reduction of the corresponding acyl-CoA (Eq. 23-48). A special thioesterase releases a myristoyl group from an acyl carrier protein, diverting it for luminescence in *V. harveyi*.[684] There is good evidence from $^{13}C$ NMR and electronic spectra for an enzyme-bound reduced flavin hydroperoxide as in Eq. 15-31. While this hydroperoxide can decompose slowly to flavin and $H_2O_2$ in the dark, it can also carry out the oxidation of the aldehyde with emission of light.[685,685a] The luminescent emission spectrum resembles the fluorescence spectrum of the 4a –OH adduct (Eq. 23-49), which is probably the light-emitting species.[686–688]

Cells of *Vibrio fischeri*, from the light organ of the fish *Monocentrus japonicus*, emit light only in dense cultures where a chemical inducer identified as N-(2-oxocaproyl)homoserine lactone[689,690] accumulates.




(23-48)




(23-49)

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## Study Questions

1. Why is the Emerson enhancement effect (i.e., light at 650 nm plus 680 nm gives a higher rate of photosynthesis than either one alone) not observed with photosynthetic bacteria?

2. Agents that uncouple oxidative phosphorylation in mitochondria uncouple photoelectron transport and ATP formation in photosynthesis. Explain.

3. The action spectrum of photosynthesis, which describes the efficiency of photosynthesis as a function of the wavelength of incident light, has a valley around 550 nm. Why?

4. Plants exposed to $C^{18}O_2$ will have the $^{18}O$ first appear in a) carbohydrate; b) water; c) oxygen gas. (More than one answer may be true.)

5. Plants exposed to $H_2{}^{18}O$ will have the label first appear in a) oxygen gas; b) carbohydrate; c) $CO_2$. (More than one may be correct.)

6. The general equation describing the photosynthesis of glucose in higher plants is:

$$6\,CO_2 + 6\,H_2O \rightarrow C_6H_{12}O_6$$

   We know that the oxygen gas comes from water, yet the equation shows only six atoms of oxygen in water on the left-hand side vs 12 in oxygen gas on the right. Explain.

7. The fructose bisphosphatase of green plants has an amino acid sequence which is very similar to those of the corresponding enzymes isolated from other sources such as yeast or mammals, except that the plant enzyme has an additional sequence of 20 or so amino acids that has no counterpart in the enzymes found in the other species. What function might this additional sequence have in the plant enzyme?

8. The following substances are either inhibitors or activators of rubisco, the enzyme that catalyzes the condensation of $CO_2$ with ribulose bisphosphate to yield 3-phosphoglycerate. State whether the substance should be an activator or an inhibitor of the enzyme and succinctly provide the logic supporting your conclusion.
   a) Fructose 1,6-bisphosphate
   b) Visible light
   c) NADPH

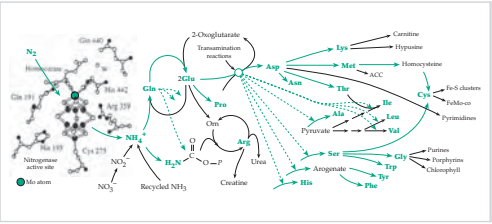
9. The reagent DCMU specifically inhibits electron transfer to plastoquinone in photosystem II. Discuss how the administration of this compound to a suspension of illuminated chloroplasts will affect the production of oxygen, ATP, and NADPH.

10. A chemical reagent is added to a solution of plant chloroplasts which immediately and specifically poisons photosystem II. What is the *short-term* effect of each of the following? Give a one-sentence defense for your conclusion.
    a) Cyclic photophosphorylation
    b) Noncyclic photophosphorylation
    c) Photorespiration
    d) NADPH production

11. If a $C_3$ and a $C_4$ plant are placed together in a sealed illuminated box, the $C_3$ plant withers and dies long before the $C_4$ plant. Explain.

12. What tricarboxylic acid cycle enzyme is analogous to the malate enzyme of bundle-sheath cells? What is the mechanism of the reaction?

13. There are two different forms of glyceraldehyde-3-phosphate dehydrogenase in higher plant cells.
    a) In which cell compartment is each one found?
    b) What are the reactions catalyzed by these two isozymes?
    c) Why are there two forms?

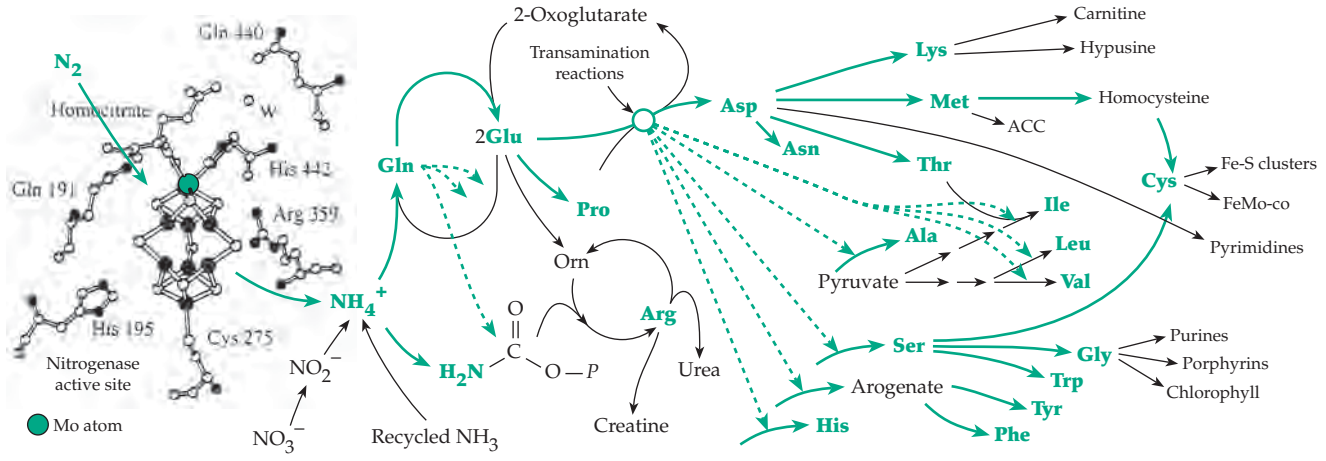



The air provides an abundant source of nitrogen for living organisms. Nitrogenase present in specialized bacteria utilizes the molybdenum- and iron-containing FeMo-co to reduce $N_2$ to two molecules of $NH_3$ (or $NH_4^+$). $NH_3$ is incorporated into the side chain of glutamine and much is transferred to the 5-carbon skeleton of 2-oxoglutarate to form glutamate. Nitrogen from glutamate and glutamine moves into the other amino acids via action of transaminases and glutamine amidotransferases. Thousands of compounds, a few of which are indicated here, are formed. (The 20 amino acid constituents of proteins are shown in green.) $NH_3$ from decaying materials is recycled, often after oxidation to nitrite or nitrate. Nitrates may also be formed by lightening and $NH_3$ industrially by catalytic reductions of $N_2$ by $H_2$ at high temperature and pressure (the Haber process).

# Contents

# The Metabolism of Nitrogen and Amino Acids

# 24




Because it is found in so many compounds and can exist in several oxidation states, nitrogen has a complex metabolism. The inorganic forms of nitrogen found in our surroundings range from the highly oxidized nitrate ion, in which N has an oxidation state of +5, to ammonia, in which the oxidation state is −3. Living cells both reduce and oxidize these inorganic forms. The organic forms of nitrogen are most often derived by incorporation of **ammonium ions** into amino groups or amide groups. Once it has been incorporated into an organic compound, nitrogen can be transferred into many other carbon compounds. Certain compounds including glutamic acid, aspartic acid, glutamine, asparagine, and carbamoyl phosphate are especially active in these transfer reactions. They constitute a **nitrogen pool** from which nitrogen can be withdrawn and to which it can be returned.

In addition to the pathways for synthesis and degradation of nitrogenous substances, many organisms have specialized metabolism for incorporation of excess nitrogen into relatively nontoxic excretion products. All of these aspects of nitrogen metabolism will be dealt with in this and the following chapter. We will look first at the reactions by which organic nitrogen compounds are formed from inorganic compounds, then at the reactions of the nitrogen pool. After that we will examine the specific reactions of synthesis and catabolism of individual nitrogenous compounds.

## A. Fixation of $N_2$ and the Nitrogen Cycle

Most of the nitrogen of the biosphere exists as the unreactive $N_2$, which makes up 80% of the molecules of air. The "fixation" of $N_2$ occurs principally by the action of a group of bacteria known as **diazotrophs** and to a lesser extent by lightning, which forms oxides of nitrogen and eventually nitrate and nitrite. Human beings also contribute a smaller but significant share through production of chemical fertilizer by the Haber process. These reactions are an important part of the **nitrogen cycle**.[1,2] Quantitatively even more important are the biochemical processes of **nitrification**, by which ammonium ions from decaying organic materials are oxidized to $NO_2^-$ and $NO_3^-$ by soil bacteria (Fig. 24-1), and reactions of reduction and **assimilation** of nitrate and nitrite by bacteria, fungi, and green plants. Another reductive process catalyzed by **denitrifying bacteria** returns $N_2$ to the atmosphere (Fig. 24-1).

### 1. Reduction of Elemental Nitrogen

One of the most remarkable reactions of nitrogen metabolism is the conversion of dinitrogen ($N_2$) to ammonia. It was estimated that in 1974 this biological nitrogen fixation added $17 \times 10^{10}$ kg of nitrogen to the earth (compared with $4 \times 10^{10}$ kg fixed by chemical reactions).[3] The quantitative significance can be more easily appreciated by the realization that one square meter of land planted to nodulated legumes such as soybeans can fix 10–30 g of nitrogen per year.

Fixation of $N_2$ by *Clostridium pasteurianum* and a few other species was recognized by Winogradsky[4] in 1893. Subsequent nutritional studies indicated that both iron and molybdenum were required for the process. Inhibition by CO and $N_2O$ was observed. While ammonia was the suggested product, the possibility remained that more oxidized compounds such

as hydroxylamine were the ones first incorporated into organic substances. When cell-free preparations capable of fixing nitrogen were obtained in 1960 rapid progress became possible.[5] It was discovered that nitrogen-fixing bacteria are invariably able to reduce acetylene to ethylene, a catalytic ability that goes hand in hand with the ability to reduce $N_2$. A simple, sensitive **acetylene reduction test** permits easy measurement of the nitrogen-fixing potential of cells.

Application of this test revealed that nitrogen fixation is not restricted to a few species, but is a widespread ability of many prokaryotes. Most studied are *Azotobacter vinelandii*, Winogradsky's *C. pasteurianum*, *Klebsiella pneumoniae* (a close relative of *E. coli*), and several species of *Rhizobium*, the symbiotic bacterium of root nodules of legumes. The latter deserves special attention. Although some free-living rhizobia reduce $N_2$, the reaction usually takes place only in nodules developed by infected roots. Within these nodules the bacteria degenerate into **bacteroids**; and the special hemoglobin **leghemoglobin**,[6,7] whose sequence is specified by a plant gene,[8] is synthesized.

Legumes are not the only plants with nitrogen-fixing symbionts.[9] Some other angiosperms are hosts to nitrogen-fixing actinomycetes and some gymnosperms contain nitrogen-fixing blue-green algae. Leaf nodules of certain plants infected with *Klebsiella* fix nitrogen. While the nutritional significance is uncertain, nitrogen-fixing strains of *Klebsiella* have also been found in the intestinal tracts of humans in New Guinea. Of the free-living nitrogen-fixing organisms, cyanobacteria appear to be of most importance quantitatively. For example, in rice paddy fields cyanobacteria may fix from 2.4 to 10 g of nitrogen per square meter per year. Cyanobacteria in the oceans fix enormous amounts of nitrogen.[9a]

## 2. Nitrogenases

Cell-free nitrogenases have been isolated from a number of organisms. These enzymes all share the property of being inactivated by oxygen, a fact that impeded early work. Apparently nitrogen fixation occurs in anaerobic regions of cells. Leghemoglobin may protect the nitrogen-fixing enzymes in root nodules from oxygen. It probably also functions to deliver $O_2$ by facilitated diffusion to the aerobic mitochondria of the bacteroids at a stable, low partial pressure.[6,10] Some bacteria utilize protective proteins to shield the nitrogenase molecules when the $O_2$ pressure is too high.[10a]

Nitrogenases catalyze the six-electron reduction of $N_2$ to ammonia (Eq. 24-1) and are also able to reduce

$$N_2 + 6\,H^+ + 6\,e^- \rightarrow 2\,NH_3 \qquad (24\text{-}1)$$

many other compounds. For example, the reduction of acetylene to ethylene (Eq. 24-2) is a two-electron process. Azide is reduced to $N_2$ and $NH_4^+$ in another two-electron reduction (Eq. 24-3). Cyanide ions yield methane and ammonia (Eq. 24-4).[11] Alkyl nitriles as well as $N_2O$ and carbonyl sulfide (COS) are also reduced. Carbon dioxide is reduced slowly to CO[12], and nitrogenases invariably catalyze reduction of protons to $H_2$ (Eq. 24-5).

$$HC{\equiv}CH + 2\,H^+ + 2\,e^- \rightarrow H_2C{=}CH_2 \qquad (24\text{-}2)$$

$$N{=}N{=}N^- + 4\,H^+ + 2\,e^- \rightarrow N_2 + NH_4^+ \qquad (24\text{-}3)$$

$$^-C{\equiv}N + 8\,H^+ + 6\,e^- \rightarrow CH_4 + NH_4^+ \qquad (24\text{-}4)$$

$$2\,H^+ + 2\,e^- \rightarrow H_2 \qquad (24\text{-}5)$$

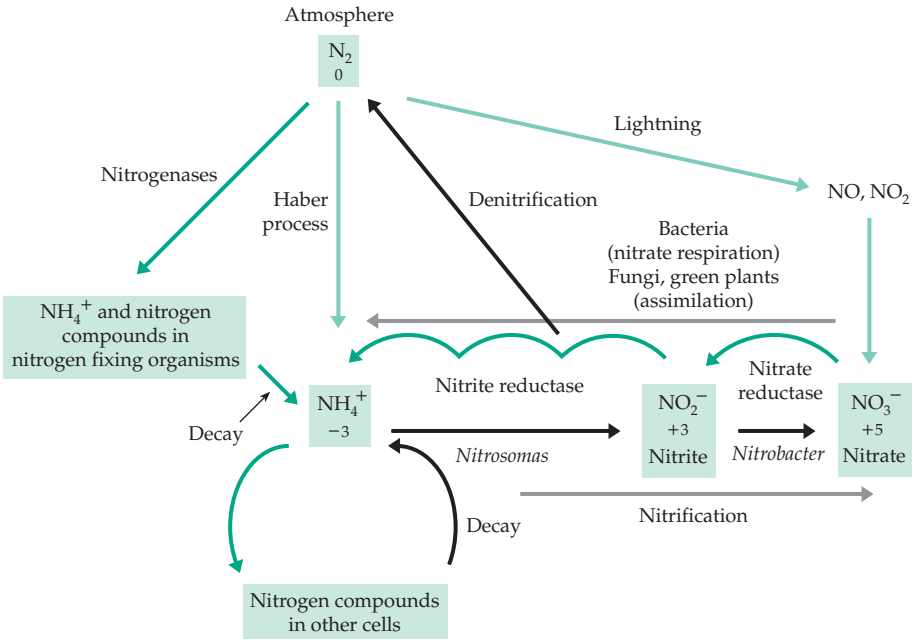



**Figure 24-1** The nitrogen cycle. Conversion of $N_2$ (oxidation state 0) to $NH_4^+$ by nitrogen-fixing bacteria, assimilation of $NH_4^+$ by other organisms, decay of organic matter, oxidation of $NH_4^+$ by the nitrifying bacteria *Nitrosomas* and *Nitrobacter*, reduction of $NO_3^-$ and $NO_2^-$ back to $NH_4^+$, and release of nitrogen as $N_2$ by denitrifying bacteria are all part of this complex cycle.[1]

In early experiments it was found that sodium pyruvate was required for fixation of $N_2$ in cell-free extracts, and that large amounts of $CO_2$ and $H_2$ accumulated. Investigation showed that cleavage of pyruvate supplies cells with two important products: ATP and reduced ferredoxin. Pyruvate can be replaced by a mixture of ATP plus $Mg^{2+}$ and reduced ferredoxin ($Fd_{red}$). Furthermore, the nonbiological reductant dithionite ($S_2O_4^{2-}$) can replace the reduced ferredoxin. Since ADP is inhibitory to the nitrogenase system, it is best in laboratory studies to supply ATP from an ATP-generating system such as a mixture of creatine phosphate, creatine kinase, and a small amount of ADP (Eqs. 6-65, 6-67).

The commonest type of nitrogenase can be separated easily into two components (Fig. 24-2). One of these, the **iron protein** (dinitrogenase reductase, azoferredoxin, or component II), is an extremely oxygen-sensitive iron-sulfur protein. It consists of two identical ~32-kDa peptide chains; those of *A. vinlandii* each contain 189 amino acid residues. The three-dimensional structure of the dimeric protein[13–16] shows that each subunit forms a nucleotide-binding domain with an ATP-binding site. About 2 nm away from this site is *a single $Fe_4S_4$ cluster which is shared symmetrically by the two subunits of the protein*. Each subunit contributes two thiolate groups from Cys 97 and Cys 132 as well as three N–H--S hydrogen bonds from NH groups at helix ends.[13]

The other component, the **molybdenum–iron protein** (dinitrogenase, molybdoferredoxin, or component I), contains both iron and molybdenum as well as labile sulfide. It is a mixed ($\alpha_2\beta_2$) tetramer of ~240-kDa mass and an analytical metal ion composition ~$Mo_2Fe_{30}S_{26}$. However, the X-ray structure[16–19a] suggests the composition $Mo_2Fe_{34}S_{36}$. The MoFe protein is a symmetric molecule in which each αβ subunit contains two types of complex metal clusters. The active sites for $N_2$ reduction, which are embedded in the α subunits, contain the **FeMo-coenzyme** molecules, each with the metal composition $MoFe_7S_9$ and also containing a molecule of **homocitrate**.[20–22a] The other clusters, known as **P-clusters**, are shared between the α and β subunits, which for *A. vinlandii* contain 491 and 522

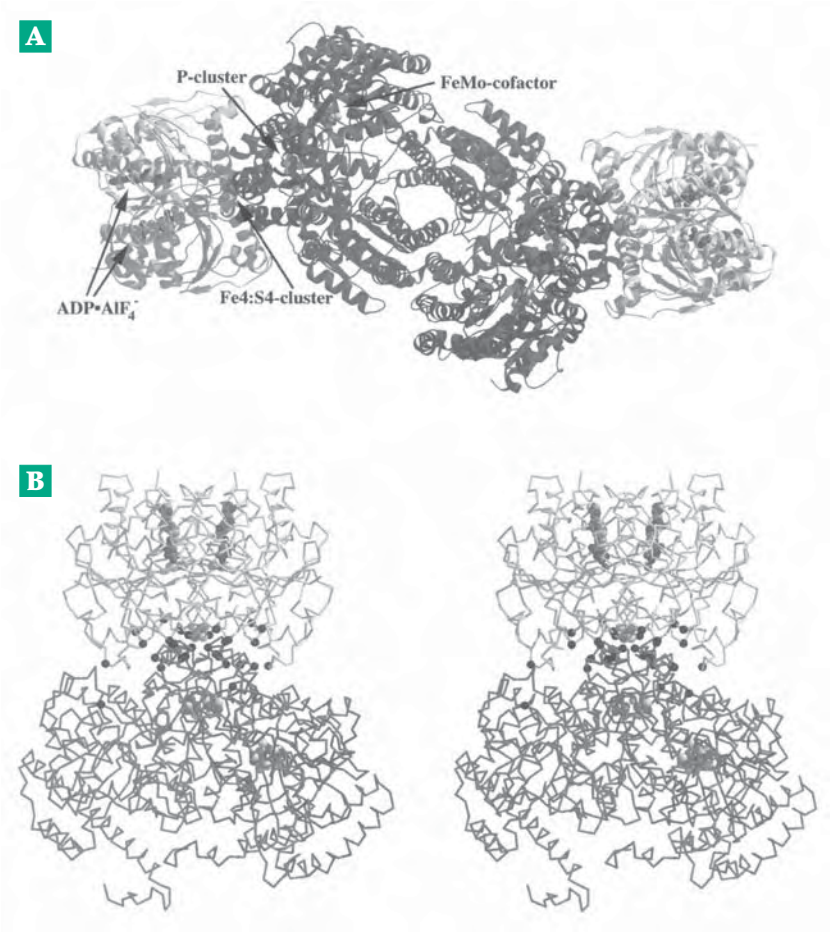
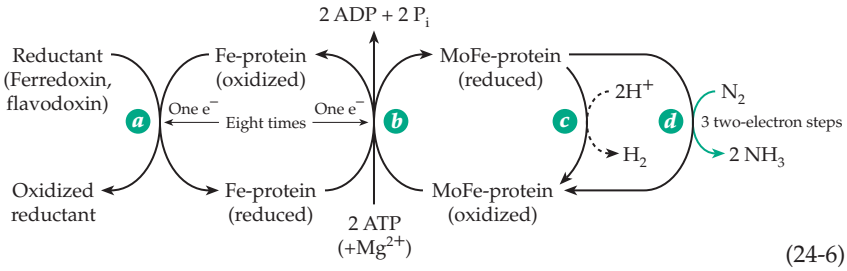



**Figure 24-2** (A) Ribbon drawing of the three-dimensional structure of the nitrogenase from *Azotobacter vinlandii* viewed down the twofold axis of the molecule. The αβ subunits of the MoFe-protein are in the center while the Fe-protein subunits are at the outer ends. The $Fe_4S_4$ cluster of the Fe-protein and the FeMo-co and P-cluster of the MoFe-protein are marked for the left-hand complex. The site of binding of ATP is identified by the bound ADP•$AlF_4^-$ complex. (B) Stereoscopic view of one complete half of the nitrogenase complex at a 90° angle to the view in (A). The Fe-protein is at the top, and the MoFe-protein is below. The ADP•$AlF_4^-$ complex is visible in the two symmetrically located binding sites of the Fe-protein. The shared $Fe_4S_4$ cluster is in the center above the P-cluster. The small black spheres mark α-carbons of residues that interact in forming the complex of Fe-protein and MoFe-proteins. When the $Fe_4S_4$ cluster accepts one electron from a molecule of ferredoxin or flavodoxin, the Fe-protein binds to the MoFe-protein and donates an electron to one of the two nearby P-clusters (one of which is in each of the αβ subunits). At the same time both of the molecules of ATP bound to the Fe-protein are hydrolyzed. The oxidized Fe-protein then dissociates from the complex and is replaced by another reduced Fe-protein–ATP complex. The net result is that each electron is "pumped" from the $Fe_4S_4$ cluster of the Fe-protein into a P-cluster of one of the αβ units of the MoFe-protein. Electrons then move from the P-clusters into the FeMo-coenzyme. From Schindelin *et al.*[19] Courtesy of Douglas C. Rees.

amino acid residues, respectively. Each P-cluster is actually a *joined pair* of cubane-type clusters, one $Fe_4S_4$ and one $Fe_4S_3$ with two bridging cysteine –SH groups and one iron atom bonded to three sulfide sulfur atoms (Fig. 24-3).[17,23] The FeMo-coenzyme can be released from the MoFe-protein by acid denaturation followed by extraction with dimethylformamide.[24] While homocitrate was identified as a component of the isolated coenzyme, the three-dimensional structure of FeMo-co was deduced from X-ray crystallography of the intact molybdenum–iron protein.[14,17,18]




(24-6)

When the Fe-protein is reduced an EPR signal at $g = 1.94$, typical of iron–sulfur proteins (Fig. 16-17), is observed. This signal is altered by interaction with Mg and ATP, whereas ATP has no effect on the complex EPR signals produced upon reduction of MoFd. These are among the observations that led to the concept that the Fe-protein is an electron carrier responsible for reduction of the molybdenum in the MoFe-protein. The Mo(IV) or two atoms of Mo(III) formed in this way could then reduce $N_2$ in three two-electron steps with formation of Mo(VI) (Eq. 24-6). Three successive two-electron steps are required to completely reduce $N_2$ to two molecules of ammonia. An unexpected feature of nitrogenase action is that there is inevitably what was once regarded as a side reaction, the reduction of protons to $H_2$.[25,26] The amount of $H_2$ formed is variable and may be much greater than that of $N_2$ reduced. However, at high pressures of $N_2$ the ratio of $H_2$ formed to $N_2$ reduced is 1:1. This led to the suggestion[28] that $H_2$ formation is not a side reaction but an essential step in preparing the active site for the binding of $N_2$. Two protons that are bound somewhere on the reduced MoFe-protein could be reduced to $H_2$ in an obligatory step (Eq. 24-6, step *c*) that would, for example, cause a conformational change required for binding of $N_2$. If no reducible substrate ($N_2$, $C_2H_2$, etc.) is present, $H_2$ would still be formed slowly. Reducible substrates inhibit $H_2$ formation. However, addition of $N_2$ or any other reducible substrate causes an initial "burst" of $H_2$ to be released. This can be measured readily when a slow substrate such as $CN^-$ is used as the inhibitor. The amount of $H_2$ released in the burst is stoichiometric with one $H_2$ per Mo being formed.[26] The overall stoichiometry for reduction of one $N_2$ becomes:

$$N_2 + 8\ e^- + 8\ H^+ + 16\ MgATP \rightarrow 2\ NH_3 + H_2 + 16\ MgADP + 16\ P_i \quad (24\text{-}7)$$

A second remarkable feature of nitrogenase is a requirement for hydrolysis of MgATP that is coupled to reduction of the MoFe-protein (Eq. 24-6). Two molecules of ATP are hydrolyzed to ADP and inorganic phosphate for each electron transferred. This large ATP requirement seems surprising in view of the fact that reduction of $N_2$ by reduced ferredoxin (Eq. 24-8) is thermodynamically spontaneous:

$$N_2 + 6\ Fd_{red} + 8\ H^+ \rightarrow 2\ NH_4^+ + 6\ Fd_{ox}^+$$
$$\Delta G'\ (pH\ 7) = -89.3\ kJ\ mol^{-1} \quad (24\text{-}8)$$

However, $N_2$ is exceedingly unreactive. In the commercial Haber process high pressure and temperature are needed to cause $H_2$ and $N_2$ to combine. Evidently cleavage of 16 molecules of ATP must be coupled to the nitrogenase reduction system to overcome the very high activation energy.

Not only are two molecules of ATP hydrolyzed to pump each electron, but the Fe-protein must receive electrons from a powerful (low $E^{\circ\prime}$) reductant such as reduced ferredoxin, reduced flavodoxin, or dithionite. *Klebsiella pneumoniae* contains a **pyruvate:flavodoxin oxidoreductase** (Eq. 15-35) that reduces either flavodoxin or ferredoxin to provide the low potential electron donor.[29,30] In some bacteria, e.g., the strictly aerobic *Azotobacter*, NADPH is the electron donor for reduction of $N_2$. The Fe-protein is thought to accept electrons from a chain that includes at least the ordinary bacterial ferredoxin (Fd) and a special one-electron-accepting **azotoflavin,** a flavoprotein that is somewhat larger than the flavodoxins (Chapter 15) and appears to play a specific role in $N_2$ fixation.[31] In *Clostridium* and *Rhizobium* reduced ferredoxins generated by cleavage of pyruvate reduce nitrogenase directly.[32]

***The mechanism of nitrogenase action.*** The one-electron reduction of the $Fe_4S_4$ cluster of the Fe-protein (step *a* of Eq. 24-6) initiates the action. This reaction occurs before the Fe-protein forms a complex with the MoFe-protein. Following this initial reduction step the two molecules of ATP required for step *b* of Eq. 24-6 bind to the Fe-protein. One is bound to each subunit of this protein but neither is immediately adjacent to the shared $Fe_4S_4$ cluster, as can be seen from Fig. 14-3B. The binding to MgATP appears to induce a conformational change that permits the "docking" of the Fe-protein with the MoFe-protein to

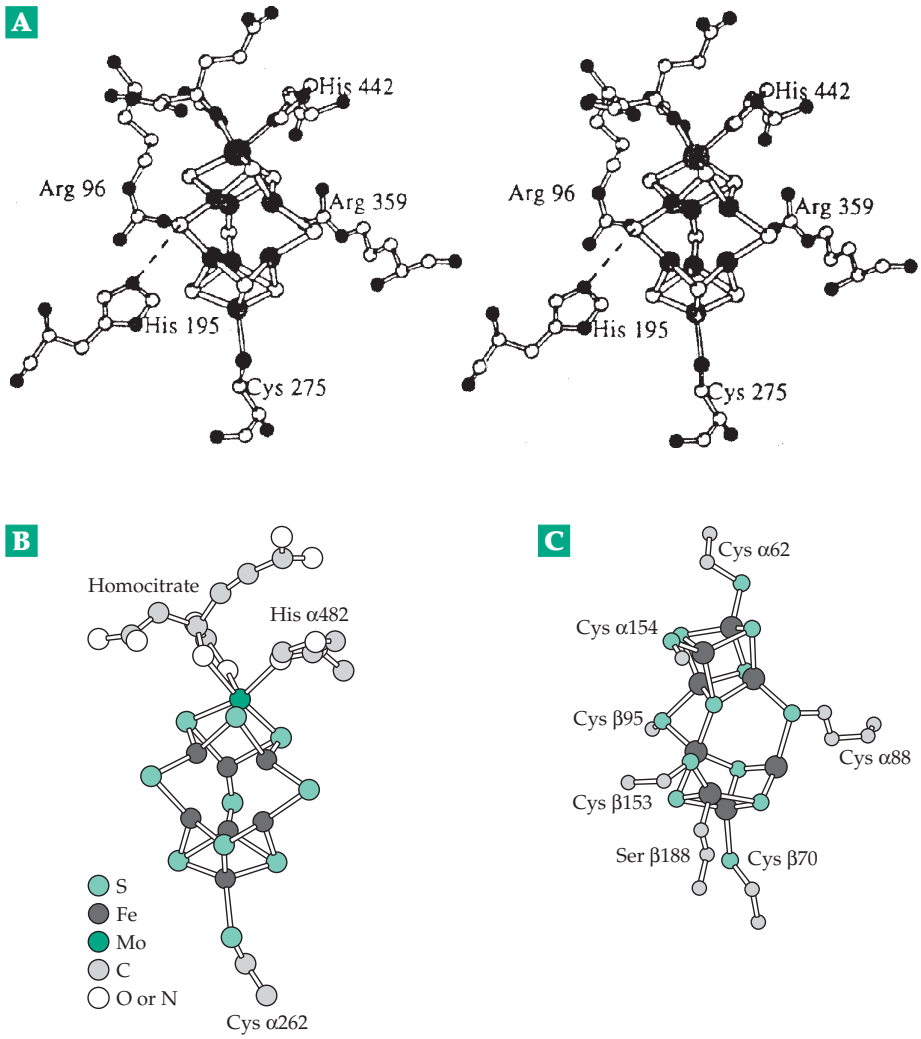



**Figure 24-3** Structures of the metal-sulfide clusters of the MoFe-protein. (A) Stereoscopic view of the FeMo-co coenzyme with interacting side chains from the MoFe-protein of *A. vinlandii*. After Kim *et al.*[27] (B) FeMo-co with atom labels. From Kim *et al.*[18] (C) The structure of the oxidized form of the P-cluster. From Peters *et al.*[23] Recent studies by Einsle *et al.*[22a] indicate that the cluster probably also contains a nitrogen atom that is held within the cluster by coordination to six of the iron atoms.

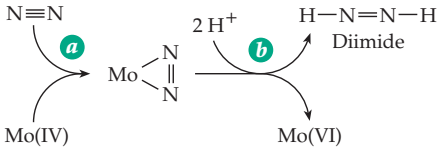
form the complex in which the electron transfer of step *b* (Eq. 24-6) occurs. Abundant evidence indicates that electron transfer does not occur without the binding of MgATP.[33–35d] The electron transfer is coupled to the hydrolysis of the ATP, but the two reactions appear to be consecutive events. In a deletion mutant of the Fe-protein (lacking Leu 127) the hydrolysis of ATP does not occur, but the complex between Fe-protein and MoFe-protein is formed and electron transfer to the MoFe-protein takes place.[36–37a] The binding of the MgATP causes the midpoint redox potential to drop from −0.42 V to −0.62 V, assisting the transfer.[38] X-ray crystallographic studies reveal a distinct conformational change similar to those observed with G-proteins (Chapter 11) and involving movement of the $Fe_4S_4$ center into a better position for electron transfer.[19,38a]

After electron transfer the complex of the two proteins is thought to be tightly bonded when unhydrolyzed ATP is present.[35] This has allowed the direct observation and imaging of the complex at low resolution (~1.5 nm) using rapid synchrotron X-ray scattering measurements.[39] The ATP is hydrolyzed, and the Fe-proton is released from the complex.

Only one electron is transferred to the MoFe-protein in each catalytic cycle of the Fe-protein. Thus, the cycle must be repeated eight times to accomplish the reduction of $N_2 + 2\ H^+$. Where in the MoFe-protein does a transferred electron go? EPR spectroscopic and other experiments with incomplete and catalytically inactive molybdenum coenzyme[40] have provided a clear answer. The electron is transferred first to one of the two P-clusters, both of which are close to the $Fe_4S_4$ cluster of the Fe-protein. The transfer causes an observable change both in the spectroscopic properties and in the three-dimensional structure of the P-cluster.[23,40a] Since protons are needed at the active site for the reduction reactions (the FeMo-coenzyme), it is probable that hydrolysis of ATP in the Fe-protein is accompanied by transport of protons across the interface with the MoFe-protein. The electron transfer from the P-cluster on to the FeMo-co center would be assisted by a protic force resulting from ATP cleavage.

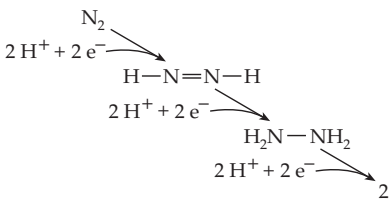
With defective FeMo-co (apparently lacking homocitrate) no reduction of $N_2$, acetylene, or protons is observed.[40] If intact FeMo-co is present, reduction of the cofactor can be observed. An $s = 3/2$ EPR signal arising from the Mo is seen,[40] and EXAFS measurements reveal decreased Mo–Fe distances as the coenzyme is reduced.[41] The molybdenum is probably present as Mo(VI) in the oxidized state of nitrogenase,[42] but after reduction it isn't clear whether it is Mo(III) or Mo(IV). Isolated FeMo-co exists in three identified oxidation states related by $E^{\circ\prime}$ values of −0.17 and −0.465 V.[43] Only the middle state is EPR-active, but it is the most reduced state that is involved in $N_2$ reduction.[42,43] With its P-cluster and FeMo-co center each αβ unit of the MoFe-protein could store several electrons. Two or more might be stored in a P-cluster, and Mo(VI) could, in principle, accept three electrons to form Mo(III). However, it is a little hard to imagine storage of the eight electrons needed to reduce both $N_2$ and $H_2$ (Eq. 24-8). The reduction of $N_2$ may begin before all eight electrons have been transferred into the MoFe-protein.

Another uncertainty lies in the mode of binding of $N_2$ and other substrates. Does $N_2$ bind end-on to Mo, does it slide between Fe atoms within the coenzyme, or does it bind in some other way? While $N_2$ is unreactive, it forms nitrides with metals and complexes with some metal chelates. These complexes are generally of an end-on nature, e.g., N≡N–Fe. Stiefel suggested that $N_2$ first forms a complex of this type with an iron atom of the MoFe-protein.[44] Then an atom of Mo(IV) could donate two electrons to the $N_2$ (Eq. 24-9, step *a*) to form a complex of $N_2$ and Mo(VI). Addition of two protons (Eq. 24-9, step *b*) would yield a molecule of **diimide**, which would stay bound at the



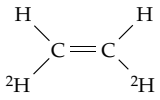

(24-9)

iron site while the molybdenum underwent another round of reduction. The diimide could be reduced to hydrazine and finally to ammonia (Eq. 24-10):




(24-10)

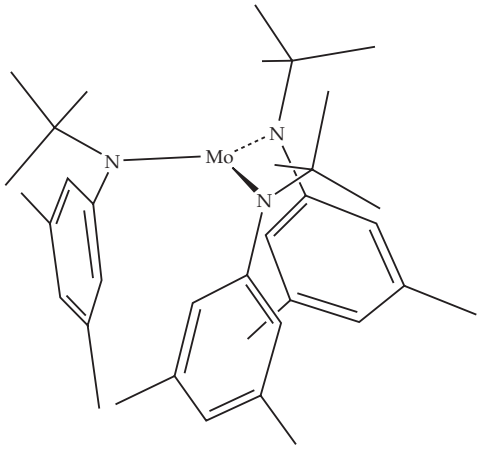
Mo(VI) attracts electrons sufficiently strongly that protons bound to surrounding ligands, such as $H_2O$, tend to dissociate completely. Thus, the molybdate ion $MoO_4^{2-}$ is not protonated. The same would be true of nitrogenous ligands of a protein that might be coordinated with the bound molybdenum. On the other hand, reduction to Mo(IV) would tend to favor protonation of ligands such as the His 442 imidazole seen in Fig. 24-3A. Concurrently with the electron transfer from molybdenum to $N_2$ these protons could be transferred to the $N_2$ molecule (Eq. 24-10). The fact that strictly *cis*-dideuteroethylene is formed from acetylene in the presence of $^2H_2O$ is in accord with this idea.




However, looking at the FeMo-co molecule and the crowded surroundings of the Mo atom it may be more likely that reduction of $N_2$ occurs while it is bound to iron. Theoretical calculations as well as experimental data support this possibility.[44a] Recent crystallographic studies at a resolution of 0.12 nm revealed the presence of an atom, probably N, coordinated to six Fe atoms of FeMo-Co. This suggets, as previously proposed by Thorneley and Lowe,[44b,c] that a nitride ion ($N_3^-$) may be an intermediate in the formation of $N_2$.

Many mutant forms of nitrogenase have been investigated. Substitutions of His 195, Lys 191, and Gly 69 of the α chain affect reactions with various substrates.[45–45d] For example, the mutant obtained by substitution of His 195, whose imidazole forms an N–H--S hydrogen bond to a central bridging sulfide atom of FeMo-co (Fig. 24-3A), with glutamine (H195Q mutant) reduces $N_2$ only very slowly.[45] However, it still reduces both acetylene and protons.[27,44a,45b] Thus, it may be that different modes of substrate binding are needed for the individual steps of Eq. 24-10.

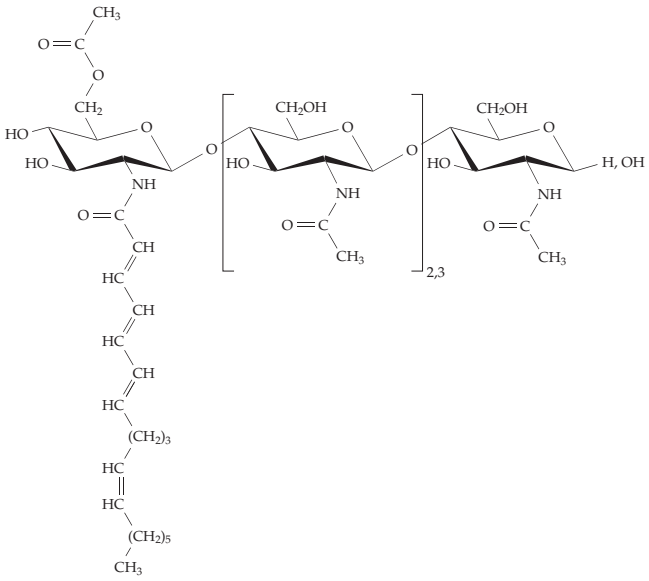
Because of the practical significance to agriculture there is interest in devising better nonenzymatic processes for fixing nitrogen using nitrogenase models that mimic the natural biological reaction.[42,46–49a] One interesting catalyst is the following molybdenum complex Mo(III)$(NRAr)_3$ where R = $C(C_2H_3)_2CH_3$ and Ar = 3,5-dimethylphenyl.[47,50,51]



Many other synthetic complexes have been studied including cubic $MoFe_3S_4$ clusters.[46] However, no exact chemical model for the FeMo-co coenzyme has been developed, and the rates of reaction for all of the model reactions are much slower than those of nitrogenases.

***Nitrogen fixation genes.*** At least 17 genes needed for nitrogen fixation are present in the 23-kb *nif* region of the *Klebsiella* chromosome[52,53] (Fig. 24-4). A similar gene cluster in *A. vinlandii*[54] contains five polygenic transcriptional units and one monogenic unit. The nitrogenase structural genes are *nifK, D*, and *H* as is indicated in Fig. 24-4. The *nifF* and *J* genes encode associated electron-transport proteins. *NifM* is needed to activate the Fe-protein in an unknown fashion. *NifS* encodes a cysteine desulfhydrase needed for assembly of Fe–S clusters in the nitrogenase and the nifU and nifY proteins assist the assembly.[54a,b] The chaperone GroEL is also required.[54c] *Nif Q, B, V, X, N, $E_1$*, and *H* are needed for synthesis of FeMo-co and for its incorporation into the MoFe-protein.[55,55a] *NifA* is an activator gene for the whole cluster including the *nifL* gene product, which is altered by the presence of $O_2$ or of glutamine. Accumulation of the latter in cells (see Section B,2) strongly represses transcription of the nitrogenase genes.

***Legume nodules and cyanobacterial heterocysts.*** Nitrogen fixation requires an anaerobic environment. Free-living bacteria fix nitrogen only when anaerobic. However, Rhizobia produce their own anaerobic environment by symbiotic association with the roots of legumes.[10,57–59] Formation of root nodules is a genetically determined process, several nodulation (*nod*) genes of the bacterium being required along with an unknown number of plant genes.[57,58] Initiation of nodulation results from a two-way molecular conversation between root hairs of the plant and bacterial cells.[60,61] The roots secrete **flavonoid compounds** (Chapter 21) which are recognized by bacterial sensors and induce transcription of the *nod* genes. Several of these genes encode enzymes required for synthesis of **Nod factors**,[62,63] small β-linked *N*-acetyl-D-glucosamine oligosaccharides containing 3–5 sugar residues and an *N*-linked long-chain fatty-acyl substituent at the nonreducing terminus (**lipochitooligosaccharides**). See also Box 20-E. Genes *nodA, B, C* specify enzymes needed for synthesis of the oligosaccharide core present in all Nod factors.



Structure of a Nod factor secreted by *Rhizobium leguminosarum*.[64]

Other Nod genes provide for modifications that restrict infection to specific species of legumes. For example, *nodS* encodes a methyltransferase and *nodU* a carbomoyltransferase.[65] NodH is a sulfotransferase.[66] NodD is a transcriptional activator that binds to DNA and induces the synthesis of the other Nod factors needed to initiate nodulation.[67] When an appropriate Nod factor is recognized, the root hairs on the legume

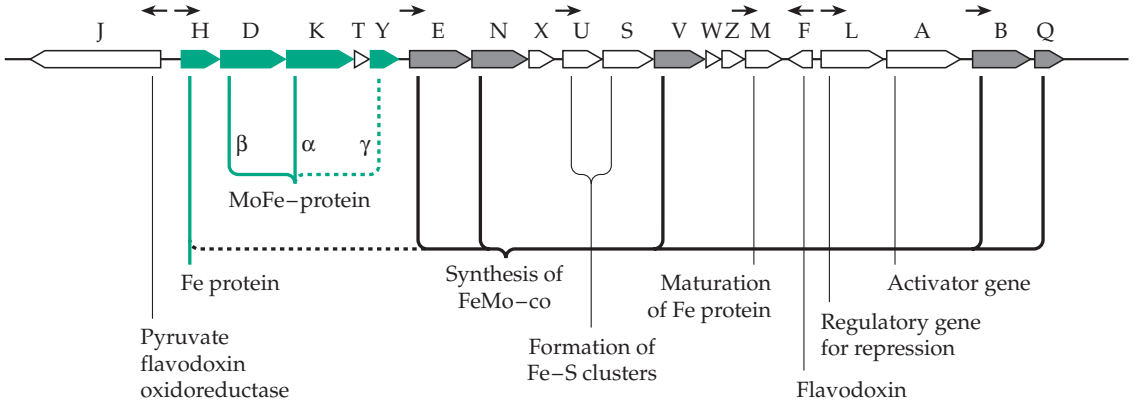



**Figure 24-4** Sequence of *nif* genes of *Klebsiella pneumoniae*.[56] These precede the *his* operon directly at the right side. The nitrogenase structural genes are marked with green.

curl around the bacteria to initiate nodulation.[60,61] However, there are other factors. Infecting bacteria must reach a region of low oxygen in the plant. A hemoprotein **FixL** is a sensor kinase that regulates phosphorylation of transcription factor **FixJ**. This two-component system induces transcription of *nifA* (Fig. 24-4) and others.[68–69a] Nitrogen-fixing nodules, which are filled with the bacteroids derived from the infecting bacteria, synthesize leghemoglobin. The polypeptide chain of this protein is encoded by the plant, but its heme may be synthesized by bacteroid enzymes.[10,57] In at least one strain of *Rhizobium* the *nod* genes as well as the *fix* and *nif* genes are all carried on a 536-kb plasmid, which is almost as large as the whole 580-kb genome of *Mycoplasma genitalium* (Table 1-3).[70,71] This arrangement seems to have allowed these bacteria to form an unusually large number of Nod factors and to colonize a wider variety of hosts including a non-leguminous tree.

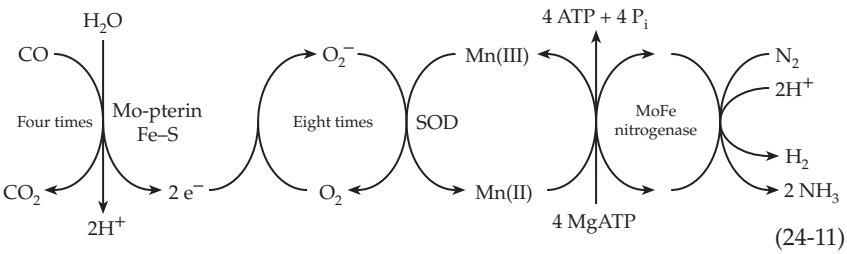
The $H_2$ that is produced in Eq. 24-6 (step *c*) may be used by bacteroids or by the plant cells. Some nodules evolve $H_2$, but in others it is utilized by hydrogenases as a source of energy.[57] From Eq. 24-6 it can be seen that up to 1/4 of the ATP utilized can, ideally, be recovered by use of the $H_2$ in this manner.

In cyanobacteria nitrogen fixation occurs in the **heterocysts**, specialized cells with thickened cell envelopes. They supply $NH_4^+$ to other cells in the filament of which they are a part. The cell envelopes prevent rapid diffusion of $O_2$ into the cells but do permit rapid enough entry of $N_2$ to maintain the observed rate of fixation of $N_2$.[72] In actinomycetes of the genus *Frankia*, which forms root nodules with woody plants, nitrogen fixation occurs in vesicles that are sheathed by multiple layers of **hopanoid lipids** (see Chapter 22).[73]

***Genetic engineering.*** Because of the high cost of nitrogen fertilizers there is intense interest in improving biological nitrogen fixation. Ideas range from increasing the efficiency of nitrogenase by using fewer molecules of ATP, by limiting excessive evolution of $H_2$, or transferring the whole *nif* region of a bacterial genome into nonleguminous plants. The last proposal has generated much publicity, but it will probably be difficult because of the need to create an anaerobic environment suitable for nitrogen fixation. A crop plant engineered in this way might not resemble the hoped-for product. It would have an enormous energy requirement for nitrogen fixation, which would have to be met by photosynthesis. At present genetic engineering on *nif* genes to increase efficiency seems most likely to succeed.

***Other nitrogenases.*** Although the well-characterized Mo-containing nitrogenase is responsible for most of their nitrogen fixation, bacteria often have alternative nitrogen fixation systems.[74] *Azotobacter vinlandii* produces three different nitrogenases in response to varying metal compositions in its surroundings.[75–76a] When the molybdenum level is adequate nitrogenase 1 is formed with its FeMo-coenzyme. In a low-molybdenum environment containing vanadium nitrogenase 2 is formed with an FeV-coenzyme.[75–77] If both molybdenum and vanadium are lacking, the bacteria form nitrogenase-3, which has an iron-only FeFe-coenzyme.

An unusual nitrogenase is formed by the chemolithotrophic *Streptomyces thermoautotrophicus*, which obtains energy from reduction of $CO_2$ or CO by $H_2$ (Eq. 17-50). These organisms form a MoFe nitrogenase that utilizes a manganese-containing superoxide dismutase to generate superoxide anion radicals. The latter transfer electrons to the MoFe protein in an ATP-dependent process. Electrons for generation of superoxide are formed using another molybdenum enzyme, a CO dehydrogenase containing molybdpterin cytosine dinucleotide (Fig. 16-31) and Fe–S centers.[78] The two systems function together as indicated by Eq. 24-11.




(24-11)

## 3. Interconversion of Nitrate, Nitrite, and Ammonium Ions

As is indicated in Fig. 24-1, the interconversions of nitrate and nitrite with ammonia and with organic nitrogen compounds are active biological processes. Two genera of nitrifying soil bacteria, which are discussed in Chapter 18, oxidize ammonium ions to nitrate. *Nitrosomas* carries out the six-electron oxidation to nitrite (Eq. 18-17) and *Nitrobacter* the two-electron oxidation of nitrite to nitrate (Eq. 18-18).[79]

The opposite sequence, reduction of nitrate and nitrite ions, provides a major route of acquisition of ammonia for incorporation into cells by bacteria, fungi, and green plants (Fig. 24-1). **Assimilatory** (biosynthetic) **nitrate reductases** catalyze the two-electron reduction of nitrate to nitrite (Eq. 16-61). This is thought to occur at the molybdenum atom of the large ~900-residue highly regulated[79a] molybdopterin-dependent enzyme. In green plants the reductant is

usually NADH while in fungi it is more often NADPH.[80–82] In all cases the cofactors FAD, heme, and molybdopterin are bound to a single polypeptide chain with the molybdopterin domain near the N terminus, and the heme in the middle. The electron-accepting FAD domain is near the C terminus[83–85a] and is thought to transfer the two electrons through the following chain.

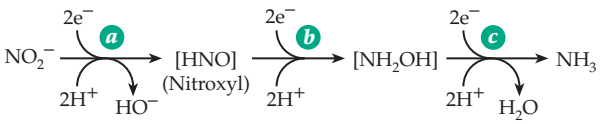
$$\text{NAD(P)H} \rightarrow \text{FAD} \rightarrow \text{Cyt } b \rightarrow \text{Mo-pterin} \rightarrow NO_3^- \qquad (24\text{-}12)$$

Bacterial assimilatory nitrate reductases have similar properties.[86,86a] In addition, many bacteria, including *E. coli*, are able to use nitrate ions as an oxidant for **nitrate respiration** under anaerobic conditions (Chapter 18). The **dissimilatory nitrate reductases** involved also contain molybdenum as well as Fe–S centers.[85] The *E. coli* enzyme receives electrons from reduced quinones in the plasma membrane, passing them through cytochrome *b*, Fe–S centers, and molybdopterin to nitrate. The three-subunit αβγ enzyme contains cytochrome *b* in one subunit, an $Fe_3S_4$ center as well as three $Fe_4S_4$ clusters in another, and the molybdenum cofactor in the third.[87] Nitrate reduction to nitrite is also on the pathway of denitrification, which can lead to release of nitrogen as NO, $N_2O$, and $N_2$ by the action of **dissimilatory nitrite reductases**. These enzymes[87a] have been discussed in Chapters 16 and 18.

**Assimilatory nitrite reductases** of plants, fungi, and bacteria carry out the six-electron reduction of nitrite to ammonium ions (Eq. 24-13) using electron donors such as reduced ferredoxins or NADPH.

$$NO_2^- + 6\,e^- + 8\,H^+ \rightarrow NH_4^+ + 2\,H_2O \qquad (24\text{-}13)$$

The enzymes from green plants and fungi are large multifunctional proteins,[80] which may resemble assimilatory sulfite reductases (Fig. 16-19). These contain **siroheme** (Fig. 16-6), which accepts electrons from either reduced ferredoxin (in photosynthetic organisms) or from NADH or NADPH. FAD acts as an intermediate carrier. It seems likely that the nitrite N binds to Fe of the siroheme and remains there during the entire six-electron reduction to $NH_3$. Nitroxyl (NOH) and hydroxylamine ($NH_2OH$) may be bound intermediates as is suggested in steps *a*–*c* of Eq. 24-14.




(24-14)

## B. Incorporation of $NH_3$ into Amino Acids and Proteins

Prior to 1935, amino acids were generally regarded as relatively stable nutrient building blocks. That concept was abandoned as a result of studies of the metabolism of $^{15}NH_3$ and of $^{15}N$-containing amino acids by Schoenheimer and Rittenberg[88] and more recent studies using $^{13}N$ by Cooper *et al.*[89,90] These investigations showed that nitrogen could often be shifted rapidly between one carbon skeleton and another. This confirmed proposals put forth earlier by Braunstein, Meister, and others who had pointed out that the $C_4$ and $C_5$ amino acids, aspartate and glutamate, which are closely related to the tricarboxylic acid cycle, are able to exchange their amino groups rapidly with those of other amino acids via transamination (Fig. 24-5, step *d*). Since ammonia can be incorporated readily into glutamate (Fig. 24-5, step *a*; see next section), a general means is available for the biosynthesis of amino acids. The citric acid cycle is able to provide any needed amount of 2-oxoglutarate for the synthesis of both glutamate and glutamine.[91–94]

Glutamine, and to a lesser extent asparagine, act as soluble, nontoxic carriers of additional ammonia in the form of their amide groups. An active synthase converts glutamate and ammonia to glutamine (Fig. 24-5, step *d*), and another enzyme transfers the amide nitrogen into aspartate, in an ATP-dependent reaction, to form asparagine (Fig. 24-5, step *e*). The amide nitrogen of glutamine is incorporated in a similar way into a great variety of other biochemical compounds, including carbamoyl phosphate (Fig. 24-5, step *f*; Section C,2), glucosamine (Eq. 20-5), $NAD^+$, *p*-aminobenzoate,

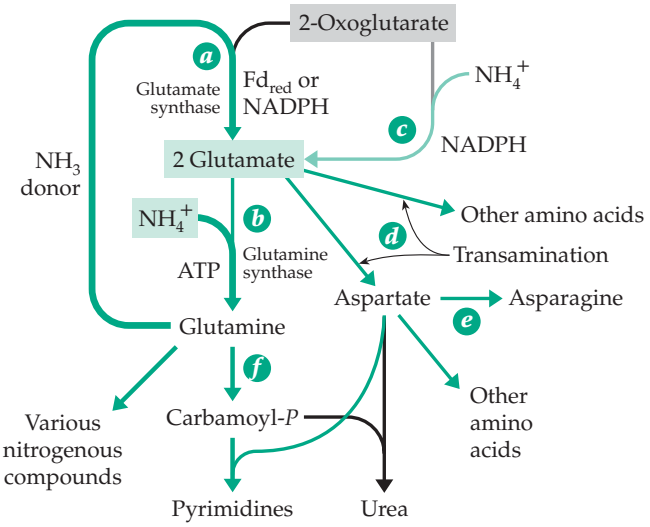



**Figure 24-5** Major pathways of incorporation of nitrogen from ammonium ions into organic compounds, traced by green arrows.

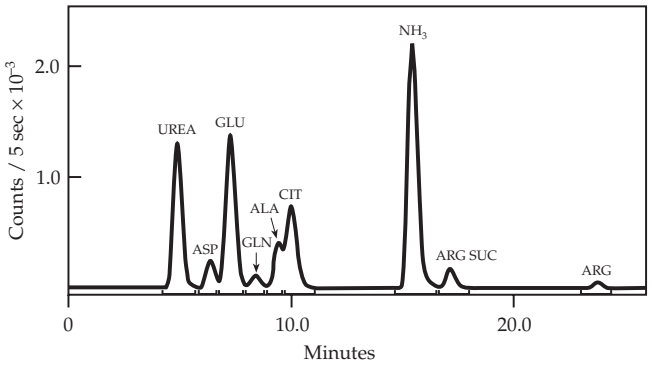
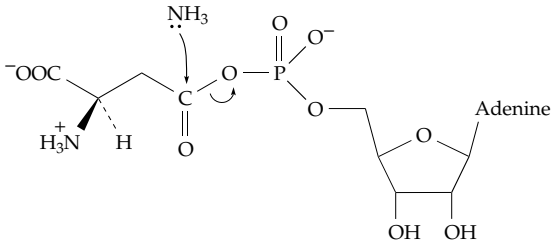



**Figure 24-6** Elution profile of $^{13}$N-containing metabolites extracted from liver 15 s after injection of $^{13}NH_3$ into the portal vein of an anesthetized adult male rat. CIT, citrulline; ARG SUC, argininosuccinate. From Cooper *et al.*[89]

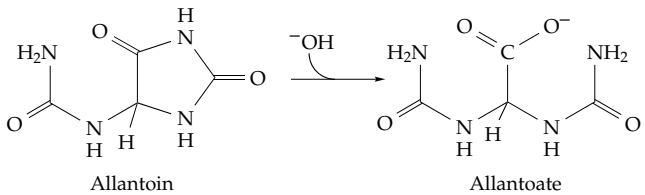
histidine, CTP, and purines (Chapter 25). These reactions are catalyzed by a family of **amidotransferases**,[95–97d] which hydrolyze the glutamine to glutamate and $NH_3$. The last is entrapped until it reacts with the second substrate. **Asparagine synthetase** apparently first binds ATP and aspartate, which probably react to form β-aspartyl adenylate (β-aspartyl-AMP). Glutamine then binds and is hydrolyzed.[98,98a]




β-Aspartyl adenylate

The liberated $NH_3$ can attack the β-aspartyl-AMP as indicated in the accompanying diagram to form asparagine. However, it is also possible that $NH_3$ is transferred via covalently bonded complexes[99] and is never free $NH_3$. An asparagine synthetase that utilizes free $NH_3$ as a nitrogen donor is also present in many organisms.

A third mechanism of synthesis, which was only recently recognized, appears to provide the sole source of asparagine for many bacteria.[98b] The asparagine-specific transfer RNA $tRNA^{Asn}$ is "mischarged" with aspartic acid to form Asp-$tRNA^{Asn}$. This compound is then converted to the properly aminoacylated Asn-$tRNA^{Asn}$ by a glutamine-dependent amidotransferase. (The entire ATP-dependent sequence is shown in Eq. 29-6.) The activated asparaginyl group is then transferred from Asn-$tRNA^{Asn}$ into proteins as they are synthesized.




Allantoin Allantoate

(24-15)

Most green plants transport nitrogen from roots to growing shoots as asparagine. However, in peanuts **β-methyleneaspartate** is the major nitrogen carrier,[100] and in some legumes, including soybeans, **allantoin** and **allantoate** (Eq. 24-15) play this role. Allantoin arises from hydrolysis of purines (see Fig. 25-18), which are synthesized in root nodules of nitrogen-fixing plants.[101]

Glutamate, glutamine, and aspartate also play central roles in *removal* of nitrogen from organic compounds.[102] Transamination is reversible and is often the first step in catabolism of excess amino acids. 2-Oxoglutarate is the recipient of the nitrogen, and the glutamate that is formed can be deaminated to form ammonia which can then be incorporated into glutamine. Glutamate can also donate its nitrogen to form aspartate. In the brain glutamate is a major neurotransmitter but is toxic in excess. The astrocyte glial cells take up glutamate from the synaptic clefts between neurons, converting it to glutamine, which is then released into the extracellular space for reuptake by neurons.[103,104] In the animal body both aspartate and glutamine (via carbamoyl phosphate) are precursors of **urea**, the principal nitrogenous excretion product. These relationships are also summarized in Fig. 24-5, and details are provided in later sections.

While reductive amination of glutamate via glutamate synthase appears to be the major pathway for incorporation of nitrogen into amino groups, some direct amination of pyruvate and other 2-oxoacids in reactions analogous to that of glutamate dehydrogenase occurs in bacteria.[105,106] Another bacterial enzyme catalyzes reversible addition of ammonia to fumarate to form aspartate (p. 685).

An initially surprising conclusion drawn from the studies of Schoenheimer and Rittenberg was that proteins within cells are in a continuous steady state of synthesis and degradation. The initial biosynthesis, the processing, oxidative and hydrolytic degradative reactions of peptides, and further catabolism of amino acids all combine to form a series of metabolic loops as discussed in Chapter 17 and dealt with further in Chapters 12 and 29. Within cells some proteins are degraded much more rapidly than others, an important aspect of metabolic control. This is accomplished with the aid of the ubiquitin system (Box 10-C) and proteasomes (Box 7-A).[107] Proteins secreted into extracellular fluids often undergo more rapid turnover than do those that remain within cells.

### 1. Uptake of Amino Acids by Cells

While cells of autotrophic organisms can make all of their own amino acids, other organisms utilize many preformed amino acids. Human beings and other higher animals require several **essential amino acids** in their diets. Additional amounts of "nonessential" amino acids are also needed. It is true that amino groups can be transferred from one carbon skeleton to another among most of the amino acids. However, the body must take in enough amino groups to supply its need for all of the 20 amino acid components of proteins.[107a,b] Because of an unfavorable equilibrium constant, and the normally low concentration of $NH_4^+$, glutamate dehydrogenase (step *c* in Fig. 24-5) does not normally synthesize glutamate in the animal body. Its function is to deaminate excess glutamate. Furthermore, cells of some tissues take up amino acids that are made in other tissues. The active transport systems of bacteria have been described in Chapter 8. In mammals the absorption of amino acids takes place through epithelial cells of the intestinal tract, kidney tubules, and the brain (blood–brain barrier). Both $Na^+$-dependent transport (as for sugars; see Chapter 8) and $Na^+$-independent processes occur.[107c] Among the latter is the proposed **γ-glutamyl cycle,** which is described in Box 11-B. The cycle makes use of the γ-carboxyl group of glutamate, the same carboxyl that carries ammonia in the form of glutamine. Glutathione supplies the activated γ-glutamyl group. The amino acid to be transported reacts on the membrane surface by **transpeptidation**[108–109a] to form a **γ-glutamylamino acid** which enters the cytoplasm. It releases the free amino acid through an internal displacement by the free amino group of the glutamyl group. The natural tendency of the 5-carbon glutamate to undergo cyclization is used to provide the driving force for release of the bound amino acid. The cyclic product 5-oxoproline is then opened hydrolytically in an ATP-requiring reaction.[110] Cysteinylglycine formed in the initial transpeptidation is hydrolyzed by a peptidase, and glutathione is regenerated in two ATP-dependent steps as indicated in the scheme in Box 11-B.

The significance of the γ-glutamyl cycle is not fully understood. However, the finding of a mentally retarded individual who excretes 25–50 g / day of 5-oxoproline in the urine (possibly because of a defective 5-oxoprolinase) suggests that the pathway is a very active one.[111] A few persons deficient in γ-glutamyl transpeptidase have been found. They excrete glutathione and have a variety of medical problems.[109]

### 2. Glutamate Dehydrogenase and Glutamate Synthase

In animal tissues and in some bacteria the **glutamate dehydrogenase** reaction (Fig. 24-5, step *c*; see also Chapter 15)[112–115] provides a means of incorporating ammonia reversibly into glutamic acid. In eukaryotic cells the allosteric enzyme is found largely in the mitochondria.[115a] Glutamate dehydrogenase is also found in chloroplasts where it may function in glutamate synthesis when ammonia is present in excess.[116] The action of aminotransferases, both within and without mitochondria, distributes nitrogen from glutamate into most of the other amino acids. Especially active is aspartate aminotransferase (Eq. 14-24; Fig. 24-5, step *d*) which equilibrates aspartate and oxaloacetate with the 2-oxoglutarate–glutamate couple. However, the body obtains glutamate, as well as other amino acids, from foods, the initial source being largely green plants.

In plants as well as in *E. coli* and many other bacteria most glutamate is formed by **glutamate synthase**, which carries out reductive amination of 2-oxoglutarate (Fig. 24-5, reaction *a*). Glutamate synthase (also called **GOGAT**) utilizes the amide side chain of glutamine as the nitrogen donor. It is one of the previously mentioned amidotransferases in which glutamine is hydrolyzed to glutamate and $NH_3$ within the active site of the enzyme. Formation of a Schiff base and reduction probably occurs as in the reverse of reaction B of Table 15-1. However, one of the two glutamate molecules formed in reaction *a* of Fig. 24-5 must be reconverted by glutamine synthase to glutamine with the utilization of a molecule of ATP (Fig. 24-5, step *b*). Because of this coupling of ATP cleavage to the reaction the equilibrium in reaction *a* lies far toward the synthesis of glutamate. The low value of $K_m$ for $NH_4^+$ that is characteristic of glutamine synthase favors glutamate synthesis even when little nitrogen is available.

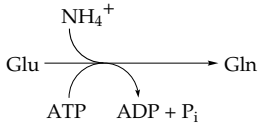
Bacterial glutamate synthases are large oligomeric proteins containing flavin and Fe–S centers. That of *Azospirillum brasilense* consists of αβ units in which the 53-kDa β chains contain FAD and an NADPH binding site.[117–118a] The NADPH evidently transfers electrons to the FAD, which transfers them to an $Fe_3S_4$ center in the large 162-kDA α subunit. A molecule of bound FMN receives the electrons and reduces the iminoglutarate to glutamate. The site of binding and hydrolysis of glutamine is also present in subunit α. Chloroplasts of higher plants contain two glutamate synthases. One resembles the bacterial enzyme and utilizes NADPH as the reductant. The other requires reduced ferredoxin.[118–120]

Bacteria utilize both D-alanine and D-glutamate in the synthesis of their peptidoglycan layers (Fig. 8-29). Both D-amino acids are formed by racemases. That of

alanine uses PLP (Chapter 15) but **glutamate racemase**[121–122a] does not. It may be able to remove the α-H of glutamate by utilizing the –COOH of the substrate, rather than the PLP ring, as an electron sink. Small amounts of D-amino acids occur also in animals.[123] Animal livers and kidneys contain **D-amino acid oxidase** and **D-aspartate oxidase,** which apparently function to metabolize D-amino acids from foods or those formed by brain activity (Chapter 30) or by aging.

## 3. Glutamine Synthetase

The formation of glutamine from glutamate (Eq. 24-16) also depends upon a coupled cleavage of ATP:




(24-16)

Glutamine synthase, as isolated from *E. coli*, contains 12 identical 51.6-kDa subunits arranged in the form of two rings of six subunits each with a center-to-center spacing of 4.5 nm. The units in one layer lie almost directly above those in the next,[104,124,125] the center-to-center spacing between the two layers is also 4.5 nm, and the array has 622 dihedral symmetry. The enzyme displays complex regulatory properties,[112,126–129] which are summarized in Fig. 24-7. The enzyme exists in two forms. **Active glutamine synthase** requires $Mg^{2+}$ in addition to the three substrates glutamate, $NH_4^+$, and ATP. If the glutamate precursor, 2-oxoglutarate, is present in excess, the enzyme tends to remain in the active form because conversion to a modified form is inhibited; when the oxoglutarate concentration falls to a low value and glutamine accumulates, alteration is favored. The modifying enzyme **adenylyltransferase** (AT) in its active form $AT_A$ transfers an adenylyl group from ATP to a tyrosine hydroxyl on glutamine synthase to give an adenylyl enzyme (GS-AMP). This **modified enzyme** requires $Mn^{2+}$ instead of $Mg^{2+}$ and is far more sensitive than the original enzyme to feedback inhibition by a series of end products of glutamine metabolism. All nine of the feedback inhibitors (serine, alanine, glycine, histidine, tryptophan, CTP, AMP, carbamoyl-*P*, and glucosamine 6-*P*) seem to bind to specific sites on the enzyme surface and to exert a cumulative inhibition. Serine, alanine, and glycine appear to be competitive inhibitors at the glutamate binding site.[130]

Relaxation of adenylylated glutamine synthase to the unmodified form is not catalyzed by a separate hydrolase but is promoted by a modified form of the adenylyltransferase $AT_D$. The active enzyme $AT_A$ is

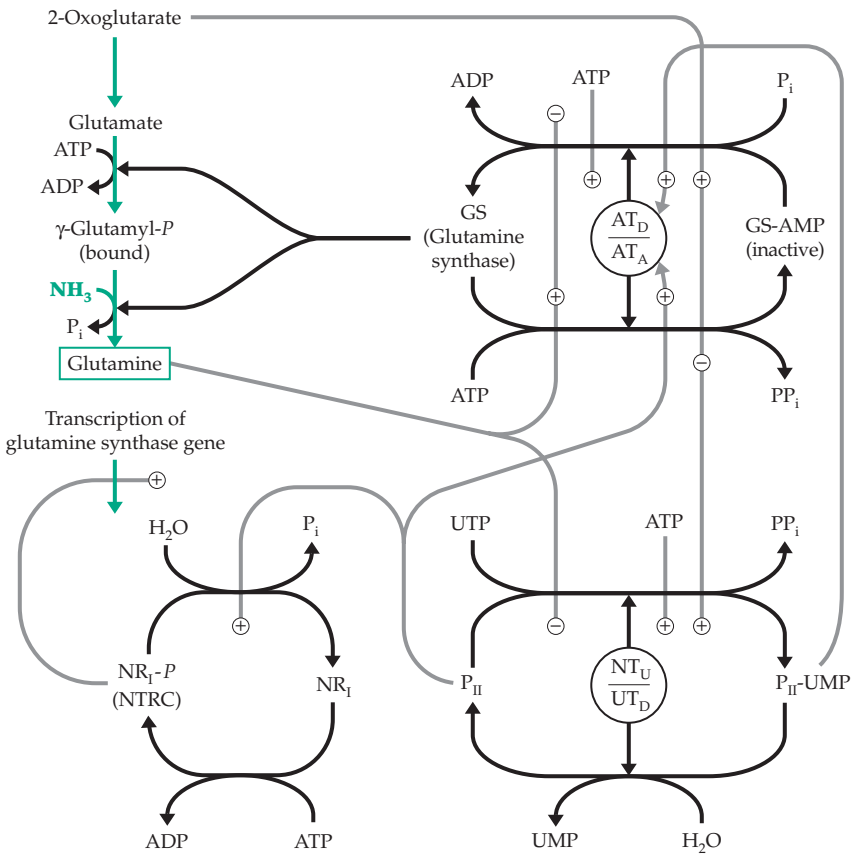
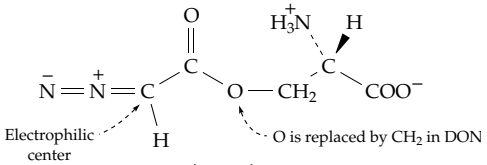



**Figure 24-7** Regulation of glutamine synthase of *E. coli* using activation (+) and inhibition (–). Glutamine synthase (GS upper center) is converted by adenylylation of Tyr 397 into an inactive form GS-AMP by the action of an adenylyltransferase $AT_A$ in complex with regulatory protein PII. PII is uridylylated at up to four sites by action of uridylyltransferase $UT_U$, which resides in the same polypeptide chain as a uridylyl removing enzyme $UT_D$ (or UR). When PII carries a uridylyl group (PII-UMP), $AT_A$ is transformed to $AT_D$, which reconverts the inactive GS-AMP to active GS by phosphorolytic removal of the adenylyl group. The ratios of $AT_A / AT_D$ and $UT_U / UT_D$ are controlled by the concentrations of the metabolites 2-oxoglutarate, a precursor, and glutamine, the immediate product. The amount of GS formed is controlled at the transcriptional level by an enhancer-binding transcription factor called NRI or NtrC (lower left). It is active when phosphorylated. Dephosphorylation of NRI-*P* is catalyzed by yet another protein and is stimulated by PII. Thus, PII both decreases synthesis of GS and promotes conversion of GS to its inactive form.

actually a complex AT • PII containing the regulatory protein PII. Subunit PII can be uridylylated on a tyrosine side chain by action of a 95-kDa **uridylyltransferase** (UT)[127,131] to form the modified glutamine synthetase AT • PII-UMP or$AT_D$. This form catalyzes phosphorolytic deadenylylation of glutamine synthetase, $P_i$ displacing the adenylyl group to form ADP. Removal of the uridylyl group from PII-UMP is catalyzed by a fourth enzyme, $UT_D$ (or UR), which is part of the same polypeptide chain as $UT_U$.[127] The cycle of interconversions of PII catalyzed by the $UT_U$ and $UT_D$ activities is shown at the lower right side of Fig. 24-7. From the allosteric modification reactions indicated by the gray lines, it is seen that glutamine not only promotes the adenylylation of glutamine synthetase but also inhibits the uridylylation of PII, thereby preventing $AT_D$ from removing the adenylyl group from the synthetase. Furthermore, it allosterically inhibits the deadenylylation reaction itself. On the other hand, 2-oxoglutarate acts in the opposite way.

The glutamine synthase regulatory system has another important function. Protein PII stimulates the dephosphorylation of the enhancer-binding transcriptional regulator NRI-*P* (NtrC-*P*).[131,132] This slows transcription of the glutamine synthase gene (see Fig. 24-7) as well as a variety of other genes including those for the nitrogenase proteins in organisms that have them.[133] As a consequence, a deficiency of glutamine turns on a number of genes involved in nitrogen metabolism. Accumulation of glutamine promotes PII accumulation, modification of the synthase, and loss of gene activation.

Nitrogen can be transferred from glutamine into many other substrates.[102] Several antibiotic analogs of glutamine have been useful in studying these processes. Examples are the streptomyces antibiotics **L-azaserine** and 6-diazo-5-oxo-L-norleucine (DON).




L-Azaserine

These compounds act as alkylating agents; $N_2$ is released and a nucleophilic group from the enzyme becomes attached at the carbon atom indicated.[134] Other inhibitors bind noncovalently to form dead-end complexes.[134a]

## 4. Catabolism of Glutamine, Glutamate, and Other Amino Acids

Glutamine is hydrolyzed back to glutamate by glutaminases that are found both in eukaryotic tissues and in bacteria.[135,136] Liver contains an isozyme whose function appears to be to release $NH_3$ from glutamine for urea synthesis.[135,137] Glutamate dehydrogenase deaminates excess glutamate back to 2-oxoglutarate, which is degraded to succinyl-CoA and via β oxidation to malate, pyruvate, and acetyl-CoA. The last can reenter the citric acid cycle and be oxidized to $CO_2$ (Eq. 24-17). In fact, in mammalian tissues glutamate is essentially in equilibrium with 2-oxoglutarate and other citric acid cycle intermediates (see Box 17-C).

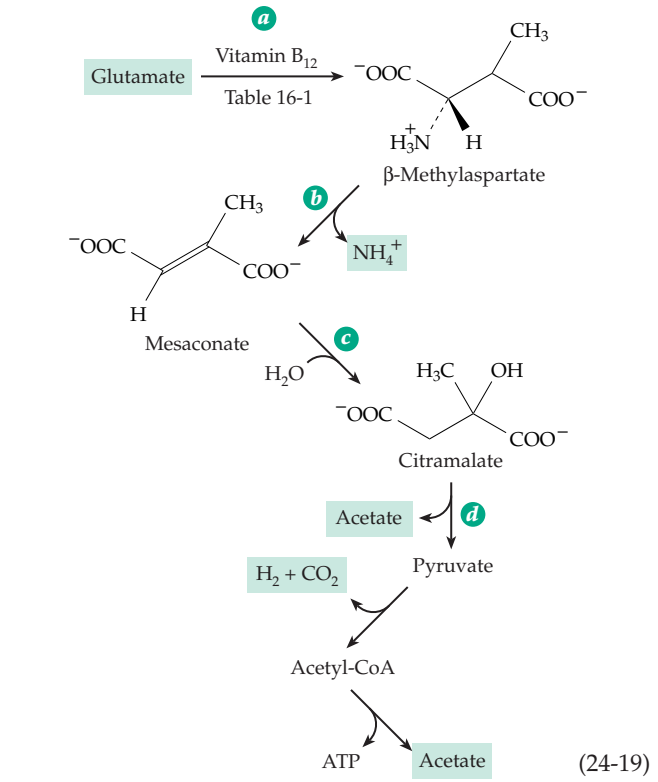
$$\text{Glu} \rightarrow \text{2-oxoglutarate} \rightarrow \text{succinyl-CoA} \rightarrow \rightarrow \rightarrow \text{malate} \rightarrow \text{pyruvate} \rightarrow \text{acetyl-CoA} \rightarrow CO_2 \qquad (24\text{-}17)$$

Many other amino acids are degraded in similar ways. In most cases the sequence is initiated by transamination to the corresponding 2-oxoacid. Beta oxidation and breakdown to such compounds as pyruvate and acetyl-CoA follows.

***Catabolism initiated by decarboxylation.*** An alternative pathway for glutamate degradation is through the γ-aminobutyrate shunt (Fig. 17-5). This pathway is initiated by a PLP-dependent decarboxylation rather than by a deamination or transamination. Since decarboxylases are known for most amino acids, there are usually alternative breakdown pathways initiated by decarboxylation. In many cases these pathways lead to important products. For example, γ-aminobutyrate functions in the brain as an important neurotransmitter. Dihydroxyphenylalanine is converted to noradrenaline and adrenaline, tryptophan to serotonin, and histidine to histamine. All of these are neurotransmitters (Chapter 30) and/or have other hormonal functions. A calmodulin-dependent glutamate decarboxylase occurs in higher plants, which accumulate γ-aminobutyrate in response to a variety of stresses.[138] However, the significance of this accumulation is unclear.

***Fermentation of glutamate.*** Special problems face anaerobic bacteria subsisting on amino acids. Their energy needs must be met by balanced fermentations.[138a] For example, glutamate may be converted to $CO_2$, ammonia, acetate$^-$, and butyrate$^-$ according to the reactions of Fig. 24-8. The end result is described by Eq. 24-18.

$$2\ \text{Glutamate}^- + 2\ H_2O + H^+ \rightarrow 2\ CO_2 + 2\ NH_4^+ + 2\ \text{acetate}^- + \text{butyrate}^- \qquad \Delta G'\ (\text{pH } 7) = -131\ \text{kJ} \qquad (24\text{-}18)$$




(24-19)

The sequence begins with the γ-aminobutyrate shunt reactions (Fig. 24-8, steps *a* and *b*), but succinic semialdehyde is reduced to γ-hydroxybutyric acid using the NADH generated in the trans-deamination process of step *c*. With the aid of a CoA-transferase (step *d*) two molecules of the CoA ester of this hydroxy acid are formed at the expense of two molecules of acetyl-CoA. Use is then made of a β,γ elimination of water (step *e*), analogous to that involved in the formation of vaccenic acid (Eq. 21-2). Isomerization (perhaps by the same enzyme that catalyzes elimination) forms crotonyl-CoA (step *f*). The latter undergoes dismutation, one-half being reduced to butyryl-CoA and one-half being hydrated and oxidized to acetoacetyl-CoA in the standard β-oxidation sequence. Acetoacetyl-CoA is cleaved to regenerate the two molecules of acetyl-CoA. The organism can gain one molecule of ATP through cleavage of the butyryl-CoA. Perhaps a second can be gained by oxidative phosphorylation between the NADH produced in the formation of acetoacetyl-CoA and the reduction of crotonyl-CoA to butyryl-CoA. The two processes take place at sufficiently different redox potentials to permit this kind of coupling.

Another fermentation of glutamate is initiated by the vitamin $B_{12}$-dependent isomerization of glutamate to β-methylaspartate (Eq. 24-19, step *a*).[138b] This rearrangement of structure permits α,β elimination of ammonia (step *b*), a process not possible in the original glutamate. Hydration to **citramalate** (step *c*) and aldol cleavage yields acetate and pyruvate. Acetate is one of the usual end products of the fermentation. The pyruvate can be cleaved to $H_2$, $CO_2$, and acetyl-CoA by the pyruvate-formate-lyase system (Fig. 15-16; Eq. 17-25), and cleavage of the acetyl-CoA can provide ATP. Alternatively, two molecules of acetyl-CoA can be coupled and reduced to butyryl-CoA. The reducing power generated in the cleavage of pyruvate is used to reduce crotonyl-CoA rather than being released as $H_2$. The stoichiometry is identical to that in Fig. 24-8. Still other fermentation mechanisms are used by some Clostridia to degrade glutamate.[138a] See study question number 16 at the end of this chapter.

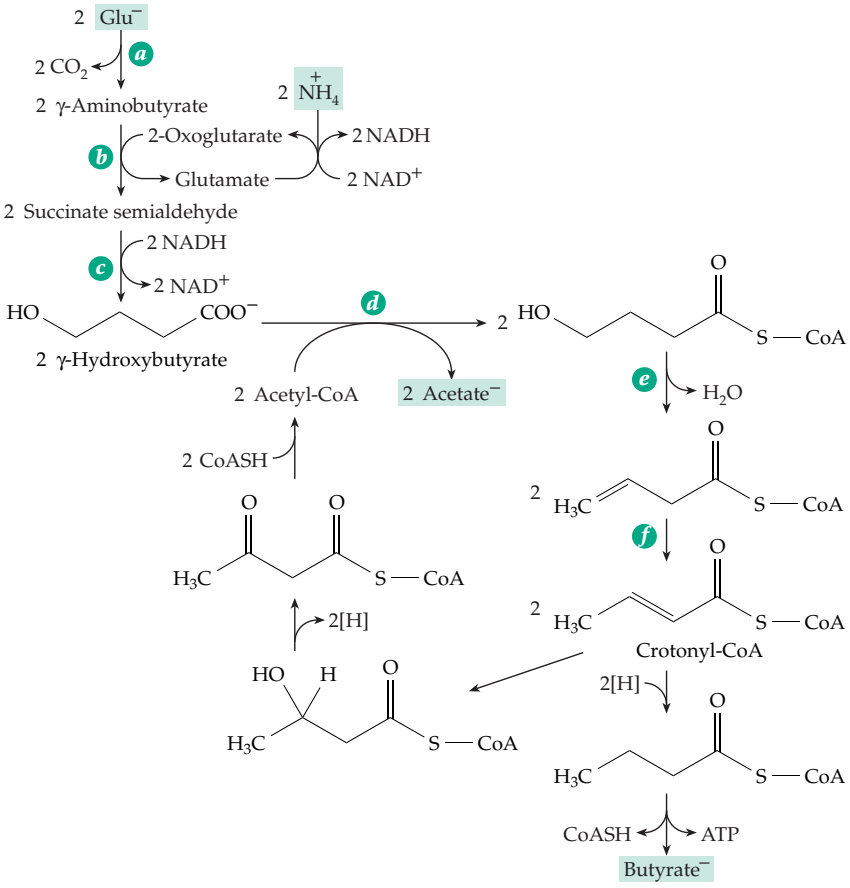



**Figure 24-8** Fermentation of glutamate by *Clostridium aminobutylicum.*

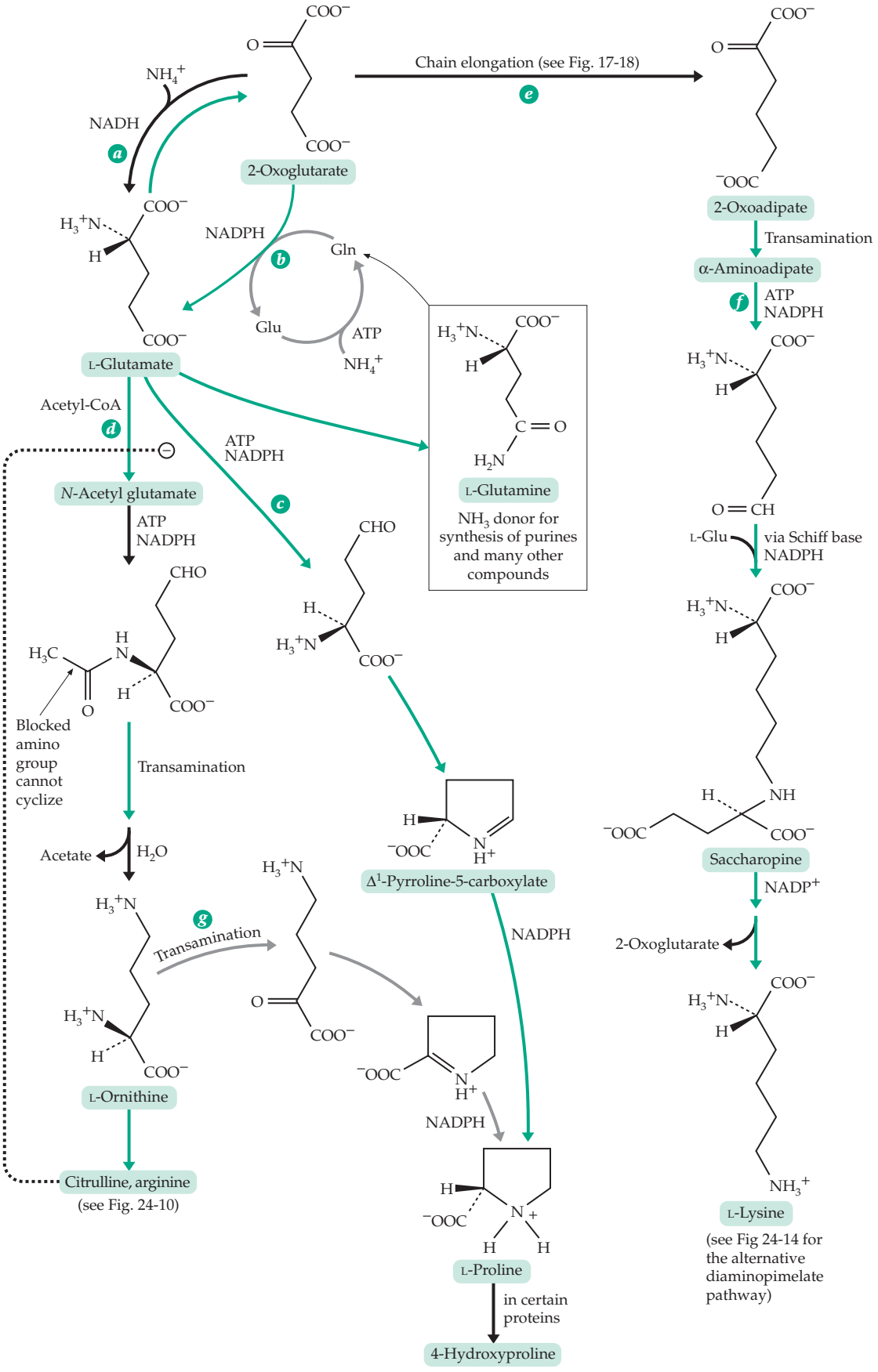



**Figure 24-9** Biosynthesis of glutamate, glutamine, proline, and lysine from 2-oxoglutarate.

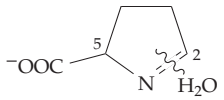
## C. Synthesis and Catabolism of Proline, Ornithine, Arginine, and Polyamines

The 5-carbon skeleton of glutamic acid gives rise directly to those of proline, ornithine, and arginine. The reactions are outlined in Fig. 24-9. Arginine, in turn, is involved in the urea cycle, which is shown in detail in Fig. 24-10. Arginine is also a biosynthetic precursor of the polyamines. Another important biosynthetic product of glutamate metabolism is δ-aminolevulinate, a precursor to porphyrins (Eq. 24-44) in some organisms.[139]

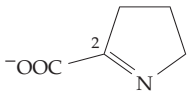
### 1. Synthesis and Catabolism of Proline

The ATP-dependent reduction of the γ-carboxyl group of glutamate to an aldehyde by NADPH (reaction *c*, Fig. 24-9) is of a standard biosynthetic reaction type, the opposite of the oxidation reaction of Fig. 15-6. Like the latter it is thought to occur via an acyl phosphate intermediate.[140,141] The oxidation product, **glutamate semialdehyde**, cyclizes and can be converted to proline by further reduction (Fig. 24-9, step *c* and subsequent reactions in center of scheme). The pathway has been well established in bacteria and yeast by both biochemical and genetic experiments. In plants both the initial reduction and the cyclization are catalyzed by a bifunctional enzyme.[142,143] An alternative pathway important in animals is initiated by transamination of ornithine to the corresponding 2-oxoacid, spontaneous cyclization, and reduction to proline (Fig. 24-9, step *g*).[140,144] Selected prolines in collagen and in plant glycoproteins[145] are oxygenated to form 4-hydroxyproline (Eqs. 8-6, 18-51).

One route of catabolism of proline is essentially the reverse of its formation from glutamate. **Proline oxidase** yields $\Delta^1$-pyrroline 5-carboxylate.[145a,b]



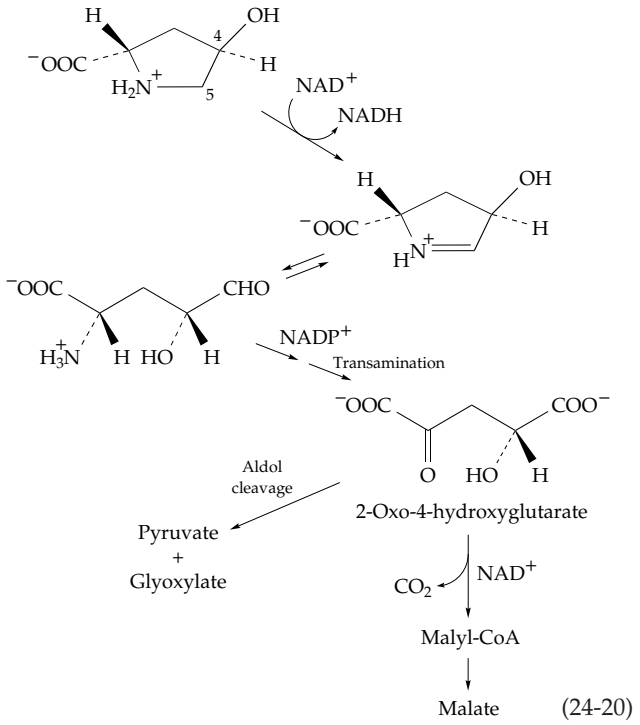

$\Delta^1$-Pyrroline 5-carboxylate




$\Delta^1$-Pyrroline 2-carboxylate

The corresponding open-chain aldehyde, formed by hydrolysis, can be oxidized back to glutamate by pyrroline 5-carboxylate dehydrogenase.[145a–147] Lack of this enzyme is associated with the human genetic deficiency causing **hyperprolinemia**.[147–148a]

Alternatively, degradation can be initiated by oxidation on the other side of the ring nitrogen to form $\Delta^1$-pyrroline 2-carboxylate. The metabolic fate of this compound is uncertain. A corresponding pathway for breakdown of 4-hydroxy-L-proline of collagen yields glyoxylate and pyruvate or malate and $CO_2$ (Eq. 24-20).[149] Oxidation on the other side of the ring nitrogen




(24-20)

of hydroxyproline is utilized by some pseudomonads to convert the amino acid into 2-oxoglutarate. Anaerobic bacteria may reduce proline to 5-aminovalerate and couple this reaction to the oxidative degradation of another amino acid (Stickland reaction).

### 2. Synthesis of Arginine and Ornithine and the Urea Cycle

If the amino group of glutamate is blocked by acetylation prior to the reduction to the semialdehyde (Fig. 24-9, step *d*) cyclization is prevented. The γ-aldehyde group can be transaminated to an amino group and the acetyl blocking group removed to form **ornithine**. Ornithine is not usually a constituent of proteins, but it is sometimes formed by hydrolytic modification of arginine at specific sites in a protein. A 67-kDa urate-binding glycoprotein of plasma is reported to contain 43 residues of ornithine.[150,151] It is postulated that a special arginase is needed to form these residues, and that it may be lacking in some cases of gout in which the urate-binding capacity of blood is impaired. Ornithine appears to be present in specific sites in a few other proteins as well.[151] *Neurospora* grown in a minimal medium accumulates large amounts of both ornithine and arginine, over 98% of which is sequestered in vesicles within the cytoplasm.[152,153] This appears to be a way of accumulating a store of arginine that is protected from the active catabolism of that amino acid by the fungus. However, accumulation of ornithine in the human body, as a result of lack of **ornithine aminotransferase**

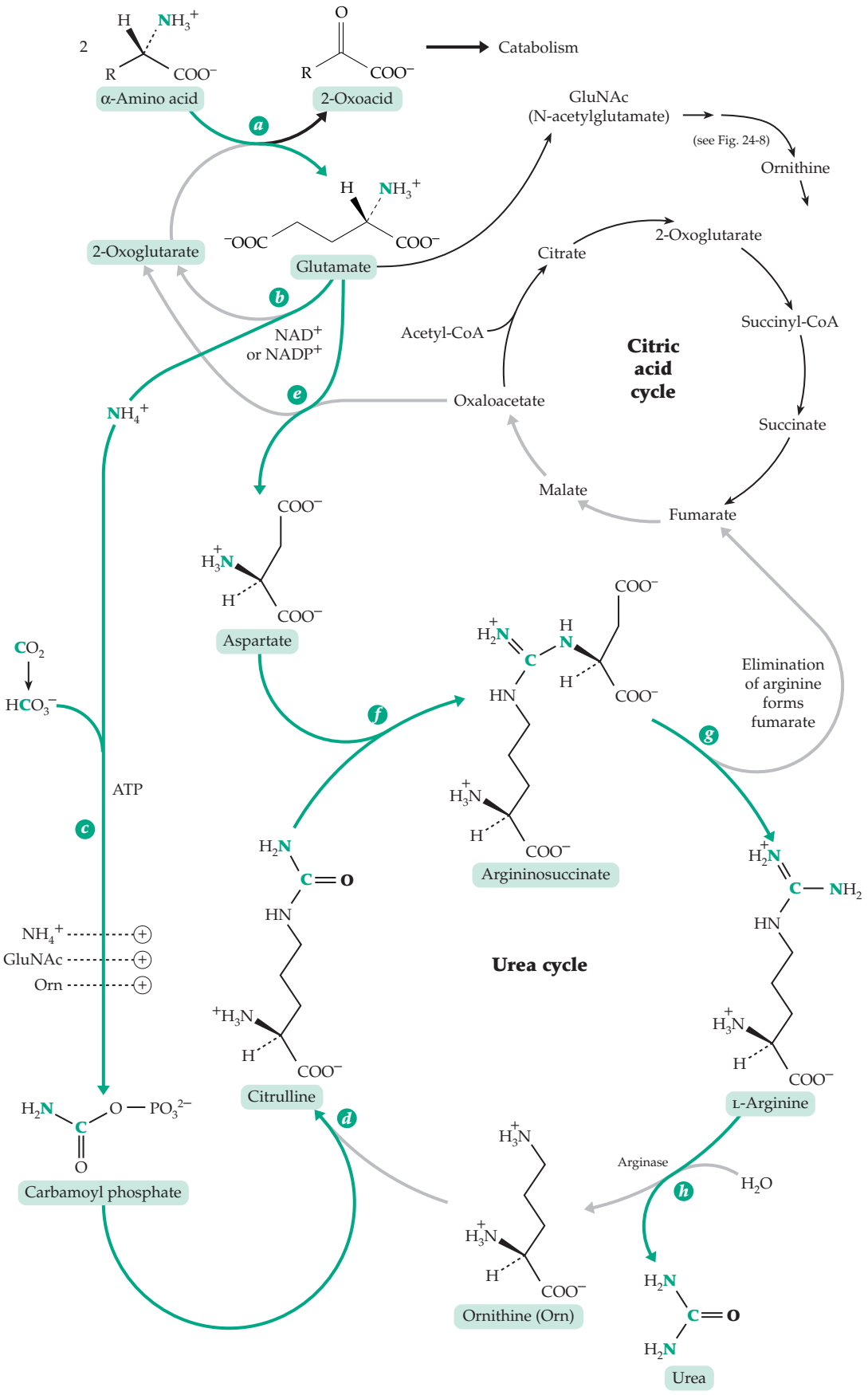


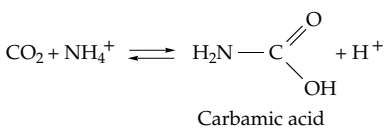

**Figure 24-10** Biosynthesis of citrulline, arginine, and urea. The green arrows indicate reactions directly involved in deamination of amino acids and the synthesis of urea. N from amino acids and C from $CO_2$ are traced in green.

(Fig. 24-9, step *g*), causes gyrate atrophy of the choroid and retina, a disease that results in tunnel vision and blindness.[154,155] A major interest in arginine metabolism asises from its role in formation of urea in the human body. Study of arginine biosynthesis in bacteria has also been important in developing our understanding of regulation of gene expression.[156]

***The urea cycle.*** In 1932, Krebs and Henseleit proposed that urea is formed in the liver by a cyclic process in which ornithine is converted first to **citrulline** and then to arginine.[157,158] The hydrolytic cleavage of arginine produces the urea and regenerates ornithine (Fig. 24-10, bottom). Subsequent experiments fully confirmed this proposal. Urea is the principal nitrogenous end product of metabolism in mammals and many other organisms, but the urea cycle reactions have other functions. As with the citric acid cycle, products other than urea can be withdrawn in any needed quantity. Most notably, the reactions of Fig. 24-10 provides for the biosynthesis of arginine in all organisms.[159,160] Also of physiological importance is the fact that the urea cycle involves both mitochondria and cytosolic enzymes.[161,162] This is illustrated in Fig. 24-11.

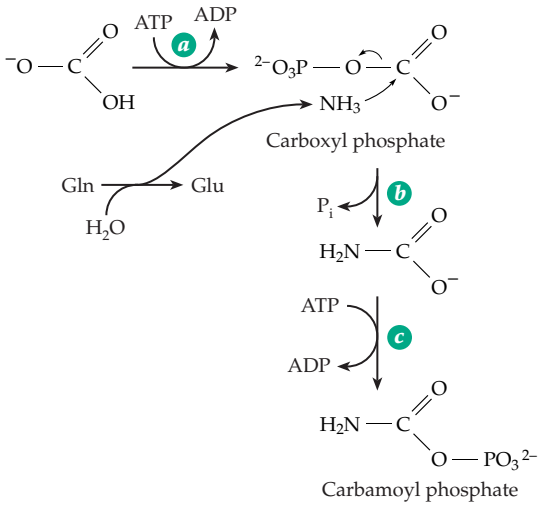
Let us trace the entire route of nitrogen removed by the liver from excess amino acids. Transaminases (step *a*, Fig. 24-10) transfer nitrogen to 2-oxoglutarate to form glutamate. Since urea contains two nitrogen atoms, two molecules of glutamate must donate their amino groups. One molecule is deaminated directly by glutamate dehydrogenase to form ammonia (step *b*). This ammonia is combined with bicarbonate (step *c*) to form carbamoyl phosphate, which transfers its carbamoyl group onto ornithine to form citrulline (step *d*). The second molecule of glutamate transfers its nitrogen by transamination to oxaloacetate (reaction *e*) to form aspartate. The aspartate molecule is incorporated intact into **argininosuccinate** by reaction with citrulline (reaction *f*). Undergoing a simple elimination reaction, the 4-carbon chain of argininosuccinate is converted to fumarate (step *g*) with arginine appearing as the elimination product. Finally, the hydrolysis of arginine (step *h*) yields urea and regenerates ornithine.

***Carbamoyl phosphate synthetases.*** The first of the individual steps in the urea cycle is the formation of carbamoyl phosphate.[163] Carbon dioxide and ammonia equilibrate spontaneously with carbamic acid:




Carbamic acid (24-21)

Some bacteria have a kinase able to convert carbamate into carbamoyl phosphate starting with step *a* of Eq. 24-22. However, the equilibrium constant is low (0.04 at pH 9, 10°C), and it is now believed that carbamate kinase functions in the opposite direction, providing a means of synthesis of ATP for bacteria degrading arginine (Section C,5,d). The biosynthetic carbamoyl phosphate synthases harness the cleavage of *two* molecules of ATP to formation of one molecule of carbamoyl phosphate (reaction *c*, Fig. 24-10).[163] In bacteria such as *E. coli*, a single synthase provides carbamoyl phosphate for biosynthesis of both arginine and pyrimidines (Fig. 25-14). However, fungi and higher animals have at least two carbamoyl-*P* synthases. Synthase I provides substrate for formation of citrulline from ornithine (Fig. 24-10), while carbamoyl-*P* synthase II, which is part of a larger multifunctional protein,[164] functions in pyrimidine synthesis. Synthase I is found in mitochondria and synthase II in the cytoplasm. Mammalian carbamoyl phosphate sythase I consists of a single 160-kDa peptide.[163] A powerful allosteric effector for the liver synthase is ***N*-acetylglutamate** (Fig. 24-10), a precursor of ornithine.[165] The enzyme from certain marine elasmobranchs, such as the spiny dogfish *Squalus acanthias*, have carbamoyl-*P* synthase III, an enzyme with somewhat different molecular properties.[166] It probably functions in synthesis of urea, which is used by these animals to regulate osmotic pressure.[167,168] Synthase I utilizes only free $NH_3$. The others are amidotransferases and prefer glutamine as the ammonia donor. Carbamoyl-*P* synthase from *E. coli* consists of two subunits (~42 and 118 kDa, respectively) and can utilize *either* free ammonia or glutamine.[169,170] The light subunit has **glutaminase** activity; i.e., it is able to hydrolyze glutamine to ammonia. All of these synthatases presumably act by first phosphorylating bicarbonate to an enzyme-bound carboxyl phosphate,[163,171,172] which can then undergo a displacement of phosphate by $NH_3$ to give enzyme-



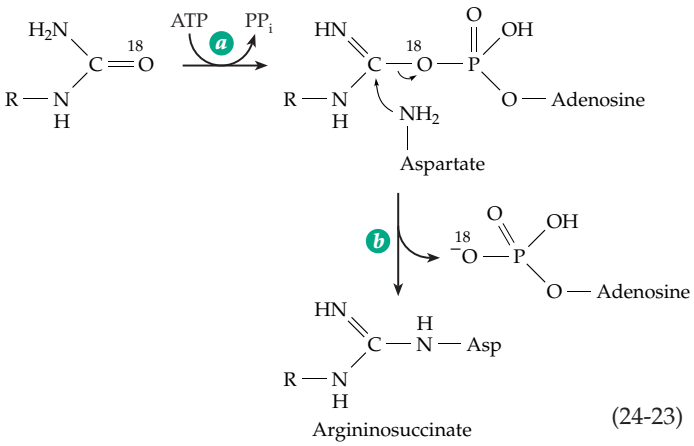

(24-22)

bound carbamate (Eq. 24-22, step *a*). Phosphorylation of the latter by ATP completes the reaction. In the single-chain enzymes the amidotransferase domain is at the N terminus.

Crystallographic study of a mutant form of the *E. coli* enzyme unable to act rapidly on glutamine showed that the latter released its ammonia to form a thioester with cysteine 269,[173,173a] suggesting a mechanism resembling that of serine proteases or papain (Chapter 12) for the glutaminase action. The X-ray crystallography also showed that the released $NH_3$ must travel 4.5 nm through the interior of the protein to the site of carbamate formation. The carbamate must travel another ~4.5 nm to the site from which carbamoyl phosphate is released.[170,172,173b] The C-terminal regions of the synthases undergo allosteric modification by a number of effectors.[163,173c] Both ornithine and IMP are activators for the *E. coli* enzyme, whereas UMP, a pyrimidine end product, exerts feedback inhibition. Phosphoribosyl pyrophosphate activates synthase II, and *N*-acetylglutamate activates the mammalian liver synthase I by binding near the C terminus.[165]

***Citrulline and argininosuccinate.*** One $NH_3$ and one $HCO_3^-$ for urea formation are provided by the carbamoyl group, which is transferred from carbamoyl-*P* to ornithine to form citrulline. The second nitrogen atom is transferred from glutamate to aspartate into argininosuccinate (steps *d* and *f*, Fig. 24-10). The equilibrium constant for ornithine transcarbamoylase (reaction *d*) is very high so that ornithine is completely converted to citrulline. The trimeric human enzyme is a trimer of 36-kDa subunits[174–175a] whose structural gene is on the X chromosome. Like many other mitochondrial matrix enzymes it is synthesized as a larger (40 kDa) pre-cursor, which enters the mitochondria in an energy-dependent process.[176] A genetic defect in this sex- linked gene is often lethal to boys, and even girls, heterozygous for the defect, sometimes have serious problems with accumulation of ammonia in the brain.[162,174,177]

The conversion of citrulline to argininosuccinate and the subsequent breakdown to fumarate and arginine take place in the cytosol (Fig. 24-11). The ureido

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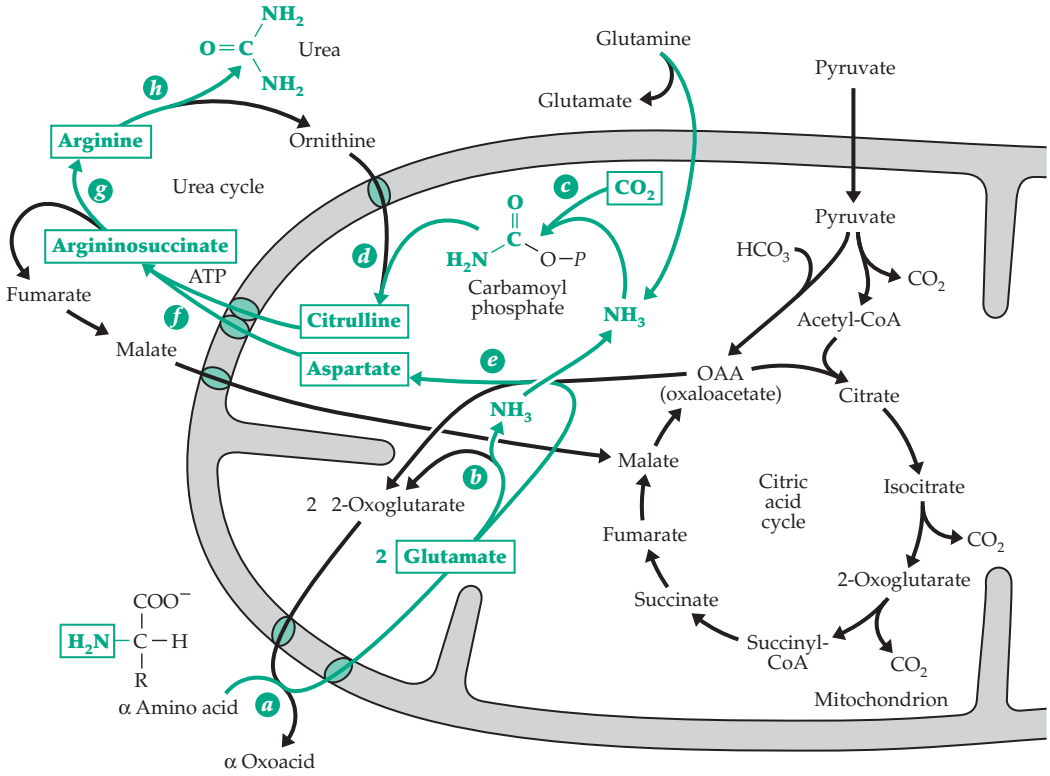



**Figure 24-11** Integration of the urea cycle with mitochondrial metabolism. Green lines trace the flow of nitrogen into urea upon deamination of amino acids or upon removal of nitrogen from the side chain of glutamine.

group of citrulline is activated by ATP for the argininosuccinate synthase reaction (Eq. 24-23, step *a*). Thus, $^{18}O$ present in this group is transferred into AMP. A citrulline adenylate intermediate (center) is likely. **Argininosuccinate lyase** (reaction *g*, Fig. 24-10)[177a] catalyzes the elimination of arginine with formation of fumarate. It is entirely analogous to the bacterial aspartase that eliminates ammonia from aspartate to form fumarate.[178] Like the latter enzyme and fumarate hydratase (Chapter 13), argininosuccinase promotes a trans elimination.[179] The fumarate produced can be reconverted through reactions of the citric acid cycle to oxaloacetate, which can be reaminated to aspartate (Fig. 24-11). Aspartate is used to introduce amino groups in an entirely similar way in other metabolic sequences such as in the formation of adenylic acid from inosinic acid (Fig. 25-16).

The cleavage of arginine to ornithine and urea by the $Mn^{2+}$-containing **arginase** (Chapter 16)[180,181] converts the biosynthetic route to arginine into a cycle for the synthesis of urea. This cyclic pathway is unique to organisms that excrete nitrogenous wastes as urea, but the biosynthetic path is nearly ubiquitous.[182] Human adults excrete approximately 20 g of urea nitrogen per day. If this rate decreases, ammonia accumulates in the blood to toxic levels. Normally, plasma contains 0.03 mM ammonia, and only 2–3 times this level is required to produce toxic symptoms. Therefore, it is not surprising that five different well-documented hereditary enzyme deficiencies affect the urea cycle.[162,183] One of the most common, **argininosuccinic aciduria**, is a deficiency of the breakdown of argininosuccinic acid.[178] Both lethal and nonlethal variants of this disease are known. Human argininosuccinate lyase consists of two subunits. Defects may occur in either subunit but considerable genetic heterogeneity exists and intragenic complementation between the two subunits accounts for many of the nonlethal forms of the disorder.[177a,178a] A common feature of all of the hereditary defects of the urea cycle is an intolerance to high protein intake and mental symptoms. Toxic accumulation of ammonia in blood is often seen also in **alcoholic liver cirrhosis** as a result of a decreased capacity of the liver for synthesis of urea.

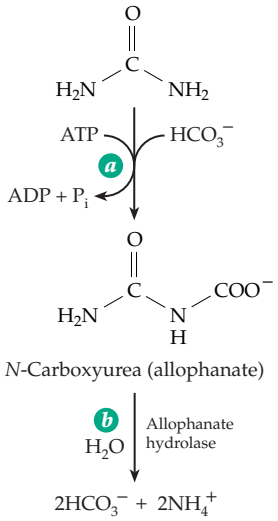
For some urea cycle defects a combination of a low-protein diet together with an arginine supplement prevents the ammonia intoxication while allowing normal growth. In other cases it is necessary to replace the natural dietary protein with a mixture of essential amino acids or with the corresponding 2-oxoacids, which can be converted to amino acids in the body with utilization of endogenous ammonia.[183] A specific treatment for lack of *N*-acetylglutamate synthetase, which forms the carbamoyl phosphate synthase activator *N*-acetylglutamate, is administration of the analog *N*-carbamoylglutamate. This also activates carbamoyl phosphate synthase and is not cleaved by acylases that would prevent the natural activator from being supplied artifically via the blood.[158]

Although the primary function of the urea cycle is usually regarded as the removal of $NH_4^+$ from the body, it also removes $HCO_3^-$ in equal amounts (Eq. 24-24). This is essential for maintenance of neutral pH,

$$2\,HCO_3^- + 2\,NH_4^+ \longrightarrow H_2NCONH_2 + CO_2 + 3\,H_2O \qquad (24\text{-}24)$$

and Atkinson and Bourke suggested that removal of $HCO_3^-$ is as important a function of the cycle as removal of $NH_4^+$.[184] However, there are strong arguments against this concept.[162]

***Excretion of ammonia.*** While mammals excrete urea, many invertebrate organisms that live in water as well as some fishes simply excrete $NH_3$. Other organisms hydrolyze urea to $NH_3$. Even green plants recycle nitrogen via urea and the $Ni^{2+}$-dependent urease (Eq. 16-47). Two compounds that can be hydrolyzed by cells to urea and glyoxylate are allantoin and allantoic acid (Eq. 24-15). If cells of *Saccharomyces cerevisiae* are grown on either of these compounds as a sole source of nitrogen, they make a biotin-dependent **urea carboxylase** (Eq. 24-25). This enzyme facilitates the hydrolysis of urea by conversion to the more easily degraded allophanate (Eq. 24-25).

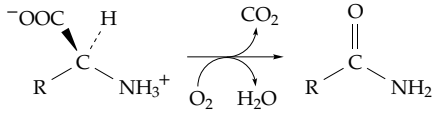



(24-25)

***Catabolism of arginine.*** Arginine can also be converted back to glutamate and 2-oxoglutarate. The initial step is removal of the guanidino group to form ornithine. This occurs in the urea cycle and also in many bacteria[185] by the action of arginase (Fig. 24-5, step *h*). A parallel pathway involving conversion of arginine to $N^1$-succinylarginine, then on to succinylglutamate, and to free glutamate and succinate is used by some pseudomonads.[186] The alternative **arginine**

**dihydrolase** pathway, used by some bacteria and a few protozoa such as *Giardia*, is initiated by a different hydrolase that cleaves arginine to citrulline and ammonia.[187] Phosphorolysis of citrulline yields carbamoyl phosphate whose breakdown to $CO_2$ and ammonia (catalyzed by carbamate kinase, Eq. 24-22) can be utilized for generation of ATP by microorganisms that subsist on arginine.[188]

Degradation of L-arginine by *Streptomyces griseus* is initiated by a hydroxylase that causes decarboxylation and conversion of the amino acid into an amide (Eq. 24-26), a reaction analogous to that catalyzed by the flavin-dependent lysine oxygenase (Eq. 18-41). The

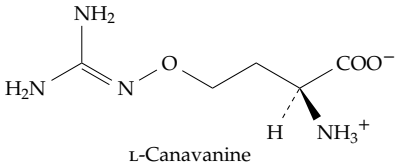



(24-26)

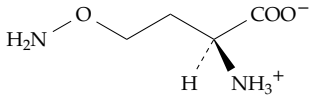
product formed from arginine is γ-guanidinobutyramide, which is further degraded by the hydrolysis of the amide group and cleavage of the guanidino group to form urea and γ-aminobutyrate. *Pseudomonas putida* initiates degradation of arginine by decarboxylation to the corresponding 2-oxoacid and oxidative decarboxylation with a thiamin diphosphate-requiring enzyme to γ-guanidinobutyraldehyde. Dehydrogenation and hydrolysis lead, again, to γ-aminobutyrate.[189]

Specific arginine residues in proteins are methylated on their guanidino groups to give monomethylated and both symmetrically and asymmetrically dimethylated derivatives.[190,191] These methylated arginines also occur free in various mammalian tissues, where they may serve as endogenous regulators of nitric oxide synthases. A $Zn^{2+}$-containing dimethylarginase hydrolyzes the monomethyl and dimethyl arginines to citrulline and monomethyl or dimethyl amines.[191]

***Insecticidal analogs of arginine.*** The toxic amino acid **L-canavanine** is synthesized by more than 1500 species of legumes including alfalfa and clover.[192–194] It is structurally similar to arginine, the 5-$CH_2$ group being replaced by O. However, the guanidino group is much less basic than in arginine. Canavanine is a natural insecticide, which in some plants accumulates to a level of 13% of the total dry matter.[193] Plants that store canavanine hydrolyze it to **canaline** and urea, which they use as a nitrogen source. Canaline is a toxic derivative of hydroxylamine and forms oximes with 2-oxoglutarate, other oxoacids, and PLP-containing enzymes. Although canavanine and canaline are effective insecticides, some beetles are adapted to these compounds to the extent that they feed exclusively on canavanine-containing seeds. The tobacco budworm is likewise resistant to these toxins and produces a **canavanine hydrolase** that converts canavanine to L-homoserine, a normal intermediate in




L-Canavanine



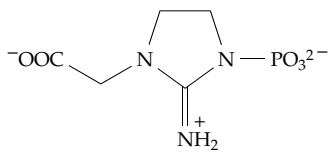

L-Canaline

the threonine, isoleucine, methionine biosynthetic pathway (Fig. 24-13), and **hydroxyguanidine**. The latter undergoes NADH-dependent reduction to guanidine[194] which can be catabolized.

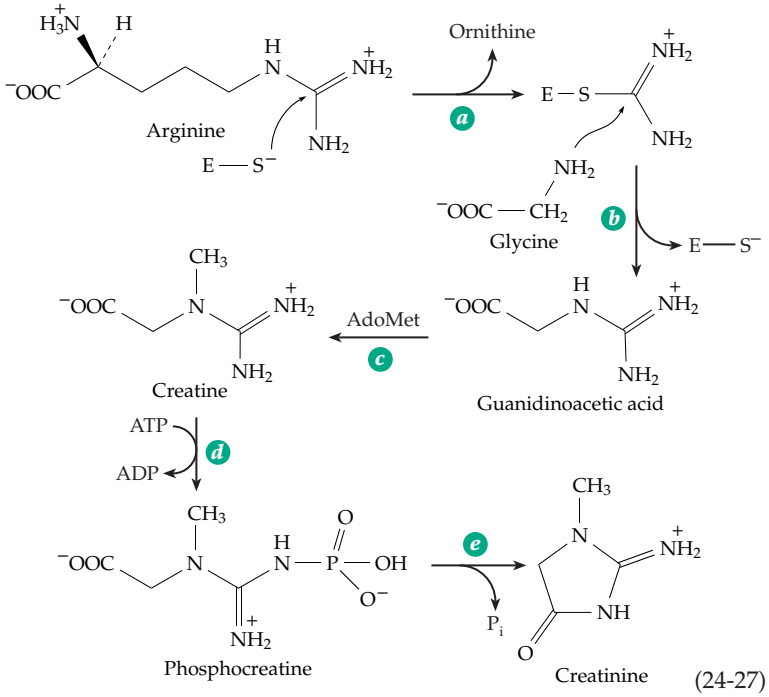
## 3. Amidino Transfer and Creatine Synthesis

The terminal amidino group of arginine is transferred intact to a number of other substances in simple displacement reactions. An example is the formation of **guanidinoacetic acid** (Eq. 24-27, steps *a* and *b*). The amidino group appears to be transferred first to the SH group of cysteine 407 then to glycine in a double displacement mechanism.[195–197] Transmethylation from *S*-adenosylmethionine (Eq. 24-27, step *c*) converts guanidinoacetic acid to **creatine**, a compound of special importance in muscle. Creatine kinase reversibly transfers the phospho group of ATP to creatine to form the *N*-phosphate (Eq. 24-27, step *d*). **Creatine phosphate**, and in some invertebrates phosphoarginine,[198] serves as an important "energy buffer" for muscular contraction (Chapter 19). Through the reversible action of creatine kinase it is able rapidly to transfer its phospho group back onto ADP as fast as the latter is formed during the hydrolysis of ATP in the contraction process. An end product of creatine phosphate metabolism is the anhydride **creatinine** formed from creatine phosphate as is indicated in Eq. 24-27, step *e* as well as directly from creatine. The urinary creatinine excretion for a given individual is extremely constant from day to day, the amount excreted apparently being directly related to the muscle mass of the person. Another example of the transfer of amidino groups from arginine is found in the synthesis of streptomycin (Box 20-B).

A cyclic analog of creatine, **cyclocreatine**, when fed to animals, accumulates in large amounts in muscle, heart, and brain and is a long-acting phosphagen.[199]




Cyclocreatine phosphate



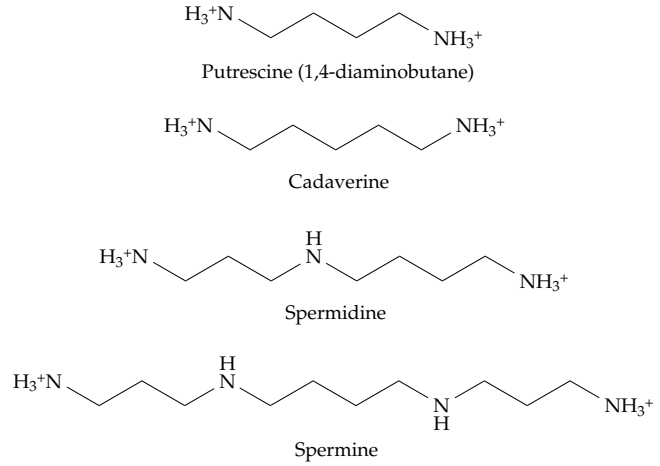

(24-27)

## 4. The Polyamines

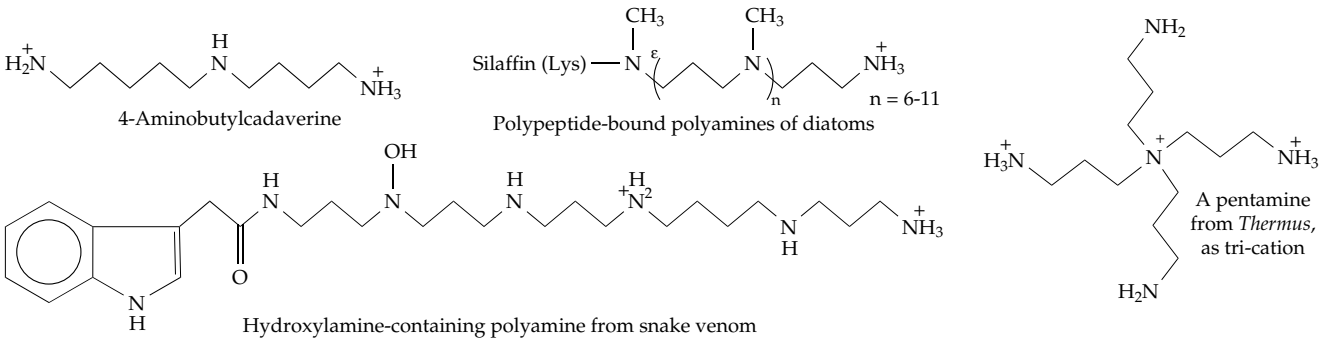
A series of related polyamino compounds, which are derived in part from arginine, are present in all cells in relatively high, often millimolar, concentrations.[200–203] The content of polyamines in cells tends to be stoichiometric with that of RNA, and the polyamines are concentrated in the ribosomes and also in the nucleus. Two moles of polyamine are usually present per mole of any isolated tRNA.[202,204] The first satisfactory crystals of tRNA for X-ray structure determination were obtained in the presence of spermine. Spermidine is associated with RNA in the turnip yellow mosaic virus.[205] The T-even bacteriophage and most bacteria contain polyamines in association with DNA. Polyamines are able to interact with double helical nucleic acids by bridging between strands, the positively charged amino groups interacting with the phosphates of the nucleic acid backbones. Tsuboi suggested that the tetramethylene portion of the polyamine lies in the minor groove bridging three base pairs, and the trimethylene portions (one in spermidine, two in spermine) bridge adjacent phosphate groups in one strand.[206] Polyamines may also stabilize supercoiled or folded DNA.

The structures of polyamines are shown here as di- and tri-cations, but it should be realized that there are multiple positions for protonation and therefore various tautomers. Also, polyamines show extreme anticooperativity in proton binding, i.e., successive p$K_a$ values range from very low to very high for the last proton to leave. Polyamines are thought to have several functions. They can substitute to some extent for cellular $K^+$ and $Mg^{2+}$, and they may play essential controlling roles in nucleic acid and protein synthesis. A specific role of spermidine in cell division seems likely.[207,207a] An absolute requirement for polyamines has been demonstrated for some bacteria such as *Hemophilus parainfluenzae*[208] and for mutants of *Aspergillus* and *Neurospora*. Polyamines are also essential for mammalian cells. Polyamines activate some enzymes including the serine/threonine protein kinase CK2.[209]

Mutants of *E. coli* have been constructed in which enzymes of all known biosynthetic pathways for polyamines are blocked by deletion of the genes for arginine decarboxylase (*SpeA*), agmatine ureahydrolase (*SpeB*), ornithine decarboxylase (*SpeC*), and adenosylmethionine decarboxylase (*SpeD*).[210] Even though polyamines cannot be detected in these cells they grow at one-third the normal rate. However, yeast cells require both putrescine and spermidine or spermine for growth.[211,211a] Another effect is seen in strains of yeast carrying the **"killer plasmid,"** a 1500-kDa double-stranded RNA plasmid that encodes a toxic protein, which is secreted and kills other susceptible strains of yeast. Yeast cells carrying the killer plasmid lose it when made deficient in polyamines.[212] The bacterial outer membrane porins OmF and OmC (Fig. 8-20) bind polyamines, especially spermine, and inhibit passage of ions. Polyamines may also modulate ion channels of heart, muscle, and neurons.[213] Both prokaryotic and eukaryotic cells have transporters that allow uptake of polyamines from their surroundings.[214,215]




Major naturally occurring polyamines (as dications)




***Biosynthesis.*** The 4-carbon putrescine arises most directly by decarboxylation of ornithine (Fig. 24-12, step *b*),[216] but it can also be formed by decarboxylation of arginine to agmatine followed by hydrolysis of the latter (Fig. 24-12, steps *c*,*d*). An alternative pathway utilizes an "agmatine cycle" in which agmatine is first hydrolyzed to ammonium ions and *N*-carbamoylputrescine. The latter transfers its ureido group to ornithine to form citrulline and releases free putrescine (Fig. 24-12, steps *f*,*g*). The citrulline is reconverted to arginine. This pathway appears to be important in plants.[217] Putrescine is normally present in all cells, and all cells are able to convert it on to spermidine. This is accomplished by decarboxylation of *S*-adenosylmethionine (Fig. 24-12, step *a*) and transfer of the propylamine group from the resulting decarboxylation product onto an amino group of putrescine (Fig. 24-12, step *h*).[218–221]:

The more complex spermine is found only in eukaryotes. It is formed by transfer of a second propylamine group onto spermidine (Fig. 24-12, step *i*). A historical note is that Anthony von Leeuwenhoek with one of his first microscopes observed crystals of the phosphate salt of spermine in human semen in 1678. The 5-carbon diamine cadaverine arises from decarboxylation of lysine (Fig. 24-12, step *d*). The extremely thermophilic bacterium *Thermus thermophilis* produces several additional polyamines including a pentamine (see above), a quaternary nitrogen compound.[222] Many other polyamines are known.[223–224c] Among these are a 4-aminobutylcadaverine isolated from root nodules of the adzuki bean[223] and very long partially aromatic hydroxylamine derivatives from venom of common funnel-web spiders (structures at top of page).[225] Cationic polypeptides called **silaffins**, with masses of ~3 kDa, apparently initiate the growth of the silica cell walls of diatoms (Box 4-B). These peptides contain polyamines consisting of 6 to 11 repeated *N*-methylpropylamine units covalently attached to lysine residues[224a,224b] and also many phosphoserines.[224d]

The synthesis of polyamines is tightly regulated. The PLP-dependent ornithine decarboxylase is present in very low concentrations[226] and apparently has the shortest half-life (~10 min) of any mammalian

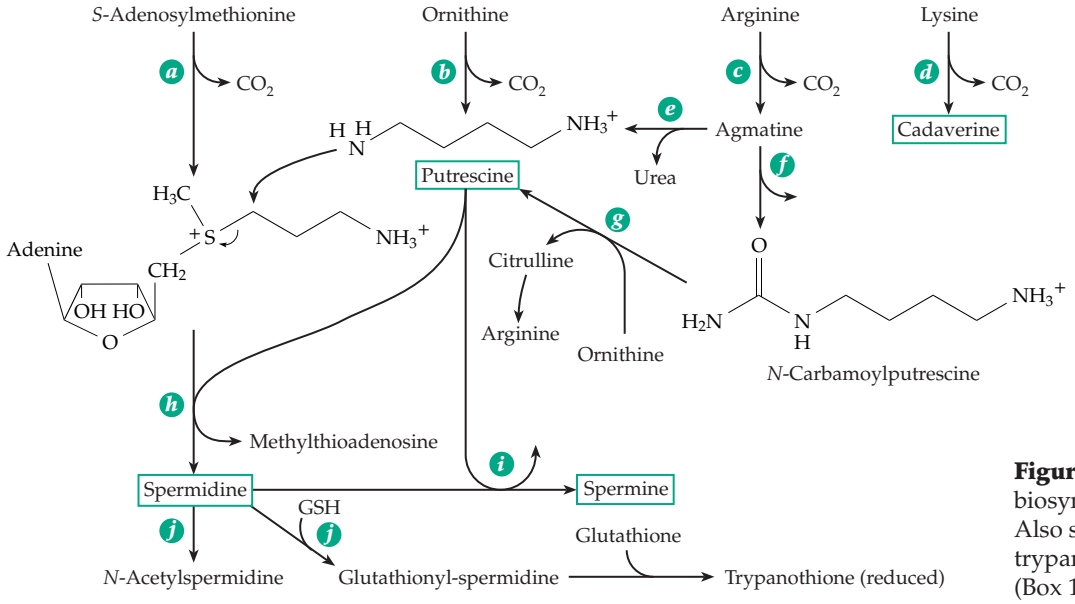
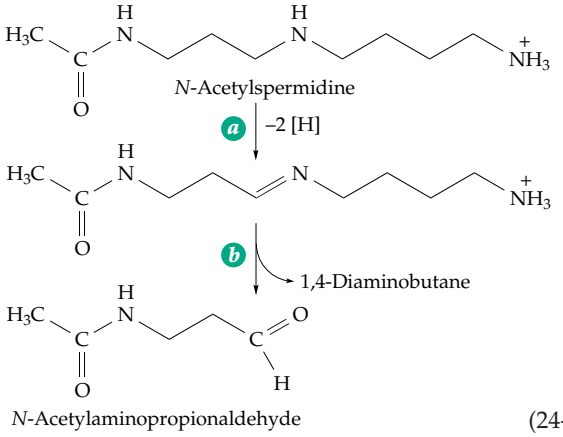



**Figure 24-12** Pathways of biosynthesis of polyamines. Also shown is the formation by trypanosomes of trypanothione (Box 11-B).

enzyme.[227,228] Its concentration increases rapidly in most species with the onset of rapid growth, transformation to a neoplastic state, or initiation of cell differentiation. The rate of synthesis of the enzyme appears to be regulated by feedback repression by spermidine and by inactivation in response to a buildup of putrescine.[229] One mechanism of inactivation is the synthesis of a 26-kDa specific inhibitor called an **antizyme** in response to the presence of putrescine, spermidine, or spermine. The antizyme is ubiquitous in both prokaryotes and eukaryotes and keeps most of the ornithine decarboxylase bound and inactive and also promotes its degradation by 26S proteosomes.[230–230b] A polyamine-dependent protein kinase in *Physarum* phosphorylates the decarboxylase thereby inhibiting its activity.[231]

***Breakdown.*** The catabolism of polyamines is less well understood than is their biosynthesis. Oxidative cleavages of spermine to spermidine and of the latter to 1,4-diaminobutane appear to occur in the animal body, and a substantial amount of this diamine is excreted in the urine.[201] Spermidine is acetylated on $N^1$ by acetyl-CoA and a spermidine *N*-acetyltransferase.[232,233] The resulting $N^1$-acetylspermidine is more readily cleaved by hepatic polyamine oxidase[233a] than is free spermine; again 1,4-diaminobutane is reformed together with an *N*-acetylaminopropionaldehyde (Eq. 24-28). This and other aldehydes formed from polyamines are very toxic but they may play essential roles in regulation of metabolism.[201] Transamination of 1,4-diaminobutane yields γ-aminobutyraldehyde which cyclizes (Eq. 24-29). Diamine oxidases of animal tissues oxidize 1,4-diaminobutane with formation of the same products.[234] Further metabolism of $\Delta^1$-pyrroline yields γ-aminobutyrate, which can undergo transamination and oxidative metabolism as shown in Fig. 17-5. Other products[235] are also indicated in Eq. 24-29. Metabolism of other polyamines also begins by oxidation at the primary amino termini.[236] Formation of β-alanine, needed for synthesis of pantothenic acid, can also occur by oxidation of spermine.[236a]

When *E. coli* cells enter the stationary phase of the growth curve (Box 9-B), most of the spermidine is converted to **glutathionylspermidine** (γ-glutamylcysteinylglycylspermidine) in which glutathione and spermidine are joined by an amide linkage.[237–239] Trypanosomes join a second glutathione at the other end of the spermidine to form reduced **trypathione**,[238] a compound also considered in Box 11-B. $N^1$-γ-Glutamylspermidine and related compounds have been found in proteolytic digests of certain proteins, suggesting that polyamines may be physiological substrates for transglutaminases.[240] Portions of polyamines are incorporated into a variety of products including **nicotine** (Fig. 30-22)[241] and the unusual

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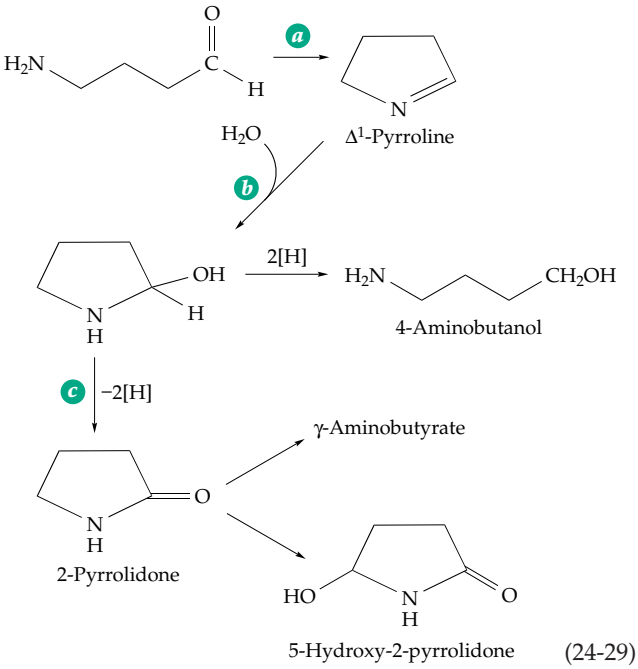



(24-29)

amino acid **hypusine** (see p. 1386).[242]

Ornithine decarboxylase is specifically inhibited by the enzyme-activated inhibitor α-difluoromethylornithine, which can cure human infection with *Trypanosoma brucei* (African sleeping sickness) by interfering with polyamine synthesis.[243–244a] In combination with inhibitors of spermidine synthase or *S*-adenosylmethionine decarboxylase,[245] it can reduce polyamine levels and growth rates of cells. Another powerful inhibitor that acts on both ornithine and adenosylmethionine decarboxylases is the hydroxylamine derivative 1-aminooxy-3-aminopropane.[246]

$$H_2N{-}O{-}CH_2CH_2CH_2{-}NH_2$$

1-Aminooxy-3-aminopropane

Like difluoromethylornithine the compound at low concentrations is not toxic to cells but inhibits growth. It is hoped that adequate inhibition of growth of

normal cells may allow more aggressive chemotherapeutic treatment of cancer.

## D. Compounds Derived from Aspartate

The 4-carbon aspartate molecule is the starting point for synthesis of **pyrimidines** and of the amino acids **lysine**, **methionine**, **threonine**, **isoleucine**, and **asparagine**.[247,248] The pathways are summarized in Fig. 24-13. There are several branch points, and aspartate can be converted directly to asparagine, to carbamoylaspartate (the precursor of pyrimidines), or to β-aspartyl phosphate and aspartate semialdehyde. The latter can be converted in one pathway to lysine and in another to homoserine. Homoserine can yield either homocysteine and methionine or threonine. Although threonine is one of the end products and a constituent of proteins, it can also be converted further to 2-oxobutyrate, a precursor of isoleucine.

Most of the chemistry has been considered already. The reduction of aspartate via β-aspartyl phosphate[249,249a] and aspartate β-semialdehyde[250] is a standard one. Conversion to methionine can occur in two ways. In *E. coli* homoserine is succinylated with succinyl-CoA. The γ-succinyl group is then replaced by the cysteine molecule in a PLP-dependent γ-replacement reaction (Fig. 24-13). The product **cystathionine** (Eq. 14-33) undergoes elimination to form homocysteine. A similar pathway via *O*-phosphohomoserine occurs in chloroplasts of green plants.[251] A more direct γ replacement of the hydroxyl of homocysteine or *O*-phosphohomoserine by a sulfide ion has also been reported for both *Neurospora* and green plants.[252] Methylation of homocysteine to methionine (Fig. 24-13) has been considered previously, as has the conversion of homoserine to threonine by homoserine kinase[253] and the PLP-dependent **threonine synthase** (p. 746, Fig. 14-7).[254–255a] A standard PLP-requiring β elimination converts threonine to **2-oxobutyrate**, a precursor to isoleucine (Fig. 24-13).[256]

Formation of **asparagine** has been discussed in section B. Asparagine synthase of *E. coli*[98–99] cleaves ATP to AMP and $PP_i$ rather than to ADP via an aspartyladenylate intermediate. In higher animals glutamine serves as the ammonia donor for synthesis of asparagine, but $NH_4^+$ can also function.[257] **L-Asparaginase**, a bacterial hydrolase, is an experimental antileukemic drug. It acts to deprive fast-growing tumor cells of the exogenous asparagine needed for rapid growth.[136,257a] Tissues with a low asparagine synthase activity are also damaged, limiting the clinical usefulness.

Aspartate can be decarboxylated either to α-alanine by a PLP-dependent enzyme[258] or to β-alanine by a pyruvoyl group-containing enzyme (Chapter 14). Beta-alanine is not only a component of the vitamin pantothenic acid but is found in the dipeptides carnosine (β-alanylhistidine) and anserine (β-alanyl-$N^{\delta}$-methylhistidine) present in vertebrate muscles.[259] It is a crosslinking agent in insect cuticle.

Aspartate can be deaminated to fumarate by bacterial L-**aspartate oxidase**.[259a] This flavoprotein is structurally and mechanistically related to succinate dehydrogenase and can function as a soluble fumarate reductase (p. 1027). However, its main function appears to be to permit the intermediate iminoaspartate to react with dihydroxyacetone-*P* to form quinolinate, which can be converted to **NAD** (see Fig. 25-11).[259b]

### 1. Control of Biosynthetic Reactions of Aspartate

In *E. coli* there are three **aspartokinases** that catalyze the conversion of aspartate to β-aspartyl phosphate. All three catalyze the same reaction, but they have very different regulatory properties, as is indicated in Fig. 24-13. Each enzyme is responsive to a different set of end products.[247,260] The same is true for the two **aspartate semialdehyde reductases** which catalyze the third step. Both repression of transcription and feedback inhibition of the enzymes are involved. Two of the aspartokinases of *E. coli* are parts of bifunctional enzymes, which also contain the homoserine dehydrogenases that are needed to reduce aspartate semialdehyde in the third step. These aspartokinase–homoserine dehydrogenases I and II (Fig. 24-13) are encoded by *E. coli* genes *thrA* and *metL*, respectively, and have homologous sequences.[247,261–262a] The N-terminal portions are also homologous to the lysine-sensitive aspartokinase III which is encoded by the *lysC* gene.[263] In *Bacillus subtilis* the lysine-sensitive enzyme is known as aspartokinase II. It has an $\alpha_2\beta_2$ oligomeric structure and both α and β chains are encoded within a single gene.[264] There is no associated homoserine dehydrogenase. Both genetic organization and processing of the synthesized protein are thus different in these two bacteria.

### 2. Lysine Diaminopimelate, Dipicolinic Acid, and Carnitine

Lysine cannot be made at all by animals but is nutritionally essential. There are two distinct pathways for its formation in other organisms. The **α-aminoadipate pathway** (shown in Fig. 24-9) occurs in a few lower fungi, the higher fungi, and euglenids. The 5-carbon 2-oxoglutarate is the starting compound. Bacteria, other lower fungi, and green plants all use the **diaminopimelate** pathway (Fig. 24-14) which originates with the 4-carbon aspartate.

The α-aminoadipate pathway (Fig. 24-9) parallels

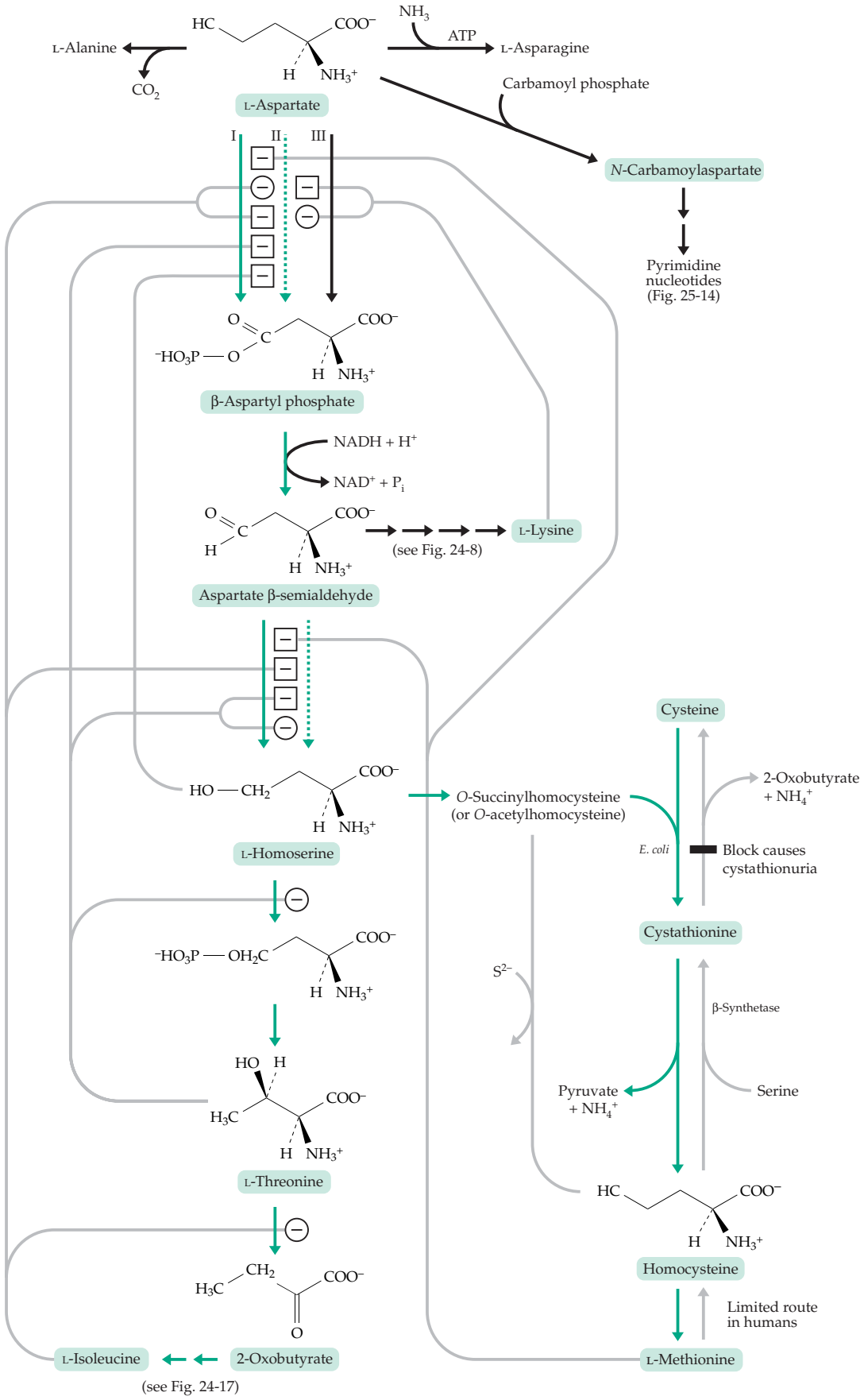
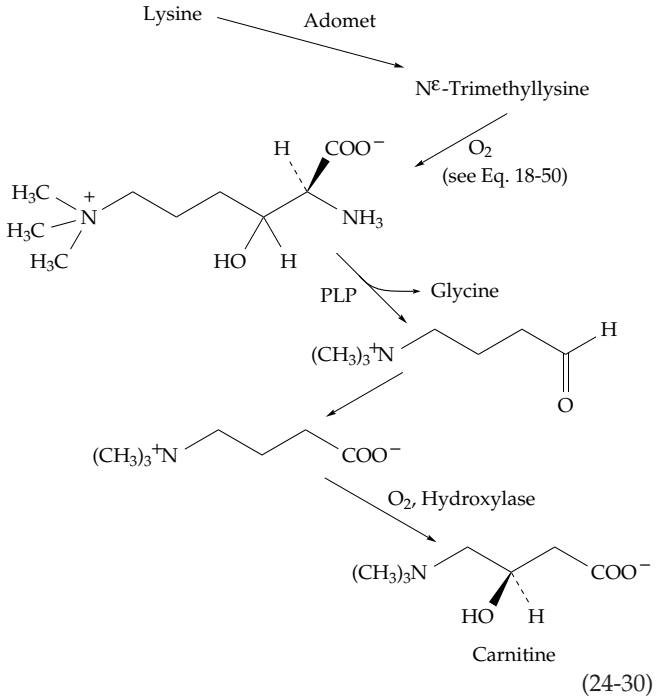



**Figure 24-13** Some biosynthetic reactions of aspartate: ⊖, feedback inhibition and ⊟, feedback repression.

that of ornithine biosynthesis, 2-oxoglutarate undergoing chain elongation (Fig. 17-18) to 2-oxoadipate followed by transamination to α-aminoadipate. This is followed by ATP-dependent reduction to the aldehyde.[264a] The final step of transamination is not accomplished in the usual way (with a PLP-dependent enzyme), but through formation of a Schiff base with glutamate and reduction to **saccharopine**.[265] Oxidation now produces the Schiff base of lysine with 2-oxoglutarate.

In the diaminopimelate pathway of lysine synthesis (Fig. 24-14) aspartate is converted to aspartate semialdehyde, and a two-carbon unit is added via aldol condensation with pyruvate.[266–269] Decarboxylation at the end of the sequence yields lysine. A series of cyclic intermediates exist, but it is noteworthy that the initial product of the aldol condensation (bracketed in Fig. 24-14) is converted to diaminopimelic acid by a simple sequence involving α,β elimination of the hydroxy group, reduction with NADPH, and transamination.[267,268,270,271] The process is complicated by the natural tendency for ring closure. All gram-negative and many gram-positive bacteria use the succinylase pathway shown in Fig. 24-14. Succinylation serves to shift the equilibrium back in favor of open-chain compounds.[272–274] Some species of *Bacillus* use acetylation in the same way, while a few bacteria manage to use a dehydrogenase to reductively aminate tetrahydropimelate to diaminopimelate.[275,276] The diaminopimelate pathway is of special significance to prokaryotic organisms for the reason that **dipicolinic acid** is formed as an important side product and because of formation of **diaminopimelic acids**. The cyclic




(24-30)

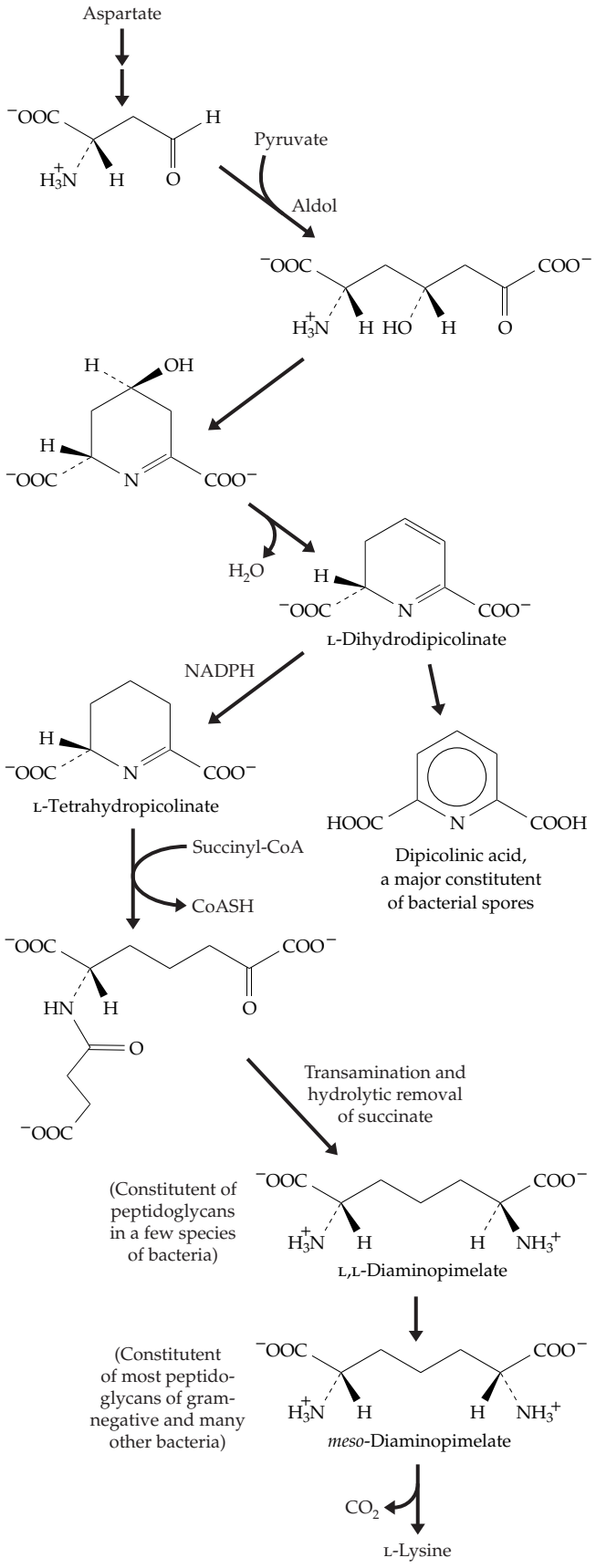
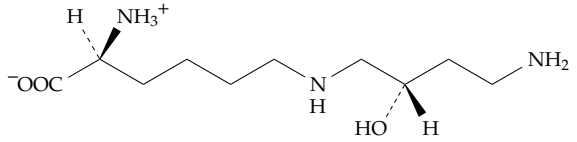



**Figure 24-14** The biosynthesis of lysine by the diaminopimelate pathway.

dipicolinic acid is a major constituent of bacterial spores[277] but is rarely found elsewhere in nature. Both L, L- and meso-diaminopimelic acids[276,276a] are constituents of peptidoglycans of bacterial cell walls (Fig. 8-29).

Lysine is not only a constituent of proteins. It can also be trimethylated and converted to **carnitine** (p. 944). In mammals some specific lysyl side chains of proteins undergo *N*-trimethylation and proteolytic degradation with release of free trimethyllysine (Eq. 24-30).[278,279] The free trimethyllysine then undergoes hydroxylation by a 2-oxoglutarate–$Fe^{2+}$–ascorbate-dependent hydroxylase (Eq. 18-51) to form β-hydroxytrimethyllysine, which is cleaved by a PLP-dependent enzyme (Chapter 14). The resulting aldehyde is oxidized to the carboxylic acid and is converted by a second 2-oxoglutarate–$Fe^{2+}$–ascorbate-dependent hydroxylase to carnitine (Eq. 24-30; see also Eq. 18-50).

**Hypusine** ($N^{\varepsilon}$-(4-amino-2-hydroxybutyl)lysine)[242] occurs in mammalian initiation factor 4D, which is utilized in protein synthesis (Chapter 29) and is formed by transfer of the 4-carbon butylamine group from spermidine to a lysine side chain followed by hydroxylation.[280–282a] The lupine alkaloid lupinine[283] is formed from two $C_5$ units of cadaverine which arises by decarboxylation of lysine. Silaffins (pp. 178, 1381) also contain modified lysines.




Hypusine: $N^{\varepsilon}$-(4-amino-2-hydroxybutyl) lysine

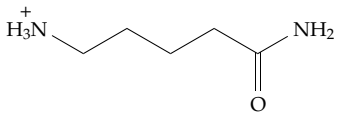
## 3. The Catabolism of Lysine

An unusual feature of lysine metabolism is that the α-amino group does not equilibrate with the "nitrogen pool." Catabolism is initiated by deamination and proceeds by β oxidation.[284] At least six variations of the β-oxidation process have been proposed. The evolutionary differences concern the manner in which the two amino groups are moved from the carbon skeleton. In the seemingly simplest pathway (A in Fig. 24-15), which is used by *Flavobacterium fuscum*,[285] the ε-amino group is removed in a direct (but atypical) transamination. The resulting α-aminoadipate semialdehyde is oxidized to α-aminoadipate, which *is degraded in a sequence characteristic for the catabolism of amino acids.* Transamination is followed by oxidative decarboxylation of the resulting 2-oxoacid and β oxidation of the coenzyme A derivative. A decarboxylation step by which the terminal carboxyl group is removed is interposed in the β-oxidation sequence for lysine degradation.

Perhaps the initial transamination in pathway A is chemically difficult, for most organisms use more complex sequences to form 2-oxoadipate. In pathway B (which takes place in liver mitochondria and is believed to be the predominant pathway in mammals),[286,287] the ε-amino group is reductively coupled with 2-oxoglutarate to form saccharopine. The latter is in turn oxidized on the opposite side of the bridge nitrogen to form glutamic acid and α-aminoadipate semialdehyde. The overall process is the same as direct transamination and just the opposite of that occurring in the aminoadipate pathway of biosynthesis (Fig. 24-9). Absence of one or both of these dehydrogenases causes familial hyperlysinemia.[287,288]

Pathway C has been established for *Pseudomonus putida*[289] and is also followed to some extent in both plants and animals. In most animal tissues it may be used principally for degradation of D-lysine.[290] However, it is the major L-lysine oxidation pathway in brain.[291] In a fungal parasitic species of *Rhizoctonia* L-lysine is converted to saccharopine via pathway B; then using an $NADP^+$-dependent saccharopine oxidase the sequence is shunted to pathway C.[292] L-Pipicolic acid formed in this way also gives rise to various alkaloids including the α-mannosidase inhibitor swainsonine (Fig. 20-7).[290] Pathway C, like pathway B, makes use of transamination via a reduction-oxidation sequence. It is strictly internal, the oxidizing carbonyl group being formed by transamination of the α-amino group of lysine. Pathway D, apparently used by yeasts,[293] avoids cyclic intermediates by acetylation of the ε-amino group prior to transamination. The 2-oxo group is then effectively blocked by reduction to an alcohol, the blocking group is removed from the ε-amino group, and that end of the molecule is oxidized in a straightforward way to a carboxyl group. Now the hydroxyl introduced at position 2 is presumably oxidized back to the ketone, which again can be converted to give 2-oxoadipate.

Some bacteria, e.g., *Pseudomonas putida*,[294] degrade L-lysine with a flavin-dependent oxygenase (Eq. 18-41) to δ-aminovaleramide:




The product is hydrolyzed and oxidized to **glutaryl-CoA**, rejoining the pathways shown in Fig. 24-15. A remarkable and very different approach to lysine breakdown has been developed by clostridia which obtain energy from the fermentation of Eq. 24-31:

$$\text{L-Lysine} + 2\ H_2O \longrightarrow \text{butyrate}^- + \text{acetate}^- + 2\ NH_4^+ \qquad (24\text{-}31)$$

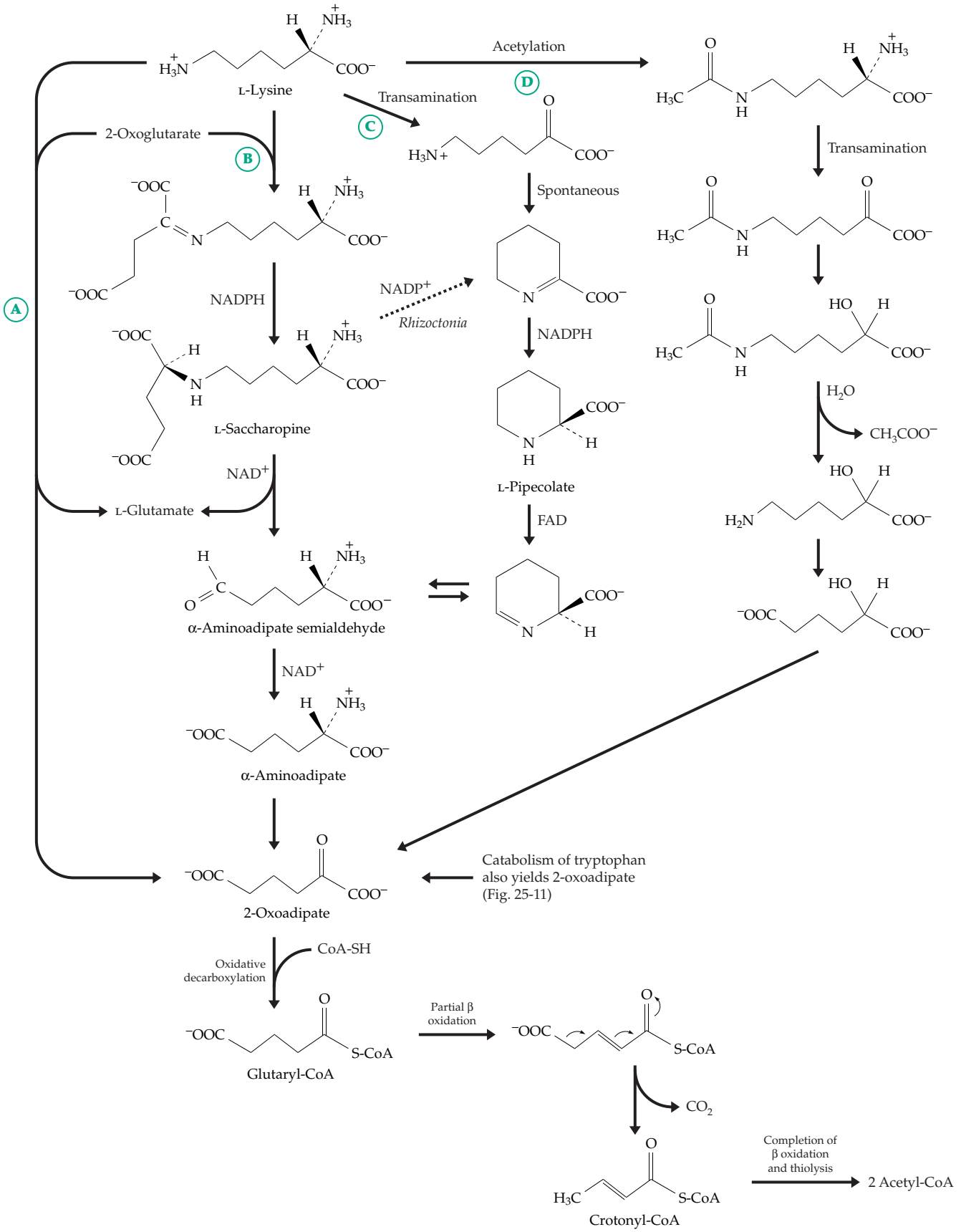
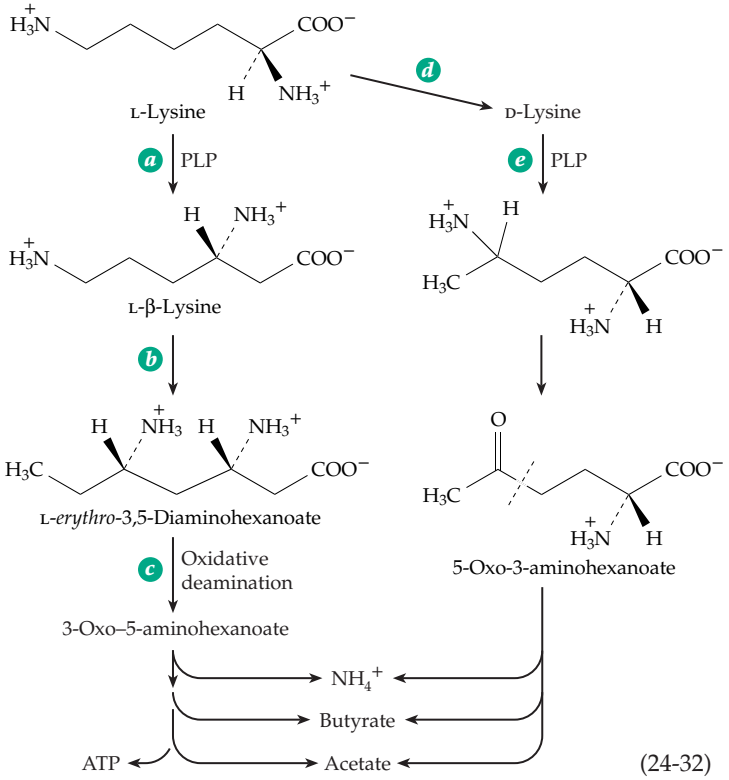



**Figure 24-15** Catabolism of lysine.

The reaction is coupled to formation of one molecule of ATP from ADP and $P_i$. Two pathways have been worked out. In the first lysine is acted upon by a PLP-dependent **L-lysine 2,3-aminomutase** (Eq. 24-32, step *a*) to convert it to β-lysine (3,6-diaminohexanoate). The latter is further isomerized (Eq. 24-32, step *b*) by the vitamin $B_{12}$ and PLP-dependent β-lysine mutase. Oxidative deamination to a 3-oxo compound (Eq. 24-32, step *c*) permits chain cleavage. The reader can easily propose the remaining reactions of chain cleavage, ATP synthesis, elimination of ammonia, and balancing of the redox steps. An alternative pathway begins with a racemase (Eq. 24-32, step *d*) and isomerization of the resulting D-lysine by another $B_{12}$ and PLP-dependent enzyme (Eq. 24-32, step *e*).[294a] Oxidative deamination presumably occurs, but the mechanism for chain cleavage is not so obvious. It does occur between C-4 and C-5 as indicated by the dashed line in Eq. 24-32.

Another variation is used by *Pseudomonas* β4 (Eq. 24-31). Beta-lysine is acetylated on N-6, then undergoes transamination to a 2-oxo acid and removal of the first two carbons as acetyl-CoA. The resulting 4-aminobutyrate is then converted to succinate via succinate semialdehyde.[295]

Why are there so many pathways of lysine breakdown? The answer is probably related to the ease of spontaneous formation of cyclic intermediates as occurs in the pipecolate pathway (pathway C, Fig. 24-15). These intermediates may be too stable for efficient metabolism so the indirect pathways evolved. In the fermentation reactions additional constraints are imposed on the pathways by the need for balanced redox processes and a net Gibbs energy decrease.



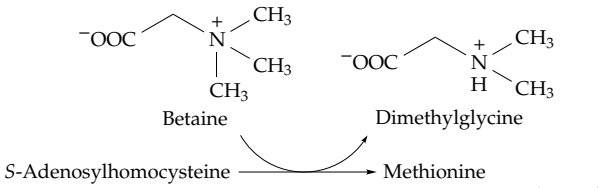

(24-32)

## 4. Metabolism of Homocysteine and Methionine

Autotrophic organisms synthesize methionine from aspartate as shown in the lower right side of Fig. 24-13. This involves transfer of a sulfur atom from cysteine into homocysteine, using the carbon skeleton of homoserine, the intermediate **cystathionine**, and two PLP-dependent enzymes, **cystathionine γ-synthase**[296,296a] and **cystathionine β-lyase**.[297] This **transsulfuration** sequence (Fig. 24-13, Eq. 14-33) is essentially irreversible because of the cleavage to pyruvate and $NH_4^+$ by the β-lyase. Nevertheless, this transsulfuration pathway operates in reverse in the animal body, which uses two different PLP enzymes, **cystathionine β-synthase** (which also contains a bound heme)[298–299c] and **cystathionine γ-lyase**[300] (Figs. 24-13, 24-16, steps *h* and *i*), in a pathway that metabolizes excess methionine.

For human beings methionine is nutritionally essential and comes entirely from the diet. However, the oxoacid analog of methionine can be used as a nutritional supplement. Dietary homocysteine can also be converted into methionine to a limited extent. Methionine is incorporated into proteins as such and as ***N*-formylmethionine** at the N-terminal ends of bacterial proteins (steps *a* and *b*, Fig. 24-16). In addition to its function in proteins methionine plays a major role in biological methylation reactions in all organisms. It is converted into ***S*-adenosylmethionine** (AdoMet or SAM; Fig. 24-16, step *e*; see also Eq. 17-37),[301–302b] which is the most widely used methyl group donor for numerous biological methylation reactions (Eq. 12-3). *S*-Adenosylmethionine is also the precursor of the special "wobble base" **queuine** (Fig. 5-33).[312]

The product of transmethylation, ***S*-adenosylhomocysteine**, is converted (step *g*) into homocysteine in an unusual NAD-dependent hydrolytic reaction (Eq. 15-14) by which adenosine is removed (step *g*).[302c] Homocysteine can be reconverted to methionine, as indicated by the dashed line in Fig. 24-16. This can be accomplished by the vitamin $B_{12}$- and tetrahydrofolate-dependent **methionine synthase**, (Eq. 16-43), which transfers a methyl group from methyl-tetrahydrofolate[303–303b]; by transfer of a methyl group from **betaine**, a trimethylated glycine (Eq. 24-33)[304], or by remethylation with AdoMet (Fig. 24-16).[304a]

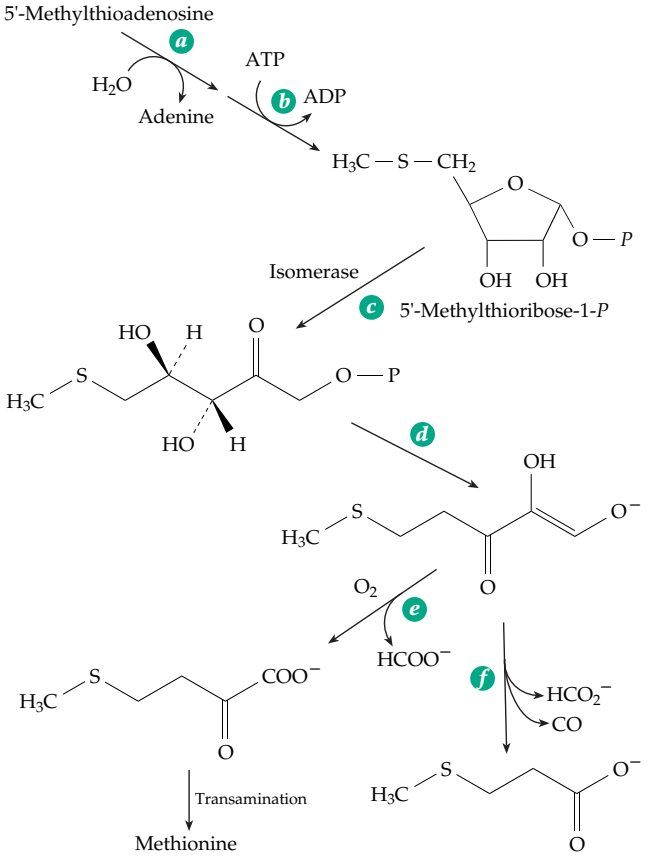



(24-33)

When present in excess methionine is toxic and must be removed. Transamination to the corresponding 2-oxoacid (Fig. 24-16, step *c*) occurs in both animals and plants. Oxidative decarboxylation of this oxoacid initiates a major catabolic pathway,[305] which probably involves β oxidation of the resulting acyl-CoA. In bacteria another catabolic reaction of methionine is γ-elimination of methanethiol and deamination to 2-oxobutyrate (reaction *d*, Fig. 24-16; Fig. 14-7).[306] Conversion to homocysteine, via the transmethylation pathway, is also a major catabolic route which is especially important because of the toxicity of excess homocysteine. A hereditary deficiency of cystathionine β-synthase is associated with greatly elevated homocysteine concentrations in blood and urine and often disastrous early cardiovascular disease.[299,307–309b] About 5–7% of the general population has an increased level of homocysteine and is also at increased risk of artery disease. An adequate intake of vitamin $B_6$ and especially of folic acid, which is needed for recycling of homocysteine to methionine, is helpful. However, if methionine is in excess it must be removed via the previously discussed transsulfuration pathway (Fig. 24-16, steps *h* and *i*).[310] The products are cysteine and 2-oxobutyrate. The latter can be oxidatively decarboxylated to propionyl-CoA and further metabolized, or it can be converted into leucine (Fig. 24-17) and cysteine may be converted to glutathione.[299a]

Methionine in plants can be converted to the sulfonium compound *S*-methyl-L-methionine, also called vitamin U. It has strong osmoprotectant activity and accumulates in many marine algae and some flowering plants.[311] Other organisms, including mammals, can use *S*-methylmethionine to methylate homocysteine, converting both reactants back to methionine[311a] enabling animals to meet some of their methionine need from this source.

***A salvage pathway.*** Another product of *S*-adenosylmethionine is **5'-methylthioadenosine**, which can be formed by an internal displacement on the γ-methylene group by the carboxylate group (step *l*, Fig. 24-16). Methylthioadenosine also arises during formation of the compounds spermidine (Fig. 24-12) and ACC (Fig. 24-16). Mammalian tissues convert methylthioadenosine back to methionine by the sequence shown in Eq. 24-34. It undergoes phosphorolysis to 5'-methylthioribose whose ring is opened and




(24-34)

converted to the 2-oxoacid analog of methionine.[313,314]

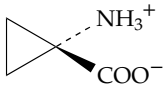
Step *c* of Eq. 24-34 may occur by ring opening to an enol phosphate which ketonizes to the observed product, but step *e* is a more complex multistep oxidative process.[314a,b] The last step is transamination to methionine with a glutamine-specific aminotransferase. Another enzyme from *Klebsiella* converts the same intermediate anion to methylthiopropionate, formate, and CO (Eq. 24-34, step *f*).[315]

***The plant hormone ethylene.*** A major reaction of *S*-adenosylmethionine in plants is the formation of **ethylene**.[316,317] Ethylene has been recognized since 1858 as causing a thickening of stems of plants and a depression in the rate of elongation. In 1917, it was established that ethylene is formed in fruit and that addition of this gaseous compound hastened ripening. Ethylene is now an established plant hormone having a variety of effects including retardation of mitosis, inhibition of photosynthesis, and stimulation of respiration and of the enzyme phenyalanine ammonia-lyase (Eq. 14-45). These effects are indirectly a result of the action of ethylene on transcription of certain genes. In *Arabidopsis*, with which genetic studies are being made, ethylene binds to the N-terminal part of at least two receptor proteins, which have intracellular histidine kinase domains in the C-terminal parts.[318,319]

DNA-binding proteins specific for **ethylene-responsive elements** (EREs), having the conserved sequence AGCCGCC, are presumably phosphorylated by this kinase[320] and affect transcription of many genes. A protein homologous to one ethylene receptor of *Arabidopsis* has been identified in the tomato. A proline to leucine mutation at position 36, near the N terminus, destroys the sensitivity to ethylene and prevents ripening of this tomato. Another component in the ethylene signaling pathway in *Arabidopsis* is a protein serine / threonine kinase that resembles the mammalian raf kinase involved in the signaling cascade shown in Fig. 11-13.[318]

The formation of ethylene is often induced by the hormone **auxin** (Chapter 30), which stimulates activity of the synthase that forms **1-aminocyclopropane-1-carboxylate** (**ACC**) from *S*-adenosyl methionine (Eq. 14-27, step *j*: Fig. 24-16).[320a,b] Although ACC has been known as a minor plant product for over 25 years, it was much more recently identified as the immediate precursor of ethylene. ACC is often produced in response to stresses such as wounding, drought, or waterlogging of roots.[316,321] In the last of these cases the ACC is transferred through the xylem from the roots upward to shoots, which respond in characteristic ways to the ethylene that is released.

The conversion of ACC to ethylene, HCN, and $CO_2$ is catalyzed by ACC oxidase, an $Fe^{2+}$-dependent enzyme of the isopenicillin-*N*-synthase (Eq. 18-52) subfamily of oxygenases. However, unlike most of



$$+ O_2 + \text{Ascorbate} \rightarrow H_2C{=}CH_2 + HCN + CO_2 + 2\,H_2O + \text{Dehydroascorbate} \quad (24\text{-}35)$$

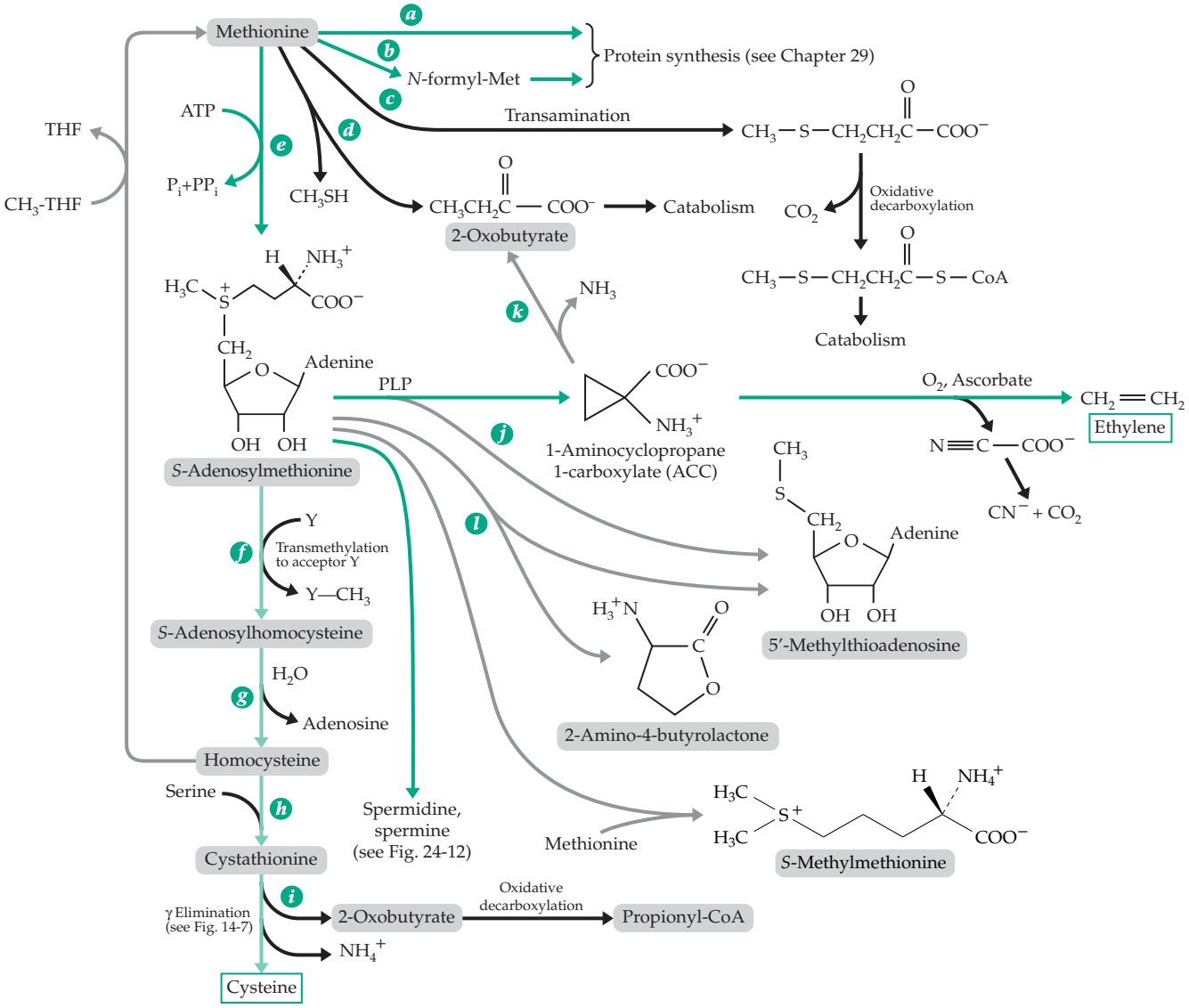
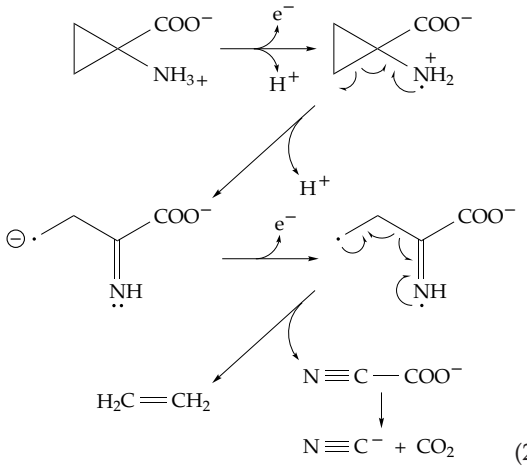


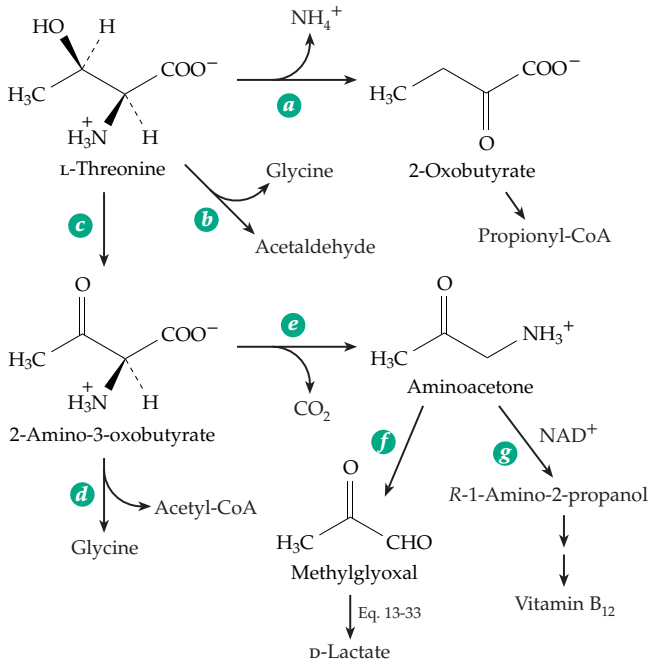

**Figure 24-16** Some metabolic reactions of methionine. Biosynthetic reactions are indicated by green arrows.




(24-36)

these enzymes, which utilize 2-oxoglutarate as a co-substrate (Eq. 18-52), ACC oxidase employs ascorbate and forms HCN or cyanide ions.[322–324b] It also requires $CO_2$ or bicarbonate as an activator.[324,325] A radical mechanism (Eq. 24-36) is probable,[317] with two electrons from ACC and two from ascorbate being utilized to reduce $O_2$ to 2 $H_2O$.

Ethylene is rather inert, but it is metabolized slowly, some of it to ethylene glycol.[326] Plants store *N*-malonyl-ACC as a metabolically inert pool. Excess ACC can be deaminated in a PLP-dependent reaction to 2-oxobutyrate (step *k*, Fig. 24-16), a process that also occurs in bacteria able to subsist on ACC.[327,327a] There may also be other mechanisms for ethylene formation, e.g., peroxidation of lipids during scenescence of leaves.[328] See also Chapter 31, Section G.




(24-37)

## 5. Metabolism of Threonine

Excess threonine is degraded in several ways, one of which is a β elimination reaction catalyzed by L-threonine dehydratase (Eq. 24-37, step *a*). This PLP-requiring enzyme is produced in high amounts in *E. coli* grown on a medium devoid of glucose and oxygen. Under these circumstances the reaction provides a source of propionyl-CoA, which can be converted to propionate with generation of ATP. This **biodegradative threonine dehydratase** (threonine deaminase)[329,330] is allosterically activated by AMP, an appropriate behavior for a key enzyme in energy metabolism. A second **biosynthetic threonine dehydratase** is also produced by *E. coli*[331,332] and is specifically required for production of 2-oxobutyrate needed in the biosynthesis of isoleucine by bacteria, plants,[333] and other autotrophic organisms. In 1956, Umbarger[334] showed that this enzyme is inhibited by isoleucine, the end product of the synthetic pathway. This discovery was instrumental in establishing the concepts of feedback inhibition in metabolic regulation (Chapter 11) and of allostery.

A second catabolic reaction of L-threonine (Eq. 24-37, step *b*) is cleavage to glycine and acetaldehyde. The reaction is catalyzed by serine hydroxymethyltransferase (Eq. 14-30). Some bacteria have a very active D-threonine aldolase.[335] A quantitatively more important route of catabolism in most organisms is dehydrogenation (Eq. 24-37, step *c*)[336] to form 2-amino-3-oxobutyrate. This intermediate can be cleaved by another PLP-dependent enzyme to acetyl-CoA plus glycine (Eq. 24-38, step *d*). It can also be decarboxylated (Eq. 24-38, step *e*) to aminoacetone, a urinary excretion product, or oxidized by amine oxidases to **methylglyoxal** (Eq. 24-37, step *f*).[337] The latter can be converted to D-lactate through the action of glyoxalase (Eq. 13-33). Aminoacetone is also the source of 1-amino-2-propanol for the biosynthesis of vitamin $B_{12}$ (Eq. 24-37, step *g*; Box 16-B).[338,338a]

## E. Alanine and the Branched-Chain Amino Acids

As indicated in Fig. 24-17, pyruvate is the starting material for the formation of both L- and D-alanine and also the branched chain amino acids **valine**, **leucine**, and **isoleucine**.[339,340] The chemistry of the reactions has been discussed in the sections indicated in the figure. The first step is catalyzed by the thiamin diphosphate-dependent **acetohydroxyacid synthase** (acetolactate synthase), which joins two molecules of pyruvate or one of pyruvate and one of 2-oxobutyrate (Fig. 24-17; Fig. 14-3).[340a,b] In *E. coli* there are two isoenzymes encoded by genes *ilv B* and *ilv HI*. Both are regulated by feedback inhibition by valine, probably

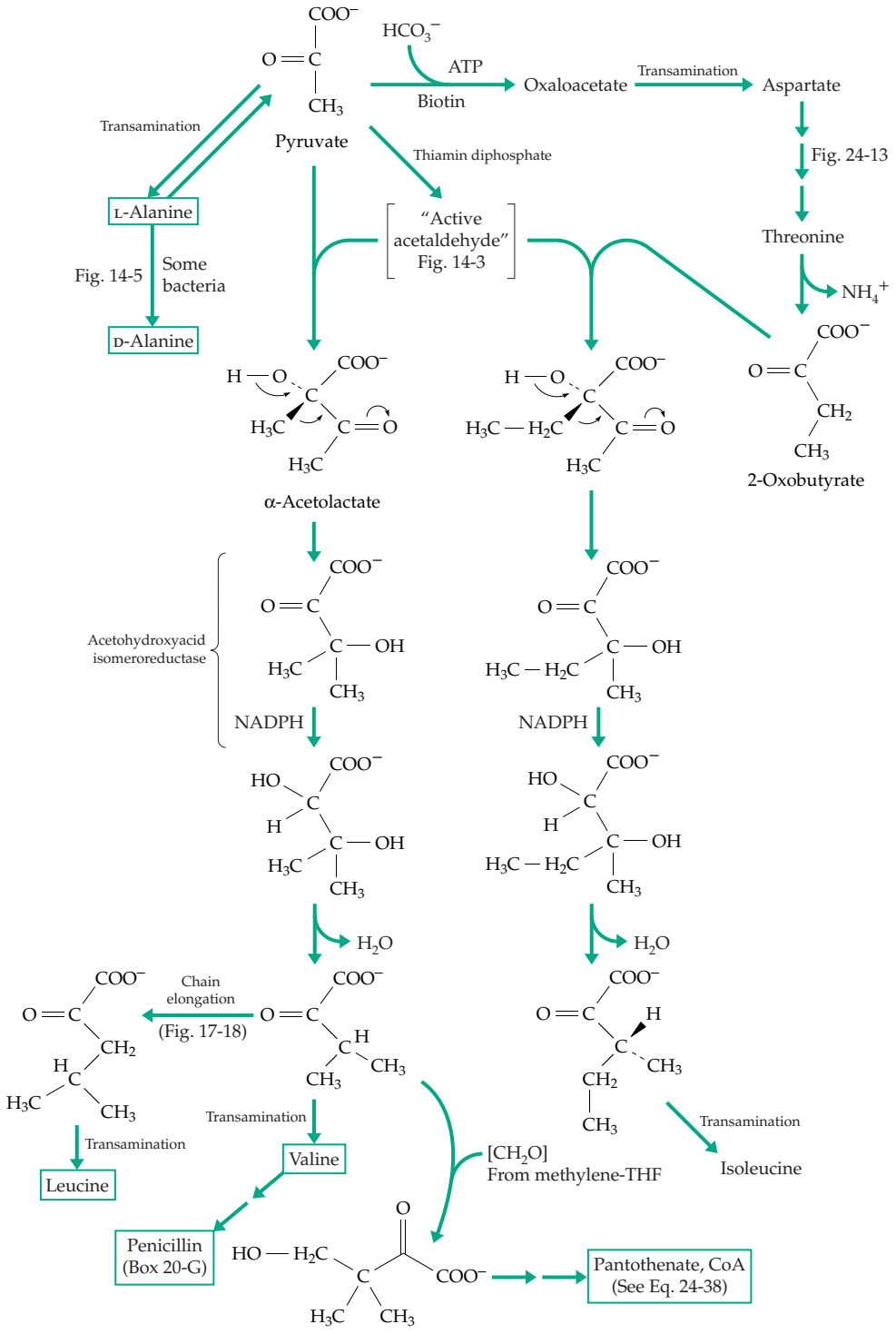
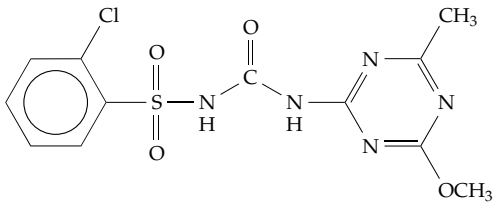
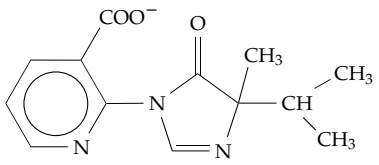



**Figure 24-17** Biosynthesis of leucine, isoleucine, valine, and coenzyme A.



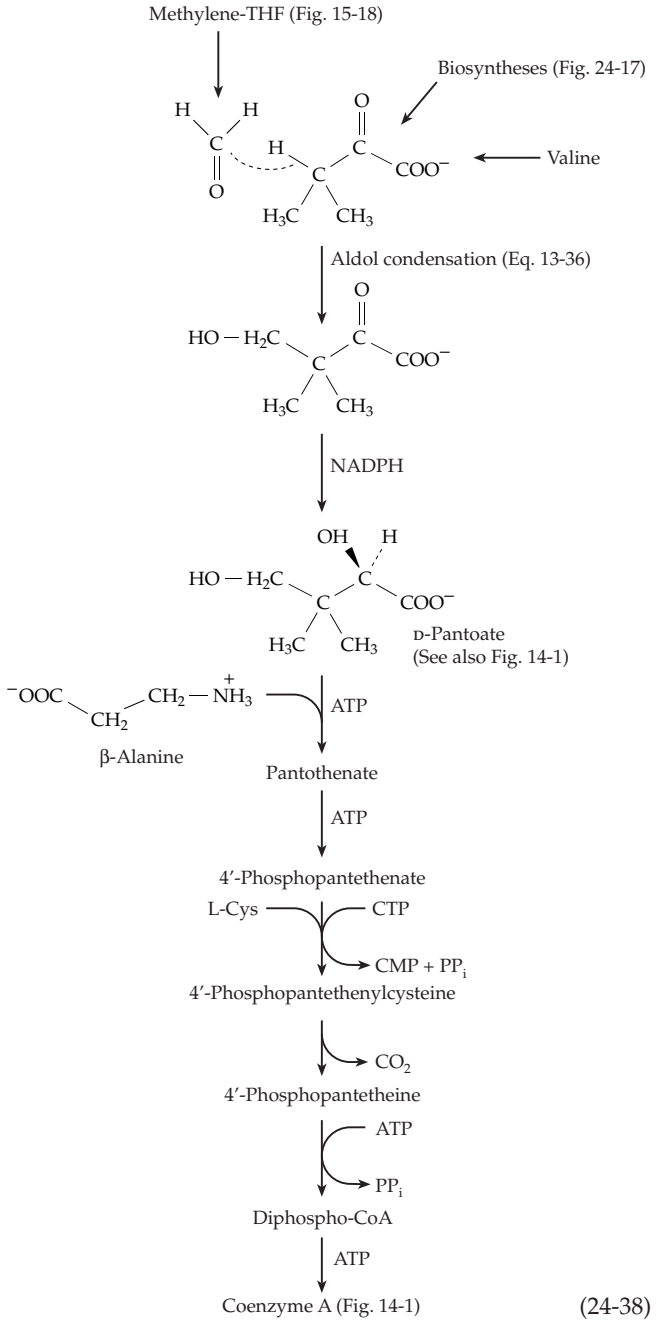
Chlorsulfuron



An imidazolinone herbicide

by an **attenuation** mechanism[341] (explained in Chapter 28). The enzymes are of some practical interest because they are specifically inhibited by two classes of herbicides, the **sulfonylureas**, of which chlorsulfuron is an example, and the **imidazolinones**.[342–345]

The second step in the synthesis, catalyzed by **acetohydroxyacid isomeroreductase**, involves shift of an alkyl group (Fig. 24-17). Neither this reaction nor the preceding one occurs in mammals. For this reason, the enzymes required are both attractive targets for herbicide design.[343,346] The third enzyme, **dihydroxy acid dehydratase**, catalyzes dehydration followed by tautomerization, resembling 6-phosphogluconate dehydratase (Eq. 13-32). The dihydroxyacid dehydratase from spinach contains an $Fe_2S_2$ cluster and may function by an aconitase type mechanism (Eq. 13-17).[347] In *Neurospora* isoleucine and valine are synthesized in the mitochondria.




(24-38)

While the 2-oxobutyrate needed for isoleucine formation is shown as originating from threonine in Fig. 24-17, bacteria can often make it in other ways,[348] e.g., from glutamate via β-methylaspartate (Fig. 24-8) and transamination to the corresponding 2-oxoacid. It can also be made from pyruvate by chain elongation using acetyl-CoA (Fig. 17-18); citramalate and mesaconate (Fig. 24-8) are intermediates. This latter pathway is used by some methanogens as are other alternative routes.[348] The first step unique to the biosynthetic pathway to leucine is the reaction of the 2-oxo analog of valine with acetyl-Co*A* to form **α-isopropylmalate**, the first step in a chain elongation sequence leading to the oxoacid precursor of leucine (Figs. 17-18; 24-17). The third enzyme required in the chain elongation is a decarboxylating dehydrogenase similar to isocitrate dehydrogenase.[349]

An additional series of reactions,[350] which are shown in Eq. 24-38, leads to **pantoic acid**, **pantetheine**, **coenzyme A**, and related cofactors.[350a–j] The initial reactions of the sequence do not occur in the animal body, explaining our need for pantothenic acid as a vitamin.

Alanine also gives rise to a precursor of the vitamin **biotin** (Eq. 24-39) after a PLP-dependent decarboxylative condensation with the 7-carbon dicarboxylic acid unit of pimeloyl-CoA in a reaction analogous to that of Eq. 14-32.[351] The resulting alcohol is reduced to 7-oxo-8-aminopelargonic acid which is converted by transamination, with *S*-adenosylmethionine as the nitrogen donor,[351a] to 7,8-diaminopelargonic acid. This compound undergoes a two-step ATP-dependent cyclization[352–355] to form **dethiobiotin**. The final step, insertion of sulfur into dethiobiotin, is catalyzed by **biotin synthase**, a free-radical-dependent enzyme related to pyruvate formate lyase (Fig. 15-16). It transfers the sulfur from cysteine via an Fe–S cluster.[355a–c] Biosynthesis of **lipoic acid** involves a similar insertion of two sulfur atoms into octanoic acid.[356] See also p. 1410.

## 1. Catabolism

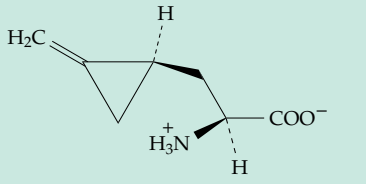
Degradation of amino acids most often begins with conversion, either by transamination[356a] or by $NAD^+$-dependent dehydrogenation,[357] to the corresponding 2-oxoacid and oxidative decarboxylation of the latter (Fig. 15-16). Alanine, valine, leucine, and isoleucine are all treated this way in the animal body. Alanine gives pyruvate and acetyl-CoA directly, but the others yield CoA derivatives that undergo

## BOX 24-A MAPLE SYRUP URINE DISEASE AND JAMAICAN VOMITING SICKNESS

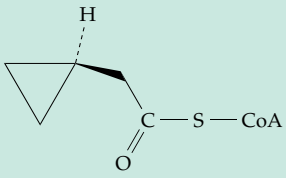
In a rare autosomal recessive condition (discovered in 1954) the urine and perspiration has a maple syrup odor.[a–c] High concentrations of the branched-chain 2-oxoacids formed by transamination of valine, leucine, and isoleucine are present, and the odor arises from decomposition products of these acids. The branched-chain amino acids as well as the related alcohols also accumulate in the blood and are found in the urine. The biochemical defect lies in the enzyme catalyzing oxidative decarboxylation of the oxoacids, as is indicated in Fig. 24-18. Insertions, deletions, and substitutions may be present in any of the subunits (Figs. 15-14, 15-15). The disease which may affect one person in ~200,000, is usually fatal in early childhood if untreated. Children suffer seizures, mental retardation, and coma. They may survive on a low-protein (gelatin) diet supplemented with essential amino acids, but treatment is difficult and a sudden relapse is apt to prove fatal. Some patients respond to administration of thiamin at 20 times the normal daily requirement. The branched-chain oxoacid dehydrogenase from some of these children shows a reduced affinity for the essential coenzyme thiamin diphosphate.[d]

Polled hereford calves in Australia develop maple syrup urine disease relatively often.[a,e] One cause was established as a mutation that introduces a stop codon that causes premature termination within the leader peptide during synthesis of the thiamin diphosphate-dependent E1 subunit. A similar biochemical defect in a mutant of *Bacillus subtilis*[f] causes difficulties for this bacterium, which requires branched-chain fatty acids in its membranes. Branched acyl-CoA derivatives are needed as starter pieces for their synthesis (Chapter 29). With the oxidative decarboxylation of the necessary oxoacids blocked, the mutant is unable to grow unless supplemented with branched-chain fatty acids.

Because persons may be born with defects in almost any gene, a variety of other problems leading to accumulation of organic acids are also known. **Methylmalonic aciduria** and propionic acidemia are discussed in Box 17-B. **Lactic acidemia** (Box 17-F) often results from a defect in pyruvate dehydrogenase. A rare defect of catabolism of leucine is **isovaleric acidemia**, a failure in oxidation of isovaleryl-CoA.[g] The symptoms of this disease are also present in the Jamaican vomiting sickness, caused by eating unripe ackee fruit. Although the ripe fruit is safe to eat, unripe fruit contains a toxin **hypoglycin A** with the following structure.[h–j] It is metabolized to an acyl-CoA derivative as shown.




Hypoglycin A




Toxic metabolite

This is an enzyme-activated inhibitor of the medium-chain fatty acyl-CoA dehydrogenase required for β oxidation of fatty acids.[j,k] The compound also inhibits isovaleryl-CoA dehydrogenase, causing an accumulation of isovaleric acid in the blood. Depression of the central nervous system by isovaleric acid in the blood could be responsible for some symptoms.[h,i] However, death from the highly fatal Jamaican vomiting sickness comes from the hypoglycemic effect. Blood glucose levels may fall as low as 0.5 mM, one-tenth the normal concentration and patients must be treated by infusion of glucose.

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[a] Patel, M. S., and Harris, R. A. (1995) *FASEB J.* **9**, 1164–1172

[b] Chuang, D. T., and Shih, V. E. (1995) in *The Metabolic and Molecular Bases of Inherited Disease*, 7th ed., Vol. 1 (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., eds), pp. 1239–1278, McGraw-Hill, New York

[c] Mamer, O. A., and Reimer, M. L. J. (1992) *J. Biol. Chem.* **267**, 22141–22147

[d] Chuang, D. T., Ku, L. S., and Cox, R. P. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 3300–3304

[e] Zhang, B., Healy, P. J., Zhao, Y., Crabb, D. W., and Harris, R. A. (1990) *J. Biol. Chem.* **265**, 2425–2427

[f] Willecke, K., and Pardee, A. B. (1971) *J. Biol. Chem.* **246**, 5264–5272

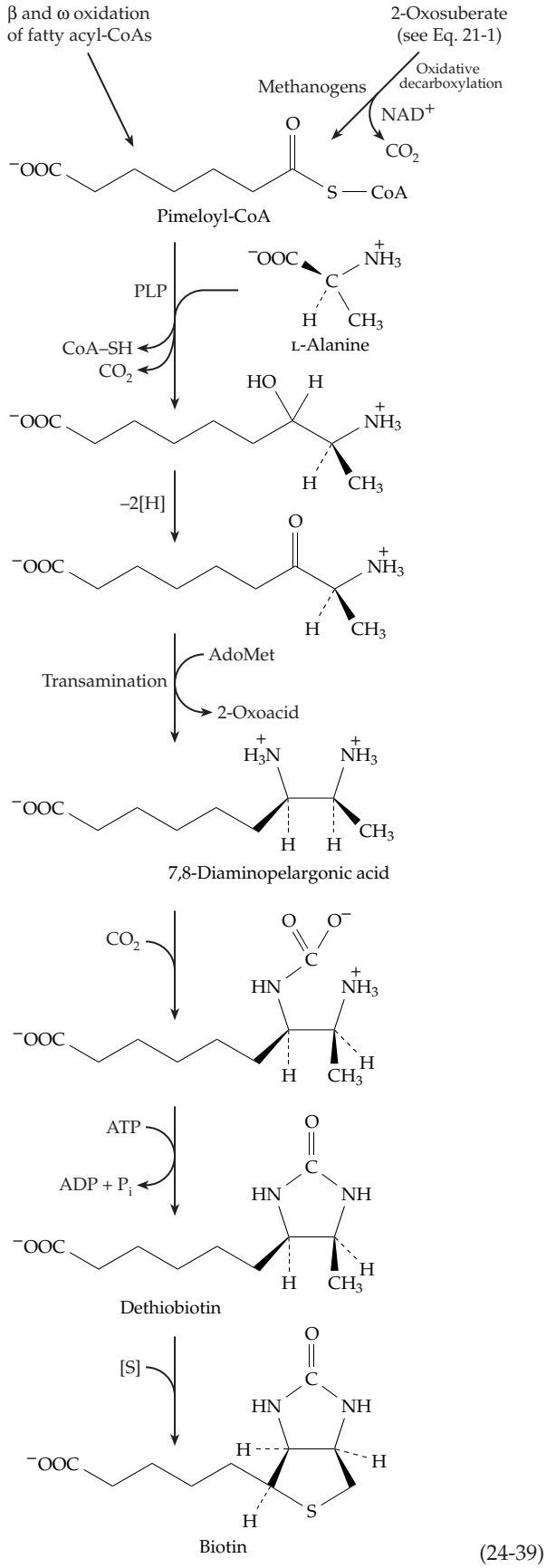
[g] Mohsen, A.-W. A., and Vockley, J. (1995) *Biochemistry* **34**, 10146–10152

[h] Tanaka, K., Isselbacher, K. J., and Shih, V. (1972) *Science* **175**, 69–71

[i] Tanaka, K. (1972) *J. Biol. Chem.* **247**, 7465–7478

[j] Lai, M.-t, Liu, L.-d, and Liu, H.-w. (1991) *J. Am. Chem. Soc.* **113**, 7388–7397

[k] Lai, M.-t, Li, D., Oh, E., and Liu, H.-w. (1993) *J. Am. Chem. Soc.* **115**, 1619–1628

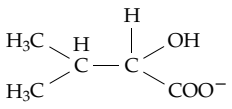



(24-39)

β-oxidation within the mitochondria[358] via the schemes shown in Fig. 24-18. There are some variations from the standard β oxidation sequence for fatty acids shown in Fig. 17-1. In the case of valine the sequence proceeds only to the stage of addition of water to form the β-hydroxy derivative. The latter is converted to free 3-hydroxyisobutyrate, and β oxidation is then completed by oxidation to methylmalonate semialdehyde.[359] The latter is oxidatively decarboxylated to form *S*-methylmalonyl-CoA.[360] Further metabolism of the latter is indicated in Fig. 17-3. However, some methylmalonate semialdehyde may be decarboxylated to propionaldehyde, which could be oxidized to propionate. Either of these compounds could then be metabolized to propionyl-CoA.[361]

In the degradation of isoleucine, β oxidation proceeds to completion in the normal way with generation of acetyl-CoA and propionyl-CoA. However, in the catabolism of leucine after the initial dehydrogenation in the β-oxidation sequence, carbon dioxide is added using a biotin enzyme (Chapter 14). The double bond conjugated with the carbonyl of the thioester makes this carboxylation analogous to a standard β-carboxylation reaction. Why add the extra $CO_2$? The methyl group in the β position blocks complete β oxidation, but an aldol cleavage would be possible to give acetyl-CoA and acetone. However, acetone is not readily metabolized further. By addition of $CO_2$ the product becomes acetoacetate, which can readily be completely metabolized through conversion to acetyl-CoA.

An alternative pathway of leucine degradation in the liver is oxidative decarboxylation by a cytosolic oxygenase to form α-hydroxyisovalerate.[362]



α-Hydroxyisovalerate

This compound may be metabolized via the valine catabolic pathway of Fig. 24-18. A third pathway, present in some bacteria, begins with the vitamin $B_{12}$-dependent isomerization of leucine to β-leucine (Chapter 16), which can undergo transamination to 3-oxoisocaproate. This can be converted to its CoA ester by a CoA transferase and can undergo β cleavage by free CoA-SH to form acetyl-CoA and isobutyryl-CoA. The latter may enter the valine catabolic pathway (Fig. 24-18). Leucine has long been known as a regulator of protein degradation in muscle.[362a–e] Dietary protein deficiency leads to especially rapid degradation of the branched-chain amino acids. The daily turnover of proteins for a 70-kg adult ingesting 70 g of protein per day has been estimated as 280 g, most of which must be reused.[362d] This large turnover can lead to

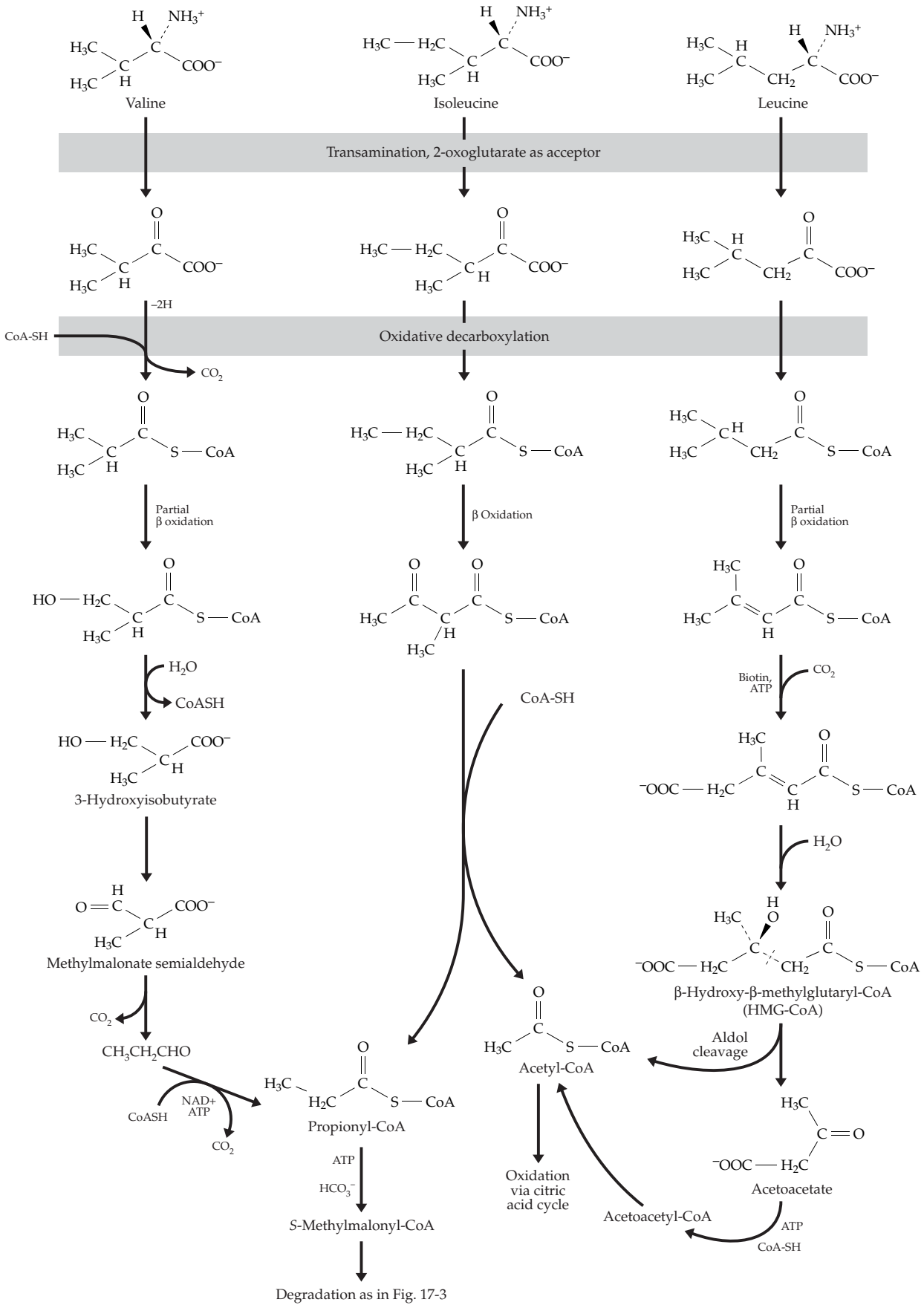



**Figure 24-18** Catabolism of valine, leucine, and isoleucine.

excessive muscle wasting in disease states. A minor leucine metabolite found in muscle, β-hydroxy β-methylbutyrate has been proposed as a possible endogenous inhibitor of muscle breakdown.[362e,f] (See study question 17.)

*Clostridium propionicum* can use alanine as substrate for a balanced fermentation to form ammonium propionate, acetate, and $CO_2$ (Fig. 24-19).

## 2. Ketogenic and Glucogenic Amino Acids

According to a long-used classification amino acids are **ketogenic** if (like leucine) they are converted to acetyl-CoA (or acetyl-CoA and acetoacetate). When fed to a starved animal, ketogenic amino acids cause an increased concentration of acetoacetate and other ketone bodies in the blood and urine. On the other hand, **glucogenic** amino acids such as valine, when fed to a starved animal, promote the synthesis of glycogen (in the case of valine via methylmalonyl-CoA, succinate, and oxaloacetate). Examination of Fig. 24-18 shows that isoleucine is both ketogenic and glucogenic, a fact that was known long before the pathway of catabolism was worked out.

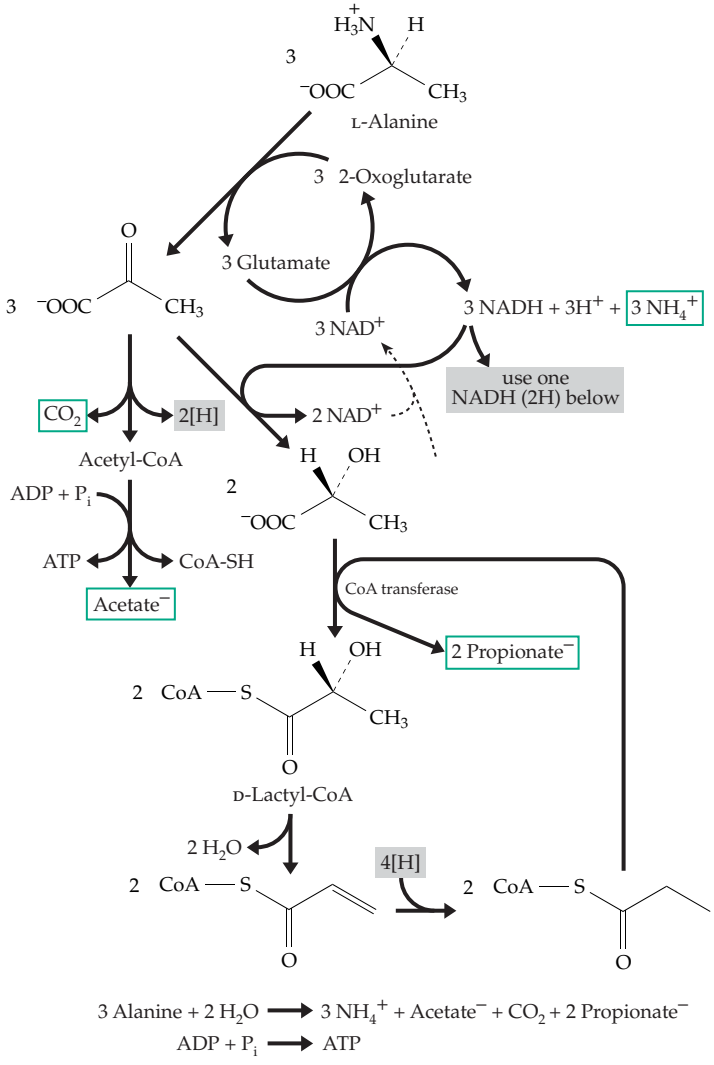



**Figure 24-19** Fermentation of L-alanine by *Clostridium propionicum*. After Buckel.[363]

## F. Serine and Glycine

Serine originates in a direct pathway from 3-phosphoglycerate (pathway *a*, Fig. 24-20) that involves dehydrogenation, transamination, and hydrolysis by a phosphatase. It can also be formed from glycine by the action of serine hydroxymethyltransferase (Eq. 14-30). This occurs in chloroplasts during photorespiration (Fig. 23-37)[364] and also with some methanogens and other autotropic bacteria and methylotrophs (Fig. 17-15). The glycine decarboxylase cycle shown in Fig. 15-20 provides another mechanism available in bacteria, plants, and animal mitochondria for reversible interconversion of glycine and serine. The principal route of catabolism of serine in many microorganisms is deamination to pyruvate (Fig. 24-20, step *b*),[364a] a reaction also discussed in Chapter 14 (Eq. 14-29). An alternative catabolic pathway is transamination to **hydroxypyruvate,** which as in plants (Fig. 23-37) can be reduced to D-glycerate and back to 3-phosphoglycerate.[365] That this pathway is important in human beings is suggested by the occurrence of a rare metabolic defect **L-glyceric aciduria** (or primary hyperoxaluria type II).[365–367] The biochemical defect may lie in the lack of reduction of hydroxypyruvate to D-glycerate. When hydroxypyruvate accumulates, lactate dehydrogenase effects its reduction to L-glycerate, which is excreted in large amounts (0.3–0.6 g / 24 h) in the urine. Surprisingly, the defect is accompanied by excessive production of oxalate from glyoxylate. This is apparently an indirect result of the primary defect in utilization of hydroxypyruvate. It has been suggested that oxidation of glyoxylate by $NAD^+$ is coupled to the reduction of hydroxypyruvate by NADH.[366] This and other hyperoxalurias are very serious diseases characterized by the formation of calcium oxalate crystals in tissues and often death from kidney failure before the age of 20.

### 1. Biosynthetic Pathways from Serine

L-Serine gives rise to many other substances (Fig. 24-20) including **sphingosine** and the **phosphatides**. In many bacteria conversion to

*O*-acetyl-L-serine (step *c*, Fig. 24-20) provides for the formation of **cysteine** by a β-replacement reaction.[368–369a] Serine is also the major source of glycine (step *d*) and of the single-carbon units needed for the synthesis of methyl and formyl groups. The enzyme **serine hydroxymethyltranferase** (step *d*) also provides the principal route of formation of glycine from serine,[370,371] but a lesser portion comes via phosphatidylserine, phosphatidylethanolamine, phosphatidylcholine, and free choline (step *e*). This pathway includes decarboxylation of phosphatidylserine by a pyruvoyl group-dependent enzyme (pp. 753–755). In contrast, in green plants the major source of ethanolamine is a direct PLP-dependent decarboxylation of serine (Fig. 24-20).[371a] Because the body's capacity to generate methyl groups is limited, **choline** under many circumstances is a dietary essential and has been classified as a vitamin. However, in the presence of adequate amounts of folic acid and vitamin $B_{12}$, it is not absolutely required. Choline can be used to reform phosphatidylcholine (Fig. 21-5), while an excess can be dehydrogenated to **glycine betaine,** which is one of the osmoprotectant substances in plants (Eq. 24-40).[372,373] This quaternary nitrogen compound is one of a small number of substances that, like methionine, are able to donate methyl groups to other compounds and which are also capable of methylating homocysteine to form methionine. However, the product of transmethylation from betaine, dimethylglycine, is no longer a methylating agent. The two methyl groups are removed oxidatively as formic acid to produce glycine (Eq. 24-40). A third source of glycine is transamination of glyoxylate (step *f*, Fig. 24-20). The equilibrium constant for the reaction favors glycine strongly for almost any amino donor.

## 2. Metabolism of Glycine

While glycine may be formed from glyoxylate by transamination, the oxidation of glycine by an amino acid oxidase (Table 15-2) permits excess glycine to be converted to glyoxylate. That this pathway, too, is quantitatively important in humans is suggested by the existence of **type 1 hyperoxaluria**.[366] It is thought that a normal pathway for utilization of glyoxylate is blocked in this condition leading to its oxidation to oxalate. The biochemical defect is frequently the absence of a liver-specific alanine:glyoxylate aminotransferase that efficiently converts accumulating glyoxylate back to glycine. In some cases the disease arises because, as a result of

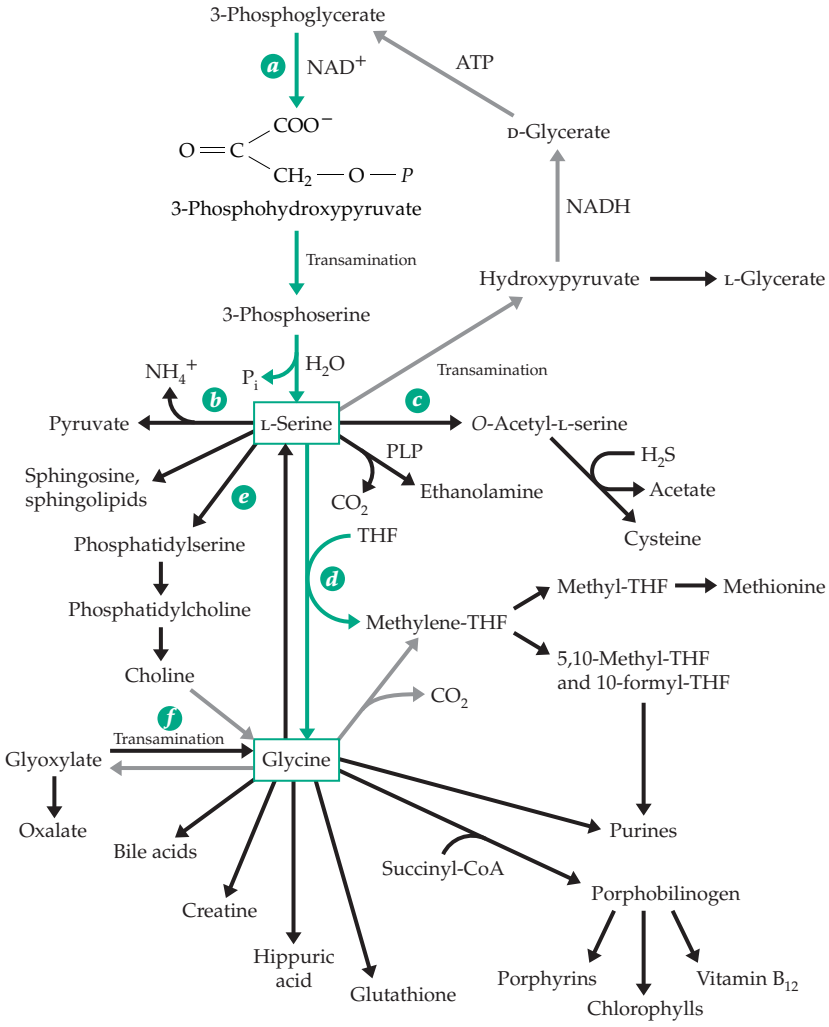
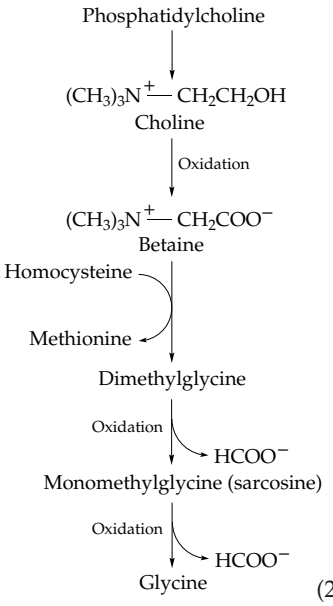


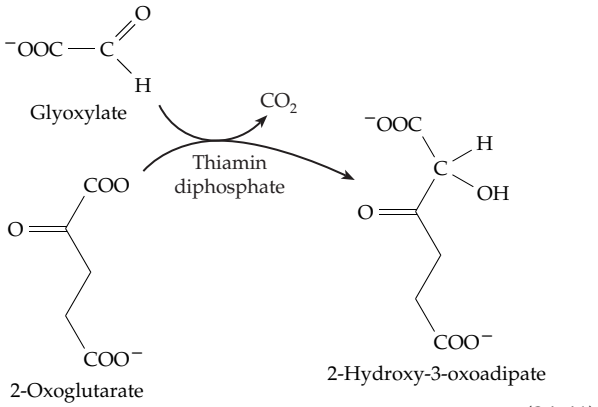

**Figure 24-20** Metabolism of serine and glycine.



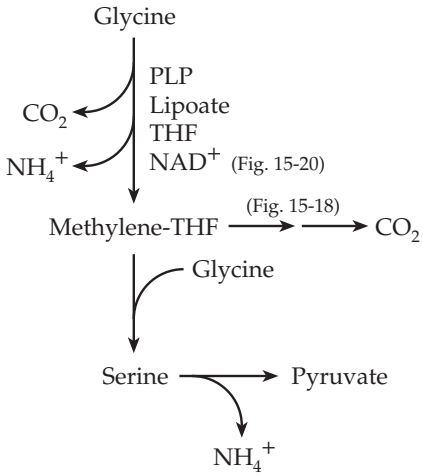

(24-40)

a mutation in its N-terminal targeting sequence the aminotransferase is targeted to mitochondria, where it functions less efficiently.[366a] Another possible defect lies in a thiamin-dependent enzyme that condenses glyoxylate with 2-oxoglutarate to form 2-hydroxy-3-oxoadipate (Eq. 24-41). The function of this reaction is uncertain, but the product could undergo decarboxylation and oxidation to regenerate 2-oxoglutarate. This would provide a cyclic pathway (closely paralleling the dicarboxylic acid cycle, Fig. 17-6) for oxidation of glyoxylate without formation of oxalate. Bear in mind that the demonstrated enzymatic condensation reactions of the rather toxic glyoxylate are numerous, and that its metabolism in most organisms is still not well understood.

An alternative route of catabolism is used by organisms such as *Diplococcus glycinophilus*, which are able to grow on glycine as a sole source of energy, of carbon, and of nitrogen,[374] and is also used in mitochondria of plants and animals.[374a] This glycine cleavage system, depicted in Fig. 15-20, involves decarboxylation, oxidation by $NAD^+$, release of ammonia, and transfer of the decarboxylated α-carbon of glycine to tetrahydrofolic acid (THF) to form methylene-THF. The C-1 methylene unit of the latter is used primarily for purine biosynthesis but can also



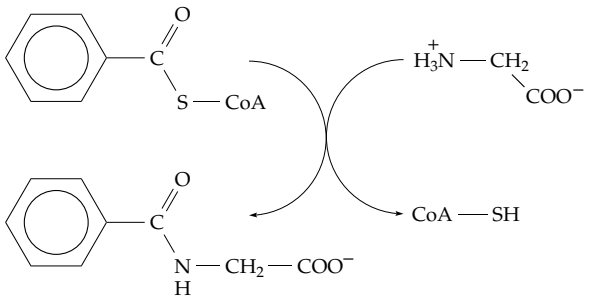

(24-41)




(24-42)

be oxidized to $CO_2$ or can condense with another molecule of glycine (Fig. 15-18, step *c*, reverse) to form serine. This can in turn be converted to pyruvate and utilized for biosynthetic processes (Eq. 24-42).

Glycine can be reduced to acetate and ammonia by the selenium-dependent clostridial glycine reductase system (Eq. 15-61). A variety of additional products can be formed from glycine as is indicated in Fig. 24-20. **Hippuric acid** (Box 10-A), the usual urinary excretion product in the "detoxication" of benzoic acid, is formed via benzoyl-CoA (Eq. 24-43):




(24-43)

*N*-Methylation yields the monomethyl derivative **sarcosine**[375] and also dimethylglycine, compounds that may function as osmoprotectants (Box 20-C). Many bacteria produce **sarcosine oxidase**, a flavoprotein that oxidizes its substrate back to glycine and formaldehyde, which can react with tetrahydrofolate.[376–377a] The formation of porphobilinogen and the various pyrrole pigments derived from it and the synthesis of the purine ring (Chapter 25) represent two other major routes for glycine metabolism.

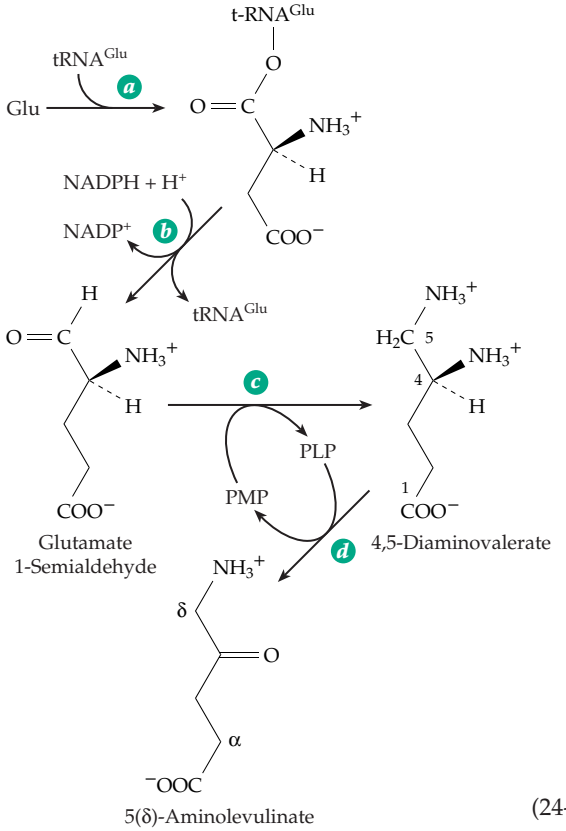
## 3. Porphobilinogen, Porphyrins, and Related Substances

In 1946, Shemin and Rittenberg[378] described one of the first successful uses of radiotracers in the study of metabolism. They reported that the atoms of the porphyrin ring in heme have their origins in the simple compounds acetate and glycine. As we now know, acetate is converted to succinyl-CoA in the citric acid cycle. Within the mitochondrial matrix of animal cells succinyl-CoA condenses with glycine to form **5(δ)-aminolevulinic acid** (Eq. 14-32),[379–381a] which is converted to **porphobilinogen** (Fig. 24-21), the immediate precursor to the porphyrins. The same pathways lead also to other tetrapyrroles including chlorophyll, the nickel-containing $F_{430}$, vitamin $B_{12}$, and other corrins.[382,383]

By degradation of $^{14}C$-labeled porphyrins formed from labeled acetate and glycine molecules, Shemin and Rittenberg established the labeling pattern for the

pyrrole ring that is indicated for porphobilinogen in Fig. 24-21. The solid circles mark those atoms that were found to be derived from methyl carbon atoms of acetate (bear in mind that acetyl groups of acetyl-CoA pass around the citric acid cycle more than once to introduce label from the methyl group of acetate into both the 2 and 3 positions of succinyl-CoA). Those atoms marked with open circles in Fig. 24-21 were found to be derived mainly from the methyl carbon of acetate and in small part from the carboxyl carbon. Atoms marked with asterisks came from glycine, while unmarked carbon atoms came from the carboxyl carbon of acetate.[384]

In cyanobacteria and in chloroplasts the intact 5-carbon skeleton of glutamate enters δ-aminolevulinate.[139,385,385a] A surprising finding was that the glutamate becomes coupled to one of the three known glutamate isoacceptor tRNAs that are utilized for protein synthesis. The aminoacyl-tRNA is formed in the usual manner with an ester linkage to the CCA end of the tRNA (Eq. 24-44; see also Eq. 17-36). This ester linkage can be reductively cleaved by NADPH to form glutamate 1-semialdehyde.[386] Isomerization of the glutamate semialdehyde to δ-aminolevulinate is accomplished by an aminomutase that is structurally and functionally related to aminotransferases.[139,387] The enzyme utilizes pyridoxamine phosphate (PMP) to transaminate the substrate carbonyl group to form 4,5-diaminovalerate plus bound PLP. A second transamination step yields the product and regenerates the PMP (Eq. 24-44, steps *c* and *d*).[387]




(24-44)

***Porphyrins.*** As indicated in Fig. 24-21, the conversion of two molecules of 5-aminolevulinate into porphobilinogen is a multistep reaction initiated by **5-aminolevulinate dehydratase** (porphobilinogen synthase).[381,388–390b] The enzyme binds two molecules of substrate in distinct sites known as the A site and the P site (Fig. 24-21). The substrate in the P site forms a Schiff base with a lysine side chain (K247 in the *E. coli* enzyme), while a bound $Zn^{2+}$ is thought to polarize the carbonyl of the substrate in the A site. An aldol condensation (Fig. 24-21, step *a*) ensues and is followed by dehydration to form a carbon–carbon double bond and ring closure (step *b*). Tautomerization step (*c*) leads to porphobilinogen. The enzyme is a sensitive target for poisoning by lead ions.[381,390c] Condensation to form porphyrins requires two enzymes, **porphobilinogen deaminase** (hydroxymethylbilane synthase) and **uroporphyrinogen III cosynthase**. Porphobilinogen deaminase has a bound coenzyme (prosthetic group) consisting of two linked pyrromethane groups, also derived from porphobilinogen.[391] The first step in assembling the porphyrin ring is condensation of porphobilinogen with this coenzyme (Fig. 24-21, step *d*). To initiate this step ammonia is eliminated, probably not by the direct displacements, but by electron flow from the adjacent nitrogen in the same pyrrole ring as indicated in the figure to give an exocyclic double bond. The terminal ring of the coenzyme then adds to the double bond. The condensation process is repeated four times to produce **preuroporphyrinogen** (hydroxymethylbilane).[392–394a] This intermediate is a precursor of the symmetric uroporphyrin I (Fig. 16-5). In the presence of the cosynthase a different ring-closure reaction takes place. The five-membered ring in porphobilinogen has a symmetric arrangement of double bonds. Thus, a condensation reaction can occur at either of the positions α to the ring nitrogen. A sequence of condensation, tautomerization, cleavage, and reformation of the ring as shown in steps *e* to *h* of Fig. 24-21 leads to the unsymmetric uroporphyrinogen III with its characteristic pattern of the carboxymethyl and carboxyethyl side chains. A series of decarboxylation and oxidation reactions then leads directly to protoporphyrin IX.

The first of these decarboxylations is catalyzed by the cytoplasmic **uroporphyrinogen decarboxylase,** which removes the carboxylate groups of the four acetate side chains sequentially from the D, A, B, and C rings.[395–396a] A possible mechanism, utilizing a tautomerized ring, is illustrated in the accompanying structural formula.

The decarboxylated product, coproporphyrinogen (Fig. 16-5), enters the mitochondria and is acted upon by **coproporphyrinogen oxidase,** which oxidatively

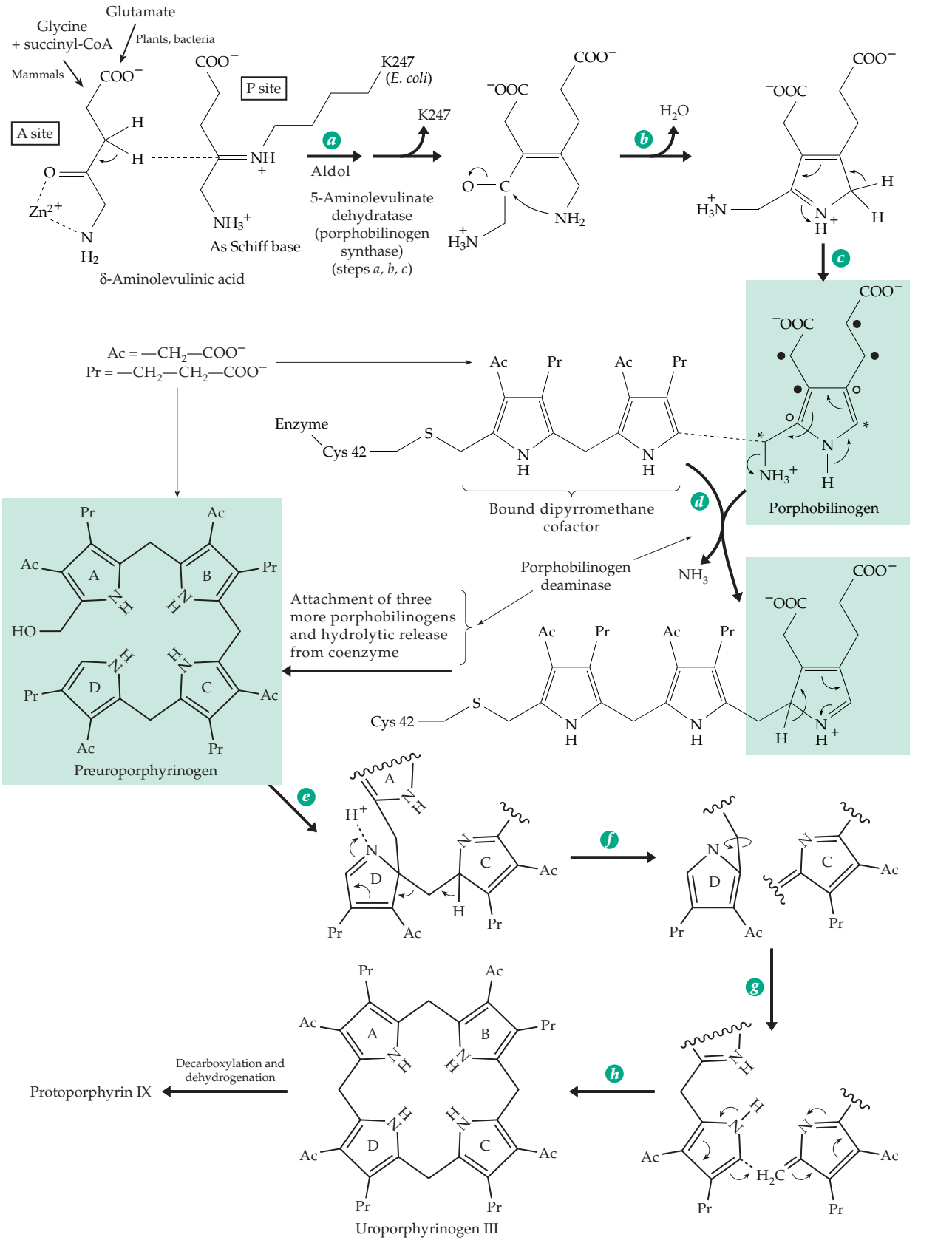
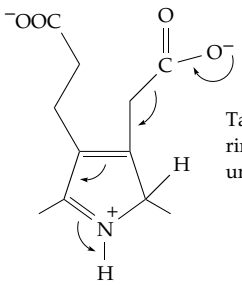



**Figure 24-21** Biosynthesis of porphyrins, chlorins, and related compounds.




Tautomerized pyrrole ring of uroporphyrinogen undergoing decarboxylation

decarboxylates and oxidizes two of the propyl side chains to vinyl groups.[397] A flavoprotein, **protoporphyrinogen oxidase**, oxidizes the methylene bridges between the pyrrole rings[398,399] to form protoporphyrin IX. A somewhat different pathway from uroporphyrinogen is followed by sulfate-reducing bacteria.[400]

**Ferrochelatase** (protoheme ferro-lyase)[401–403] inserts $Fe^{2+}$ into protoporphyrin IX to form heme. The enzyme is found firmly bound to the inner membrane of mitochondria of animal cells, chloroplasts of plants, and chromatophores of bacteria. While $Fe^{2+}$ is apparently the only metallic ion ordinarily inserted into a porphyrin, the $Zn^{2+}$ protoporphyrin chelate accumulates in substantial amounts in yeast, and $Cu^{2+}$–heme complexes are known (p. 843). Ferrochelatase, whose activity is stimulated by $Ca^{2+}$, appears to be inhibited by lead ions, a fact that may account for some of the acute toxicity of lead.[404]

Heme *b* is utilized for formation of hemoglobin, myoglobin, and many enzymes. It reacts with appropriate protein precursors to form the cytochromes *c*. Heme *b* is converted by prenylation to heme *o*[405] and by prenylation and oxidation to heme *a*.[405a] The porphyrin biosynthetic pathway also has a number of branches that lead to formation of corrins, chlorins, and chlorophylls as shown schematically in Fig. 24-22.

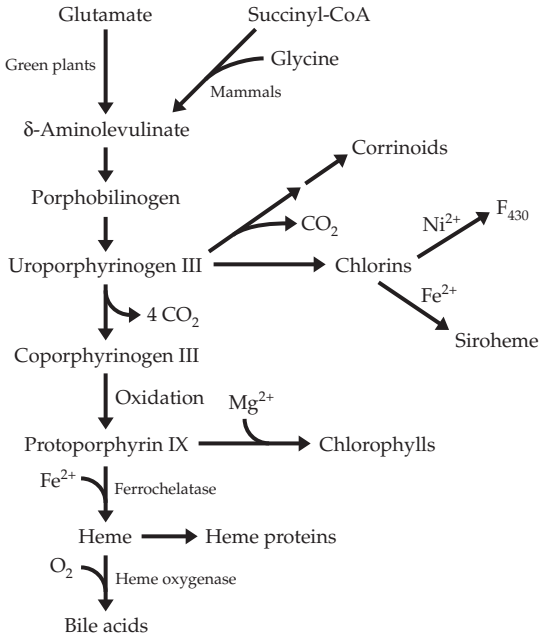



**Figure 24-22** Abbreviated biosynthetic pathways from δ-aminolevulinate to heme proteins, corrins, chlorophylls, and related substances.

***Corrins.*** The formation of vitamin $B_{12}$, other corrins, siroheme, and related chlorin chelates[406,407] requires a ring contraction with elimination of the methine bridge between rings A and D of the porphyrins (see Box 16-B). It is natural to assume that the methyl group at C-1 of the corrin ring might arise from the same precursor carbon atom as does the methine bridge in porphyrins, and it is easy to visualize a modified condensation reaction by which ring closure at step *e* in Fig. 24-21 occurs by nucleophilic addition to a C=N bond of ring A. However, $^{13}C$-NMR data ruled out this possibility. When vitamin $B_{12}$ was synthesized in the presence of $^{13}C$-methyl-containing methionine, it was found that seven methyl groups contained $^{13}C$. All of the "extra" methyl groups around the periphery of the molecule as well as the one at C-1 were labeled.[408] Other experiments established uroporphyrinogen III as a precursor of vitamin $B_{12}$. Therefore, it appeared that the ring first closed in a normal way and then reopened between rings A and D with removal of the carbon that forms the methylene bridge.[409] This turned out to be true, but with some surprises.

The complex pathways of corrin synthesis have been worked out in detail.[410–415a] This has been possible because of extensive use of $^{13}C$ and $^{1}H$ NMR and because the group of ~20 enzymes required has been produced in the laboratory from genes cloned from *Pseudomonas denitrificans*.[410] The first alterations of uroporphyrinogen are AdoMet-dependent methylations on carbon atoms. Surprisingly, one of these is on the bridge atom that is later removed. The details, including the insertion of $Co^{2+}$ by a **cobaltochelatase**, are described by Battersby[410] and portrayed in Michal's *Biochemical Pathways*.[416]

***Chlorophyll.*** The pathway of chlorophyll synthesis has been elucidated through biochemical genetic studies of *Rhodobacter spheroides*[417–418a] which produces bacteriochlorophyll, from studies of cyanobacteria,[419,420] and from investigations of green algae and higher plants,[421] which make chlorophyll *a*. The first step in the conversion of protoporphyrin IX into chlorophyll is the insertion of $Mg^{2+}$ (Fig. 24-23, step *a*). This reaction does not occur readily spontaneously but is catalyzed by an ATP-dependent **magnesium protoporphyrin chelatase**.[419,422] Subsequently, the carboxyethyl side chain on ring C undergoes methylation (Fig. 24-23, step *b*) and β oxidation (step *c*).

Oxidative closure of ring E (step *d*) is followed by reduction of the vinyl group of ring B and of the double bond in ring D to form **chlorophyllide *a***. The latter is coupled with phytol, via phytyl diphosphate, to form chlorophyll *a*.[420] Chlorophyll *b* is derived from chlorophyll *a*, evidently by action of an as yet uncharacterized oxygenase, which converts the methyl group on ring B into a formyl group.[423,424] Bacteriochlorophylls also arise from chlorophyllide *a* and involve reduction of the double bond in ring B.[416,418,420] Most photosynthetic bacteria make bacteriochlorophylls esterified with the $C_{20}$ phytol, but some substitute the unsaturated $C_{20}$ geranylgeranyl group and a variety of other isoprenoid alcohols.

***The porphyrias.*** The human body does not use all of the porphobilinogen produced, and a small amount is normally excreted in the urine, principally as coproporpyrins (Fig. 16-5). In a number of hereditary and acquired conditions blood porphyrin levels are elevated and enhanced urinary excretion (porphyria) is observed.[425–427] Porphyrias may be mild and almost without symptoms, but the intensely fluorescent free porphyrins are sometimes deposited under the skin and cause photosensitivity and ulceration. In extreme cases, in which the excreted porphyrins may color the urine a wine red, patients may have acute neurological attacks and a variety of other symptoms. Lucid accounts of such symptoms, experienced by King George III of England, have been written.[426,428] However, there are doubts about the conclusion that the king suffered from porphyria.[425]

Porphyria may result from several different enzyme deficiences in the porphyrin biosynthetic pathway. The condition is often hereditary but may be induced by drugs or other xenobiotic substances and may be continuous or intermittent.[425,426,429] In one type of congenital porphyria uroporphyrin I is excreted in large quantities. The biochemical defect appears to be a deficiency of the cosynthase that is required for formation of protoporphyrin IX. Another type of porphyria results from overproduction in the liver of

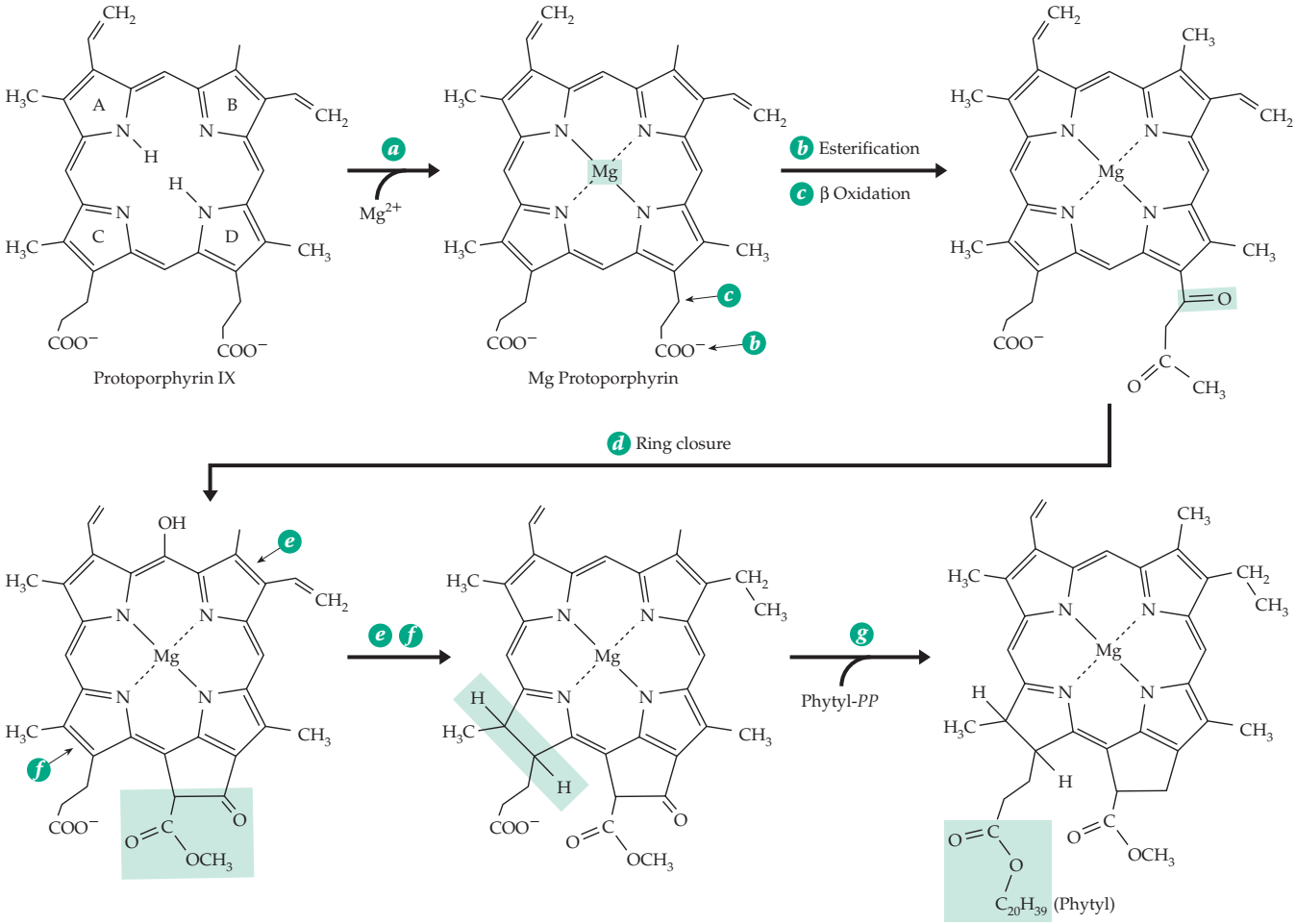
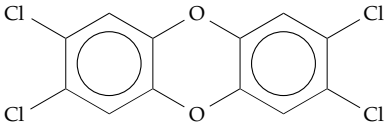



**Figure 24-23** Outline of the biosynthetic pathways for conversion of protoporphyrin IX into the chlorophylls and bacteriochlorophylls. After Bollivar *et al*.[417]

δ-aminolevulinic acid, a compound with neurotoxic properties, possibly as a result of its similarity to the neurotransmitter γ-aminobutyrate.[426,427] This may account for some of the neurological symptoms of porphyria.

Some mild forms of intermittent porphyria may go unrecognized. However, ingestion of drugs can precipitate an acute attack, probably by inducing excessive synthesis of δ-aminolevulinate synthase. Among compounds having this effect are hexachlorobenzene and tetrachlorodibenzodioxin.



2,3,7,8-Tetrachlorodibenzo-*p*-dioxin

The latter is one of the most potent inducers of the synthase known.[430] The tendency for this dioxin to be present as an impurity in the herbicide 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) has caused concern. For rodents this dioxin may be the most toxic small molecule known, the oral $LD_{50}$ for guinea pigs being only 1 μg / kg body weight.[430] However, it is over 1000 times less toxic to humans.[430a] It is also a potent teratogenic agent. Synthesis of porphyrins in the liver is controlled by δ-aminolevulinate synthase. This key enzyme is sensitive to feedback inhibition by heme, but the increased synthesis of the enzyme induced by drugs can override the inhibition. Several times as much heme is synthesized in the erythroid cells of bone than in liver, but this is not subject to feedback regulation or to stimulation by drugs.[427] Heme is a potent and toxic regulator. Malaria mosquitos, which utilize blood for food but do not have a heme oxygenase (Fig. 24-24), detoxify heme by inducing its aggregation into an insoluble hydrogen-bonded solid known as β-hematin.[431]

***The bile pigments.*** The enzymatic degradation of heme is an important metabolic process if only because it releases iron to be reutilized by the body. Some of the pathways are illustrated in Fig. 24-24. The initial oxidative attack is by the microsomal **heme oxygenases,**[432–434a] which catalyze the uptake of three molecules of $O_2$, formation of CO, and release of the chelated Fe. The electron transport protein NADPH-cytochrome P450 reductase brings electrons from NADPH to the oxygenase. An enzyme–substrate heme complex is formed with the oxygenase. Then the $Fe^{3+}$ is reduced to $Fe^{2+}$ which binds $O_2$ as in myoglobin or hemoglobin. The complex hydroxylates its own heme α-carbon (Fig. 24-24), the other oxygen atom being reduced to $OH^-$ by the $Fe^{2+}$ and an additional electron from NADPH. The same enzyme catalyzes the next steps in which the α carbon is split out as CO by reaction with two molecules of $O_2$ to form the open tetrapyrrole dicarbonyl compound **biliverdin**, one of the bile pigments (Fig. 24-24).[435–435a] When $^{18}O_2$ was used, it was found that the biliverdin contains two atoms of $^{18}O$, and that the CO contains one. Heme from the cytochromes *c* appears to be degraded by the same enzymes after proteolytic release from the proteins to which it is bound.[436]

There are two human heme oxygenases. The first (HO-1) is synthesized principally in the liver and spleen. Its formation is strongly induced by heme. The second heme oxygenase (HO-2) is distributed widely among tissues, but it is most abundant in certain neurons in the brain.[437,437a] Its major function may be to generate CO, which is now recognized as a probable neurohormone (Chapter 30). Bacteria, such as *Corynebacterium diphtheriae,* employ their own heme oxygenase as a means of recovering iron that they need for growth.[438]

A large number of other open tetrapyrroles can be formed from biliverdin by reduction or oxidation reactions. Within our bodies biliverdin is reduced to **bilirubin**, which is transported to the liver as a complex with serum albumin. In the liver bilirubin is converted into glucuronides (Eq. 20-16), glycosylation occurring on the propionic acid side chains.[439] A variety of these bilirubin conjugates are excreted into the bile. In the intestine they are hydrolyzed back to free bilirubin, which is reduced by the action of intestinal bacteria to urobilinogen, stercobilinogen, and *meso*-bilirubinogen. These compounds are colorless but are readily oxidized by oxygen to **urobilin** and **stercobilin**. Some of the urobilin and other bile pigments is reabsorbed into the blood and excreted into the urine where it provides the familiar yellow color.

The yellowing of the skin known as **jaundice** can occur if the heme degradation system is overburdened (e.g., from excessive hemolysis), if the liver fails to conjugate bilirubin, or if there is obstruction of the flow of heme breakdown products into the intestinal tract. Bilirubin is toxic, and continued exposure to excessive bilirubin levels can cause brain damage.[434,439] Bilirubin has a low water solubility and tends to form complexes with various proteins, perhaps partly because it assumes folded conformations rather than the linear one shown in Fig. 24-24.[440] These properties make it difficult to excrete. Thousands of newborn babies are treated for jaundice every year by prolonged irradiation with blue or white light which isomerizes 4Z,15Z bilirubin to forms that are more readily transported, metabolized, and excreted.[441] A more difficult problem is posed by the fatal deficiency of the glucuronosyl transferase responsible for formation of bilirubin glucuronide. Efforts are being made to develop a genetic therapy.[439]

The open tetrapyrroles of algae and the chromophore of phytochrome (Chapter 23) are all derived from **phycoerythrobilin**, which is formed from biliverdin, as indicated in Fig. 24-24. The animal bile pigments have not been found in prokaryotes. However, *Clostridium tetanomorphum*, which accumulates uroporphyrinogen III, a precursor to vitamin $B_{12}$, and does not synthesize protoporphyrin IX, makes a blue bile pigment **bactobilin**. This is a derivative of uroporphyrin rather than of protoporphyrin.[442]

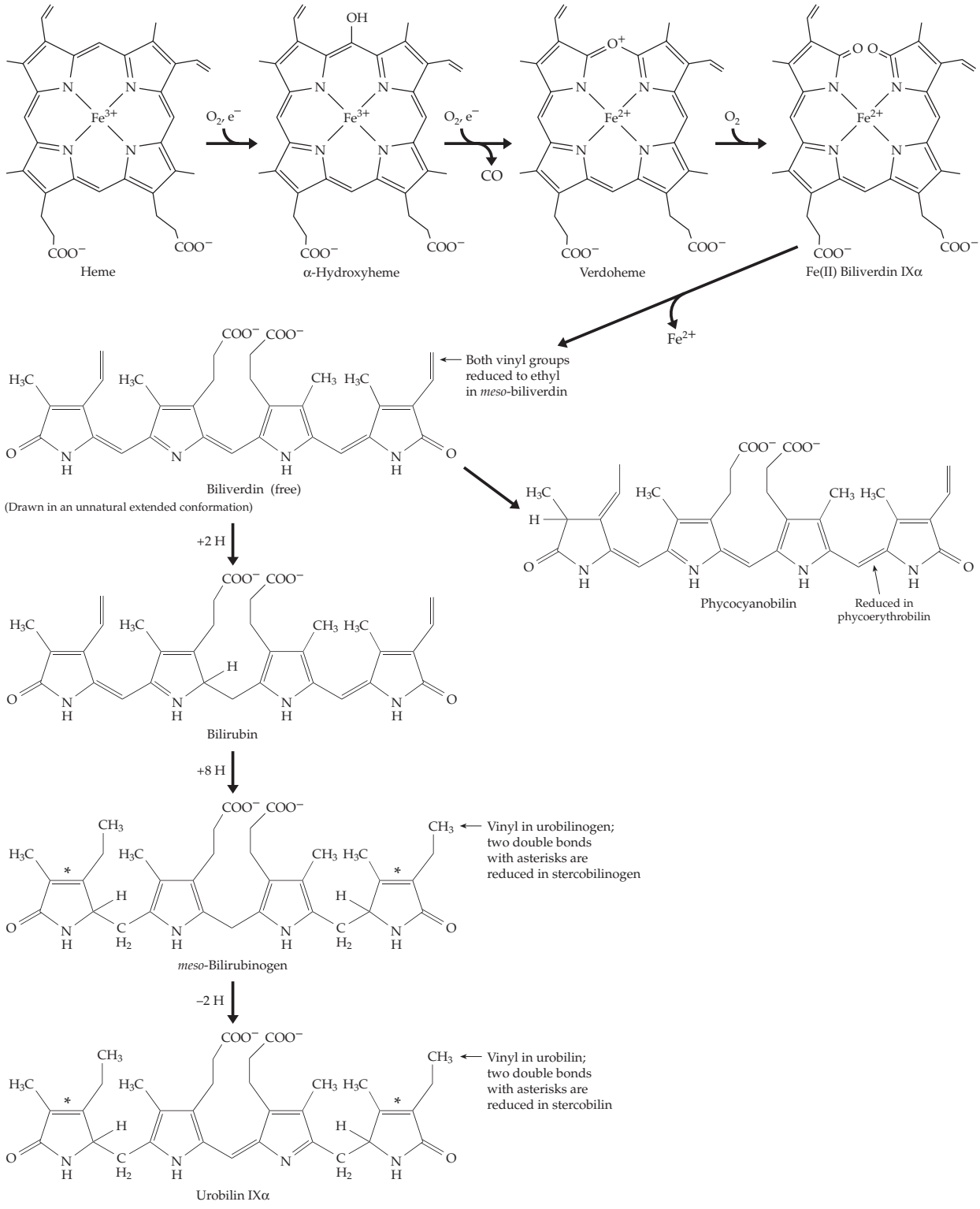



**Figure 24-24** The degradation of heme and the formation of open tetrapyrrole pigments.

## G. Cysteine and Sulfur Metabolism

Cysteine not only is an essential constituent of proteins but also lies on the major route of incorporation of inorganic sulfur into organic compounds.[443] Autotrophic organisms carry out the stepwise reduction of sulfate to sulfite and sulfide ($H_2S$). These reduced sulfur compounds are the ones that are incorporated into organic substances. Animals make use of the organic sulfur compounds formed by the autotrophs and have an active oxidative metabolism by which the compounds can be decomposed and the sulfur reoxidized to sulfate. Several aspects of cysteine metabolism are summarized in Fig. 24-25. Some of the chemistry of inorganic sulfur metabolism has been discussed in earlier chapters. Sulfate is reduced to $H_2S$ by sulfate-reducing bacteria (Chapter 18). The initial step in *assimilative* sulfate reduction, used by autotrophs including green plants and *E. coli*, is the formation of adenosine 5'-phosphosulfate (APS) (step *a*, Fig. 24-25; see also Eq. 17-38).[444–444c] The sulfate-reducing bacteria reduce adenylyl sulfate directly to sulfite (Eq. 18-32, step *b*), but the assimilative pathway of reduction in *E. coli* proceeds through 3'-phospho-5'-adenylyl sulfate (PAPS), a compound whose function as "active sulfate" has been considered in Chapter 17. Reduction of PAPS to sulfite (Fig. 24-25, step *d*) is accomplished by an NADPH-dependent enzyme.

The same pathway is found in the alga *Chlorella*, but a second route of sulfate reduction occuring in green plants may be more important.[445] Adenylyl sulfate transfers its sulfo group to a thiol group of a carrier (Eq. 24-43, step *a*). The resulting thiosulfonate is reduced by a ferredoxin-dependent reductase. Finally, a sulfide group is transferred from the –S—S⁻ group of the reduced carrier directly into cysteine in a

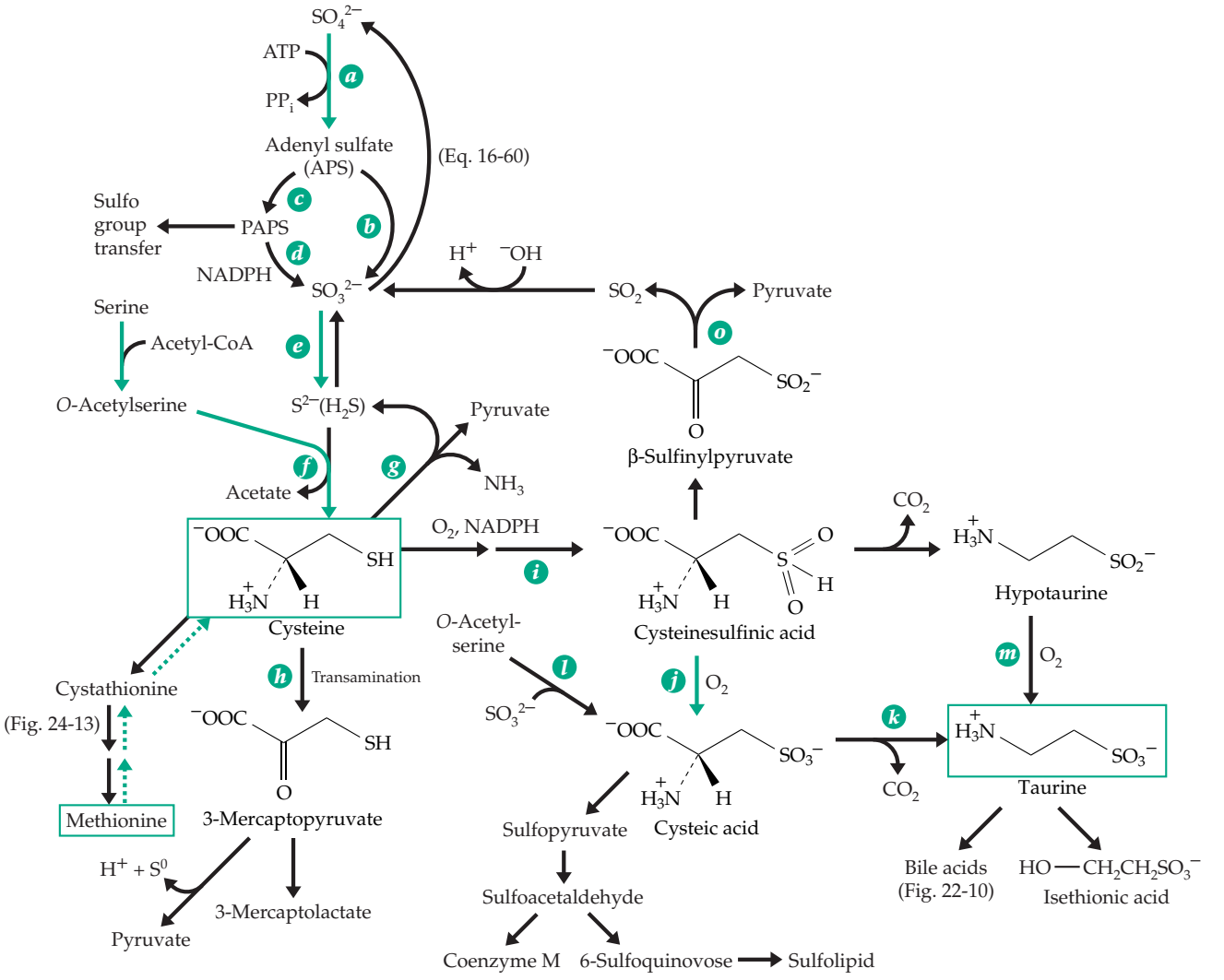
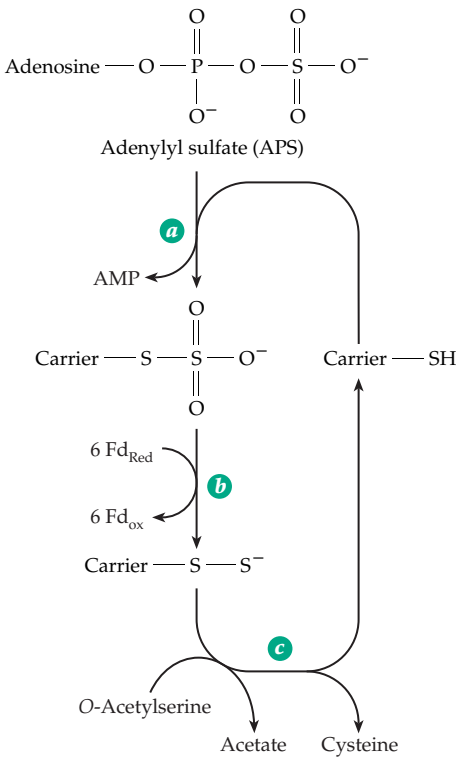



**Figure 24-25** Pathways of biosynthesis (green arrows) and catabolism of cysteine as well as other aspects of sulfur metabolism. Solid arrows are major biosynthetic pathways. The dashed arrows represent more specialized pathways; they also show processes occurring in the animal body to convert methionine to cysteine and to degrade the latter.




(24-43)

β-substitution reaction analogous to that described in the next paragraph.

## 1. Synthesis and Catabolism of Cysteine

Cysteine is formed in plants and in bacteria from sulfide and serine after the latter has been acetylated by transfer of an acetyl group from acetyl-CoA (Fig. 24-25, step *f*). This standard PLP-dependent β replacement (Chapter 14) is catalyzed by **cysteine synthase** (*O*-acetylserine sulfhydrase).[446,447] A similar enzyme is used by some cells to introduce sulfide ion directly into homocysteine, via either *O*-succinyl homoserine or *O*-acetyl homoserine (Fig. 24-13). In *E. coli* cysteine can be converted to methionine, as outlined in Eq. 16-22 and as indicated on the right side of Fig. 24-13 by the green arrows. In animals the converse process, the conversion of methionine to cysteine (gray arrows in Fig. 24-13, also Fig. 24-16), is important. Animals are unable to incorporate sulfide directly into cysteine, and this amino acid must be either provided in the diet or formed from dietary methionine. The latter process is limited, and cysteine is an essential dietary constituent for infants. The formation of cysteine from methionine occurs via the same transsulfuration pathway as in methionine synthesis in autotrophic organisms. However, the latter use cystathionine γ-synthase and β-lyase while cysteine synthesis in animals uses cystathionine β-synthase and γ-lyase.

Some bacteria degrade L-cysteine or D-cysteine[447a] via the PLP-dependent α,β elimination to form $H_2S$, pyruvate, and ammonia (reaction *g*, Fig. 24-25, Eq. 14-29). Another catabolic pathway is transamination (Fig. 24-25, rection *h*) to **3-mercaptopyruvate**.[448] The latter compound can be reductively cleaved to pyruvate and sulfide. Cysteine can also be oxidized by $NAD^+$ and lactate dehydrogenase to 3-mercaptopyruvate. An interesting PLP-dependent β-replacement reaction of cysteine leads to **β-cyanoalanine**, the lathyritic factor (Box 8-E) present in some plants.[449] This reaction also detoxifies the HCN produced during the biosynthesis of ethylene from ACC.

Cysteine and cystine are relatively insoluble and are toxic in excess.[450] Excretion is usually controlled carefully. However, in **cystinuria**, a disease recognized in the medical literature since 1810,[451] there is a greatly increased excretion of cystine and also of the dibasic amino acids.[451,452] As a consequence, stones of cystine develop in the kidneys and bladder. Patients may excrete more than 1 g of cystine in 24 h compared to a normal of 0.05 g, as well as excessive amounts of lysine, arginine, and ornithine. The defect can be fatal, but some persons with the condition remain healthy indefinitely. Cystinuria is one of several human diseases with altered membrane transport and faulty reabsorption of materials from kidney tubules or from the small intestine. Substances are taken up on one side of a cell (e.g., at the bottom of the cell in Fig. 1-6) and discharged into the bloodstream from the other side of the cell. In another rare hereditary condition, **cystinosis**, free cystine accumulates within lysosomes.[453]

## 2. Cysteine Sulfinate and Taurine

A quantitatively important pathway of cysteine catabolism in animals is oxidation to **cysteine sulfinate** (Fig. 24-25, reaction *i*),[450] a two-step hydroxylation requiring $O_2$, NADPH or NADH, and $Fe^{2+}$. Cysteine sulfinic acid can be further oxidized to **cysteic acid** (cysteine sulfonate),[454] which can be decarboxylated to **taurine**. The latter is a component of bile salts (Fig. 22-16) and is one of the most abundant free amino acids in human tissues.[455–457] Its concentration is high in excitable tissues, and it may be a neurotransmitter (Chapter 30). Taurine may have a special function in retinal photoreceptor cells. It is an essential dietary amino acid for cats, who may die of heart failure in its absence,[458] and under some conditions for humans.[459] In many marine invertebrates, teleosts, and amphibians taurine serves as a regulator of osmotic pressure, its concentration decreasing in fresh water and increasing in salt water. A similar role has been suggested for taurine in mammalian hearts. A chronically low concentration of $Na^+$ leads to increased taurine.[460] Taurine can be reduced to **isethionic acid**

(Fig. 24-25), another component of nervous tissue. Cysteic acid can arise in an alternative way from *O*-acetylserine and sulfite (reaction l, Fig. 24-25), and taurine can also be formed by decarboxylation of cysteine sulfinic acid to **hypotaurine** and oxidation of the latter (reaction *m*). Cysteic acid can be converted to the sulfolipid of chloroplasts (p. 387; Eq. 20-12).

Another route of metabolism for cysteine sulfinic acid is transamination to 3-sulfinylpyruvate, a compound that undergoes ready loss of $SO_2$ in a reaction analogous to the decarboxylation of oxaloacetate (reaction *o*, Fig. 24-25). This probably represents one of the major routes by which sulfur is removed from organic compounds in the animal body. However, before being excreted the sulfite must be oxidized to sulfate by the Mo-containing sulfite oxidase. The essentiality of sulfite oxidase is evidenced by the severe neurological defect observed in its absence (Chapter 16).

Most of the sulfate generated in the body is excreted unchanged in the urine, but a significant fraction is esterified with oligosaccharides and phenolic compounds. These sulfate esters are formed by sulfo transfer from PAPS (Eq. 17-38).

### 3. Mercaptopyruvate, Thiosulfate, and Assembly of Iron–Sulfur Centers

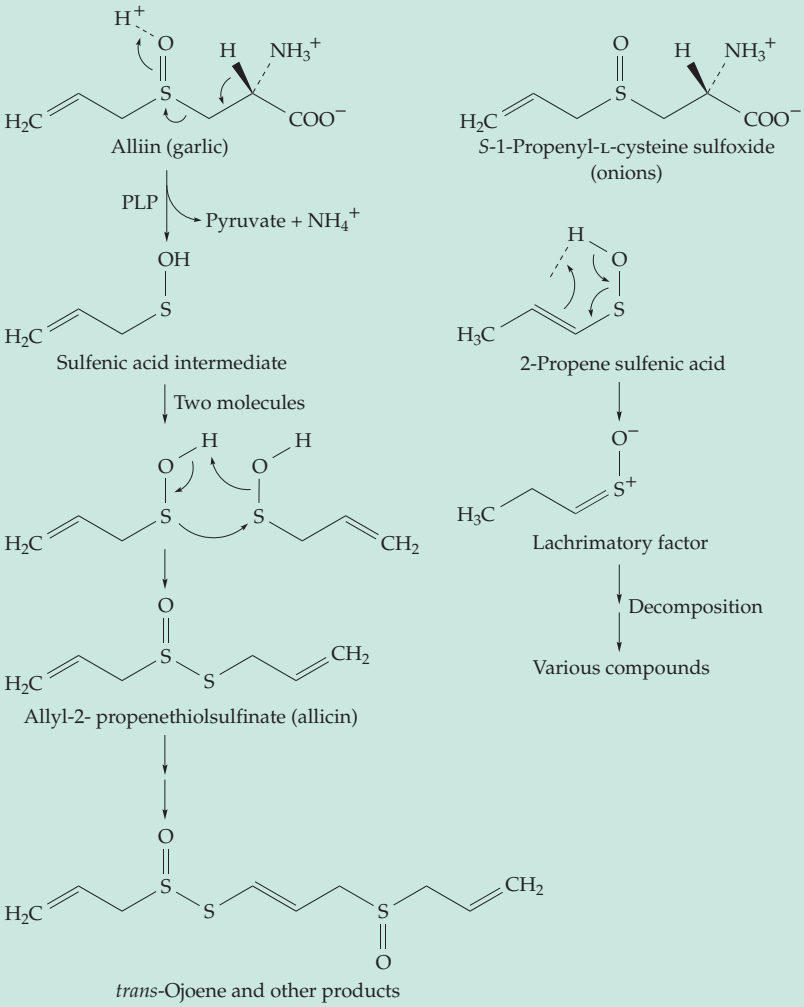
An important property of 3-mercaptopyruvate arises from electron withdrawal by the carbonyl group. This makes the SH group electrophilic and able to be transferred as $SH^+$, $S^0$, to a variety of nucleophiles (Eq. 24-44). Thus sulfite yields thiosulfate ($S_2O_3^{2-} + H^+$, Eq. 24-45, step *a*), cyanide yields thiocyanate (Eq. 24-45, step *b*), and cysteine sulfinate yields alanine thiosulfonate.[448,461] The reactions are catalyzed by **mercaptopyruvate sulfurtransferase**, an enzyme very similar to **thiosulfate sulfurtransferase**. The latter is a liver enzyme often called by the traditional

## BOX 24-B SULFUR COMPOUNDS OF GARLIC, ONIONS, SKUNKS, ETC.

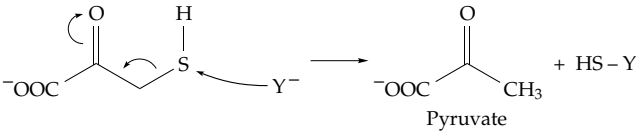
Many familiar odors and tastes come from sulfur-containing compounds. Crushing onions or garlic releases the pyridoxal phosphate-dependent enzyme **alliinase**. In garlic it acts upon the amino acid **alliin** (accompanying scheme) releasing, by β elimination, a sulfenic acid that dimerizes to form **allicin**, a chemically unstable molecule that accounts for the odor of garlic.[a,b] Among the breakdown products of allicin is the nonvolatile **ajoene**, a compound with anticoagulant activity and perhaps accounting for one aspect of the purported medical benefits of garlic. Another is an antibacterial activity.

Onions contain an amino acid that is a positional isomer of alliin. When acted upon by alliinase it produces 2-propene sulfenic acid, which isomerizes to the **lachrimatory factor** that brings tears to the eyes of onion cutters.[c] This, too, decomposes to form many other compounds.[a]

The defensive secretion of the striped skunk has intrigued chemists for over 100 years. The components were shown to contain

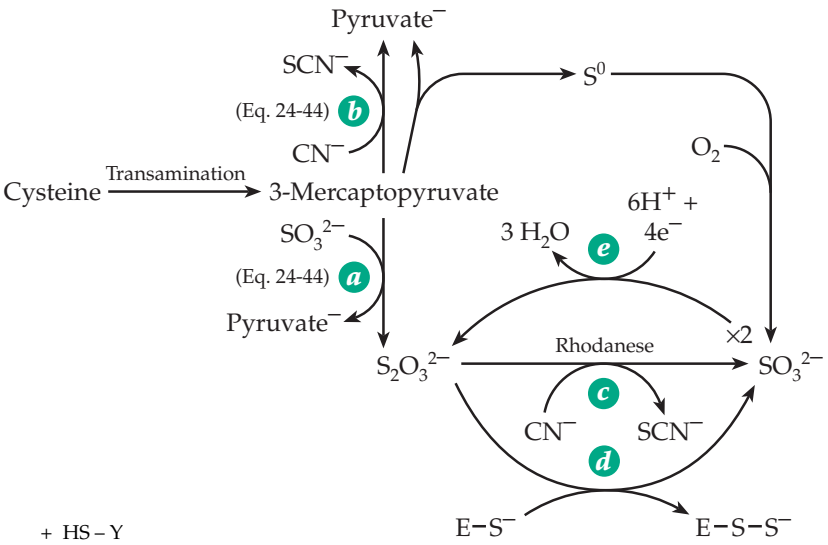


name **rhodanese**.[462–463b] It acts by a double displacement mechanism, a thiolate anion in the active site of the enzyme serving as a carrier for the S atom being transferred. Equation 24-46 illustrates the transfer of $S^0$ from thiosulfate to $CN^-$, converting that ion to the less toxic thiocyanate (also shown as step *c* in Eq. 24-45). Crystallographic studies show that the negative charge on the thiol and dithiol anions of rhodanese is balanced by the partial




Pyruvate

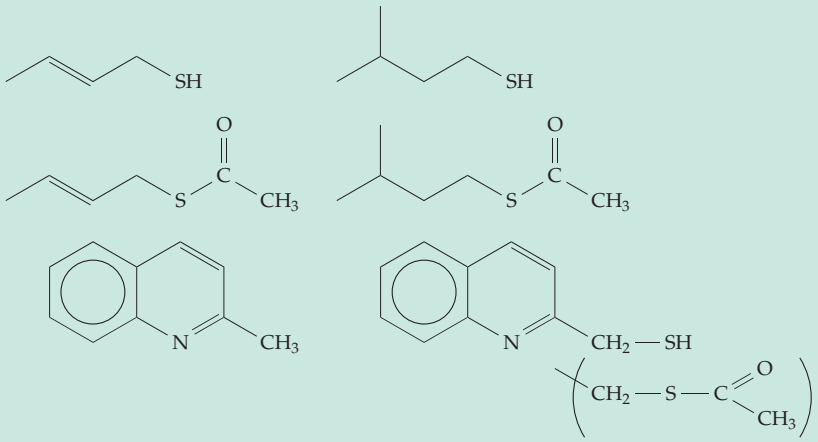
(24-44)




(24-45)

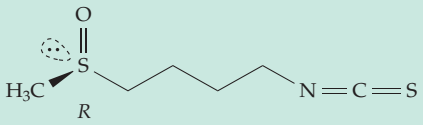
## BOX 24-B (continued)

sulfur, and one was incorrectly identified and was long accepted as being butyl mercaptan. Modern capillary gas chromatography by Wood[d] has revealed the presence of seven major components, with the indicated structures. Two are simple volatile mercaptans, but three are thioacetates which hydrolyze in water only slowly, releasing smell for days or weeks from sprayed animals. Washing with mildly basic soap hastens the hydrolysis.




Many readers (~40%) may be aware that after eating asparagus a strong odor appears in their urine. These genetic "stinkers" secrete *S*-methyl thioacrylate, and related compounds, derived from a plant constituent.[e]

The sulfur compound sulfuraphane, extracted from fresh broccoli, has received attention in recent years because of its strong action in inducing synthesis of quinone reductase and glutathione *S*-transferases that help detoxify xenobiotics and may have significant anticancer activity.[f]




Sulforaphane;
1-isothiocyanate-(4*R*)-(methylsulfinyl)butane

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[a] Block, E. (1985) *Sci. Am.* **252**(Mar), 114–119
[b] Jain, M. K., and Apitz-Castro, R. (1987) *Trends Biochem. Sci.* **12**, 252–254
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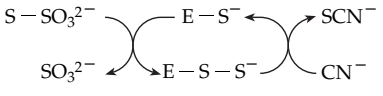
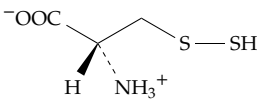
[f] Zhang, Y., Talalay, P., Cho, C.-G., and Posner, G. H. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 2399–2403




(24-46)

positive charges at the N termini of two helices (see Fig. 2-20A) and by hydrogen bonds to protons of several side chains.[462] This evidently explains how the negative thiosulfate anion can react with another anion, E–S⁻. Another interesting feature of this enzyme is that the monomer has a nearly perfect twofold axis of symmetry with respect to the protein folding pattern. However, the symmetry is lacking in the sequence and only one-half of the molecule contains an active site.[462]

Yet another enzyme able to release or transfer sulfur in the $S^0$ oxidation state is the PLP-dependent **cysteine desulfurase** that is encoded by the *nifS* gene of the nitrogenase gene cluster shown in Fig. 24-4. This enzyme releases $S^0$ from cysteine with formation of alanine[464–466] as is shown in Eq. 14-34 for release of $Se^0$ from selenocysteine. As with rhodanese an active site cysteine accepts the departing $S^0$ of cysteine to form an enzyme-bound persulfide. This protein may in turn transfer the sulfur into the forming Fe–S or Fe–S–Mo clusters.[464] Three PLP-dependent persulfide-forming sulfurtransferases related to the NifS protein have been found in *E. coli*. Similar enzymes are present in other organisms.[466a–d] A sulfur atom may be transferred from the bound persulfide anion to acceptor proteins involved in metal cluster formation. Some members of the nifS-like family act on cystine to release free thiocysteine (cysteine peroxide), which may also serve as a sulfur atom donor.[466e]

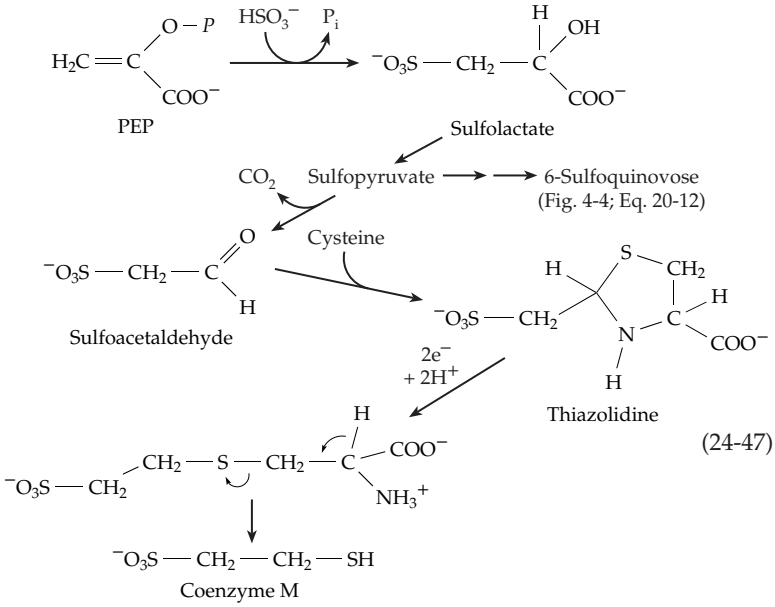



Thiocysteine (cysteine persulfide)

Thiocysteine can also arise in a similar manner through action of cystathionine β lyase on cystine. Thiocysteine is eliminated with production of pyruvate and ammonia from the rest of the cystine molecule.[467] One of the nifS-like proteins of *E. coli* is thought to transfer a selenium atom from selenocysteine (pp. 823–827) into **selenophosphate**.[466a,f] The latter can be formed by transfer of a phospho group from ATP to selenide $HSe^-$. The other products of ATP cleavage are AMP and $P_i$. Reduction of $Se^0$ to $HSe^-$ is presumably necessary.

Several additional proteins identified as necessary for metal-sulfide cluster formation are present in bacteria and in eukaryotes, both in the cytosol and in mitochondria. They may serve as intermediate sulfur carriers, as scaffolds or templates for cluster formation, or for insertion of intact Fe–S, Fe–S–Mo, or other types of clusters into proteins[468–473f] and into 2-selenouridine[473g] (see also p. 1617). Sulfurtransferases are also thought to be involved in insertion of sulfur atoms into organic molecules such as biotin, lipoic acid, or methanopterin.[474]

A reaction that is ordinarily of minor consequence in the animal body but which may be enhanced by a deficiency of sulfite oxidase is the reductive coupling of two molecules of sulfite to form thiosulfate (Eq. 24-45, step *e*). Several organic hydrodisulfide derivatives such as thiocysteine, thioglutathione, and thiotaurine occur in animals in small amounts. Another biosynthetic pathway, outlined in Eq. 24-47 converts sulfite and PEP into coenzyme M (Fig. 15-22).[475,475a] This cofactor is needed not only for methane formation (Fig. 15-2) but also for utilization of alkenes by soil bacteria.[475b]




(24-47)

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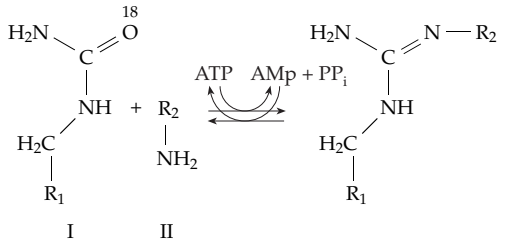
## Study Questions

1. Bacterial glutamine synthetase is feedback inhibited by serine, glycine, and alanine. Explain specifically the connection between these amino acids and glutamine that would account for the logic of this inhibition.

2. Argininosuccinate synthetase catalyzes the following reaction:




a) The reaction as shown is reversible. What metabolic stratagem is employed to drive the reaction from left to right?
b) It has been shown that the oxygen-18 from I (see structure) is transferred to the phosphate group of AMP. Propose a biochemical reaction mechanism to account for the transfer.

3. An organism has been discovered whose urea cycle does not include any reaction with aspartate. Both of the urea nitrogen atoms come directly from ammonia. All other components of the cycle are present and the net reaction is

$$CO_2 + 2\ NH_3 + 3\ ATP \rightarrow \text{urea} + 2\ ADP + 2\ P_i + AMP + PP_i$$

Explain how this is accomplished. Include plausible reaction mechanisms for any *new* steps proposed.

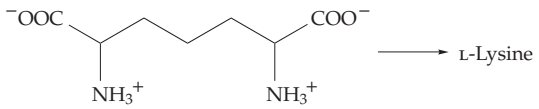
4. Which of the following compounds, if added to an active tissue preparation, might be expected to yield the greatest increase in urea production in terms of moles of urea produced per mole of added compound?

a) Ammonia
b) Bicarbonate
c) Aspartate
d) Ornithine

More than one answer may be correct. Explain how you decided.

5. On a given diet yielding 2500 kcal per day, a 70-kg man excretes 30.0 g of urea daily. What percentage of his daily energy requirement is met by protein? Assume that 1.0 g of protein yields 4.0 kcal and 0.16 g of nitrogen as urea.

6. In many organisms the immediate biosynthetic precursor of L-lysine is α,ε-diaminopimelic acid (structure below). What type of enzyme would catalyze this reaction; what coenzyme would be required; and what type of enzyme–substrate complex would be formed?




α,ε-Diaminopimelic acid

7. A possible mechanism for the action of urease is pictured in Fig. 16-25 and Eq. 16-47. Carbamate is thought to be one intermediate. Can you suggest an alternative possibility for the initial nickel ion-dependent steps. See Barrios and Lippard.[476]

8. Leucine is known as a "ketogenic" amino acid. Explain what this means.

9. In some organisms leucine is not ketogenic. Why?

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

$$\text{L-Leucine} + \text{2-oxoglutarate}^{2-} + 2\ ½\ O_2 \rightarrow \text{L-glutamate}^{-} + \text{citrate}^{3-} + H_2O + 2\ H^+$$

$\Delta G^{\circ\prime}$ (pH 7) = –1026 kJ/mol

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

11. To be practical, the fungus should convert the L-glutamate back to 2-oxoglutarate using a glutamate dehydrogenase. Here are some values for Gibbs energies of formation from the elements under standard conditions (pH = 0).

| | |
|---|---|
| 2-Oxoglutarate$^{2-}$ | –798.0 kJ/mol |
| L-Glutamate$^{-}$ | –696.8 kJ/mol |
| $NH_4^+$ | –79.5 kJ/mol |

a) Calculate the apparent Gibbs energy change $\Delta G^{\circ\prime}$ (pH 7) for the following reaction:

$$\text{L-Leucine} + 3\ O_2 \rightarrow \text{citrate}^{3-} + NH_4^+ + H_2O + 2\ H^+$$

b) Could this fungal reaction be used as a commercial process for making citric acid?

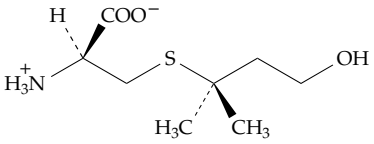
12. Two molecules of pyruvate can react to give a common precursor to valine, leucine, and pantoic acid. An isomerization step involving shift of a methyl group from one carbon to another is involved.

    a) Indicate as much as you can of the pathway for formation of valine showing coenzymes and mechanisms.
    b) Outline the reaction sequence by which the immediate oxoacid precursor of valine is converted to leucine.
    c) Outline a sequence by which the same oxoacid may be converted to pantoic acid using serine as an additional carbon atom source.

13. Explain how carboxylation can be coupled to cleavage of ATP and how this can be used to drive a metabolic sequence.

14. L-Serine is converted to pyruvate + $NH_3$ by serine dehydratase (deaminase) in a PLP-dependent reaction. However, using the same coenzyme selenocysteine is converted by selenocysteine lyase into L-alanine + elemental selenium $Se^0$. L-Cysteine may be converted by PLP-dependent enzymes into wither $H_2S$ or into $S^0$ for transfer into metal clusters. Compare the chemical mechanisms.

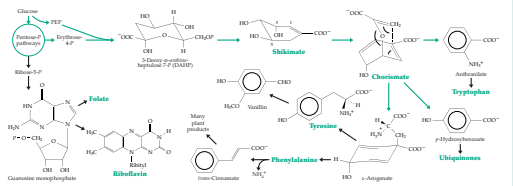
15. Felinine is found in urine of cats, the highest amounts in males. The compound arises from a reaction of glutathione. Propose a route of synthesis. See Rutherfurd *et al.*[477]



Felinine

16. Some clostridia ferment glutamate to ammonia, carbon dioxide, acetate, butyrate, and molecular hydrogen. Write a balanced equation and compare with Eq. 24-18 and Fig. 24-8. See Hans *et al.*[478]

17. How could β-hydroxy-β-methylbutyrate be formed in muscle? Could it be a physiologically important precursor to cholesterol?

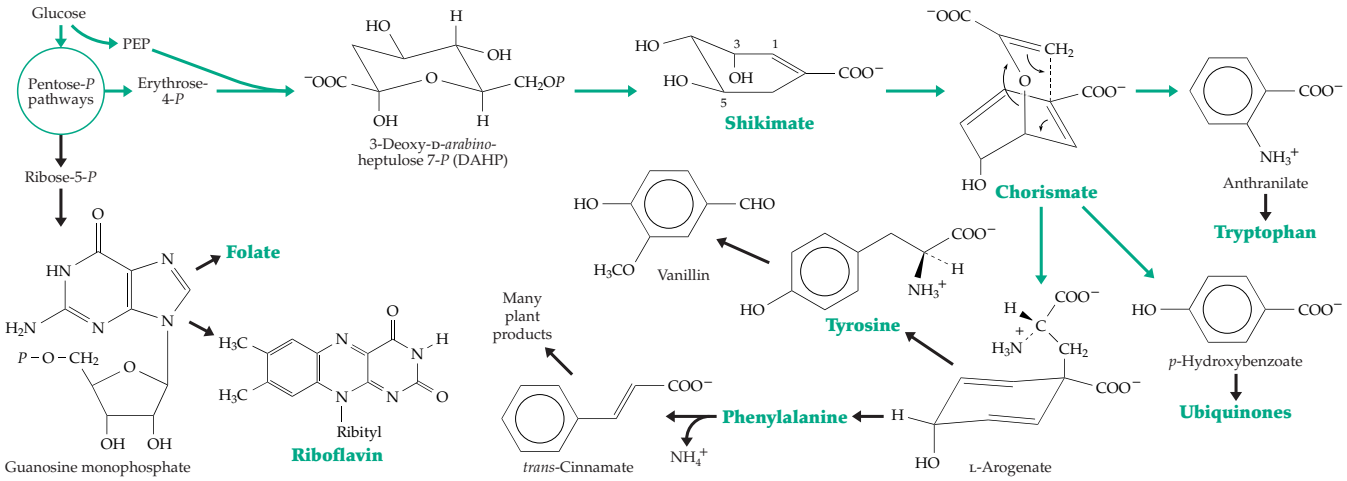



Aromatic compounds arise in several ways. The major route utilized by autotrophic organisms for synthesis of the aromatic amino acids, quinones, and tocopherols is the **shikimate pathway**. As outlined here, it starts with the glycolysis intermediate phosphoenolpyruvate (PEP) and erythrose 4-phosphate, a metabolite from the pentose phosphate pathway. Phenylalanine, tyrosine, and tryptophan are not only used for protein synthesis but are converted into a broad range of hormones, chromophores, alkaloids, and structural materials. In plants phenylalanine is deaminated to cinnamate which yields hundreds of secondary products. In another pathway ribose 5-phosphate is converted to pyrimidine and purine nucleotides and also to flavins, folates, molybdopterin, and many other pterin derivatives.

# Contents

# Metabolism of Aromatic Compounds and Nucleic Acid Bases

# 25



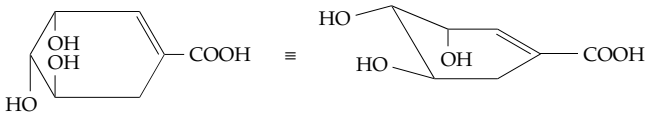

Aromatic rings are found in amino acids, purine and pyrimidine bases, vitamins, antibiotics, alkaloids, pigments of flowers and of the human skin, the lignin present in wood, and in many other substances. There are several biosynthetic pathways. One, which we met in Chapter 21, is the polyketide pathway. However, more important in most autotrophic organisms is the **shikimate pathway** which gives rise to phenylalanine, tyrosine, tryptophan, ubiquinone, plastoquinones, tocopherols, vitamin K, and other compounds.[1] The entire pathway, which is outlined in Fig. 25-1, is present in most bacteria and plants. However, animals are unable to synthesize the ring systems of the aromatic amino acids. Phenylalanine and tryptophan are dietary essentials. Tyrosine can also be formed in the animal body by the hydroxylation of phenylalanine. However, green plants lack phenylalanine hydroxylase and make tyrosine directly through the shikimate pathway (green arrows in Fig. 25-1).

There is interest in applying genetic engineering to increase the output of the shikimate pathway for production of industrially important aromatic compounds, e.g., the dye **indigo**, which is used in manufacture of blue denim (Box 25-C). Study of the enzymes involved has led to the development of potent inhibitors of the shikimate pathway which serve as widely used herbicides.[2,3]

A variety of pathways give rise to the nitrogen- and oxygen-containing heterocyclic rings of nature. All cells must be able to make pyrimidine and purine bases to be used in synthesis of nucleic acids and coenzymes. The pathway for synthesis of pyrimidine begins with aspartic acid and that for purines with glycine. In many organisms the pathway of purine formation is further enhanced because uric acid or a related substance is the major excretory product derived from excess nitrogen. This is true for both birds and reptiles, which excrete uric acid rather than urea, and for spiders which excrete guanine. In some plants, such as soy beans, the transport form of nitrogen is **allantoin** or **allantoic acid**, both of which are produced from uric acid.

## A. The Shikimate Pathway of Biosynthesis

The shikimate pathway was identified through the study of ultraviolet light-induced mutants of *E. coli*, *Aerobacter aerogenes*, and *Neurospora*. In 1950, using the penicillin enrichment technique (Chapter 26), Davis obtained a series of mutants of *E. coli* that would not grow without the addition of aromatic substances.[4,5] A number of the mutants required five compounds: tyrosine, phenylalanine, tryptophan, *p*-aminobenzoic acid, and a trace of *p*-hydroxybenzoic acid. It was a surprise to find that the requirements for all five compounds could be met by the addition of shikimic acid, an aliphatic compound that was then regarded as a rare plant acid. Thus, shikimate was implicated as an intermediate in the biosynthesis of the three aromatic amino acids and of other essential aromatic substances.[6,7]



Shikimic acid

The mutants that grew in the presence of shikimic acid evidently had the biosynthetic pathway blocked

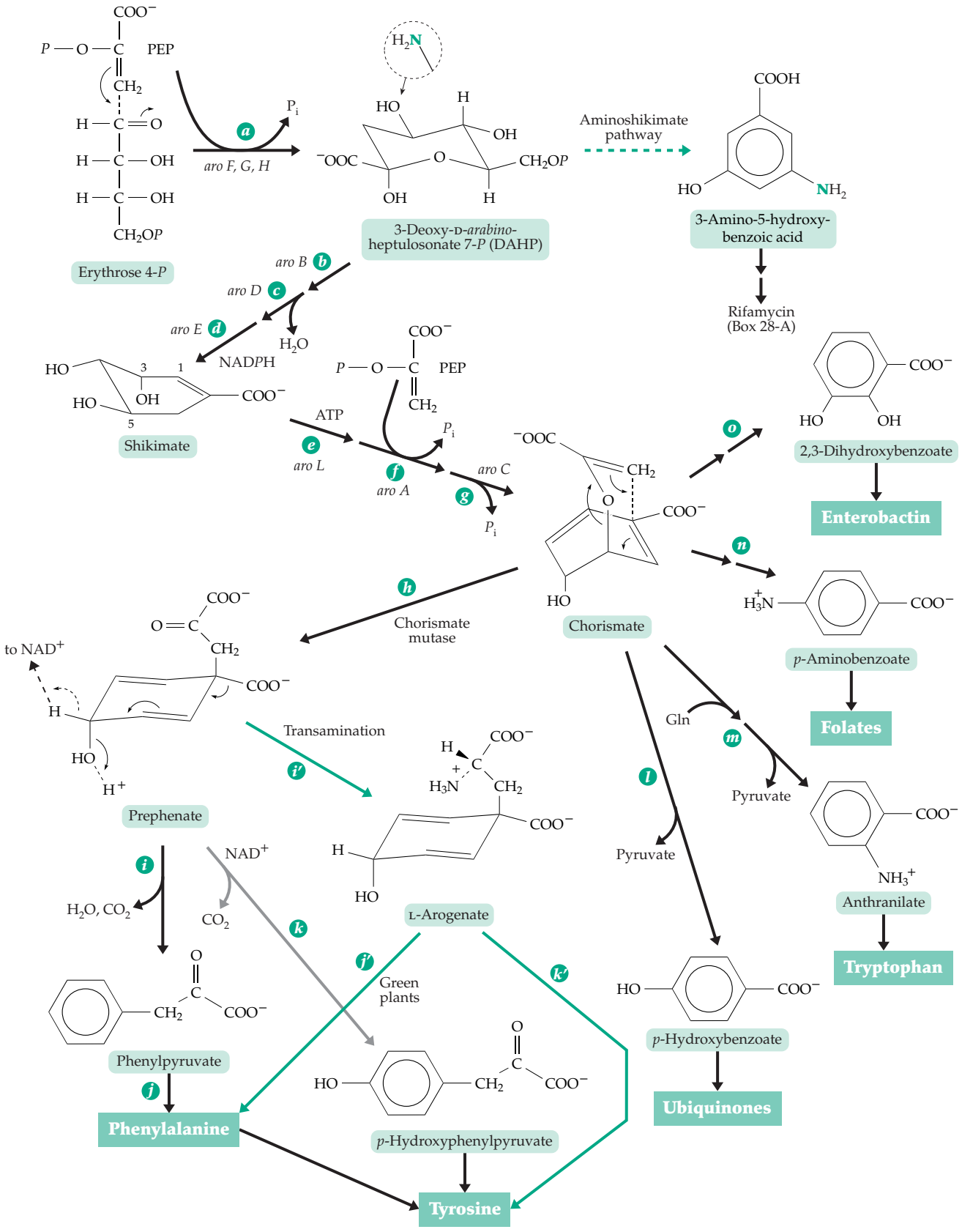
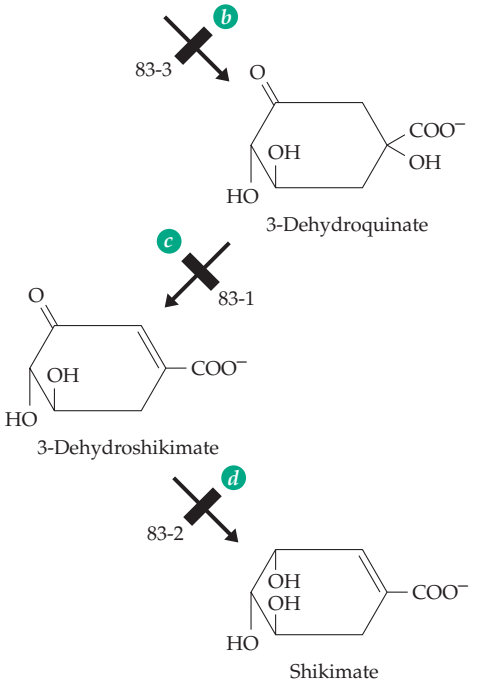



**Figure 25-1** Aromatic biosynthesis by the shikimate pathway. The symbols for several of the genes coding for the required enzymes are indicated. Their locations on the *E. coli* chromosome map are shown in Fig. 26-4. The aminoshikimate pathway which is initiated through 4-aminoDAHP leads to rifamycin and many other nitrogen-containing products.

at one or more earlier stages. Among these mutants, certain pairs were found that could not grow alone but that grew when plated together. The phenomenon is called **syntropism**. Mutant 83-2, which we now know to be blocked in the conversion of 5-dehydroshikimate to shikimate, accumulated dehydroshikimate and permitted mutant 83-1 or 83-3 to grow by providing it with a precursor that could be converted on to the end products (Eq. 25-1; the steps in this equation are lettered to correspond to those in Fig. 25-1). Eventually, the entire pathway was traced. The enzymes have all been isolated and studied[1] and the locations of the genes in the *E. coli* chromosome have been mapped[7–10] and are marked in Fig. 26-4.




(25-1)

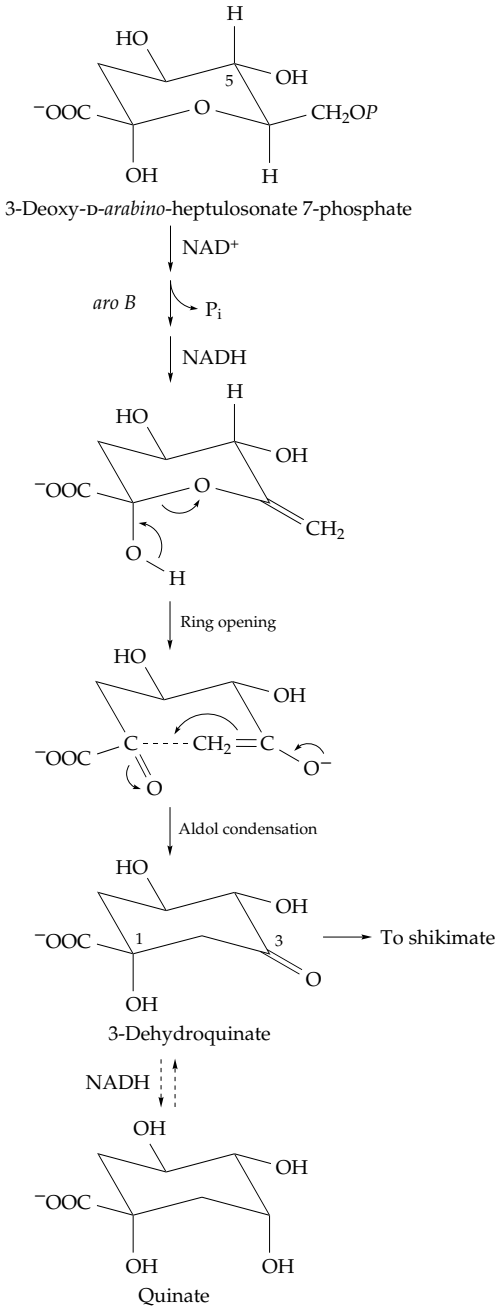
A variety of nitrogen-containing products, including rifamycin (Box 28-A), arise via the **aminoshikimate** pathway,[10a–d] which is also indicated in Fig. 25-1.

## 1. The Enzymes

The six carbons of the benzene ring of the aromatic amino acids are derived from the four carbons of erythrose 4-phosphate and two of the three carbons of phosphoenolpyruvate (PEP). The initial step in the pathway (Fig. 25-1, step *a*) is the condensation of erythrose 4-*P* with PEP and is catalyzed by 3-deoxy-D-*arabino*-heptulosonate-7-phosphate (DAHP) synthase. Closely analogous to an aldol condensation, the mechanism provides a surprise.[10] When PEP containing $^{18}O$ in the oxygen bridge to the phospho group reacts, the $^{18}O$ is retained in the eliminated phosphate; biochemical intuition would suggest that it should stay in the carbonyl group of the product. See Chapter 20, Section A,5.

Most bacteria and fungi have three isozymes of DAHP synthase, each controlled by feedback inhibition by one of the three products tyrosine, phenylalanine, or tryptophan. In *E. coli* these are encoded by genes *aro F*, *aro G*, and *aro H*, respectively.[11–12a] All of the enzymes contain one atom of iron per molecule and show spectral similarities to hemerythrin.[13]

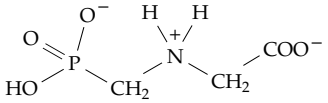
The product of the DAHP synthase, 3-deoxy-D-*arabino*-heptulosonate 7-phosphate, is shown in its cyclic hemiacetal form at the beginning of Eq. 25-2. Its conversion to 3-dehydroquinate is a multistep process that is catalyzed by a single enzyme,[14,15] which is the product of *E. coli* gene *aro B*. The elimination of




(25-2)

inorganic phosphate in the second step of the sequence is assisted by a transient oxidation of the hydroxyl group at C-5 to a carbonyl group.[6,16,17] The enzyme contains bound $NAD^+$ and is activated by $Co^{2+}$. The last step in the dehydroquinate synthase sequence is the addition of an enolic intermediate to a carbonyl group, an aldol condensation, which forms the 6-carbon ring (Eq. 25-2). Also indicated in this equation, with dashed lines, is the reversible conversion of dehydroquinate to quinate. Although it is a "side product" quinic acid accumulates to high concentrations in many plants.[18]

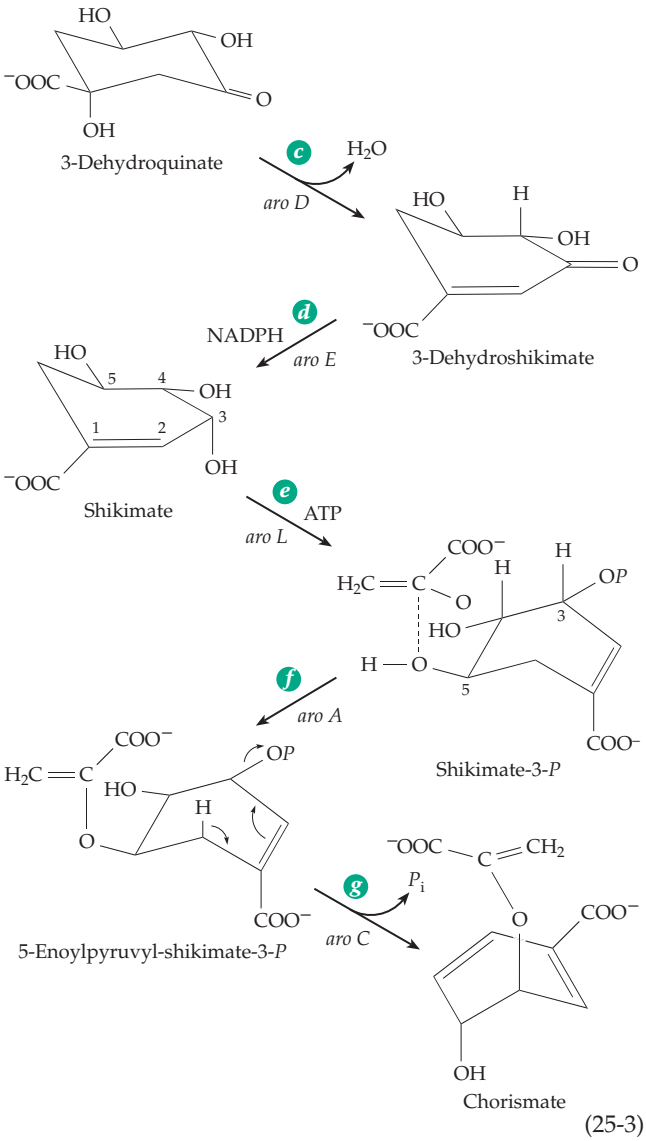
Dehydration of 3-dehydroquinate (step *c*), the first step in Eq. 25-3, is the first of three elimination reactions needed to generate the benzene ring of the end products. This dehydration is facilitated by the presence of the carbonyl group. After reduction of the product to shikimate (step *d*)[19] a phosphorylation reaction (step *e*)[20,21] sets the stage for the future elimination of $P_i$. In step *f*, condensation with PEP adds three carbon atoms that will become the α, β, and carboxyl carbon atoms of phenylalanine and tyrosine. The reaction occurs by displacement of $P_i$ from the α-carbon atom of PEP and resembles a reaction (Eq. 20-6, step *a*) in the synthesis of *N*-acetylmuramic acid.[22,23] When the reaction is carried out in $^3H$-containing water, tritium enters the methylene group,[10,24] suggesting an addition–elimination mechanism (Eq. 25-4).[25] The enzyme 5-enoylpyruvylshikimate 3-phosphate synthase (**EPSP synthase**), which catalyzes this reaction, is strongly inhibited by the commercially important herbicide ***N*-(phosphonomethyl)glycine** (glyphosate).[3,26–27a]



*N*-(Phosphonomethyl) glycine (glyphosate)

A single mutation (Pro 101 → Ser) in the 427-residue protein from *E. coli* makes the enzyme more resistant to the herbicide.[28] Other mutations affect binding and catalysis.[29]

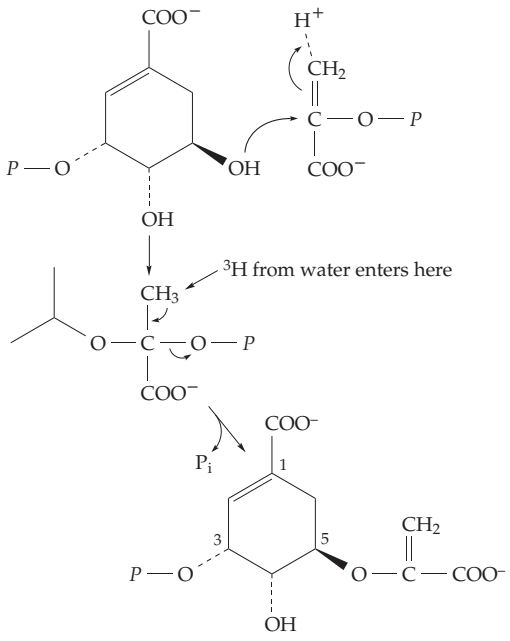
Elimination of $P_i$ from 5-enolpyruvylshikimate 3-*P* (Eq. 25-3 and Fig. 25-1, step *g*) produces chorismate.[30] The 24-kDa chorismate synthase, which catalyzes this reaction, requires for activity a reduced flavin. Although there is no obvious need for an oxidation–reduction coenzyme, there is strong evidence that the flavin may play an essential role in catalysis, perhaps via a radical mechanism.[31–33b]




(25-3)

## 2. From Chorismate to Phenylalanine and Tyrosine

Chemical properties appropriate to a compound found at a branch point of metabolism are displayed by chorismic acid. Simply warming the compound in acidic aqueous solution yields a mixture of **prephenate** and ***para*-hydroxybenzoate** (corresponding to reactions *h* and *l* of Fig. 25-1). Note that the latter reaction is a simple elimination of the enolate anion of pyruvate. As indicated in Fig. 25-1, these reactions correspond to only two of several metabolic reactions of the chorismate ion. In *E. coli* the formation of **phenylpyruvate** (steps *h* and *i*, Fig. 25-1) is catalyzed by a single protein molecule with two distinctly different enzymatic activities: **chorismate mutase** and **prephenate dehydratase**.[34–36] However, in some organisms the enzymes are separate.[37] Both of the reactions catalyzed by these enzymes also occur spontaneously upon warming chorismic acid in acidic solution. The chorismate mutase reaction, which is unique in its mechanism,[37a] is discussed in Box 9-E. Stereochemical studies indicate that the formation of phenylpyruvate in Fig. 25-1, step *i*, occurs via a

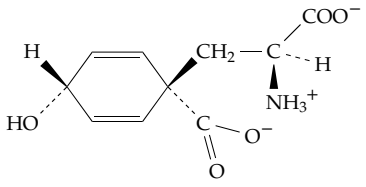



5-Enoylpyruvylshikimate 3-phosphate (25-4)

chair-like transition state.[38] Phenylpyruvate is transaminated to phenylalanine to complete the biosynthesis of that amino acid. Regulation in *E. coli* is accomplished in part by feedback inhibition of the bifunctional chorismate mutase–prephenate dehydratase.[38a]

In *E. coli* and many other bacteria a second bifunctional enzyme, **chorismate mutase-prephenate dehydrogenase** causes the isomerization of chorismate and the oxidative decarboxylation of prephenate to *p*-hydroxyphenylpyruvate (steps *h* and *k*, Fig. 25-1).[39] The latter can be converted by transamination to tyrosine.[40–42]

A slightly different pathway for tyrosine formation was found initially in cyanobacteria but has now been identified in a variety of organisms including higher green plants. In this pathway prephenate undergoes transamination to **L-arogenate** (pretyrosine), step *i*, Fig. 25-1.[43–45] In bacteria L-arogenate is oxidatively




L-Arogenate (pretyrosine)

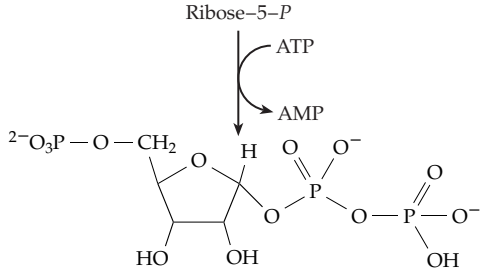
decarboxylated to tyrosine (step *k*, Fig. 25-1). However, in green plants L-arogenate undergoes decarboxylative elimination (step *j*) to give L-phenylalanine. This is a major reaction in green plants, which cannot form tyrosine by hydroxylation of phenylalanine[46,47] but which form a variety of additional products from phenylalanine by a pathway characteristic for plants (Fig. 25-8).

## 3. Anthranilate, Tryptophan, *para*-Aminobenzoate, and Dihydroxybenzoate

The three chemically similar reactions designated *m*, *n*, and *o* in Figs. 25-1 and 25-2 give rise to a variety of products. Step *m* leads to anthranilate and **L-tryptophan** and step *n* to the vitamin **folic acid.** Each of the three reactions *m*, *n*, and *o* involves addition of either $NH_3$ or $HO^-$ at a position *ortho* or *para* to the carboxyl group of chorismate with elimination of the 5-OH group. The structures of the three intermediate products are shown in Fig. 25-2.[48–50] The three enzymes have significant similarities in amino acid sequence. Anthranilate synthase and *p*-aminobenzoate synthase are both two-subunit enzymes consisting of a 20-kDa subunit glutamine amidotransferase which is presumed to generate $NH_3$ (see Chapter 24,B).[49,51–53a] The second 50-kDa subunit of anthranilate synthase catalyzes the remaining steps in the reaction. However, *p*-aminobenzoate synthesis in *E. coli* requires an additional enzyme to catalyze the elimination of pyruvate in the final step[54] of synthesis.

The product of step *o* is known as **isochorismate**.[55,56] Isochorismate gives rise to a variety of products including **vitamin K**, salicylic acid,[56a] the iron chelator **enterobactin** (Fig. 16-1), and other siderophores. These are formed in *E. coli* via 2,3-dihydroxybenzoate as indicated in Fig. 25-2.[57–59a] The genes (*ent*) for the requisite enzymes are clustered at 14 min on the *E. coli* chromosome map (Fig. 26-4).

During the conversion of anthranilate to tryptophan, two additional carbon atoms must be incorporated to form the indole ring. These are derived from **phosphoribosyl pyrophosphate (PRPP)** which is formed from ribose 5-phosphate by transfer of a *pyrophospho* group from ATP.[60,61] The –OH group on the anomeric carbon of the ribose phosphate displaces AMP by attack on $P_\beta$ of ATP (Eq. 25-5). In many organisms the enzyme that catalyzes this reaction is fused to subunit II of anthranilate synthase.[62] PRPP is also the donor of phosphoribosyl groups for biosynthesis of histidine (Fig. 25-13) and of nucleotides (Figs.




5-Phosphoribosyl 1-pyrophosphate (PRPP) (25-5)

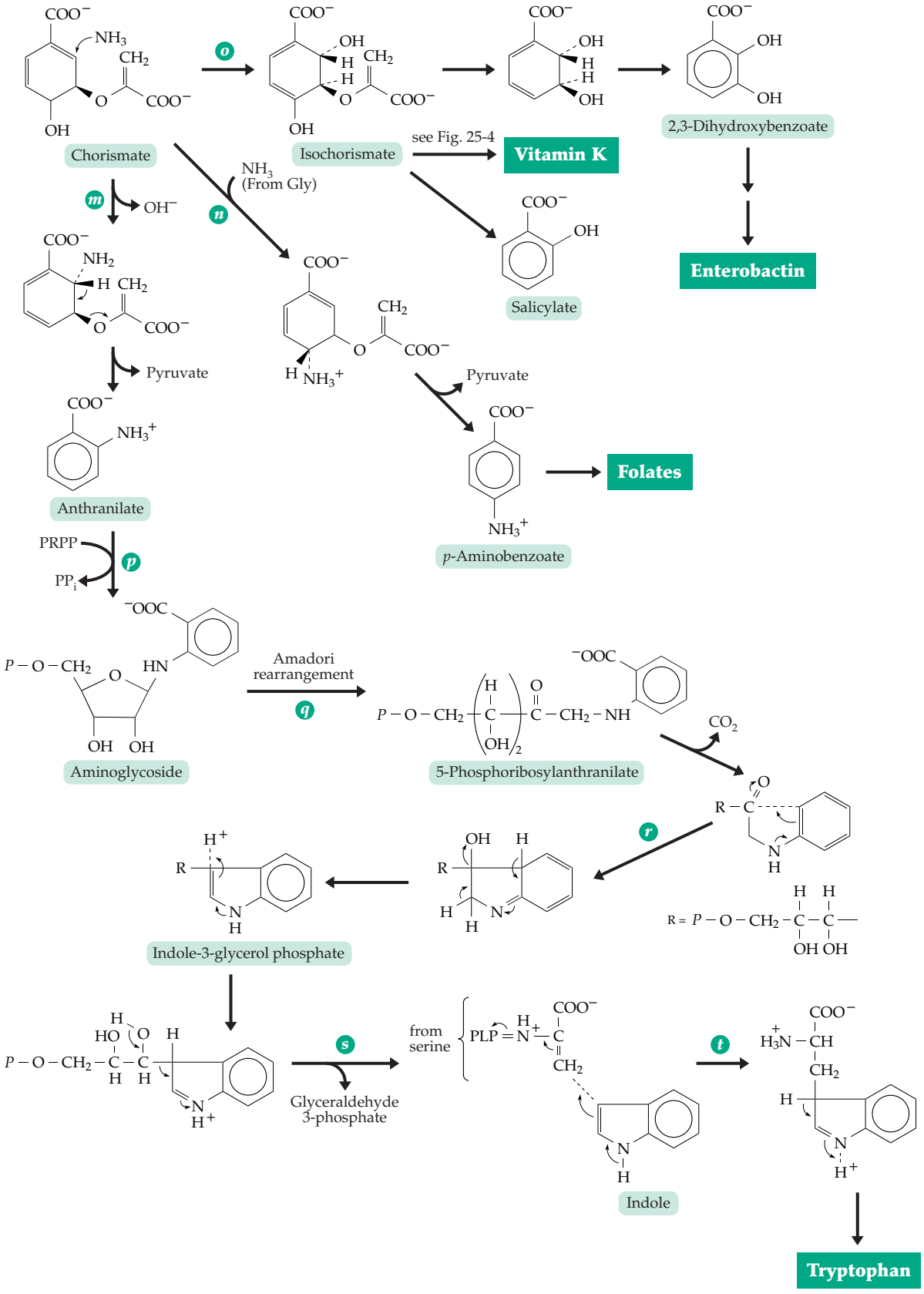



**Figure 25-2** The biosynthesis of tryptophan from chorismate and related synthetic reactions.

25-14, 15). In tryptophan biosynthesis PRPP is converted into an aminoglycoside of anthranilic acid by displacement of its pyrophospho group by the amino group of anthranilate (Fig. 25-2, step *p*). The aminoglycoside then undergoes an **Amadori rearrangement** (Eq. 4-8; Fig. 25-2, step *q*). The product has an open chain. Decarboxylation and ring closure, as indicated in this figure, yields **indoleglycerol phosphate**.[63,64]

A β replacement reaction catalyzed by the PLP-dependent **tryptophan synthase** converts indoleglycerol phosphate and serine to tryptophan. Tryptophan synthase from *E. coli* consists of two subunits associated as an $\alpha_2\beta_2$ tetramer (Fig. 25-3). The α subunit catalyzes the cleavage (essentially a reverse aldol) of indoleglycerol phosphate to glyceraldehyde 3-phosphate and free indole (Fig. 25-2, step *s*).[67] The β subunit contains PLP. It presumably generates, from serine, the Schiff base of aminoacrylate, as indicated in Fig. 25-2 (step *t*). The enzyme catalyzes the addition of the free indole to the Schiff base to form tryptophan. The indole must diffuse for a distance of 2.5 nm through a tunnel to the active site where it condenses with the aminoacrylate Schiff base.[65–68c]

The genes encoding the seven enzymes of the tryptophan biosynthetic pathway are organized as a single operon in some bacteria.[69] Its regulation in enteric bacteria is discussed in Chapter 28, Section A,5. The α subunit of tryptophan synthase and the enzymes catalyzing the preceding two steps in tryptophan synthesis are all $(\beta\alpha)_8$-barrel proteins similar to the one shown in Fig. 2-28.[69,70] The biosynthetic pathway for tryptophan in the green plant *Arabidopsis* is the same as in bacteria. The enzymes appear to be present in the chloroplasts.[71]

While the tryptophan synthase of *E. coli* is made up of two different subunits, that of *Neurospora* is a single polypeptide chain. This is one of the first proteins for which it was proposed that there were originally two separate genes, as in *E. coli*, but that they became fused during the course of evolution. After this proposal was made, gene fusion was demonstrated experimentally in *Salmonella* by introduction of two consecutive "frame shift mutations" between two genes of histidine biosynthesis (Chapter 26, Section B,1). Because of the frame shift, the stop signal for protein synthesis is no longer read, with the result that the organism makes a single long protein corresponding to both genes. Gene fusion evidently occurs in nature frequently.[63] There are many instances known in which the two distinctly different catalytic activities are possessed by the same protein in some organisms but by separate enzymes in others. The gene for the α subunit of trytophan synthase in *Salmonella* was of historical importance as it was used to establish the colinearity of the genetic code and its amino acid sequence (Chapter 26, Section B,5).

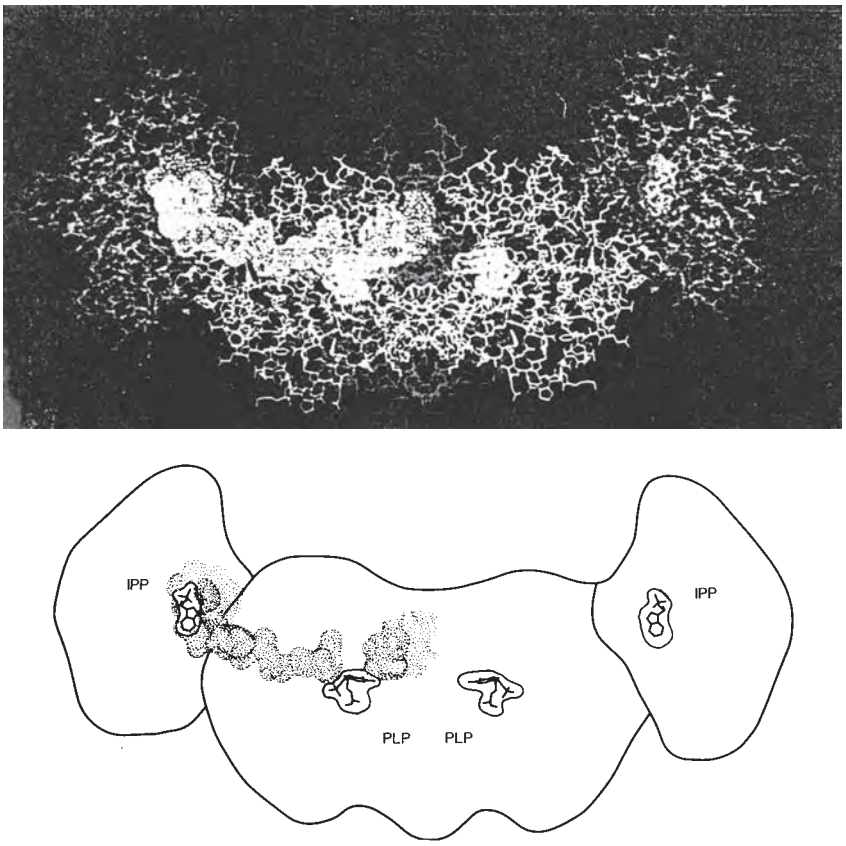



**Figure 25-3** The structure of the two-enzyme $\alpha_2\beta_2$ complex tryptophan synthase.[65,66] The view is with the twofold axis of the $\alpha_2\beta_2$ complex vertical with the two α subunits at the ends and the β subunits in the center. The tunnel through which indole molecules released from indole propanol phosphate (IPP) in the α subunits to the pyridoxal phosphate (PLP) in the β subunits is shaded. Courtesy of C. Craig Hyde and Edith Wilson Miles.

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

Radioactive carbon of [$^{14}$C]shikimate is efficiently incorporated into the quinones, vitamin K, and tocopherols. These chemically related redox agents (Fig. 15-24) also have a related biosynthetic origin, which has been elucidated in greatest detail for ubiquinone. In bacteria *p*-hydroxybenzoate is formed

directly from chorismate (Fig. 25-1), but in plants it may originate from phenylalanine, *trans*-cinnamate, or *p*-coumarate as indicated in Fig. 25-4. The conversion of *p*-hydroxybenzoate on to the ubiquinones is also shown in this figure. A polyprenyl group is transferred onto a position ortho to the hydroxyl (see Chapter 22). Then a series of consecutive hydroxylation and *S*-adenosylmethionine-dependent transmethylation reactions lead directly to the ubiquinones.[72–73b] Several qui-nones that can serve as precursors to ubiquinones have been isolated from bacteria. Two of the corresponding quinols are shown as intermediates in Fig. 25-4. Chemical considerations suggest that both the methylations and hydroxylations occur on the reduced dihydroxy derivatives. A closely similar pathway is used for synthesis of ubiquinone[74–76] in mitochondria and in the membranes of the endoplasmic reticulum of fungal, plant, and animal cells.[77,78] In bacteria the decarboxylation step occurs early, as shown in Fig. 25-4, whereas in eukaryotes it occurs later.[75] Ubiquinone is poorly absorbed from the blood and it is apparently made in all aerobic tissues.[78] Ubiquinones are thought to serve as important antioxidant compounds in cell membranes. Dietary supplementation may be of value.[73a] Curiously, the nematode *C. elegans* slows its metabolism and lives longer if it has a defect in the hydroxylase catalyzing the next to last step in biosynthesis (Fig. 25-4).[73c] Mutants of *C. elegans* that cannot form their own ubiquinone-9 (containing nine prenyl units in the side chain) are unable to grow on bacteria that make ubiquinone-8. The worms appear to have both essential mitochondrial and nonmitochondrial requirements for ubiquinone.[73d] A ubiquinone deficiency with serious consequences can sometimes be caused in humans by inhibitors of 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase) taken to lower blood cholesterol.[79]

Labeling experiments have shown that the plastoquinones of chloroplasts as well as the tocopherols each bear one methyl group (marked with an asterisk in Fig. 25-4) that originates from chorismate. The dihydroxy compound **homogentisate** is probably an intermediate.[80–83] It is a normal catabolite of tyrosine in the animal body (Fig. 25-5, Eq. 18-49). Both prenylation and methylation by AdoMet are required to complete the synthesis of the plastoquinones and tocopherols. Possible biosynthetic intermediates with one or more double bonds in the polyprenyl side chain have been found in plants and also in fish oils.[83a]

The vitamins K and other naphthoquinones arise from ***O*-succinylbenzoate**[84–86] whose synthesis from chorismate and 2-oxoglutarate depends upon a thiamine diphosphate-bound intermediate, as indicated in Fig. 25-4. Elimination of pyruvate yields *O*-succinylbenzoate. The remaining reactions of decarboxylation, methylation, and prenylation (Fig. 25-4) resemble those of ubiquinone synthesis.

## B. Metabolism of Phenylalanine and Tyrosine

Figure 25-5 shows the principal catabolic pathways, as well as a few biosynthetic reactions, of phenylalanine and tyrosine in animals. Transamination to phenylpyruvate (reaction *a*) occurs readily, and the product may be oxidatively decarboxylated to phenylacetate. The latter may be excreted after conjugation with glycine (as in Knoop's experiments in which phenylacetate was excreted by dogs after conjugation with glycine, Box 10-A). Although it does exist, this degradative pathway for phenylalanine must be of limited importance in humans, for an excess of phenylalanine is toxic unless it can be oxidized to tyrosine (reaction *b*, Fig. 25-5). Formation of phenylpyruvate may have some function in animals. The enzyme **phenylpyruvate tautomerase**, which catalyzes interconversion of enol and oxo isomers of its substrate, is also an important immunoregulatory cytokine known as **macrophage migration inhibitory factor**.[86a]

The pterin-dependent hydroxylation of phenylalanine to tyrosine (Eq. 18-45)[87,87a] has received a great deal of attention because of the occurrence of the metabolic disease **phenylketonuria (PKU)**,[88–91b] in which this reaction is blocked. Infants born with phenylketonuria appear normal but mental retardation sets in rapidly. However, if these infants are identified promptly and are reared on a low-phenylalanine diet which supplies only enough of the amino acid for essential protein synthesis, most brain damage can be prevented. Throughout most of the world every infant born is now given a simple urine test to identify phenylketonuria. Tolerance to phenylalanine increases with age, and adults may return to a near normal diet. However, there may still be problems with increased phenylalanine levels during fever and infections. A high phenylalanine level during pregnancy may damage the unborn child. Temporary insertion of multitubular reactors containing phenylalanine-ammonia lyase (Eq. 14-45) can be of value[92] as is administration of the enzyme in encapsulated form.[93] The mechanism by which phenylalanine damages the brain is uncertain.

### 1. Catabolism of Tyrosine in Animals

The major route of degradation of tyrosine in animals begins with transamination (Fig. 25-5, reaction *c*) to ***p*-hydroxyphenylpyruvate**. The enzyme tyrosine aminotransferase[94] is induced in the liver in response to the action of glucorticoid hormones (Chapter 22). The synthesis of the enzyme is also controlled at the translational level, release of the newly formed protein from liver ribosomes being stimulated by cyclic AMP. The enzyme is subject to posttranscriptional

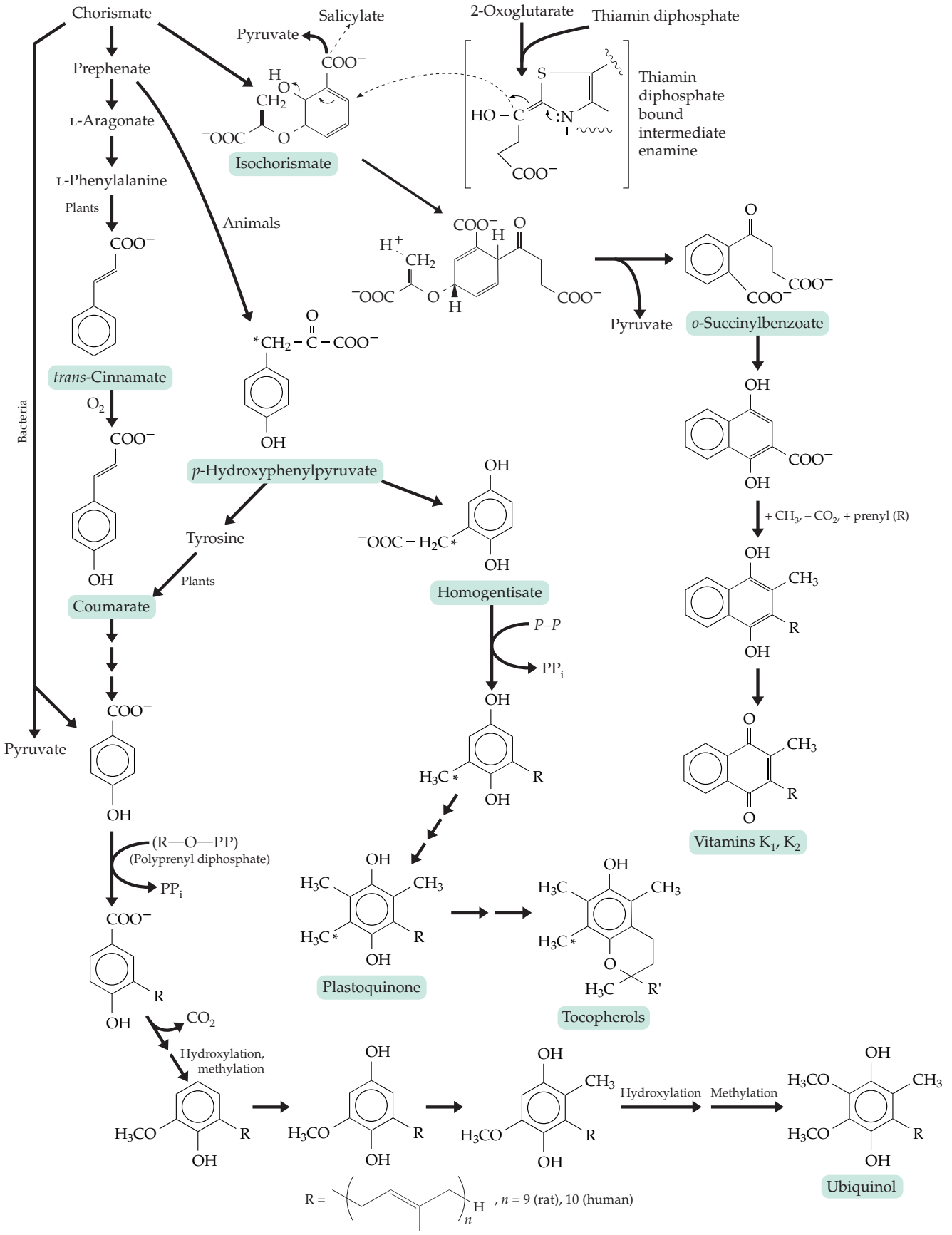
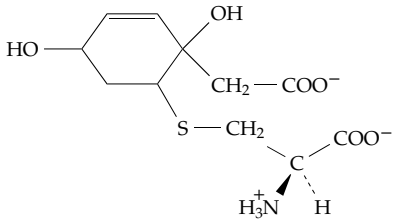



**Figure 25-4** Pathways of biosynthesis of ubiquinones, plastoquinones, tocopherols, and vitamin K.

modification including phosphorylation and it undergoes unusually rapid turnover.[95,96]

The 2-oxoacid *p*-hydroxyphenylpyruvate is decarboxylated by the action of a dioxygenase (Eq. 18-49). The product **homogentisate** is acted on by a second dioxygenase, as indicated in Fig. 25-5, with eventual conversion to fumarate and acetoacetate. A rare metabolic defect in formation of homogentisate leads to tyrosinemia and excretion of **hawkinsin**[97] a compound postulated to arise from an epoxide (arene oxide) intermediate (see Eq. 18-47) which is detoxified by a glutathione transferase (Box 11-B).

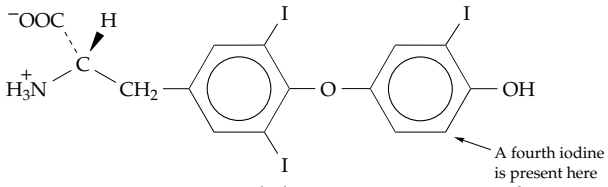


Hawkinsin

One of the first "inborn errors of metabolism" to be recognized was **alkaptonuria**, a lack of the oxygenase that cleaves the ring of homogentsic acid.[98] The condition is recognized by a darkening of the urine upon long standing (caused by oxidation of the homogentisate). Alkaptonuria was correctly characterized by Garrod (Box 1-D) in 1909 as a defect in the catabolism of tyrosine. Although relatively mild and not reducing the life span of individuals, it is nearly always accompanied by arthritis in later years and also by gray to bluish-black pigmentation of connective tissues, which may be visible through the shin or in the scleras (whites) of the eyes.[98] Absence of the next enzyme in the pathway, **maleylacetoacetate isomerase** causes one type of tyrosinemia.[99,99a] Absence of **fumarylacetoacetate hydrolase**, which acts on the product of the isomerase action causes the severe **type 1 hereditary tyrosinemia** which leads to accumulation of the toxic fumarylacetoacetate and its decarboxylation product succinylacetoacetate.[99,100]

## 2. The Thyroid Hormones

An important product of tyrosine metabolism in vetebrates is the thyroid hormone[101] of which the principal and most active forms are **thyroxine** ($T_4$) and **triiodothyronine** ($T_3$).[102] The thyroid gland is rich in iodide ion, which is actively concentrated from the plasma to ~1 μM free $I^-$.[103] This iodide reacts under the influence of a peroxidase (see Fig. 16-14 and accompanying discussion)[104] to iodinate tyrosyl residues of the very large ~660-kDa dimeric **thyroglobulin**, which is stored in large amounts in the lumen of the thyroid follicles.[105] Several of the tyrosine side chains (up to 15–25) are iodinated to form **mono-** and **diiodotyrosine** residues (Eq. 25-6), but only between four and eight of these, which are located at specific positions, are converted on to the hormones.[106,107]



Triiodothyronine

The coupling reaction by which the aromatic group from one residue of mono- or diiodotyrosine is joined in ether linkage with a second residue is also catalyzed readily by peroxidases. One dehydroalanine residue is formed for each molecule of hormone released.[108] A possible mechanism involves formation of an electron-deficient radical, which can undergo β elimination to produce a dehydroalanine residue and an aromatic radical. The latter could couple with a second radical to form triiodothyronine or thyroxine. However, as depicted in Eq. 25-6, the radical coupling may occur prior to chain cleavage. While β elimination (pathway A) has been favored, recent evidence suggests hydroxylation and cleavage to form a residue of aminomalonic semialdehyde in the thyroglobulin chain (pathway B).[108a] Alternatively, a PLP-dependent elimination of the radical could be used. Another possibility is oxidative attack on the 2-oxoacids derived from the iodotyrosines.

Thyroxine and triiodothyronine are released from thyroglobulin through the action of a series of proteases. Both the protease action and the release of the thyroid hormones into the bloodstream are stimulated by pituitary **thyrotropin (TSH)**.[109,110] Like glucagon thyrotropin is released from the pituitary in response to **thyrotropin-releasing hormone.**[111] Thyrotropin probably acts through cAMP-mediated mechanisms.[112] The hormones are carried throughout the body while bound to **thyroxine-binding globulin,** which serves as a carrier.[113] Some hormone is carried by other serum proteins such as **transthyretin** (thyroxine-binding prealbumin).[113,114] Both thyroxine and triiodothyronine have powerful hormonal effects on tissues, but the lag time for a response is shortest for triiodothyronine. Thus, it is thought that thyroxine undergoes loss of one iodine atom to form the more active triiodo form of the hormone within the target cells. Three **iodothyronine deiodinases**, all of which are selenoproteins, have been identified (Eq. 15-60).[115–116a]

Organically bound iodine is found in various invertebrates, but with one possible exception

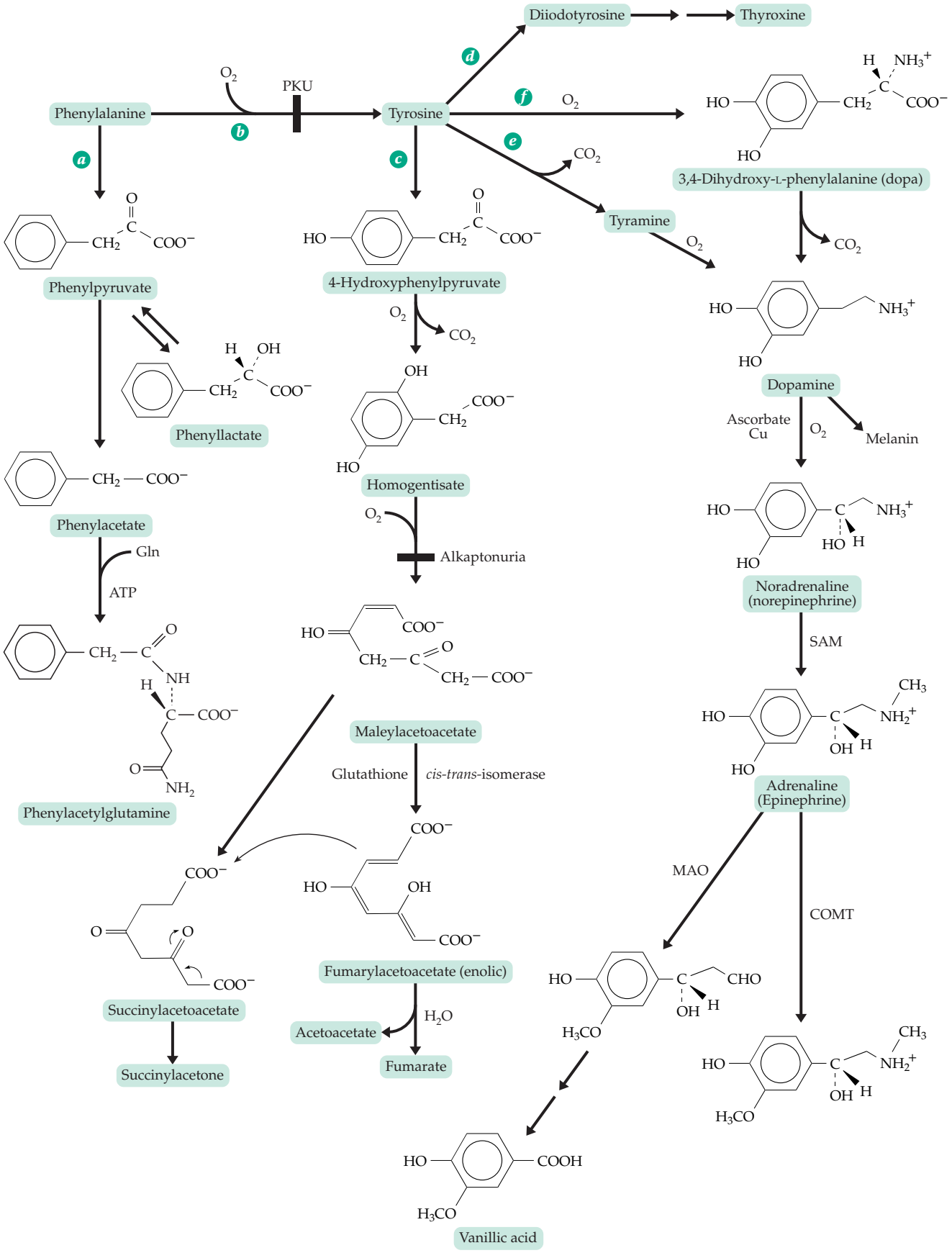
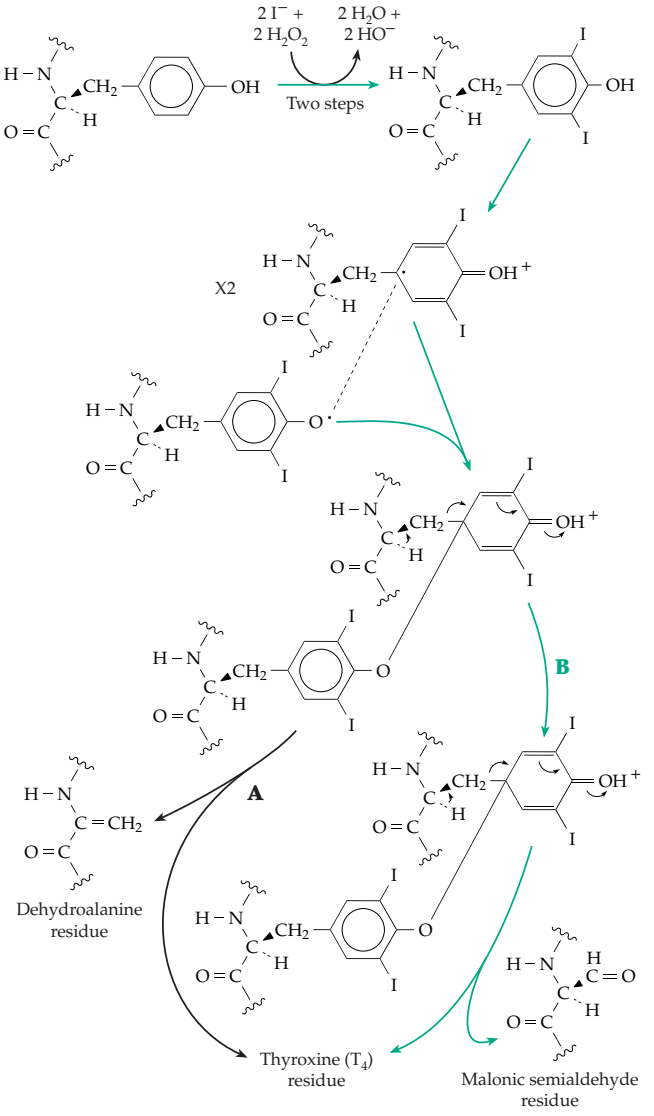



**Figure 25-5** Some routes of metabolism of phenylalanine and tyrosine in animals.




(25-6)

thyroxine is present only in vertebrates.[101] Why do we need this iodinated hormone? Halogen-free analogs in which the iodine atoms of thyroxine and triiodothyronine have been replaced by methyl or other alkyl groups are biologically active. Frieden[101] concluded that the role of iodine is related more to the evolution of biosynthetic and catabolic pathways than to chemical properties of the hormones themselves.

Thyroxine and triiodothyronine have many effects, a major one in mammals and birds being stimulation of energy metabolism in tissues. It has long been recognized that a deficiency of thyroid hormone is reflected in an overall lower basal metabolic rate (Chapter 6). Maley and Lardy observed that thyroxine uncouples oxidative phosphorylation (Chapter 18) in isolated mitochondria.[117] When mitochondria from animals receiving extra thyroxine were compared with those from control animals, an increased rate of electron transport was observed. However, there was little or no change in the P / O ratio. Thus, the hormone apparently increased the rate of electron transport without decreasing the overall efficiency of ATP synthesis.

Thyroid hormones also have a general effect on growth and development in all vertebrates. This is especially striking in amphibia in which thyroid hormones control the metamorphosis from the tadpole to adult stages.[101,118] Zebrafish, and presumably also other fishes, require thyroid hormone to complete their life cycles.[119] At present it is thought that most, if not all, effects of thyroid hormones are a result of their action on the transcription of genes.[120–123] Nuclear **thyroid hormone receptors** belong to a family of ligand-regulated transcription factors that respond to steroid, retinoid, and thyroid hormones (Table 22-1).[123–124] These proteins control many metabolic functions, often forming heterodimers with other receptors and also being activated by coactivators[125] or corepressors.[124,125a,b] Transcription of the genes for the thyroxine-synthesis proteins **thyroperoxidase**, **thyroglobulin**, and **iodide transporter**[125c] is regulated by a **thyroid transcription factor**.[126]

A number of thyroid-related diseases are known. Thyroid deficiency is often evident by enlargement of the thyroid gland (**goiter**). The deficiencies may involve inadequacy in dietary intake of iodine, transport of iodide into the thyroid, poor formation of iodinated thyroglobulin, inefficient coupling to form the iodinated thyronine residues,[127] or mutations in thyroid hormone receptors.[122,128] A major cause of goiter is a deficiency in the content of iodine in soil, a condition affecting about one billion ($10^9$) persons. A more severe effect of thyroid deficiency is the fetal brain damage called **cretinism**.[125c,129] Victims are mentally retarded, deaf-mute, and often with motor rigidity. In Grave disease, the commonest type of **hyperthyroidism**, the blood contains specific thyroid-stimulating autoantibodies.[130,131] These bind to the thyrotropin (TSH) receptors of the thyroid plasma membrane and stimulate excessive formation of thyroid hormone.

## 3. The Catecholamines

A combination of decarboxylation and hydroxylation of the ring of tyrosine produces derivatives of *o*-dihydroxybenzene (catechol), which play important roles as neurotransmitters and are also precursors to **melanin**, the black pigment of skin and hair. Catecholamines may be formed by decarboxylation of tyrosine into tyramine (step *e*, Fig. 25-5) and subsequent oxidation. However, the quantitatively more important route is hydroxylation by the reduced pterin-dependent tyrosine hydroxylase (Chapter 18) to 3,4-dihydroxyphenylalanine, better known as **dopa**. The latter is decarboxylated to **dopamine**.[131a] Hydroxylation of dopamine by an ascorbic acid and

copper-requiring enzyme (Eq. 18-53) produces the important hormone **noradrenaline** (norepinephrine), which is methylated to form **adrenaline** (epinephrine).

There are two principal catabolic routes for destruction of these catecholamines as is illustrated for adrenaline in Fig. 25-5. **Monoamine oxidase** (MAO)

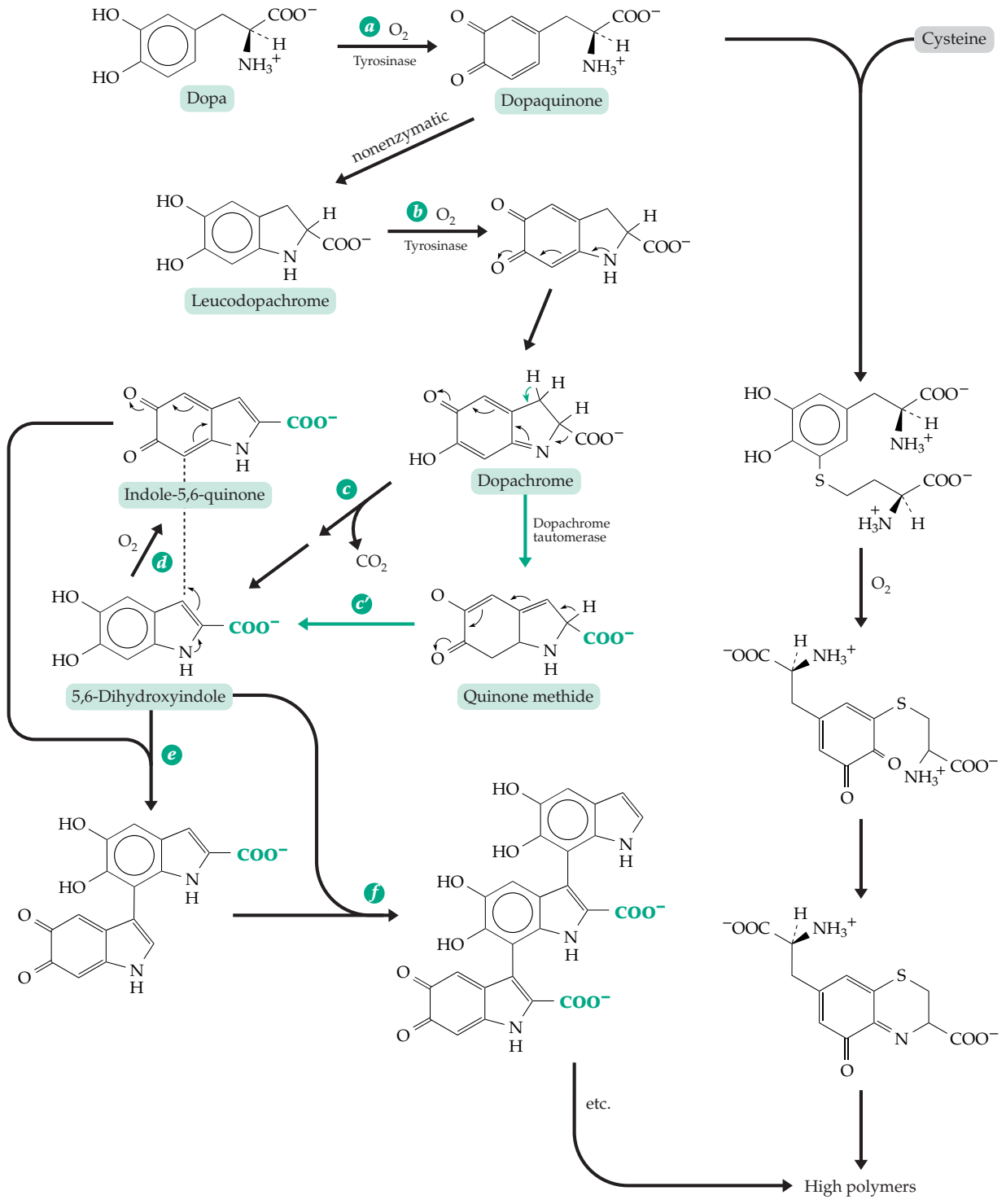


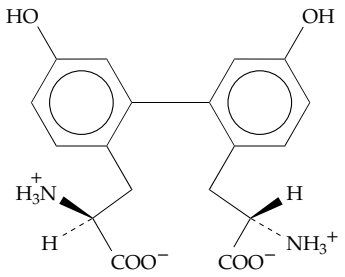

**Figure 25-6** Postulated pathways for synthesis of the black pigment melanin and pigments (phaeomelanins) of reddish hair and feathers. Dopachrome reacts in two ways, with and without decarboxylation. The pathway without decarboxylation is indicated by green arrows. To the extent that this pathway is followed the green carboxylate groups will remain in the polymer. The black eumelanin is formed by reactions at the left and center while the reddish phaeomelanin is derived from polymers with cysteine incorporated by reactions at the right.

causes oxidative cleavage with deamination. Subsequent oxidative fission of the side chain together with methylation yields such end products as vanillic acid, which is excreted in the urine. The second catabolic route is immediate O-methylation by **catecholamine *O*-methyltransferase** (COMT), an active enzyme in neural tissues. The metabolites are relatively inactive physiologically and may be secreted as such or may undergo further oxidative degradation.

## 4. The Melanins

Dihydroxyphenylalanine (dopa) darkens rapidly when exposed to oxygen. The process is hastened greatly by tyrosinase (Chapter 16), which also catalyzes reaction *f* of Fig. 25-5, the oxidation of tyrosine to dopa. Tyrosinase is found in animals only in the organelles known as **melanosomes**, which are present in the melanin-producing **melanocytes** (Boxes 8-F; 25-A).[132] A series of enzymatic and nonenzymatic oxidation, decarboxylation, and coupling reactions forms the pigments. The initial steps for one pathway are indicated in Fig. 25-6. Oxidation of dopa to dopaquinone (step *a*)[133,134] is followed by an intramolecular addition reaction, together with tautomerization to the indole derivative, leucodopachrome. A second oxidation by tyrosinase (step *b*) is followed by decarboxylation and tautomerization to 5,6-dihydroxyindole (step *c*).[135] Alternatively, the tautomerization steps may take place without decarboxylation (green arrows, step *c'*).[133,136] In either case the dihydroxyindole that is formed can undergo a third oxidation step, also catalyzed by tyrosinase, to form indole-5,6-quinone (step *d*). Coupling of the last two products as indicated in step *e* yields a dimer, which is able to continue the addition of dihydroxyindole units (step *f*, etc.) with oxidation to form a high polymer of the black true melanin (**eumelanin**). However, the structure is not regular and is crosslinked. A related series of red polymers, the **phaeomelanins** found in red hair and feathers, are formed by addition of cysteine to dopaquinone.[137,138] Addition is possible at more than one position. The resulting adducts (only one is shown) can undergo oxidative ring closure in the manner indicated. Control of melanin formation is also complex. For example, more than 50 genetic loci affect the coat color of the house mouse.[139] Melanin in some fungi is formed by oxidative reactions of tetrahydroxynaphthalene formed via the polyketide pathways (Chapter 21).[140,140a] The melanin "inks" produced by cuttlefish and other cephalopods are formed in much the same way as melanins of skin.[140a]

Dopa is converted by at least some insects into *N*-β-alanyldopamine, which is a preferred substrate for the *o*-diphenol oxidase of the insect pupal cuticle. Oxidation of this substrate plays a crucial role in the hardening and darkening of the cuticle during pupal tanning.[141–142] There are many other oxidative reactions of tyrosine side chains within proteins. These include coupling of free radicals formed by peroxidases[143] or ultraviolet light[144,144a] to form dityrosines and other products. The walls of yeast ascospores,[145,146]



Dityrosine
Other isomers are also formed

the cements formed by reef-building annelids,[147,148] and adhesive plaques of marine mussels[149,149a] all contain polyphenolic proteins. The 120-kDa "foot protein" of the mussel *Mytilus edulis* consists of tandemly repeated decapeptides, each containing 2 residues of lysine, 1–2 residues of dopa, 1–2 residues of *trans*-4-hydroxyproline, and 1 residue of *trans*-2,3, *cis*-3,4-dihydroxyproline.[149]

## 5. Microbial Catabolism of Phenylalanine, Tyrosine, and Other Aromatic Compounds

Bacteria and fungi play an essential role in the biosphere by breaking down the many aromatic products of plant metabolism.[150–153] These include vast amounts of lignin, alkaloids, flavonoid compounds, and other biochemically "inert" substances. Lignin is a major constituent of wood and a plant product second only to cellulose in abundance.

The chemical reactions used to degrade these aromatic compounds are numerous and complex. As was mentioned in Chapter 16, some fungi initiate the attack on lignin with peroxidases and produce soluble compounds that can be attacked by bacteria. In other cases elimination reactions may be used to initiate degradation. For example, some bacteria release phenol from tyrosine by β elimination (Fig. 14-5). However, more often hydroxylation and oxidative degradation of side chains lead to derivatives of benzoic acid or of the various hydroxybenzoic acids.[150,151,154–155a]

After the initial reactions many of the compounds are channeled into one of the major pathways illustrated in Fig. 25-7.[151,156,157] Dominant in aerobic bacteria is the conversion to **3-oxodipate** by one of the two convergent pathways shown. The products succinate and acetyl-CoA are readily oxidized by the citric

## BOX 25-A SKIN COLOR

The principal pigment of human skin, hair, and eyes is **melanin**, which is synthesized in specialized cells, the **melanocytes**. They lie between the epidermis (outer layer) and the dermis (inner layer) as shown in Box 8-F. Melanocytes originate from embryonic nervous tissue and migrate into the skin by the third month of fetal life. They retain the highly branched morphology of neurons. Persons of different races all have the same numbers of melanocytes but the numbers and sizes of the pigmented melanosomes (Box 8-F) vary as does the content and chemical composition of the melanin.[a–d] Melanosomes not only are found in the dendrites of the melanocytes but are transferred from them into adjacent epithelial cells.[e,f]

**Nevi** (moles) are clusters of melanocytes that start to appear in the third year of life. They gradually increase in numbers but disappear in old age. **Freckles** appear beginning at about age six in genetically susceptible individuals. They are regions in which a higher concentration of melanin is formed.[a]

Both hair and the iris of the eye are also pigmented by melanin. Although dark eyes and dark hair are more prevalent among persons with dark skin there is no direct correlation. This is only one piece of evidence that the genetics of skin, eye, and hair coloration is complex. In mice over 150 different mutations occurring at more than 50 distinct genetic loci affect pigmentation.[f] Melanin formation begins with the action of tyrosinase. The human genome contains at least three genes for tyrosinase and related proteins.[b,g] The *Tyr* gene is absent in **oculocutaneous albinism**, the lack of pigment in eyes, hair, and skin. The tyrosinase-related protein 2 (TRP2), which has been identified as **dopachrome tautomerase** (see Fig. 25-6), is also a member of the tyrosinase family. Although a key enzyme in pigment synthesis, the amount of tyrosinase or of tyrosinase mRNA is the same in all skin types and colors.[h] Thus, differences in skin color must arise from differences in regulation.

Regulation of melanin formation is achieved in part by hormones, the **melanocyte-stimulating hormone** (MSH or melanotropin) being the most important.[a,f,g] The 13-residue pituitary hormone greatly increases pigmentation and stimulates differentiation of melanocytes. Other regulatory influences arise from interleukins, prostaglandins, interferons, tetrahydrobiopterins,[h] and protein kinase C.[i] Light also has a major effect, causing rapid tanning, especially in darker skin. Release of NO and cyclic GMP may be involved.[j]

Melanin and phaeomelanins have an important role in protecting skin from sunlight. This includes protection of light-sensitive vitamins, proteins, and DNA and RNA. The correlation of high pigmentation with the high intensity of light in tropical regions may reflect this property. Light-skinned persons of northern and southern latitudes, where light intensity is weaker, are less pigmented, allowing more adequate synthesis of vitamin D in the skin (Box 22-C).

A total lack of melanin as a result of a defective *Tyr* gene is seen in oculocutaneous albinism. Lacking protection from sunlight by melanin, albino individuals must shield their skin and eyes carefully. A second type of albinism results from mutations in the P gene, known in the mouse as the "pink-eyed dilution locus." In this condition synthesis of phaeomelanin is not impaired. Mutations in the *KIT* gene, which encodes a tyrosine kinase receptor lead to **piebaldism**, with white and dark splotched skin (or fur in animals).[b,k] While piebaldism is hereditary, **vitiligo** is an acquired autoimmune disease involving spotty loss of pigment and affecting 0.5 to 4% of the world's population. Melanocytes may be present in the affected areas but are unable to make melanin.[h,l]

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acid cycle and associated reactions. Many different compounds can be converted into the starting compounds shown at the top of the figure. Both D- and L-mandelate, toluene, benzyl alcohol, L-tryptophan, phenanthrene, naphthalene, and benzene can be converted to catechol and be metabolized via the catechol branch of the pathway. Benzoate, *p*-toluate, shikimate, and quinate can be metabolized via the protocatechuate branch. Halogenated compounds, e.g., 3-chlorocatechol, may sometimes be degraded via

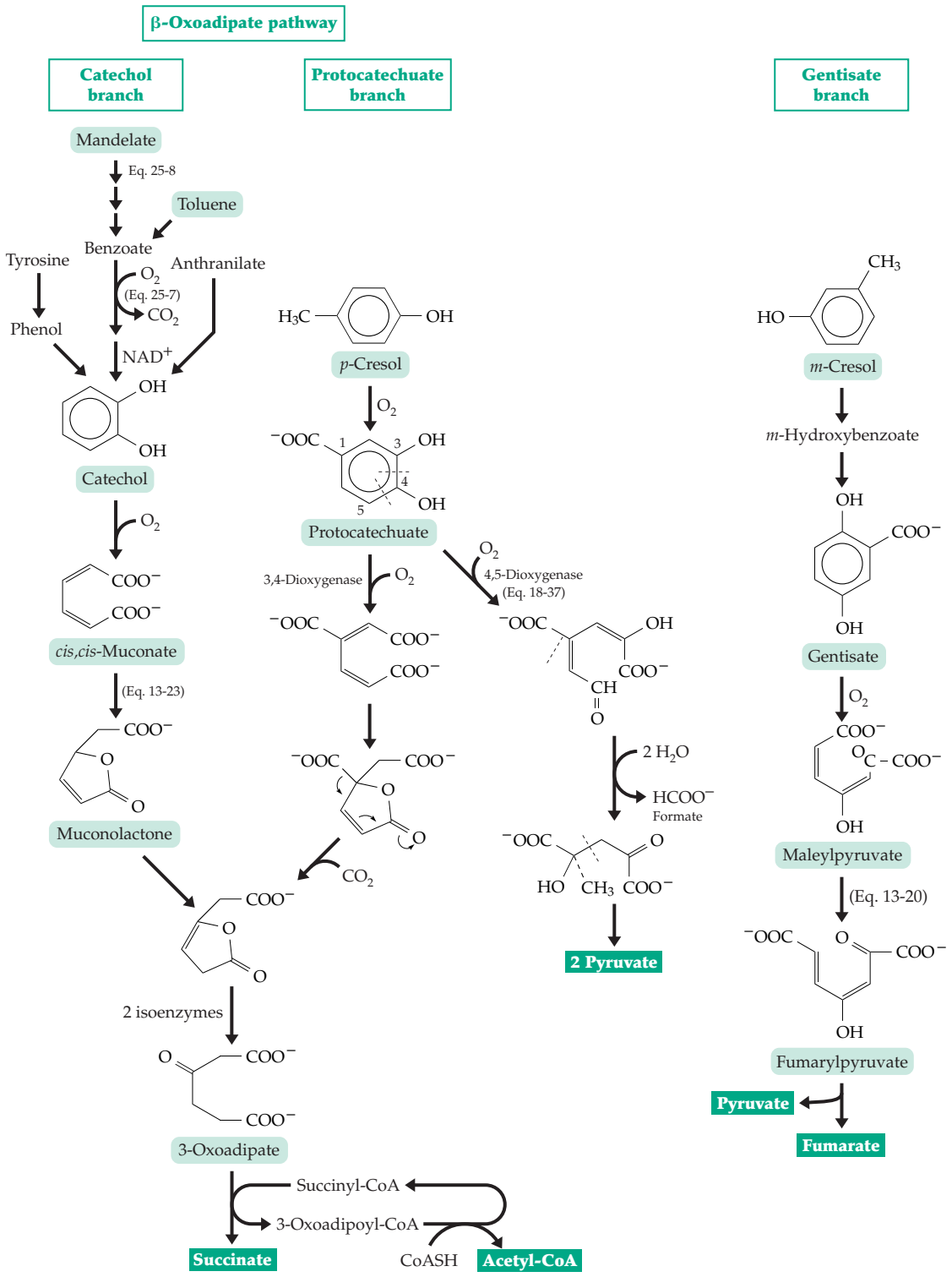
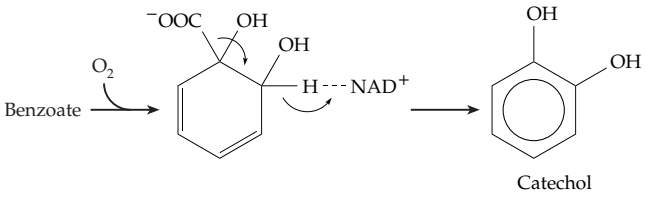



**Figure 25-7** A few pathways of catabolism of aromatic substances by bacteria.

the same pathways[151,156] or in parallel or related pathways.[157–158c] However, chlorine atoms are sometimes eliminated as $Cl^-$ at some point in the process.[159–160a] Other substrates, including *m*-hydroxybenzoate and sometimes anthranilate, are degraded via the gentisate pathway (Fig. 25-7).[157,161] Both benzoate and phenylacetate are sometimes degraded after conversion to coenzyme A thioesters.[161a,b]

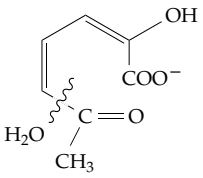
Dioxygenases play a major role in all of these pathways of aromatic catabolism. In most cases a dioxygenase (Chapter 18) is required for the opening of the benzene ring. The pathways contain interesting isomerization steps, some of which have been discussed in Chapter 13, Section B. Microorganisms often have alternative choices in the chemistry of their attack. For example, tyrosine can be converted by one bacterium to homogentisate, as in animals (Fig. 25-5), or by other bacteria to protocatechuate, homoprotocatechuate, or gentisate (Fig. 25-7) before the ring is opened.[150] A single compound can be acted upon by more than one dioxygenase. Thus protocatechuate can be opened by a 3,4-dioxygenase or by a 4,5-dioxygenase (Fig. 18-22) leading to the branch point at protocatechuate in Fig. 25-7.

The initial hydroxylation of benzene, toluene, and other alkylbenzenes is accomplished by multicomponent aromatic ring dioxygenases that introduce two oxygen atoms to form diols.[158] Dioxygenation of benzoate yields a diol that can be **oxidatively decarboxylated** by reaction with $NAD^+$ (Eq. 25-7) to form catechol.[157,162] Toluene gives 3-methylcatechol




(25-7)

whose ring is, however, opened by an extradiol 2,3-dioxygenase, a so called *meta*-cleavage.[163,164] The product, 2-hydroxy-6-oxohepta-2,4-dienoate, is cleaved hydrolytically as indicated on the structure



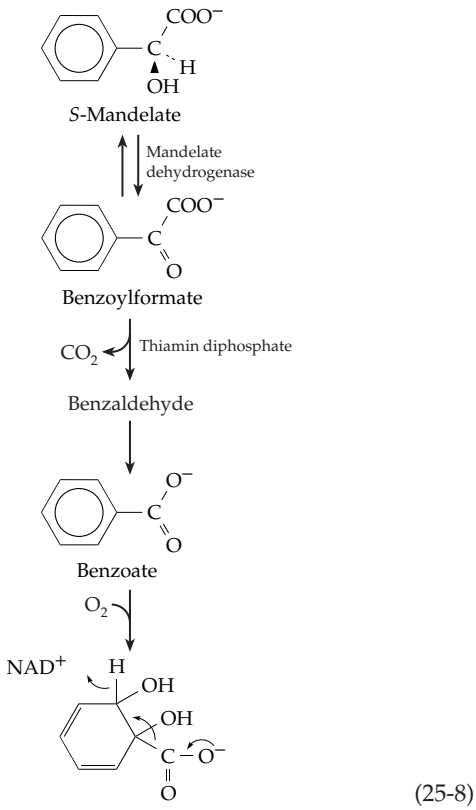

2-Hydroxy-6-oxohepta-2,4-dienoate

to give acetic acid and 2-hydroxypent-2,4-dienoate, which can be further metabolized. The hydrolytic cleavage is unusual[164] but is a β cleavage similar to the C–C bond cleavage by ribulose bisphosphate carboxylase (Eq. 13-48). Toluene can also be oxidized via benzoate through the β-oxoadipate pathway.

The plant acid *S*-mandelate must undergo conversion to *R*-mandelate by action of a racemase (Fig. 13-5) dehydrogenation, and side-chain cleavage as shown in Eq. 25-8 to form benzoate before it can be metabolized further.[165]

Although pseudomonads are well known for aerobic decomposition of aromatic compounds, some strains of *Pseudomonas*, as well as many other bacteria, are able to degrade aromatic compounds under completely anerobic conditions.[166,167] Benzoate can be converted to benzoyl-CoA and the ring can be partially reduced in two ATP- and NADH-dependent reactions (Eq. 25-9). The first of these reduction steps is unusual because ATP is apparently needed to drive the reaction.[166,166a,b] This is analogous to the need for ATP in nitrogen fixation (Eq. 24-6, step *b*).

Toluene, 3-chlorobenzoate, cinnamate, and 2-aminobenzoate can all be converted to benzoyl-CoA and be metabolized via the pathway of Eq. 25-9. Phenol, cresol, coumarate, protocatechuate, and vanillate can be converted to 4-hydroxybenzoyl-CoA and degraded in a similar fashion.[166] The breakdown of various forms of vitamin $B_6$ by bacteria is described in Section F (Eq. 25-24).

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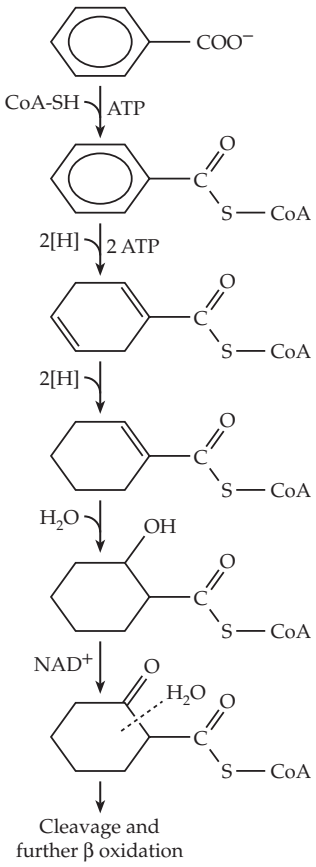
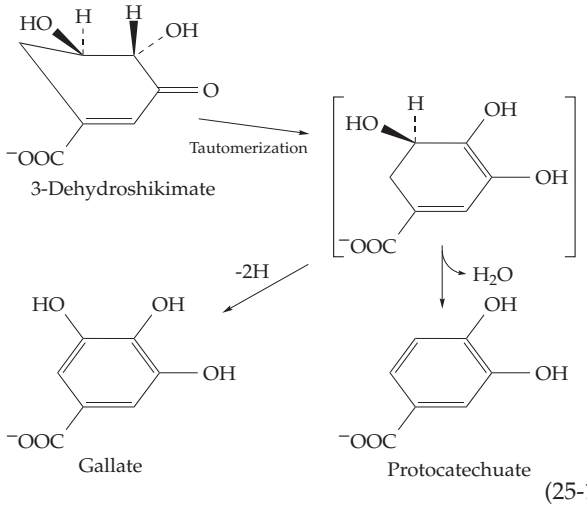



(25-9)

## 6. Quinic and Gallic Acids

Quinic acid, a compound accumulated by many green plants, can be formed by reduction of 3-dehydroquinate (Eq. 25-2) in both plants and bacteria. Quinic acid can be converted into useful industrial products such as benzoquinone and hydroquinone, and its production by bacteria provides a convenient route to these compounds.[168] In the main shikimate pathway 3-dehydroquinate is dehydrated to 3-dehydroshikimate (Eq. 25-3). The latter can be dehydrated further to **protocatechuate** (Eq. 25-10) either nonenzymatically[169] or by enzymatic action in bacteria.[170] Protocatechuate can be decarboxylated enzymatically to catechol, another compound of industrial value.[170] Nonenzymatic oxidation of 3-dehydroshikimate (Eq. 25-10) yields gallate.[169] Gallic acid derivatives are important plant constituents, but the biosynthetic origin has been obscure.[171] Gallate is probably formed from 3-dehydroshikimate as indicated in Eq. 25-10.[172] Esters and other derivatives of gallic acid constitute the **hydrolyzable tannins**. These materials accumulate in the vacuoles of the plants and are also deposited in the bark along with the **condensed tannins**, which are polymeric flavonoid compounds (Box 21-E).

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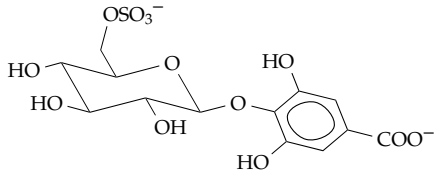



Gallic acid 4-*O*-(β-D-glucopyranosyl-6'-sulfate), the periodic leaf movement factor from *Mimosa*

## 7. The Metabolism of Phenylalanine and Tyrosine in Plants

Some of the pathways of animal and bacterial metabolism of aromatic amino acids also are used in plants. However, quantitatively more important are the reactions of the **phenylpropanoid pathway**,[173–174a] which is initiated by **phenylalanine ammonia-lyase** (Eq. 14-45).[175] As is shown at the top of Fig. 25-8, the initial product from phenylalanine is ***trans*-cinnamate**. After hydroxylation to 4-hydroxycinnamate (*p*-coumarate) and conversion to a coenzyme A ester,[175a] the resulting *p*-coumaryl-CoA is converted into mono-, di-, and trihydroxy derivatives including anthocyanins (Box 21-E) and other flavonoid compounds.[176] The dihydroxy and trihydroxy methylated products are the starting materials for formation of lignins and for a large series of other plant products, many of which impart characteristic fragrances. Some of these are illustrated in Fig. 25-8.

***Benzoic and salicylic acids.*** Two of the simplest plant acids arising from *trans*-cinnamate are **benzoic acid**, accumulated in plums and cranberries, and **salicylic acid**, present in all green plants and accumulated as methyl esters or glycosides in some plants, e.g., those of the willow family. Salicylic acid is made by hydroxylation of benzoic acid,[177] which can be formed from *trans*-cinnamate by β oxidation as depicted in Fig. 25-8, but it may also arise from isochorismate as shown in Fig. 25-2.[178] Salicylic acid plays a central role in resistance of plants to a variety of

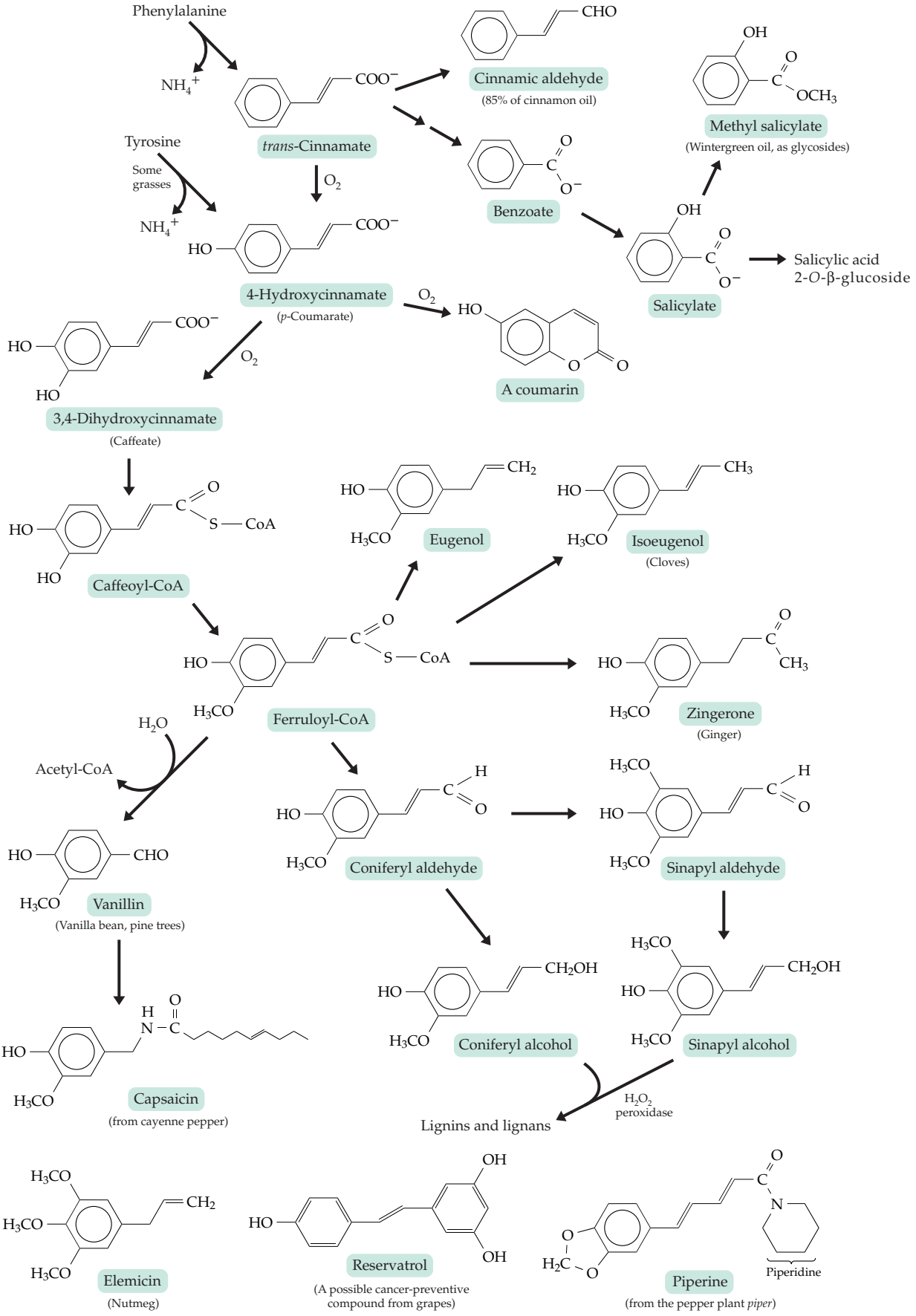
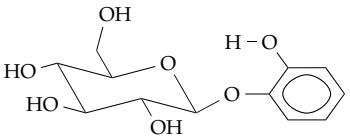



**Figure 25-8** Formation of some plant metabolites from phenylalanine and tyrosine via the phenylpropanoid pathway.

diseases, a phenomenon called **aquired systemic resistance**.[179–181] A large number of defense-related genes are induced by salicylate leading to increased synthesis of phytoalexins, proteins, and lignins. The mechanism may be to inhibit catalases allowing the level of $H_2O_2$ to rise. Hydrogen peroxide not only is a precursor to potent antimicrobial compounds as HOCl (Eqs. 16-12, 16-13) and a participant in lignin synthesis, but it may directly activate transcription of disease resistance genes.[182] This mode of action appears to parallel an **acute-phase response** of the vertebrate immune system through which $H_2O_2$ activates the transcription factor NF-kB (Fig. 5-40; Chapter 28).[182] It is possible that salicylate also has an effect on transcription in the human body.[183]

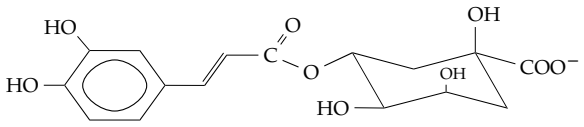
Salicylic acid derivatives, such as aspirin (acetylsalicylic acid), have long been known as pain relievers for the human body. The major effect is thought to be inhibition of a cyclooxygenase (Eq. 21-16). Willow bark has been known since the 18th century to contain a pain reliever, which was identified as salicylate esters and salicyl alcohol derivatives such as salicin.



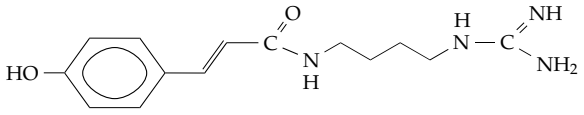

Salicin, a glucoside of salicyl alcohol

***Vanilla and other plant products.*** One of the most widely used natural plant products is **vanilla extract**, which is obtained from cured, unripe fruit of the orchid *Vanilla planifolia*. The curing process releases **vanillin** and related compounds such as vanillic acid and 4-hydroxybenzaldehyde from glycosides. Because the flowers must be hand pollinated every day, natural vanilla extract is extremely expensive.[184,185] Most vanillin used in flavoring is obtained by hydrolysis of lignin, but production from glucose using bacterially produced enzyme reactors is possible.[185] Prince and Gunson, in an interesting article,[184] described the use of mass spectrometry and $^{13}C$-enriched synthetic vanillin in the battles to distinguish natural vanilla extract from artificial mixtures of vanillin and other compounds and to camouflage the latter. Conversion of ferulic acid to vanillin in plants is apparently accomplished by β oxidation of acyl-CoA derivatives[186] as indicated in Fig. 25-8.

The ubiquitous plant compound **chlorogenic acid** (isolated from green coffee beans) is formed by transesterification with the glycoside cinnamoyl-glucose.[187] Coumaroyl-CoA is converted into monomeric and dimeric amides with **agmatine**, which provides barley plants with resistance to mildew.[188] Similar compounds with various polyamines and derived from *p*-coumaric, caffeic, ferulic, or sinapic acid appear to function in plant development. For example, caffeoylputrescine and caffeoyl-γ-aminobutyrate are accumulated specifically in sex organs of tobacco flowers.[189]

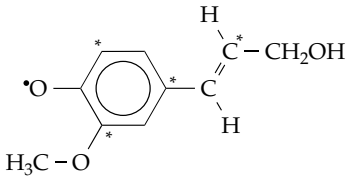



Chlorogenic acid
(3-*O*-caffeoylquinic acid)

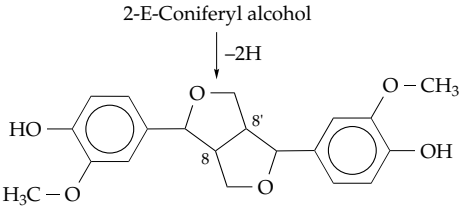



*P*-Coumaroylagmatine

***Lignin, lignols, lignans, and phenolic coupling.*** Lignin is a complex material of relative molecular mass greater than 10,000. It is remarkably stable, being insoluble in hot 70% sulfuric acid. Lignin may be described as a "statistical polymer of oxyphenylpropane units." It arises from oxidative coupling of **coniferyl and sinapyl alcohols** (Figs. 25-8, 25-9) and related monomers known as **lignols**.[190–190c] The enzyme responsible for the polymerization may be a peroxidase, which catalyzes formation of lignin from the monomeric alcohols and $H_2O_2$. A radical generated by loss of an electron from a phenolate anion of coniferyl alcohol consists of a number of resonance forms in which the unpaired electron may be present not only on the oxygen but also at the positions marked by asterisks in the following structure:




Coupling of such radicals yields a great variety of products. One type of dimerization gives the stable ether linked **pinoresorcinol** (Eq. 25-11). Through a complex sequence of reactions, it can be converted into other plant compounds including the phytoalexin **plicatic acid**, a major component of western

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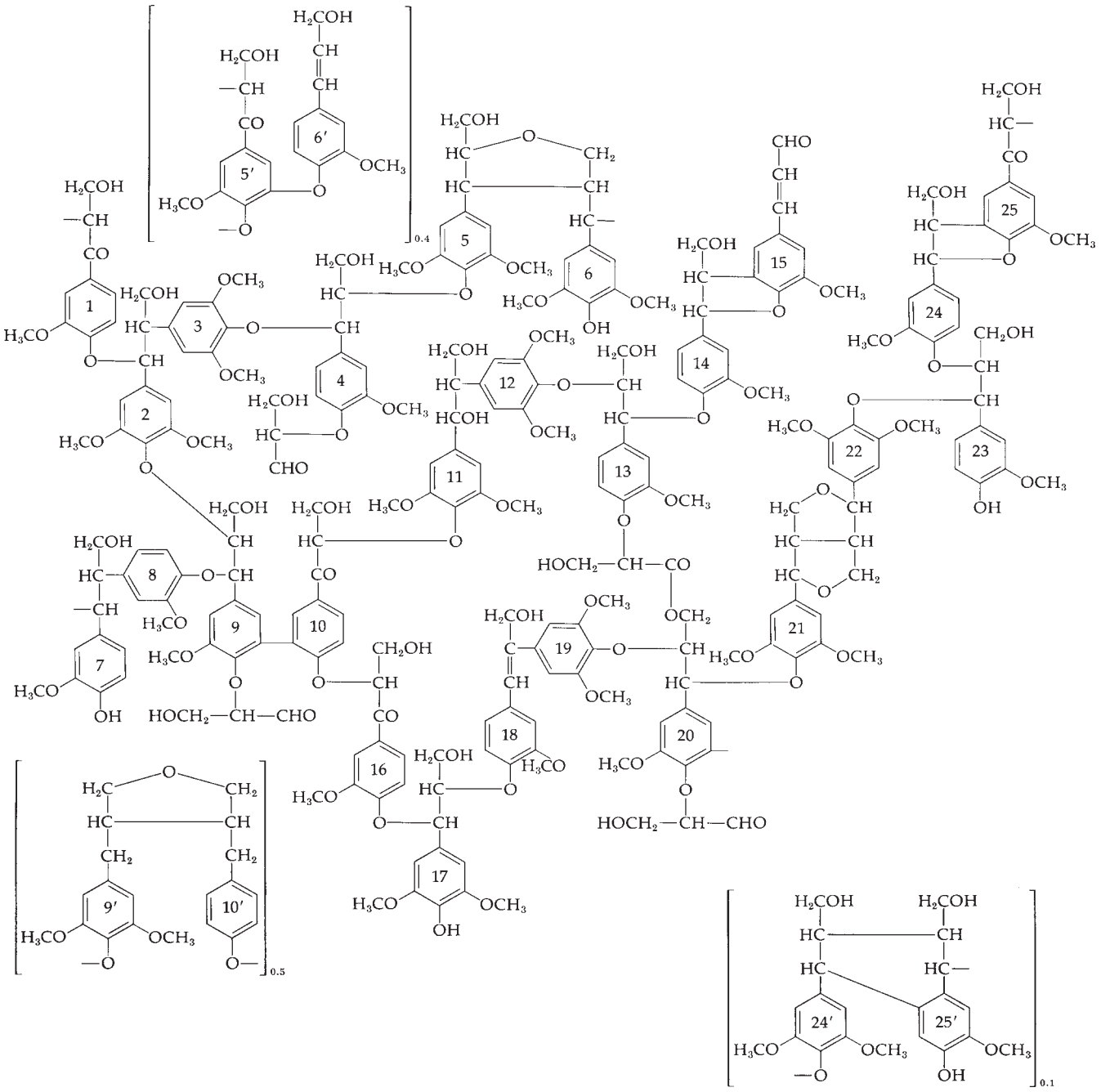
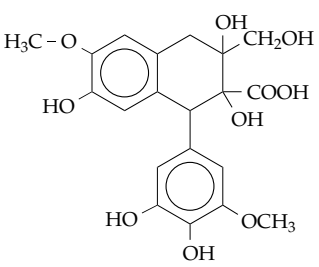
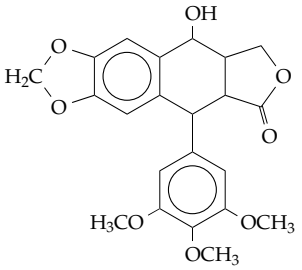


**Figure 25-9** Proposed structure of beech lignin. There are 25 different $C_9$ units, of which several can, to some extent, be replaced by the three dimeric units in brackets. Redrawn from Nimz,[194] p. 317.

red cedar heartwood, and **podophyllotoxin**, found in the poisonous roots of the May apple (*Podophyllum peltatum*).[191] The little yellow "apple" is edible. Podophyllotoxin is used in cancer treatment. These dimeric 8,8'-carbon linked derivatives of lignols are called **lignans**[191a] while oligomers linked in other ways are **neolignans**.[192] The lignols are also incorporated covalently into **suberin**, a waxy layer of plant cell walls.[193]



Plicatic acid



Podophyllotoxin

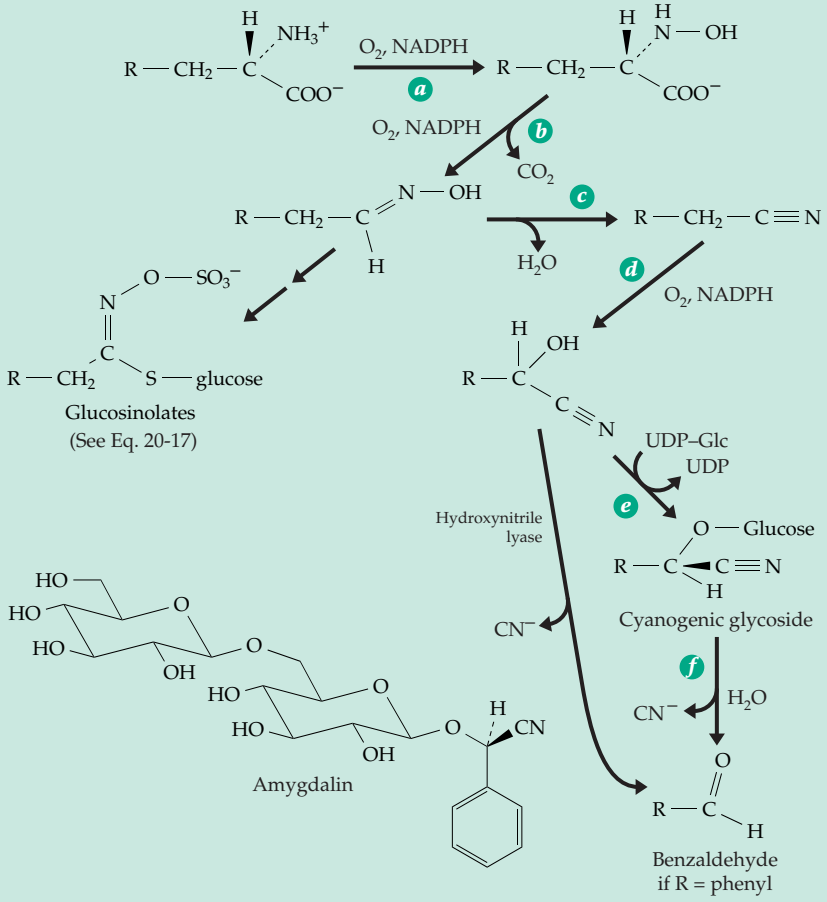
The lignols are synthesized within cells and are thought to move out into cell walls, possibly as phenolic glycosides.[190c,195] Cell wall peroxidases or laccases initiate polymerization.[173] The previously discussed lignans do not tend to polymerize, but other dimeric forms do. The dimers still contain hydroxyl groups capable of radical formation and addition to other units. At least ten types of intermonomer linkage other than that in the lignans are shown in Fig. 25-9. Lignin represents an enormous potentially valuable

## BOX 25-B THE CYANOGENIC GLYCOSIDES

Cyanide-containing glycosides are synthesized by many higher plants including such crop plants as sorghum, cassava, and white clover.[a] The starting compounds are L-amino acids, most often phenylalanine, tyrosine, valine, or isoleucine.[b] The following sequence was proposed by Conn and others.[b–f] The conversion to an *N*-hydroxyamino acid in the first reaction (step *a*) is catalyzed by a cytochrome P450 hydroxylase system requiring NADPH and $O_2$. The same enzyme catalyzes a second hydroxylation that is followed by dehydration and decarboxylation (step *b*) to form an oxime.[f] The oxime is dehydrated to form a nitrile (step *c*) and a third hydroxylation (step *d*) produces an α-hydroxynitrile (cyanohydrin). Glycosylation by transfer from UDP-Glc or other sugar nucleotide (step *e*) forms the cyanogenic glycoside. If R = *p*-hydroxyphenyl in the foregoing equation and the sugar is glucose the product is **dhurrin**, present in **sorghum**. In **amygdalin**, present in bitter almonds and in pits of apricots, peaches, cherries, etc., two glucosyl units in β-1,6 linkage (gentiobiose) are attached to mandelonitrile.

Cyanogenic glycosides generate free cyanide by elimination when the glycosidic linkage is hydrolyzed.[a] This occurs with dhurrin at high pH and with others at pH=1, 70–100°C. Elimination of cyanide from the hydroxynitriles is catalyzed enzymatically.[g,h] Another cytochrome P450 dependent process utilizes oxidation to an oxime, as in the foregoing scheme, but converts the oxime to a **glucosinolate** in a two-step process.[i]

At one time amygdalin, sold as **Laetrile**, was promoted as a treatment for cancer, presumably based on the hope that the cancer cells would be poisoned by the released cyanide.[j] The tubers and leaves of the cassava plant provide a major source of food in many tropical countries. However, unless the cyanogenic glycosides are removed by boiling the tubers and pulping the leaves cassava is very toxic.[e,k]





[a] Vennesland, B., Castric, P. A., Conn, E. E., Solomonson, L. P., Volini, M., and Westley, J. (1982) *Fed. Proc.* **41**, 2639–

[b] Conn, E. E. (1979) *Nateuwissenschaften* **66**, 28–34

[c] Moller, B. L., and Conn, E. E. (1980) *J. Biol. Chem.* **255**, 3049–3056

[d] Moller, B. L., and Conn, E. E. (1979) *J. Biol. Chem.* **254**, 8575–8583

[e] Andersen, M. D., Busk, P. K., Svendsen, I., and Moller, B. L. (2000) *J. Biol. Chem.* **275**, 1966–1975

[f] Sibbesen, O., Koch, B., Halkier, B. A., and Moller, B. L. (1995) *J. Biol. Chem.* **270**, 3506–3511

[g] Lauble, H., Miehlich, B., Förster, S., Wajant, H., and Effenberger, F. (2001) *Protein Sci.* **10**, 1015–1022

[h] Lauble, H., Miehlich, B., Förster, S., Wajant, H., and Effenberger, F. (2002) *Biochemistry* **41**, 12043 – 12050

[i] Du, L., Lykkesfeld, J., Olsen, C. E., and Halkier, B. A. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 12505–12509

[j] Newmark, J., Brady, R. O., Grimley, P. M., Gal, A. E., Waller, S. G., and Thistlethwaite, J. R. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 6513–6516

[k] Ononogbu, I. C. (1980) *Trends Biochem. Sci.* **5**, X

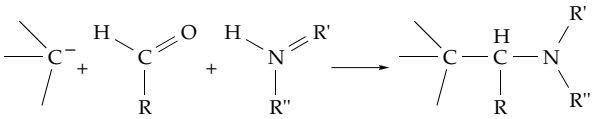
industrial source of aromatic raw materials, whose utilization has proved difficult. Oxidative degradation of lignin produces **humic acid**, an important organic constituent of soils.[196]

Oxidative coupling of radicals derived from phenols has a much wider role in nature than in lignin formation. Many alkaloids and other plant and fungal metabolites are synthesized using this reaction.[197] Tyrosine radicals are thought to be involved in formation of thyroxine (Eq. 25-6), melanin (Fig. 25-6), crosslinkages of ferulic acid with polysaccharides of plant cell walls,[197a] dityrosine, and other protein crosslinkages (Section B,4). Autocatalytic reaction of tyrosine-derived aminoquinone radicals in the $Cu^{2+}$-containing active site of amine oxidases apparently generates the mature prosthetic group topaquinone (Eq. 15-53).[198–198b] Oxidative coupling of tryptophanyl or cysteinyl side chains generates tryptophan tryptophylquinone (p. 817) or **cysteine tryptophylquinone** (CTA).[198b,c] As discussed on pp. 885–886 the tyrosine-cysteine thioether-bridged prosthetic groups of galactose oxidase (Fig. 16-29) and several other enzymes are also self-processing.[198d,e]

## 8. Alkaloids

More than 12,000 miscellaneous nitrogen-containing compounds, known as alkaloids, are produced by plants.[174a,199] Alkaloids are often thought of simply as end products of nitrogen metabolism in plants. However, most plants do not make alkaloids, whereas certain families of plants make many. There are probably ecological reasons.[200] Alkaloids often have potent physiological effects on animals, and many have been used as medicines from ancient times. Some have been prized through centuries as hallucinogens and intoxicants.

There are several classes of alkaloids. Among these are purines such as xanthine and caffeine, terpenes (Chapter 22), polyketides (Chapter 21), and alkaloids derived from amino acids. The basic amino acids ornithine, arginine, histidine, and lysine as well as the aromatic amino acids, anthranilate, and nicotinate are some of the starting materials.[199,201] Robinson[202,203] in 1917 recognized that many alkaloids are derived directly from aromatic amino acids. He proposed that alkaloids arise from **Mannich reactions** (Eq. 25-12) in which an amine and an aldehyde (probably through a Schiff base) react with a nucleophilic carbon such as that of an enolate anion. Many of the



(25-12)

amines are formed by decarboxylation of amino acids, and the aldehydes may arise by oxidative decarboxylation (transamination and decarboxylation) of amino acids. Thus, amino acids can provide both of the major reactants for alkaloid synthesis. Furthermore, nucleophilic centers in the aromatic rings, e.g., in positions para to hydroxyl substituents, are frequent participants in the proposed Mannich condensations. While Robinson's ideas on alkaloid biosynthesis were initially speculative, they have been confirmed by isotopic labeling experiments and more recently by isolation of the enzymes involved. Nevertheless, many questions remain. The postulated aldehydes are not proved intermediates. The condensations with 2-oxo acids may occur prior to decarboxylation.

An example is shown in Fig. 25-10. Dopa is decarboxylated to dopamine and is oxidized to 3,4-dihydroxybenzaldehyde. A Mannich reaction (via the Schiff base as shown) leads to ring closure. Oxidation of the ring produces an **isoquinoline** ring, a structural characteristic of a large group of alkaloids. Methylation produces **papaverine**, found in the opium poppy. A related alkaloid **morphine** (Fig. 25-10), at first glance, appears dissimilar. However, the biosynthetic route is similar. The initial Schiff base is formed from tyramine. Closure of the third ring together with hydroxylation and methylation yield ***R*-reticuline**, a precursor to many alkaloids. Its two rings are then oxidatively coupled through a C–C bond and an ether linkage.[204,204a] *S*-Reticuline is the precursor to another large family of alkaloids.[205] The six-membered ring of another alkaloid, **colchicine** (Box 7-D), originates from phenylalanine, while the seven-membered tropolone ring is formed from tyrosine by ring expansion.

# C. Metabolism of Tryptophan and Histidine

The biosynthesis of tryptophan is outlined in Fig. 25-2. This amino acid not only assumes great importance in the structure and functioning of proteins but is converted into hormones, both in animals and plants, and into alkaloids in some plants. Some of the pathways are indicated in Figs. 25-11 and 25-12.

## 1. The Catabolism of Tryptophan

The primary catabolic pathway for tryptophan in animal cells is initiated (step *a*, Fig. 25-11) by **tryptophan 2,3-dioxygenase** (tryptophan pyrrolase; Eq. 18-38).[206] The enzyme is induced both by glucocorticoids and by tryptophan.[207] The related **indolamine 2,3-dioxygenase** catalyzes the same reaction of L-tryptophan but also acts on D-tryptophan and other substrates. It has different tissue distribution and reg- ulatory properties[208] and may play a role in

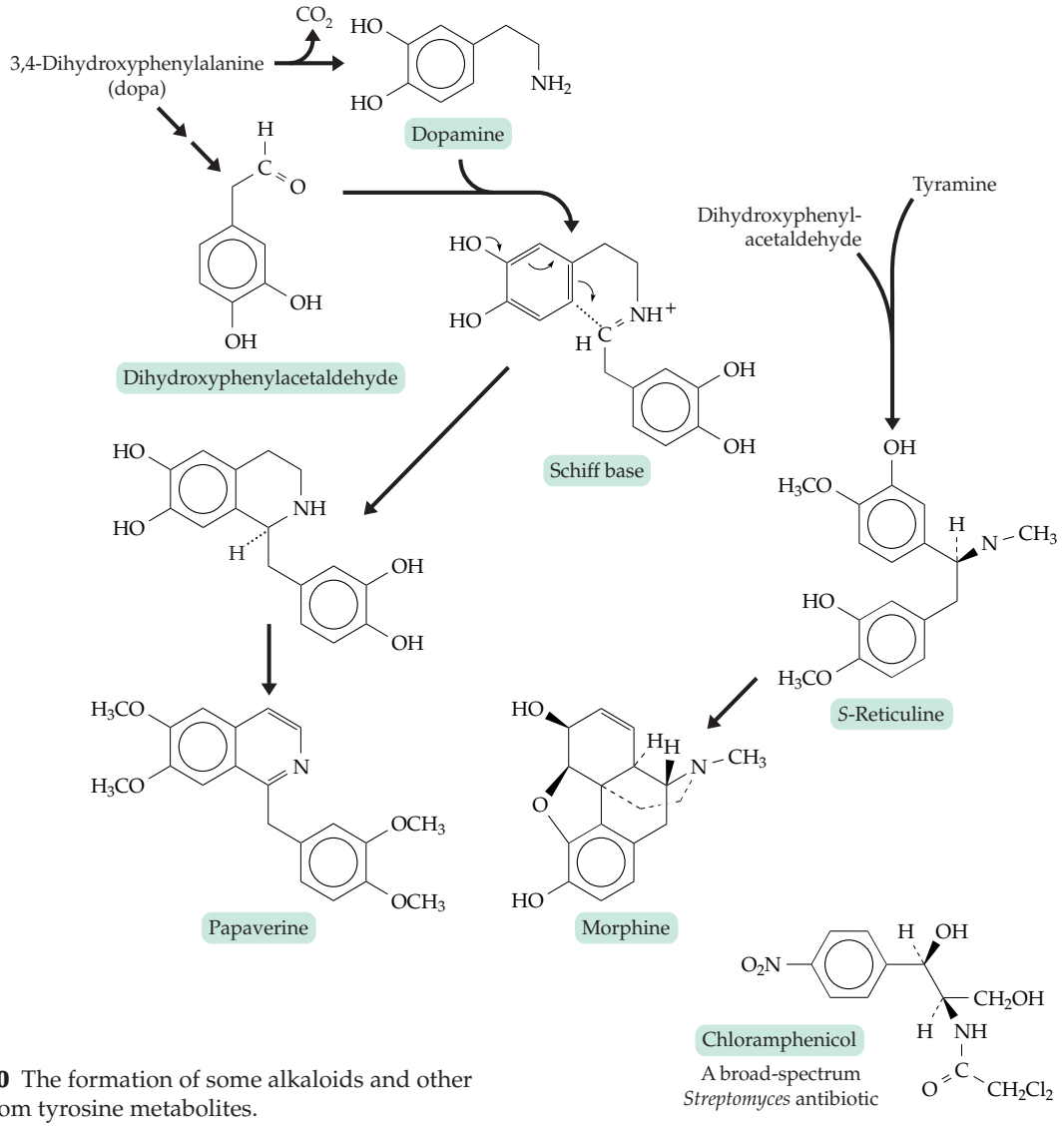



**Figure 25-10** The formation of some alkaloids and other substances from tyrosine metabolites.

inflammatory responses.[206,209] An alternative pathway of tryptophan breakdown takes place in intestinal bacteria, which utilize tryptophan indolelyase (tryptophanase) to eliminate indole (step *b*, Fig. 25-11).[209a] The indole is hydroxylated to **indoxyl**, some of which is absorbed into the bloodstream and excreted in the urine as indoxyl sulfate.

Returning to the major tryptophan catabolic pathway, marked by green arrows in Fig. 25-11, formate is removed hydrolytically (step *c*) from the product of tryptophan dioxygenase action to form **kynurenine**, a compound that is acted upon by a number of enzymes. Kynureninase (Eq. 14-35) cleaves the compound to anthranilate and alanine (step *d*), while transamination leads to the cyclic **kynurenic acid** (step *e*). The latter is dehydroxylated in an unusual reaction to **quinaldic acid**, a prominent urinary excretion product.

Another major pathway of kynurenine metabolism (step *f*, Fig. 25-11) is hydroxylation to **3-hydroxykynurenine**, which in turn can undergo transamination to the cyclic **xanthurenic acid**. Xanthurenic acid is excreted from the human body, but in the malaria mosquito *Anopheles gambia* it acts as a mating factor for the malaria parasite *Plasmodium*.[210,211] In many insects, including *Anopheles*, 3-hydroxykynurenine is a precursor of insect eye pigments or "**omnochromes**."[210,212–213a] 3-Hydroxykynurenine also has neurotoxic properties.[213a] (See p. 1796.)

Cleavage of 3-hydroxykynurenine by kynureninase (step *g*, Fig. 25-11) forms 3-hydroxyanthranilate, which is opened under the action of another dioxygenase (step *h*) with eventual degradation to acetyl-CoA, as indicated. In insects the reactive 3-hydroxyanthranilate is utilized in "tanning" reactions, e.g., coupling to tyrosine residues to toughen insect cuticles and walls of cocoons.[214]

Tryptophan is hydroxylated to 5-hydroxytryptophan[213b] which is decarboxylated to **serotonin** (5-hydroxytryptamine), an important neurotransmitter

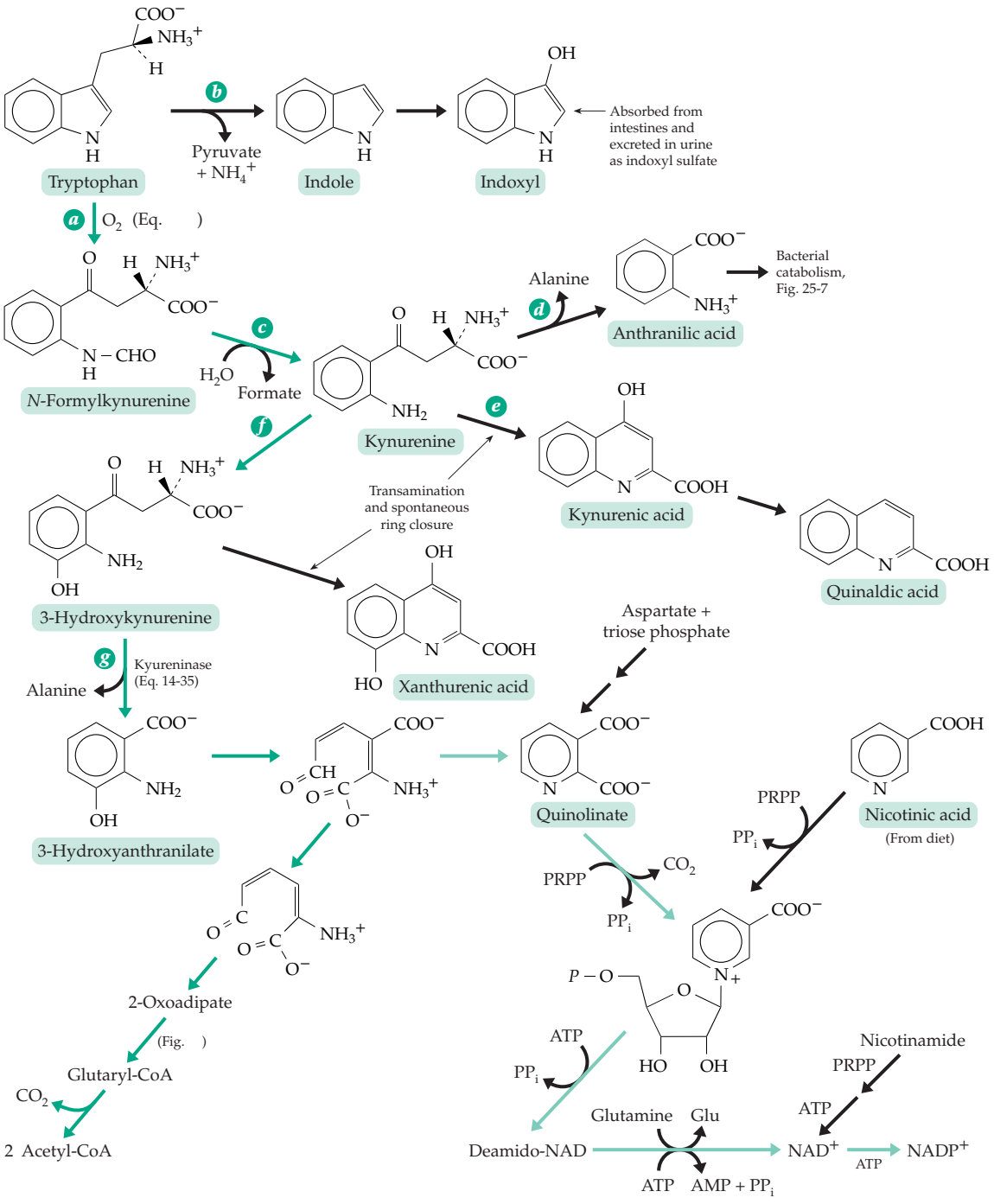
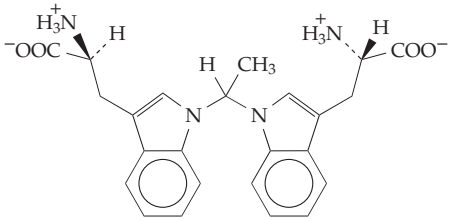



**Figure 25-11** Some catabolic reactions of tryptophan and synthetic reactions leading to NAD and NADP.

substance[215,215a] and a regulatory component of plants and animals alike.[216] In the pineal gland serotonin is methylated and acetylated to **melatonin**, the pineal hormone[217–221] (Fig. 25-12).

The following dangerous tryptophan derivative was evidently formed in a fermentation used to produce tryptophan sold as a food supplement in 1990. More than 1,500 persons became ill and 27 died, perhaps as a direct result of toxicity of this compound.[222,223]



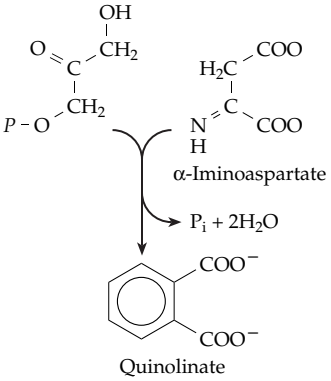
1,1'-Ethylidenebis[tryptophan]

***Formation of NAD⁺ and NADP⁺.*** An alternative pathway, marked by shaded green arrows in Fig. 25-11, allows animals to form the nicotinamide ring of $NAD^+$ and $NADP^+$ from tryptophan.[224] The aldehyde produced by the ring opening reaction of step *h* can reclose (step *i*) to a pyridine ring in the form of **quinolinic acid**.[225] The latter, in a reaction that is also accompanied by decarboxylation, is coupled with a phosphoribosyl group of PRPP to form **nicotinate mononucleotide**.[225a] Adenylylation produces deamido NAD, which is converted to **NAD** by a glutamine- and ATP-dependent amination of the carboxyl group.[226]

As indicated in Fig. 25-11, free nicotinic acid can also be used to form NAD. Not surprisingly, nicotinic acid, an essential vitamin, is about 60 times more efficient than tryptophan as a source of NAD. Nevertheless, a high-tryptophan diet partially overcomes a deficiency in dietary intake of nicotinic acid. The effectiveness of a diet containing only maize as a source of protein in inducing the deficiency disease pellagra (Box 15-A) is in part a result of the low tryptophan content of maize protein. Nicotinic acid is rapidly converted in the liver to an amide with glycine, **nicotinuric acid**. Nicotinurate can be oxidatively cleaved by peptidylglycine monoxygenase to nicotinamide[227] in another alternative synthetic route to NAD.

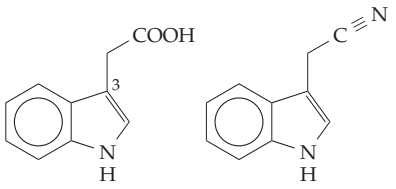
An alternative pathway for synthesis of quinolinate from aspartate and a triose phosphate exists in bacteria and in plants and provides the major route of nicotinic acid synthesis in nature. In *E. coli* the reaction is catalyzed by two enzymes, one an FAD-containing L-aspartate oxidase which oxidizes aspartate to α-iminoaspartate.[228] The latter condenses with dihydroxyacetone-*P* to form quinolinate (Eq. 25-13).[229] There are at least two other pathways for synthesis of quinolinic acid as well as five or more salvage pathways for resynthesis of degraded pyridine nucleotide coenzymes.[224,230,231]

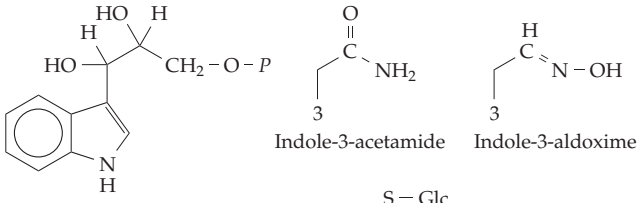
Although quinolinic acid provides an important source of nicotinamide coenzymes, in excess it is a neurotoxic **excitotoxin** (Chapter 30) that has been associated with epilepsy and with inflammatory neuropathalogical conditions resulting from encephalitis.[213,232,233]

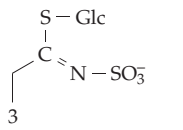



(25-13)

***Auxin.*** The important plant hormone **indole-3-acetic acid** (IAA; often called by the more general name **auxin**) is partially derived by oxidative decarboxylation (Fig. 25-12, top) catalyzed by tryptophan-2-monooxygenase,[234–234b] a flavoprotein similar to lysine monooxygenase (Eq. 18-41). The reduction product indole-3-ethanol also occurs in plants and is metabolically active.[235] However, most IAA in plants is not formed from tryptophan but from some precursor,[236,236a] perhaps indole-3-glycerol phosphate, which immediately precedes tryptophan in its biosynthesis (Fig. 25-2). Routes of synthesis from indole-3-acetonitrile, indoleacetaldoxime,[236b] indole-3-glucosinolate (glucobrassicin), indole-3-pyruvate, and tryptamine have also been reported.[237–238a] Nitrilases are found in




plants such as *Arabidopsis thaliana*[238] and also in symbiotic bacteria such as *Rhizobium*. Together with amidases they convert the acetonitrile, acetaldoximes, or acetamide derivatives to IAA,[237] which is transported throughout the plant.[238b]

About 95% of the IAA within plants is stored as conjugated forms that include amides with various amine acids and peptides[239] and glycosyl derivatives.[240] The gall-forming *Pseudomonas savastonoi* forms both IAA and conjugates such as $N^{\varepsilon}$-(indole-3-acetyl)-L-lysine, which aid these bacteria in colonizing olive and oleander plants.[241]

As a hormone IAA has a broad range of effects on plants, altering tissue differentiation, root growth, cell elongation, and cell division.[241a] The fastest observed response is an effect on cell elongation, which can be observed within 15–20 minutes.[242] In *A. thaliana* IAA

causes very rapid transcription of at least five genes, one of which encodes 1-aminocyclopropane-carboxylase (ACC) synthase (Eq. 14-27).[243]

***Alkaloids from tryptophan.*** The alkaloid **harmine**, which is found in several families of plants, can be formed from tryptophan and acetaldehyde (or pyruvate) in the same manner as is indicated for the formation of **papaverine** in Fig. 25-10. Some other characteristic plant metabolites such as **psilocybine**, an hallucinogenic material from the mushroom *Psilocybe aztecorum*, are formed directly from serotonin (Fig. 25-12). For many years **gramine** from barley was regarded as a curiosity because only one carbon atom separates the nitrogen atom from the indole ring. It is now believed that tryptophan is cleaved in a PLP-dependent reaction analogous to that of serine trans-hydroxymethylase (Eq. 14-30; Fig. 25-12). Other alkaloids arise in a more conventional fashion. Condensation of an isopentenyl group on the indole ring of tryptamine (Fig. 25-12) initiates the formation of **lysergic acid** and other ergot alkaloids.[244] The indole ring

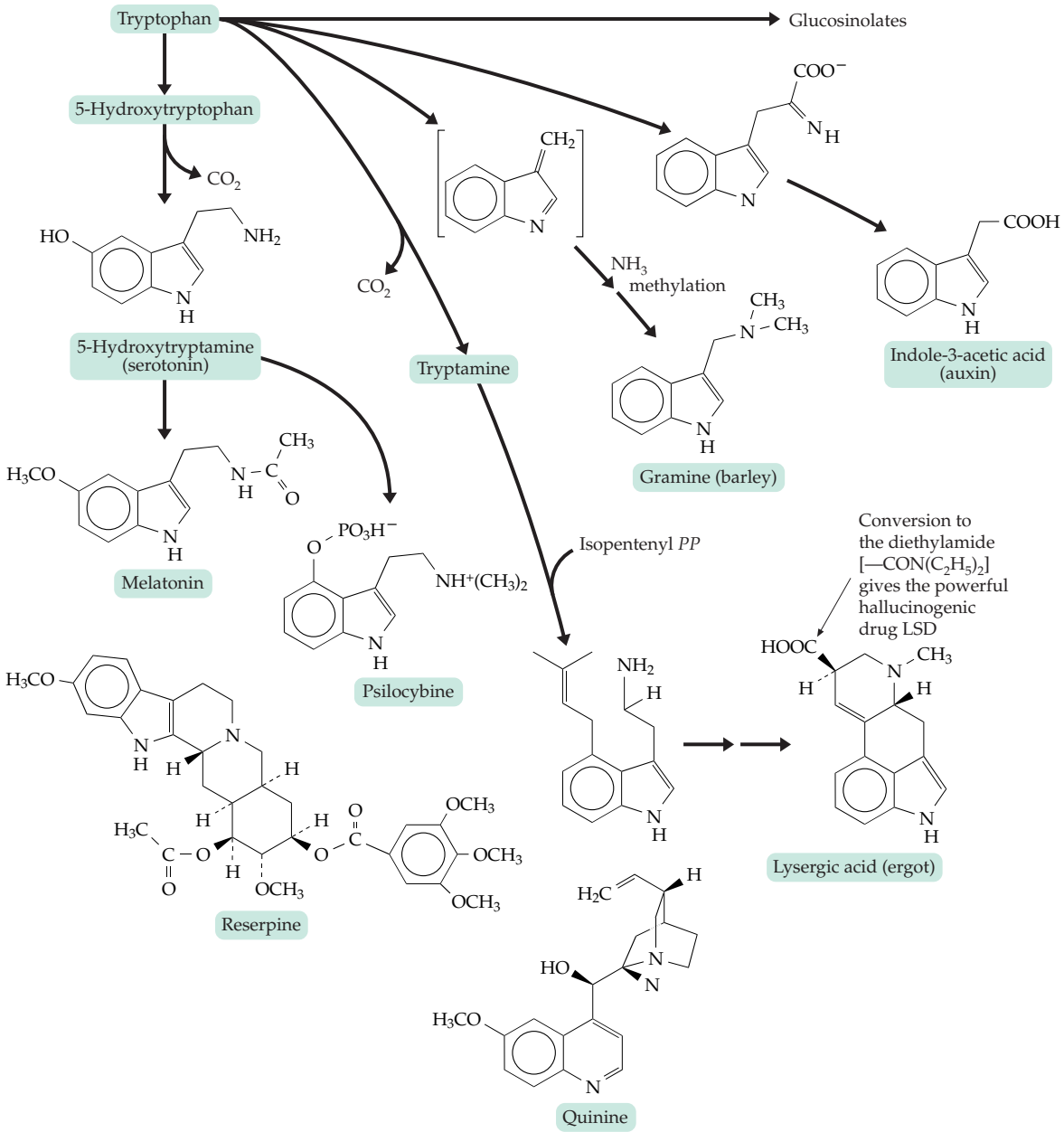
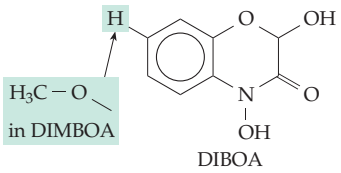



**Figure 25-12** Structures and some biosynthetic pathways for some hormones, indole alkaloids, and other metabolites of tryptophan.

of tryptophan is clearly visible in the structure of **reserpine** (Fig. 25-12). This compound from *Rauwolfia* is of medical interest because of its effect in lowering blood pressure and in depleting nervous tissues of serotonin, dopamine, and noradrenaline. Reserpine also contains a benzene ring, which is derived from tryptophan by a ring expansion. The periwinkle alkaloids, including the antitumor drug **vincristine** (see Box 7-D), are formed by condensation of tryptamine with the complex glycosidic aldehyde secologamin. Additional reactions form vincristine and more than 100 other indole alkaloids.[245,245a]

Another group of plant metabolites derived from tryptophan are cyclic hydroxamic acids whose names



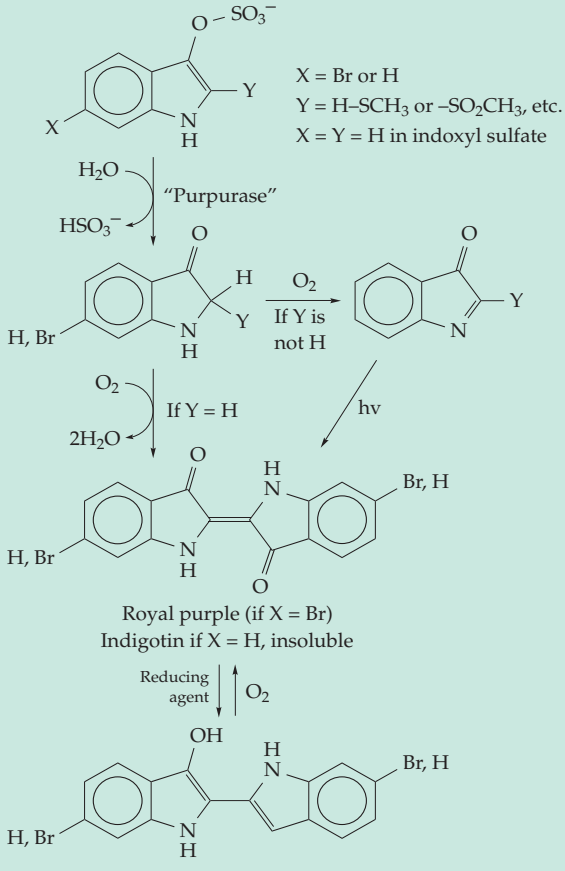

## BOX 25-C ROYAL PURPLE AND BLUE DENIM

Ancient people, including the Phoenicians, Chinese, and Peruvians, discovered a dyeing process that utilized secretions of certain marine molluscs, animals that were also eaten as food.[a] In processes that were perhaps closely guarded secrets, the molluscan secretions were heated for days in vats with water, salt, and additional additives including human urine, honey, etc. When the mixture was right, wool was dipped and allowed to air dry in sunlight to give the famous royal purple colors. In this ancient process as many as 10,000 molluscs were used to produce one gram of the dye.[a] In other parts of the world blue dyes were generated by fermentation of plants of the genus *Indigofera* and also of the European woad plant.[b,c]

By 1909 the chemical nature of the royal purple pigment dibromoindogotin (see scheme) had been established and by 1897 synthetic indigo production had already begun. Ancient indigo dyeing utilized the precursors, sulfate esters of **indoxyl** or of substituted indoxyl, metabolites of tryptophan (see Fig. 25-11). The sulfate esters were hydrolyzed by the sulfatase "purpurase" to give the tautomer of indoxyl that is shown in the accompanying scheme. Atmospheric oxygen converts these compounds to the corresponding oxidized dyes, the **indigotins**. However, they are very insoluble and unsuitable for dyeing. In modern indigo dyeing to form such fabrics as blue denim, the indigotin is reduced with sodium dithionite, about 2 kg of the latter being used to reduce 1 kg of the dye to the reduced leuco form.[c] Either wool or cotton can by dyed with this reduced form, air oxidation returning the dye to the blue oxidized form.

Ancient dyers also had to maintain the dye in the reduced form. In fermentations of the woad plant a species of thermophilic *Clostridium* apparently supplied the reducing agent. Padden *et al.* suggested that such bacterial reduction might be used today to avoid pollution by the by-products of dithionite reduction.[c] Use of engineered bacteria to form high yields of indole and indoxyl as a source of indigoid precursors has also been suggested.[b]

One more complexity needs to be considered. Some of the precursors are adducts (Y = $-SCH_3$, $-SO_2CH_3$ in the structures) and cannot be oxidized directly to the indigotins. Use of sunlight in a photochemical process was required in these cases.[a]




[a] McGovern, P. E., and Michel, R. H. (1990) *Acc. Chem. Res.* **23**, 152

[b] Ensley, B. D., Ratzkin, B. J., Osslund, T. D., Simon, M. J., Wackett, L. P., and Gibson, D. T. (1983) *Science* **222**, 167–169

[c] Padden, A. N., Dillon, V. M., John, P., Edmonds, J., Collins, M. D., and Alvarez, N. (1998) *Nature (London)* **396**, 225

are often abbreviated to DIBOA and DIMBOA. They are part of the defense system of grasses against insects and fungi. DIBOA is the main hydroxamic acid in rye while DIMBOA predominates in wheat and maize. The compounds arise from indole generated from indole-3-glycerol-*P* followed by action of four cytochrome P450 enzymes.[246]

## 2. The Metabolism of Histidine

The biosynthesis of histidine, which might be regarded as the "super catalyst" of enzyme active centers, begins with a remarkable reaction of ATP, the "super coenzyme" of cells. The reaction is a displacement by N-1 of the adenine ring on C-1 of PRPP (step *a*, Fig. 25-13). The resulting product undergoes a ring opening reaction, step *b*, followed by an Amadori rearrangement (step *c*). The rearrangement product is cleaved via reaction with ammonia released from glutamine with formation of **5-aminoimidazole-4-carboxamide**, whose ribotide is an established intermediate in the synthesis of ATP and other purines. Here it is recycled via ATP (Fig. 25-13). The other product of the cleavage contains the five carbons of the original ribosyl group of PRPP, together with one nitrogen and one carbon split out from the ATP molecule and the nitrogen donated by glutamine.[246a] Ring closure (step *e*) forms the imidazole group, which is attached to a glycerol phosphate molecule. The glycerol-*P* end of the molecule undergoes dehydration[247] and ketonization of the resulting enol to a product, which can be transaminated[247a,b] and dephosphorylated to histidinol. Dehydrogenation of this alcohol forms histidine.[248–249a]

***Regulation of histidine synthesis.*** In all, ten different genes code for the enzymes of histidine biosynthesis in *Salmonella typhimurium*. They are clustered as the **histidine operon**, a consecutive series of genes which are transcribed into messenger RNA as a unit.[250,251] The gene symbols *HisA*, *HisB*, etc., are indicated in Fig. 25-13, and their positions on the *E. coli* gene map are indicated in Fig. 26-4. The gene *HisB* codes for a complex protein with two different enzymatic activities as shown in Fig. 25-13.

The presence of an excess of histidine in a bacterial cell brings about repression of synthesis of all of the

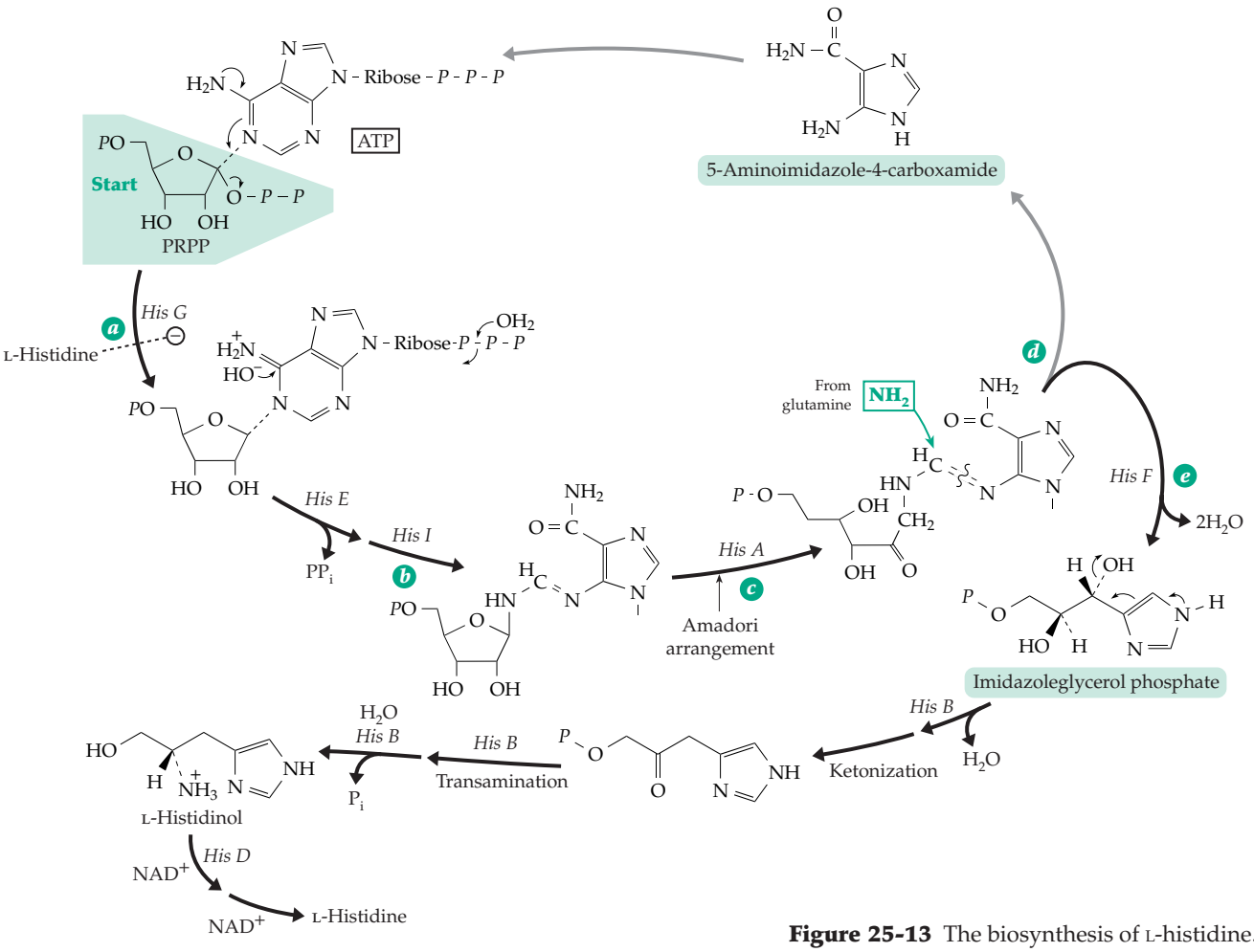
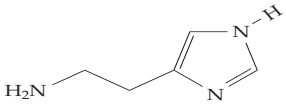



**Figure 25-13** The biosynthesis of L-histidine.

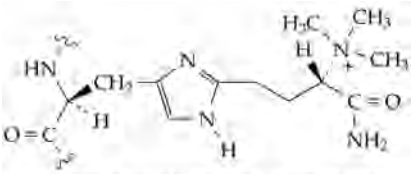
enzymes encoded in the histidine operon. Details of the functioning of this and other operons are considered in Chapter 28. Histidine is also an allosteric inhibitor for the first enzyme of the biosynthetic sequence, i.e., step *a* of Fig. 25-13. Thus, instantaneous inhibition of the biosynthesis occurs if an excess of histidine accumulates. Similar patterns of both repression and feedback inhibition exist for many of the pathways of amino acid biosynthesis (Chapter 28).

***Catabolism of histidine.*** The first steps of the major degradative pathway for histidine metabolism have already been discussed. Elimination of ammonia, followed by hydration and ring cleavage to **formiminoglutamate**, involves unusual reactions (Eq. 25-14)[252] which have been discussed earlier. Transfer of the formimino group to tetrahydrofolic acid and its further metabolism have also been considered (Chapter 15).



Histamine

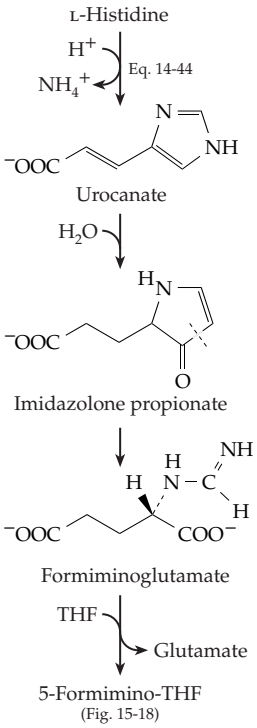
Other products from histidine include the hormonal substance **histamine** formed by decarboxylation, the oxidation product, imidazole acetic acid, and $N^{\delta}$- and $N^{\varepsilon}$-methylhistidines. Histamine plays a role in release of gastric secretions and allergic responses (Chapter 5). Drugs (antihistamines) that inhibit its release are in widespread use. The unusual amino acid **diphthamide** has an unknown function in protein synthesis, occurring within the peptide chain of eukaryotic elongation factor 2 (Chapter 29).[253] Its biosynthesis from a specific histidine in EF-2 of all eukaryotes and archaebacteria requires four molecules of *S*-adenosylmethionine. The first transfers the four-carbon backbone of AdoMet to $C^{\varepsilon 1}$ of the histidyl group, a nucleophilic displacement resembling that of AdoMet-dependent C-methylation (Eq. 12-4). This is followed by transfer of three methyl groups, each from AdoMet, and finally an ATP-dependent amidation of the carboxyl group.[253] Diphthamide is the target for attack by diphtheria toxin (Box 29-A).



Diphthamide residue within a peptide chain.

## D. Biosynthesis and Catabolism of Pyrimidines

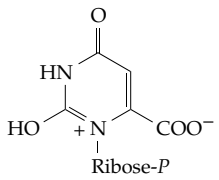
L-Aspartate contributes four of the six ring atoms of pyrimidines including the nitrogen. The α-carboxylate group is eventually lost as $CO_2$, the decarboxylation helping to drive the synthetic sequence. Six enzymatic steps are required to form the product uridine 5'-phosphate (UMP) as shown in Fig. 25-14, steps *a*–*f*. UMP is then converted on to the cytidine, uridine, and thymidine nucleotides as shown. This pathway of pyrimidine synthesis has been conserved throughout evolution and is used by all but a few specialized organisms.[254–258] The first step is synthesis of carbamoyl phosphate by the glutamine-dependent carbamoyl phosphate synthetase, an allosteric enzyme discussed in Chapter 24 (Eq. 24-22).[259–260a] The next step is transfer of the carbamoyl group to aspartate (Fig. 25-14, step *b*). The product is able to cyclize immediately (step *c*) by elimination of water to form **dihydroorotate**.[260a] The highly controlled aspartate carbamoyltransferase has been discussed in Chapters 7 and 11. Although this is a monofunctional enzyme in bacteria, it is fused with two other proteins in mammalian cells. The resulting multifunctional enzyme (with 240-kDa subunits) catalyzes three consecutive steps: the synthesis of carbamoyl phosphate, the carbamoyltransferase reaction, and the $Zn^{2+}$-dependent ring-closing reaction that forms dihydroorotate.[261,262] This cyclic product is oxidized to **orotate** (Fig. 25-14, step *d*) by a flavoprotein oxidase, which in mammals utilizes ubiquinones as electron acceptors.[263–265a] A displacement reaction with PRPP (Fig. 25-14, step *e*; see also Fig. 25-13, step *a*) converts it into **orotidine 5'-phosphate**[266,267] with release of $PP_i$.




(25-14)

## 1. Synthesis of Pyrimidine Nucleotides

Orotidine 5'-phosphate undergoes an unusual decarboxylation (Fig. 25-14, step *f*), which apparently is not assisted by any coenzyme or metal ion but is enhanced over the spontaneous decarboxylation rate $10^{17}$-fold. No covalent bond formation with the enzyme has been detected.[268] It has been suggested that the enzyme stabilizes a dipolar ionic tautomer of the substrate. Decarboxylation to form an intermediate ylid would be assisted by the adjacent positive charge.[269,270] Alternatively, a concerted mechanism may be assisted by a nearby lysine side chain.[270a–d] Hereditary absence of this decarboxylase is one cause of orotic aciduria. Treatment with uridine is of some value.[271]



Orotidine 5'-phosphate
(dipolar ionic tautomer)

We see that **uridine 5'-phosphate** (UMP) is formed from aspartate in a relatively direct and simple way. Phosphorylation with ATP in two steps produces UDP and UTP. The **cytosine nucleotides** are formed from UTP, the initial step being amination to CTP (step *h*, Fig. 25-14). This reaction resembles in many respects the conversion of citrulline to arginine, which depends upon ATP and involves transfer of the nitrogen of aspartate (Eq. 24-23). However, in the formation of CTP glutamine serves as the nitrogen donor ($NH_4^+$ can substitute). Observation of positional

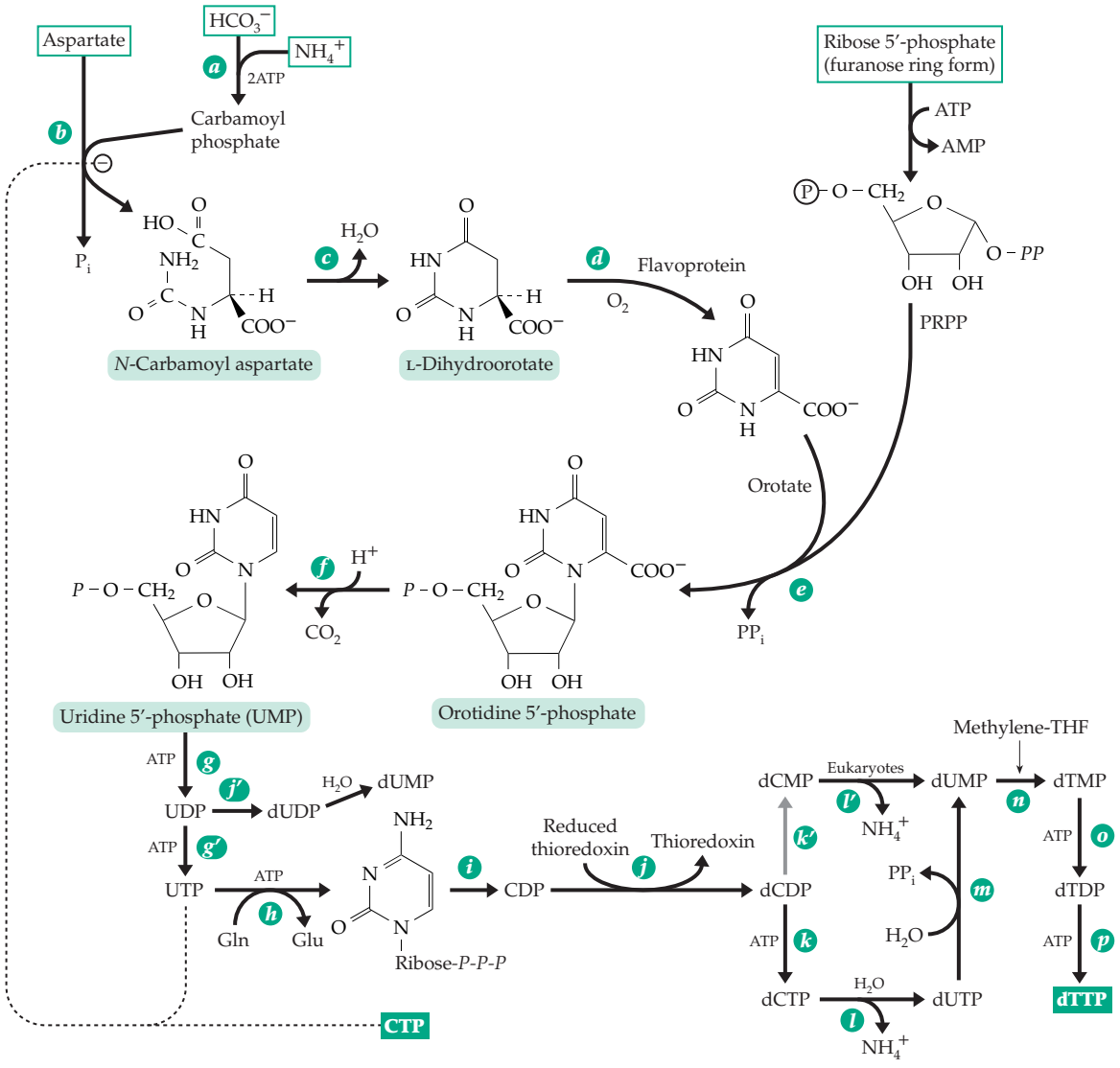
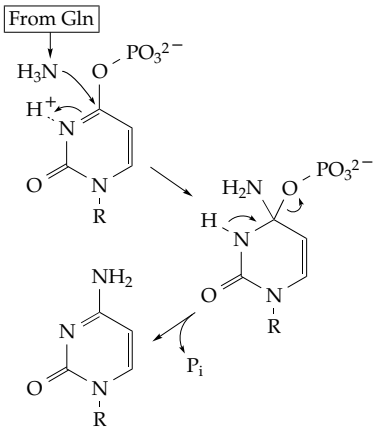


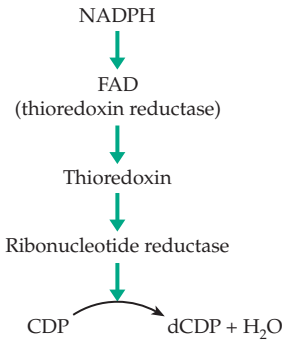

**Figure 25-14** Assembly of the pyrimidine ring and biosynthesis of the pyrimidine nucleotide precursors of RNA and DNA.

isotope exchange with [γ-$^{18}O_4$]ATP suggested the occurrence of an enolic phosphate intermediate (Eq. 25-15)[272] as had also been suggested for adenylosuccinate synthase (Fig. 25-15, step *k*). CTP is incorporated directly into RNA and into such metabolic intermediates as CDP- choline, or it can be dephosphorylated to CDP (Fig. 25-14, step *i*). It is CDP that serves as the principal precursor for the deoxyribonucleotides **dCDP** and **thymidine diphosphate** (dTTP).




(25-15)

***Deoxyribonucleotides.*** A chain involving NADPH, a flavoprotein, thioredoxin, and ribonucleotide reductase converts either the ribonucleoside diphosphates or triphosphates to the corresponding 2-deoxy forms (step *j*, Fig. 25-14) as indicated in Eq. 25-16.




(25-16)

Ribonucleotide reductases are discussed in Chapter 16. Some are iron-tyrosinate enzymes while others depend upon vitamin $B_{12}$, and reduction is at the nucleoside *triphosphate* level. Mammalian ribonucleotide reductase, which may be similar to that of *E. coli*, is regarded as an appropriate target for anticancer drugs. The enzyme is regulated by a complex set of feedback mechanisms, which apparently ensure that DNA precursors are synthesized only in amounts needed for DNA synthesis.[273] Because an excess of one deoxyribonucleotide can inhibit reduction of all ribonucleoside diphosphates, DNA synthesis can be inhibited by deoxyadenosine or by high levels of thymidine, despite the fact that both compounds are precursors of DNA.

Phosphorylation of dCDP to dCTP (step *k*, Fig. 25-14) completes the biosynthesis of the first of the pyrimidine precursors of DNA. The uridine nucleotides arise in two ways. Reduction of UDP yields dUDP (step *j'*, Fig. 25-14). However, the deoxycytidine nucleotides are more often hydrolytically deaminated (reactions *l* and *l'*).[274] Methylation of dUMP to form **thymidylate**, dTMP (step *n*, Fig. 25-14), is catalyzed by thymidylate synthase. The reaction involves transfer of a 1-carbon unit from methylene tetrahydrofolic acid with subsequent reduction using THF as the electron donor. A probable mechanism is shown in Fig. 15-21. See also Box 15-E. Some bacterial transfer RNAs contain 4-thiouridine (Fig. 5-33). The sulfur atom is introduced by a sulfurtransferase (the *ThiI* gene product in *E. coli*). The same protein is essential for thiamin biosynthesis (Fig. 25-21).[274a]

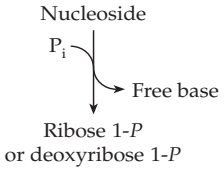
Formation of dUMP in eukaryotes may occur by hydrolytic removal of phosphate from dUDP or from the conversions dCDP → dCMP → dUMP (steps *k'* and *l'*, Fig. 25-14). A more roundabout pathway is employed by *E. coli*: dCDP → dCTP → dUTP → dUMP (steps *k*, *l*, and *m*, Fig. 25-14). One of the intermediates is dUTP. DNA polymerases are able to incorporate dUMP from this compound into polynucleotides to form uracil-containing DNA. The only reason that this does not happen extensively within cells is that dUTP is rapidly converted to dUMP by a pyrophosphatase (step *m*, Fig. 25-14). The uracil that is incorporated into DNA is later removed by a repair enzyme (Chapter 27). The presence of dUTP in DNA provides the basis for one of the most widely used methods of directed mutation of DNA (Chapter 26).

***Bacteriophage-induced alterations in metabolism.*** Interesting alterations in nucleotide metabolism occur in cells of *E. coli* infected by T-even bacteriophage. Genes carried by the phage are transcribed, and the corresponding proteins are synthesized by the host cell.[273] A number of these viral gene products are enzymes affecting nucleotide metabolism. One enzyme catalyzes the hydrolytic conversion of dCTP to dCMP, and another promotes the synthesis of 4-hydroxymethyl-dCMP. Such virus-specified enzymes may be appropriate target sites for antiviral drugs.

## 2. Catabolism of Pyrimidine Nucleotides and Nucleosides

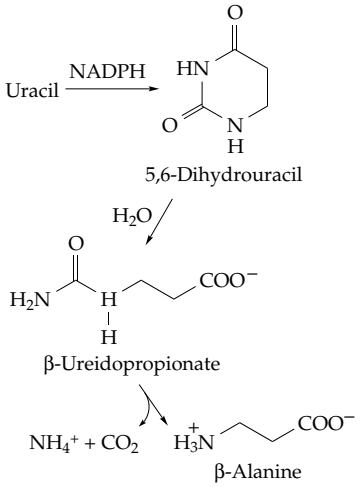
Nucleic acids within cells, as well as in the digestive tract, are continually under attack by many **nucleases**. Messenger RNA is degraded, often quite

rapidly, as an essential part of the control of protein synthesis. Although DNA is very stable, nucleases are called upon to cut out damaged segments of single strands as part of essential repair processes (Chapter 27). Thus, there is an active breakdown of polynucleotides to mononucleotides, which are hydrolyzed to nucleosides by phosphatases. Nucleosides are converted to free bases by the action of **nucleoside phosphorylases** (Eq. 25-17). The further degradation of



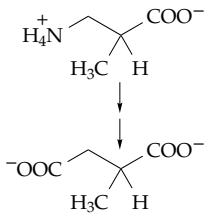

(25-17)

cytosine is initiated by deamination to uracil.[274b,c] Catabolism of uracil starts with reduction by NADPH according to Eq. 25-18 to form **β-alanine**.[275,275a] The latter can be oxidatively degraded to malonic semialdehyde and malonyl-CoA (see Fig. 17-3),[276] but it also serves as a biosynthetic precursor of pantothenic acid and coenzyme A (Eq. 24-38) and of the peptides carnosine and anserine.




(25-18)

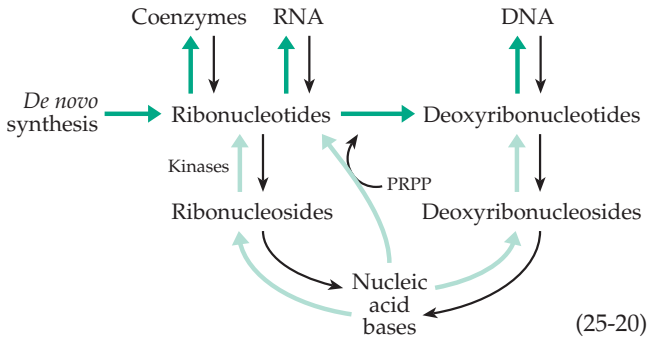
Thymine undergoes degradation in a pathway analogous to that of Eq. 25-18, but with the formation of **3-aminoisobutyrate**. The latter can be oxidatively converted to methylmalonate (Eq. 25-19), which can enter the methylmalonyl pathways (Fig. 17-3).



(25-19)

## 3. Reuse or Salvage of Nucleic Acid Bases

Almost all organisms except for protozoa synthesize nucleotides via the *de novo* pathways of Figs. 25-14 and 25-15. However, they also receive nucleotides, nucleosides, and free nucleic acid bases from catabolism of RNA and DNA. Both the synthetic and degradative pathways are carefully controlled by cells to ensure that they can grow and repair their nucleic acids and not be inhibited by accumulation of an excess of any component. Furthermore, animals receive an additional supply of preformed bases from their foods. Protozoa receive all of their bases in this way. A large set of enzymes is needed to break down the nucleotides and nucleosides. Another group of enzymes resynthesize nucleotides through **salvage pathways**.[273,277] This keeps levels of inhibitory compounds low, and at the same time produces a small constant pool of nucleotide triphosphates ready for use in nucleic acid synthesis. These pathways are summarized in a general way in Eq. 25-20, which uses dark green arrows for biosynthetic pathways, light green for salvage, and black for degradative reactions (after Kornberg and Baker[273]). The very active salvage pathways of protozoa have provided a variety of targets for inhibitors aimed at parasitic protozoa such as trypanosomes and *Toxoplasma gondii*. The target enzymes often have structures sufficiently different from the corresponding human enzymes to allow for design of selective inhibitors that can serve as drugs.[278,279]




(25-20)

Just as orotic acid is converted to a ribonucleotide in step *e* of Fig. 25-14, other free pyrimidine and purine bases can react with PRPP to give monoribonucleotides plus $PP_i$. The reversible reactions, which are catalyzed by **phosphoribosyltransferases** (ribonucleotide pyrophosphorylases), are important components of the salvage pathways by which purine and pyrimidine bases freed by the degradation of nucleic acids are recycled.[273] However, thymine is usually *not* reused. Thymine will react with deoxribose 1-*P* to form thymidine plus inorganic phosphate (thymidine phosphorylase), and thymidine is rapidly

phosphorylated by the action of successive kinases to dTTP; a substrate for DNA polymerases.[273] This has allowed biochemists to introduce radioactive thymine or thymidine into the DNA of an organism, an important experimental tool. Another important reaction of the salvage pathways for pyrimidines is the conversion of cytosine to uracil, the same kind of hydrolytic deamination represented by step *l* in Fig. 25-14.

## E. Biosynthesis and Metabolism of Purines

The first decisive experiments shedding light on the biosynthetic origins of purines were done with pigeons, which form large amounts of uric acid. Labeling experiments established the complex pattern indicated in the box in the upper left-hand corner of Fig. 25-15. Two carbon atoms were found derived from glycine, one from $CO_2$, and two from formate. One nitrogen came from glycine, two from glutamine, and one from aspartate. In the case of adenine, the 6-$NH_2$ group was also found derived from aspartate.

### 1. The Enzymatic Reactions of Purine Synthesis

The detailed biosynthetic pathway, for which enzymes have now been isolated and studied, is indicated in Fig. 25-15. The first "committed step" in purine synthesis is the reaction of PRPP with glutamine to form **phosphoribosylamine** (step *a*).[280,281] This is another glutamine-dependent amination, pyrophosphate being displaced by ammonia generated *in situ* from glutamine. The amino group of the intermediate so-formed is coupled with glycine in a standard manner (step *b*),[282] and the resulting product is formylated by 10-formyltetrahydrofolate (step *c*).[283–285b] The latter can be generated from free formate,[286] accounting for the labeling pattern indicated in the box in Fig. 25-15. For many years it was accepted, incorrectly, that 5,10-methenyl-THF was the formyl donor for this reaction.

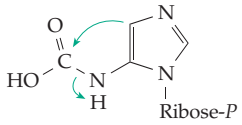
In step *d* of Fig. 25-15 a second glutamine-dependent amination takes place, possibly through aminolysis of an intermediate enol phosphate. An ATP-requiring ring closure and tautomerization (step *e*) serve to complete the formation of the imidazole ring.[287,287a] In many, perhaps all, eukaryotes a single multifunctional enzyme catalyzes steps *b*, *c*, and *e* of Fig. 25-15. The chicken enzyme has ~110-kDa subunits.[288] The product is a ribonucleotide of 5-amino-4-imidazole-4-carboxamide, (AIR), a compound that was isolated in 1945 from cultures of *E. coli* treated with sulfonamides. The latter are important drugs which are antagonists of *p*-aminobenzoate (Box 9-C) and interfere with completion of purine synthesis. This deprives the bacterial cells of essential folic acid derivatives. Its structure immediately suggested that 5-aminoimidazole-4-carboxamide might be a purine precursor. Later it was shown that it is actually the corresponding ribonucleotide (AIR or ZMP) that lies on the main route of purine synthesis. It also is an intermediate in the biosynthesis of thiamin (Fig. 25-21). Free 5-aminoimidazole-4-carboxamide participates in formation of histidine (Fig. 25-13).

The trinucleotide ZTP also accumulates, not only in bacteria but also in many eukaryotic cells. Bochner and Ames suggested it may be an **alarmone** signaling a deficit of folate coenzymes in the cell and causing a shutdown of protein synthesis. ZTP is synthesized by an unusual reaction, transfer of a pyrophosphate group from PRPP (phosphoribosyl pyrophosphate).

$$\text{ZMP} + \text{PRPP} \rightarrow \text{ZTP} + \text{Ribose 5'-}P \qquad (25\text{-}21)$$

This is similar to the reaction by which guanosine 5'-diphosphate 3'-diphosphate (ppGpp) is formed from GDP and ATP (Eq. 29-11).

In the next step of purine synthesis (Fig. 25-15, step *f*) a molecule of $CO_2$ is incorporated in an unusual type of carboxylation. It is shown in Fig. 25-15 as a single-step direct reaction of $CO_2$ with AIR. However, in many organisms it is a two-step ATP-dependent reaction to form a compound carboxylated on the 5-amino group. This rearranges[289–290a] to the product shown in Fig. 25-15.




While the arrows on the foregoing structure suggest a possible mechanism of rearrangement, the implied four-membered ring transition state makes it unlikely. The reaction resembles biotin-dependent carboxylations, suggesting the possibility that the carboxylate releases $CO_2$, which moves and rebinds while trapped within a closed active site.[290] In a two-step amination reaction (steps *g* and *h*) nitrogen is transferred from aspartate in a manner strictly comparable to that in urea synthesis in which argininosuccinic acid is an intermediate (Fig. 24-10). As in urea formation, the carbon skeleton of the aspartate molecule is eliminated as fumarate (step *h*), leaving the nitrogen in the purine precursor. The final carbon atom is added from 10-formyltetrahydrofolic acid (step *i*).[291–292b] Spontaneous ring closure is followed by dehydration to **inosine 5'-phosphate** (IMP, inosinic acid), step *j*. Steps *i* and *j* are catalyzed by a single bifunctional enzyme.

IMP is converted via two different pathways to either AMP or GTP. Conversion to AMP (Fig. 25-15, steps *k* and *l*) occurs via another two-step aspartate-dependent amination.[292c,d] The intermediate is

**adenylosuccinate**.[293–294b] Positional isotope exchange studies using [γ-$^{18}$O]GTP suggested that the enolic 6-phospho-IMP is an intermediate comparable to that in Eq. 24-23.[295,296] X-ray studies have confirmed the prediction.[297] An $NAD^+$-dependent oxidation converts IMP to the corresponding **xanthine** ribonucleotide

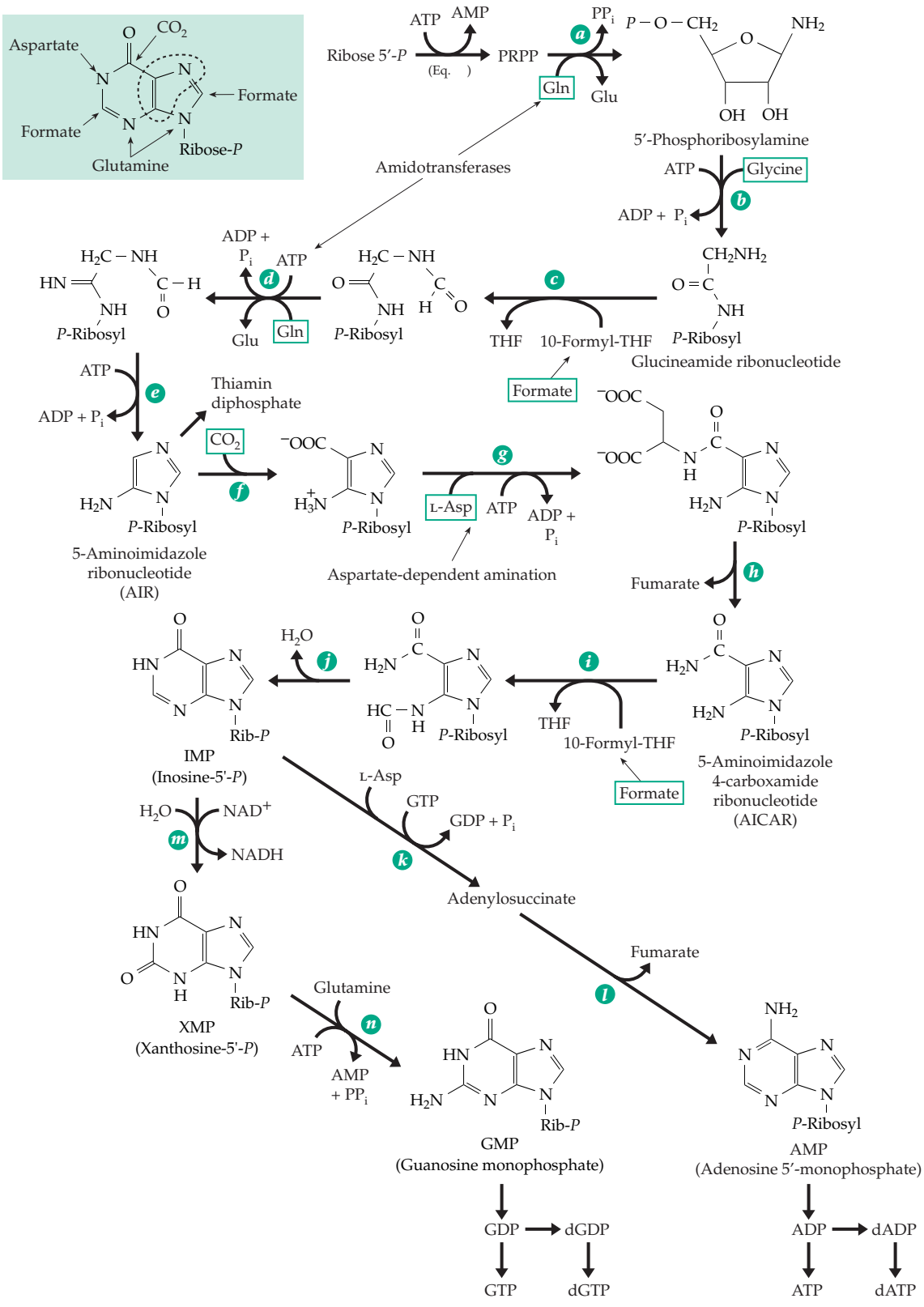



**Figure 25-15** Biosynthesis of purine nucleotides from ribose 5'-phosphate.

(step *m*),[297a–d] which is aminated in a glutamine-dependent process,[298–300] as indicated (step *n*).

Synthesis of purines is under complex control.[273] Some of the mechanisms found in bacteria are outlined in Fig. 25-16. Both feedback inhibition and activation are involved. Very important is the fact that GTP is needed in the synthesis of ATP, and that ATP is needed for synthesis of GTP. This kind of control ensures that an excess of either nucleotide will not be formed for long. In bacteria all of the final end product nucleotides inhibit the initial reaction of step *a* in Fig. 25-15.

Modified purine nucleosides are important constituents of transfer RNAs (Fig. 5-33; Chapter 28, Section A,7). Among them are the 7-deazaguanosines **queuosine** (Fig. 5-33) and **archaeosine**, which contains a 7-formamidino group. These nucleosides are incorporated into tRNAs by an exchange mechanism catalyzed by **tRNA-guanine** transglycosylase. This exchanges a precursor of queuine (7-aminomethyl-7-deazaguanine) for guanine in selected residues in the tRNAs. In eukaryotic tRNAs free queuine is exchanged into the tRNAs whereas in Archaea the archaeosine precursor is incorporated.[301a,b] The conversion of the precursors to the final modified bases occurs in the tRNAs (Eq. 28-4). Many other purine derivatives are found in nature, e.g., **puromycin** (Box 29-B), which is formed from adenosine.[301c]

## 2. The Purine Nucleotide Cycle and Salvage Pathways for Purines

Muscular work is accompanied by the production of ammonia, the immediate source of which is adenosine 5'-phosphate (AMP).[301,302] This fact led to the recognition of another substrate cycle (Chapter 11) that functions by virtue of the presence of a biosynthetic pathway and of a degradative enzyme in the same cells (cycle A, Fig. 25-17). This **purine nucleotide cycle** operates in the brain[303,304] as well as in muscle. The key enzyme 5'-AMP aminohydrolase (AMP deaminase; step *a*, Fig. 25-17) also occurs in erythrocytes and many other tissues.[304,305] Persons having normal erythrocyte levels but an absence of this enzyme in muscles suffer from muscular weakness and cramping after exercise.[306]

Purine bases from ingested foods, or formed by catabolism of nucleic acids, are able to react with PRPP under the influence of phosphoribosyltransferases.[306a] Two such enzymes are known to act on purines. One converts adenine to AMP (Fig. 25-17, step *b*) and also acts upon 5-aminoimidazole-4-carboxamide. This enzyme may be especially important to parasitic protozoa such as *Leishmania*, which lack the *de novo* pathway of purine synthesis (Fig. 25-15).[278,306b]

AMP can be converted by the action of AMP 5'-nucleotidase to adenosine (step *c*, Fig. 25-17), which is thought to be an important local hormone or second

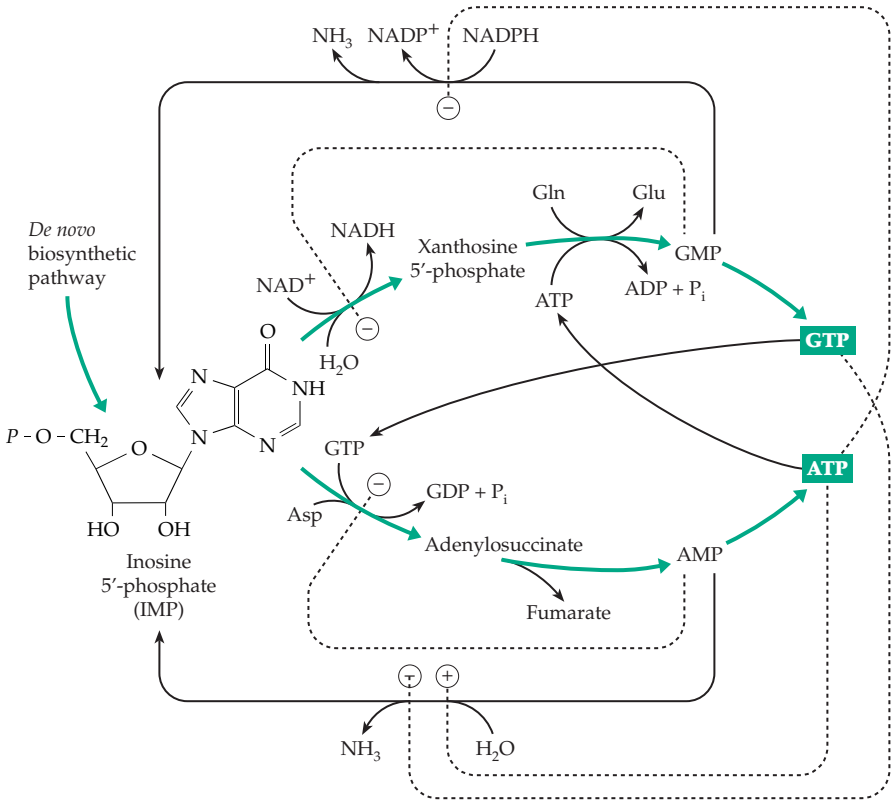



**Figure 25-16** Control of the conversion of inosine 5'-phosphate to the adenine and guanine ribonucleotides and deoxyribonucleotides in bacteria by feedback inhibition and activation.

messenger.[307–309] Adenosine has a variety of effects on all organs of an animal. It affects heart rate, smooth muscle tone, and white blood cell function. It modulates the catabolic effects of hormones such as catecholamines and stimulation of the anabolic hormone insulin.[309] Adenosine receptors of at least three types are known.[310,311] Binding of adenosine to $A_1$ receptors inhibits adenylate cyclase, while binding to $A_2$ receptors stimulates this enzyme.[307] However, effects of adenosine on $K^+$ transport are probably more important.

Deamination of adenosine (step *d*) together with reconversion of the resulting inosine to IMP (steps *e* and *f*) completes a second cycle (cycle B, Fig. 25-17). Intense interest has been focused on adenosine deaminase because hereditary lack of this enzyme is linked to a severe immunodeficiency in which the numbers of B and T lymphocytes are inadequate to combat infection.[312] Until recently bone marrow transplantation in infancy was the only possible treatment for this otherwise fatal disease. Regular injections of adenosine deaminase covalently attached to polyethylene glycol (to delay removal from the bloodstream) have been used for some patients. Since 1990 **gene therapy**, transfer of an adenosine deaminase gene into white blood cells, has also been used with apparent success.[313–315] This topic is discussed in Chapter 31. Adenosine deaminase is a 40-kDa protein,[316,317] which exists as a complex with a large 200-kDa binding protein[315] which anchors the deaminase to cell membranes.

The basic cause of the severe immunodeficiency symptoms is uncertain. However, adenosine deaminase also catalyzes hydrolysis of 2'-deoxyadenosine, and in the absence of the enzyme both this compound and its trinucleotide precursor 2'-deoxy-ATP (dATP) accumulate in tissues.[312] Ribonucleotide reductase is allosterically inhibited by dATP, and this inhibition may interfere with DNA synthesis and with the rapid growth of lymphocytes needed in response to infections. Since T lymphocytes are more severely affected than B lymphocytes, it is necessary to postulate a difference in the extent to which these two cell types accumulate dATP.

The conversions of inosine to hypoxanthine (Fig. 25-17, step *e*), of guanosine to guanine (step *g*), and of other purine ribonucleosides and deoxyribonucleosides to free purine bases are catalyzed by **purine nucleoside phosphorylase**.[318–321b] Absence of this enzyme also causes a severe immune deficiency which involves the T cells. However, B cell function is not impaired.[312,315,322]

The last enzyme in cycle B of Fig. 25-17 (catalyzing step *f* and also step *b*) is the X-linked **hypoxanthine-guanine phosphoribosyltransferase** (HGPRT or HPRT).[322a,b] Its absence causes the **Lesch–Nyhan** syndrome characterized not only by overproduction of uric acid but by a serious disorder of the central nervous system. It causes both mental retardation and a compulsive form of self-mutilation of the gums and hands by biting.[323–325] The excessive production of uric acid is easy to understand because the accumulating hypoxanthine and guanine are both readily converted to uric acid by the reactions of Fig. 25-18. Patients with a partial deficiency in HGPRT escape the worst neurological symptoms but may have severe gouty arthritis (Box 25-D ).[326] Efforts are being made to treat the disease by gene transfer.[327]

Trypanosomes and other parasitic protozoa are unable to synthesize purines and must obtain them from their hosts using salvage pathway. Selective inhibition of their HGPRT or of nucleoside hydrolases, which are absent from mammalian cells, are goals of drug development.[327a,b]

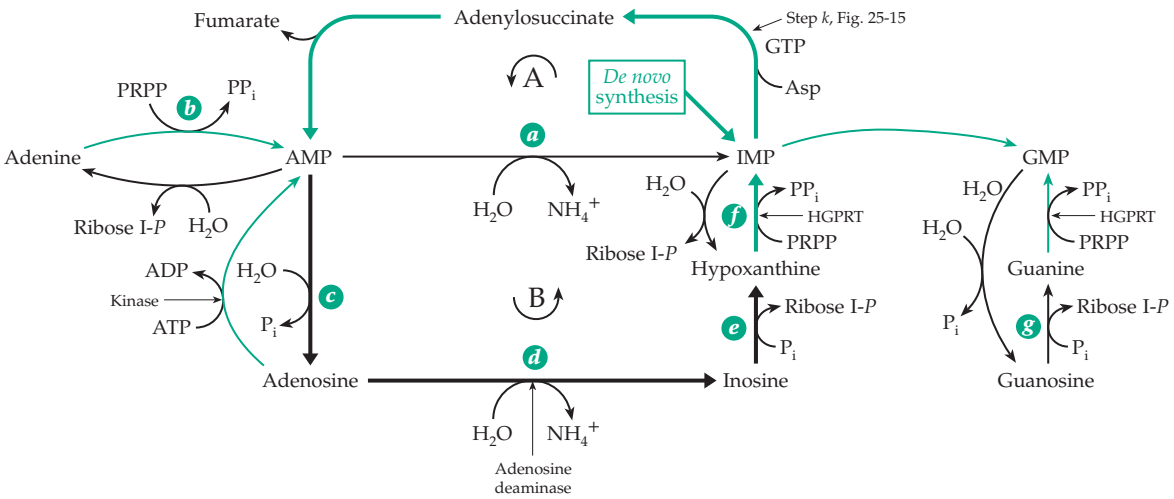



**Figure 25-17** Some purine salvage pathways and related reactions. Green lines indicate biosynthetic pathways.

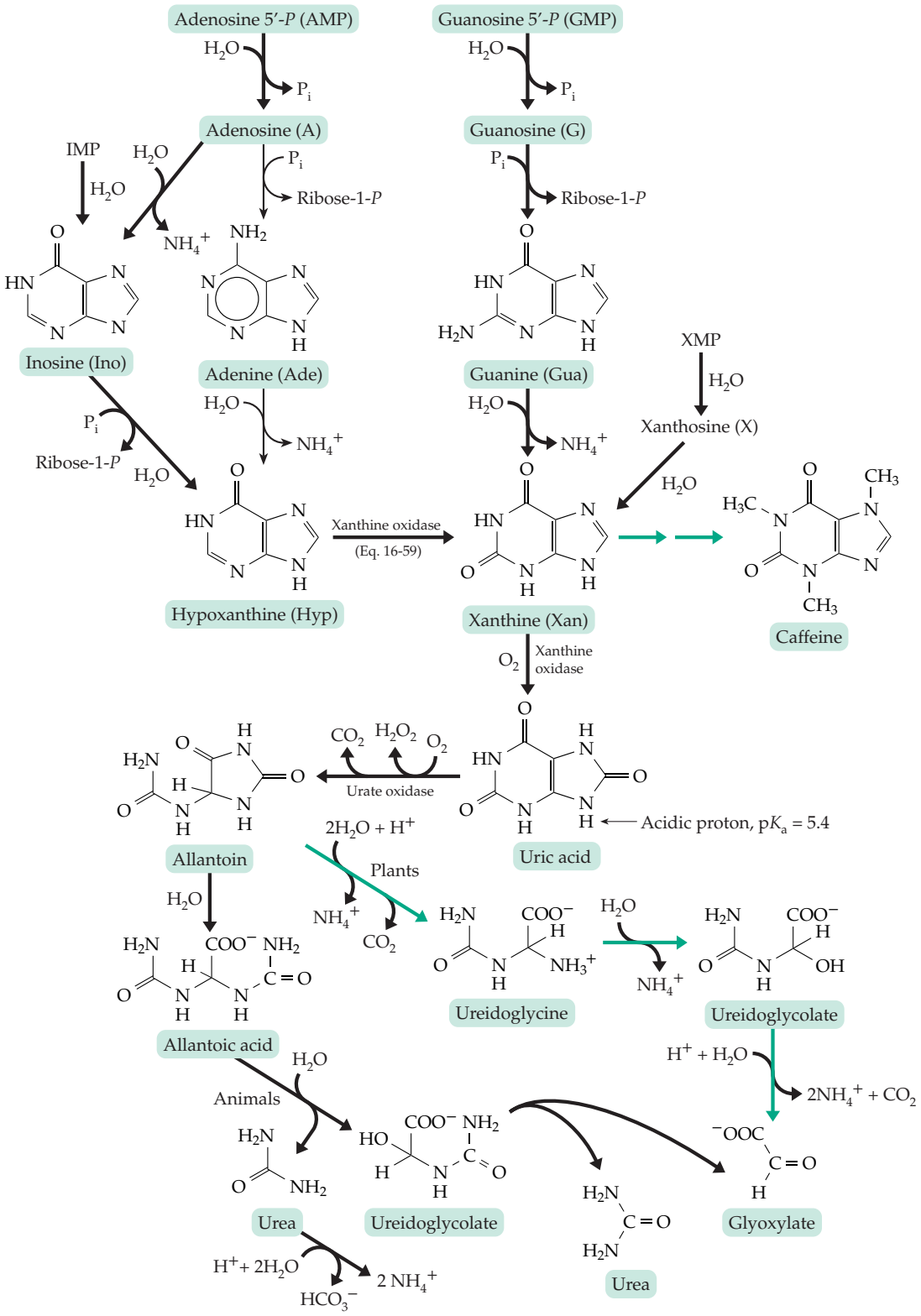



**Figure 25-18** Pathways of catabolism of purine nucleotides, nucleosides, and free bases. Spiders excrete xanthine while mammals and birds excrete uric acid. Spiders and birds convert all of their excess nitrogen via the *de novo* pathway of Fig. 25-15 into purines. Many animals excrete allantoin, urea, or $NH_4^+$. Some legumes utilize the pathway marked by green arrows in their nitrogen transport via ureides.

### 3. Oxidative Metabolism of Purines

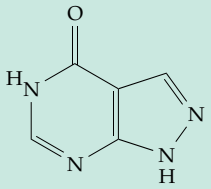
As indicated in Fig. 25-18, free adenine released from catabolism of nucleic acids can be deaminated hydrolytically to hypoxanthine, and guanine can be deaminated to xanthine.[328] The molybdenum-containing xanthine oxidase (Chapter 16) oxidizes hypoxanthine to xanthine and the latter on to uric acid. Some clostridia convert purine or hypoxanthine to xanthine by the action of a selenium-containing purine hydroxylase.[328a] Another reaction of xanthine occurring in some plants is conversion to the trimethylated derivative **caffeine**.[328b] One of the physiological effects of caffeine in animals is inhibition of pyrimidine synthesis.[329] However, the effect most sought by coffee drinkers may be an increase in blood pressure caused by occupancy of adenosine receptors by caffeine.[330]

Uric acid is the end product of purine metabolism in the human. Spiders excrete **xanthine**,[331,332] but in most animals **urate oxidase** converts uric acid to **allantoin**. Although urate oxidase contains no coenzymes or metal cofactors,[333] it catalyzes the reaction with $O_2$ to form a peroxide (Eq. 25-22),[334,335] a reaction resembling that of a reduced flavin (Eq. 15-31) or a reduced pterin. As in these other cases there may be an initial electron transfer between $O_2$ and urate to form a radical pair which couple. Elimination of $H_2O_2$, a feature also of flavoprotein oxidases, accomplishes oxidation of the urate ring. Hydration, ring opening, and decarboxylation complete the conversion to allantoin. The ease of formation of urate radicals permits uric acid to act as an effective oxidant. This may account for the fact that we long-lived human beings retain a high internal urate concentration.[335a,b]

## BOX 25-D GOUT

A common metabolic derangement with an incidence of ~3 per 1000 persons is **hyperuricemia** or **gout**.[a,b] As with most metabolic defects, there is a family of diseases ranging from mild to severe. In acute gouty arthritis, a sudden attack occurs, usually in the night, when sodium urate crystals precipitate in one or more joints. In half the cases the victim is awakened by a terrible pain in the big toe. The disease most often strikes adult males. The heredity is apparently complex and not fully understood. The primary biochemical defect in gout is usually an overproduction of uric acid which, in some cases, may result from an overactive PRPP synthase.[c] In other cases a kidney defect interferes with excretion. The less severe saturnine gout, which occurs in relatively young persons of both sexes, is a result of chronic lead poisoning. It may involve deposition of guanine in the joints as a result of inhibition by $Pb^{2+}$ of guanine aminohydrolase, the enzyme that hydrolyzes guanine to xanthine (Fig. 25-18).[d]

If properly controlled, simple gout may have few adverse effects. However, the severe neurological symptoms of Lesch–Nyhan syndrome (Section E,2 of text)[e] cannot be corrected by medication. Colchicine (Box 7-D), in a manner which is not understood, alleviates the painful symptoms of gout caused by the deposits of sodium urate in joints and tissues. It is also important to keep the dietary purine intake low and it is often necessary to inhibit xanthine oxidase. A widely used and effective inhibitor is the isomer of hypoxanthine known as **allopurinol**, which is taken daily in amounts of 100–600 mg or more.



Allopurinol

Allopurinol and its oxidation product **oxypurinol**, a xanthine analog, both inhibit xanthine oxidase and patients receiving allopurinol excrete much of their purines as xanthine and hypoxanthine. Nucleotide derivatives of oxypurinol also inhibit the *de novo* purine biosynthetic pathway. The accumulating hypoxanthine is reused to a greater extent than normal, decreasing the total purine excretion. A number of other drugs stimulate increased excretion of uric acid.[e] Although many patients tolerate allopurinol for many years, some experience dangerous side effects.

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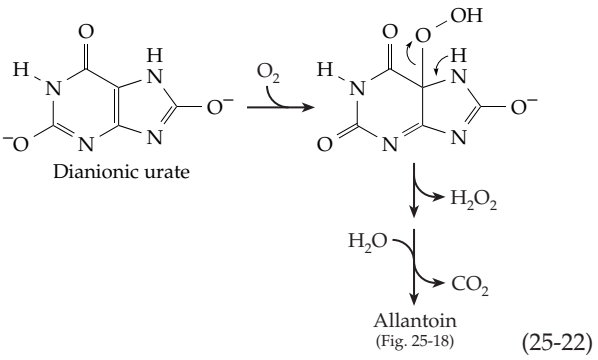
[a] Becker, M. A., and Roessler, B. J. (1995) in *The Metabolic and Molecular Bases of Inherited Disease*, 7th ed., Vol. 2 (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., eds), pp. 1655–1677, McGraw-Hill, New York

[b] Kelley, W. N., and Wyngaarden, J. B. (1972) in *The Metabolic Basis of Inherited Disease*, 3rd ed. (Stanbury, J. B., Wyngaarden, J. B., and Fredrickson, D. S., eds), pp. 969–1002, McGraw-Hill, New York

[c] Becker, M. A., Kostel, P. J., and Meyer, L. J. (1975) *J. Biol. Chem.* **250**, 6822–6830

[d] Farkas, W. T., Stanawitz, T., and Scheider, M. (1978) *Science* **199**, 786–787

[e] Rossiter, B. J. F., and Caskey, C. T. (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. 1679–1706, McGraw-Hill, New York




(25-22)

Allantoin is the excretory product in most mammals other than primates. Most fish hydrolyze allantoin to **allantoic acid,** and some excrete that compound as an end product. However, most continue the hydrolysis to form urea and glyoxylate using peroxisomal enzymes.[336] In some invertebrates the urea may be hydrolyzed further to ammonia. In organisms that hydrolyze uric acid to urea or ammonia, this pathway is used only for degradation of purines from nucleotides. Excess nitrogen from catabolism of amino acids either is excreted directly as ammonia or is converted to urea by the urea cycle (Fig. 24-10).

Plants also form the **ureides** allantoin and allantoic acid, and in some legumes, such as soy beans, these compounds account for 70–80% of the organic nitrogen in the xylem. They appear to function in nitrogen transport.[337] As indicated in Fig. 25-18, the hydrolysis to glyoxylate, $NH_4^+$, and $CO_2$ follows a different pathway than in animals. See also Chapter 24, Section C.

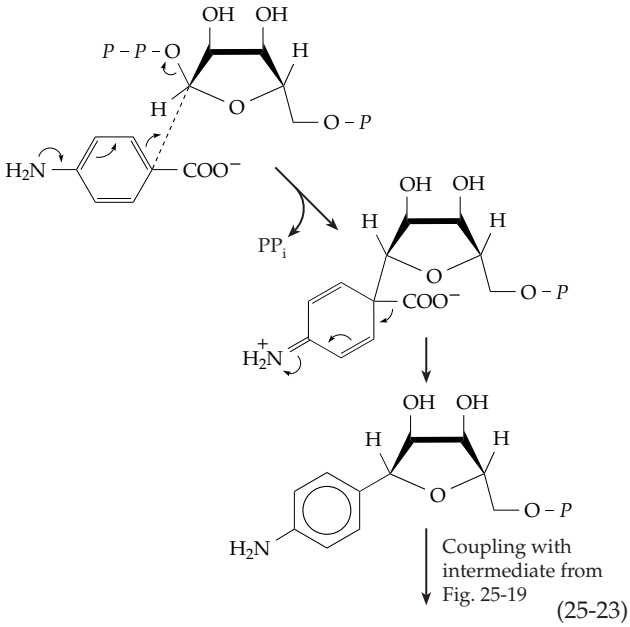
## F. Pterins, Flavins, Dimethylbenzimidazole, Thiamin, and Vitamin $B_6$

Tracer studies have established that both folic acid and riboflavin originate from guanosine phosphates (Fig. 25-19).[338–341] All of the atoms of the purine ring are conserved in the products except for C-8 of the five-membered ring. The first step is opening of the 5-membered ring and the hydrolytic removal of formate (Fig. 25-19, step *a*). This is followed by an Amadori rearrangement (step *b*) and a simple ring closure between the resulting carbonyl and adjacent amino group (step c). The product is **7,8-dihydroneopterin triphosphate**. The single enzyme **GTP cyclohydrolase** catalyzes all three steps *a*–*c*.[342,342a–c] Dihydroneopterin is a central intermediate in pterin metabolism, being converted by one route into folate and methanopterin coenzymes and by another into biopterin, drosopterin, and others.[343]

An aldol cleavage (step *d*) followed by a series of reactions, shown at the left in in Fig. 25-19, leads to folate.[344–347] The reactions include the ATP-dependent conversion of 6-hydroxy-7,8-dihydropterin to its pyrophosphate ester.[347a] This is followed by coupling to *p*-aminobenzoate with elimination of $PP_i$ to form dihydropteroic acid.[347b] ATP-dependent joining of glutamate and reduction yields **tetrahydrofolate**. Additional molecules of γ-linked glutamate are added to form the functional polyglutamate forms (p. 803).[347c,d] Gamma-glutamyl hydrolases provide essential turnover.[347e] Formation of biopterin is initiated by tautomerization of dihydroneopterin triphosphate (step *e*), a proton from the solvent binding to C-6 of the tetrahydropterin ring.[348] The single $Zn^{2+}$-dependent enzyme **6-pyruvoyltetrahydropterin synthase**[349–352] catalyzes the two consecutive tautomerization steps shown in Fig. 25-19 as well as elimination of the triphosphate group tripolyphosphate (step *f*), a reaction facilitated by the carbonyl group introduced in the preceding step.[349] The same enzyme promotes a final tautomerization to form 6-pyruvoyltetrahydropterin, a compound that is reduced by NADPH to tetrahydrobiopterin. The reaction is catalyzed by **sepiapterin reductase**.[353,354] Notice that biopterin, like the folates, is synthesized at the oxidation level of a tetrahydrobiopterin.

The *Drosophila* eye pigments **sepiapterin** and **drosopterins** (Figs. 15-17 and 25-19) arise from 6-pyruvoyltetrahydropterin.[355–357] Reduced glutathione appears to be the reducing agent needed to convert the 6-pyruvoyltetrahydropterin into the more reduced pyrimidinodiazepine (step *h*) with its 7-membered ring (Fig. 25-19).[357,358] Tetrahydrobiopterin can arise in mammalian cells, not only by the *de novo* pathway of Fig. 25-19 but also from salvage of sepiapterin.

**Methanopterin** (Fig. 15-17) is formed via a branch in the folate pathway. The 5-carbon chain that replaces the carboxyl group of *p*-aminobenzoate in the folates is derived by reaction of PRPP with *p*-aminobenzoate (Eq. 25-23).[359,360] White has proposed a

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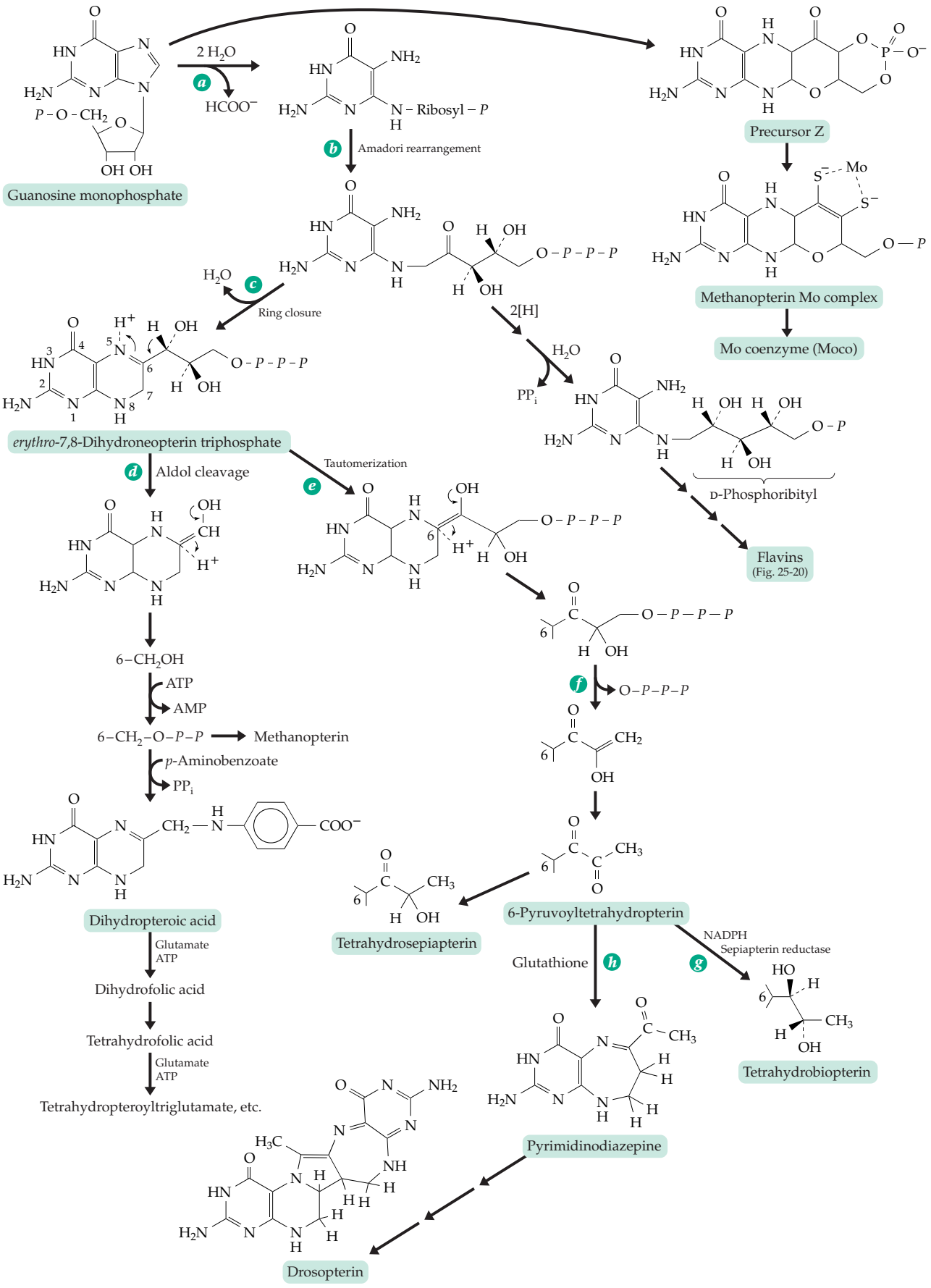



**Figure 25-19** The biosynthesis of folic acid and other pterins.

detailed pathway for completion of the methanopterin synthesis.[359] After coupling with 6-hydroxymethyl-7,8-dihydropterin pyrophosphate (shown in abbreviated form in Fig. 25-19) with loss of $PP_i$, the ribose ring in the product of Eq. 25-23 is reductively opened to give the ribityl side chain of methanopterin (see Fig. 15-17). Hydrolytic removal of the phosphate is then followed by transfer of the α-linked ribose phosphate. The *S*-hydroxyglutarate (derived by reduction of 2-oxoglutarate) is added in an ATP-dependent reaction. The extra methyl groups at positions 7 and 9 are transferred from *S*-adenosylmethione and the compound is reduced to the tetrahydropterin state.

Biosynthesis of **molybdopterin** (Fig. 15-17) also begins with a guanosine derivative, quite possibly GMP as is shown in Fig. 25-19. However, the C-8 carbon atom is not lost as formate, as in step *a* of Fig. 25-19, but is incorporated into the side chain in the molybdopterin precursor Z. A pathway was proposed by Wuebbens and Rajagopalan.[361] The first stage in the reaction sequence,[361a–d] which is identical in most organisms, is formation of the metastable compound Z, a proposed structure of which is shown in Fig. 25-19. The conversion of this compound into molybdopterin requires opening of the cyclic phosphate ring and incorporation of two atoms of sulfur. These may both be released from cysteine as $S^0$ and carried by a sulfurtransferase as an enzyme-bound persulfide group (see Chapter 24, Section G,3).[361b] A thiocarboxylate group generated from a C-terminal carboxylate of a molybdopterin synthase subunit, as in the ThiS protein (p. 1463), may be the direct sulfur donor. It is probably formed in an ATP-dependent process.[361b] Incorporation of molybdenum, perhaps from $MoO_4^{2-}$, completes the synthesis of the molybdenum cofactor Moco.[361c,e] In some molybdoenzymes, e.g., xanthine oxidase, an additional sulfur atom is bound to the Mo atom (Fig. 16-32). This is also obtained from cysteine using a PLP-dependent sulfurtransferase similar to the NifS protein.[361f,g] In many bacteria molybdopterin is joined to GMP, AMP, IMP, or CMP to form a dinucleotide.[361h,i]

Both the fungus *Eremothecium* (Box 15-B) and mutants of *Saccharomyces* have been used to deduce the pathways of **riboflavin** synthesis outlined in Figure 25-20. The first reaction (step *a*) is identical to step *a* of Fig. 25-19 but is catalyzed by a different GTP cyclohydrolase.[362] Instead of an Amadori rearrangement it catalyzes the hydrolytic deamination and dephosphorylation (step *b*) to give the flavin precursor 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine. Additional carbon atoms to build the benzene ring of riboflavin are supplied in two stages from ribulose 5-phosphate. Isotopic labeling showed that carbon atoms 1, 2, 3, and 5 of this compound are utilized as is marked in Fig. 25-20, while C-4 is eliminated as formic acid in a rearrangement (step *c*, Fig. 25-20). A plausible mechanism has been suggested.[363,363a,b] The product L-3,4-dihydroxy-2-butanone 4-phosphate condenses (step *d*) with the product formed from GMP, possibly via the Schiff base shown. Elimination of $H_2O$ and inorganic phosphate followed by tautomerization gives **6,7-dimethyl-8-ribityllumazine**.[363a,364–364c] Completion of the flavin ring requires an additional four carbon atoms, which are supplied by a second molecule of 6,7-dimethyl-8-ribityllumazine, as indicated in Fig. 25-20.[364d] This disproportionation reaction appears remarkable but is less so when one considers that the bimolecular reaction to form riboflavin occurs spontaneously under mild conditions. The precursor 4-ribitylamino-5-amino-2,6-dihydroxypyrimidine is regenerated in this process (Fig. 25-20).

The enzyme complex that catalyses steps *d* to *f* of Fig. 25-20 has an unusual composition. An $\alpha_3$ trimer of 23.5-kDa subunits is contained within an icosahedral shell of 60 16-kDa β subunits, similar to the protein coats of the icosahedral viruses (Chapter 7). The β subunits catalyze the formation of dimethylribityllumazine (steps *d*, *e*), while the $\alpha_3$ trimer catalyzes the dismutation reaction of step *f*, the final step in riboflavin formation.[365] A separate bifunctional bacterial ATP-dependent synthetase phosphorylates riboflavin and adds the adenylyl group to form FAD.[366] Two separate mammalian enzymes are required.[367] Synthesis of **deazaflavins** of methanogens (Fig. 15-22) follows pathways similar to those of riboflavin. However, the phenolic ring of the deazaflavin originates from the shikimate pathway.[368]

Dimethylbenzimidazole, a constituent of vitamin $B_{12}$ (Box 16-B), also arises from 6,7-dimethyl-8-ribityllumazine in a process resembling that of riboflavin synthesis, but in which the riboflavin formed is hydrolytically degraded to remove the pyrimidine ring and to form the imidazole ring.[369] Conversion to an α-ribazole and linkage to the aminopropyl group of the corrin ring is described by Thompson *et al*.[370] Various related cobamides are also formed by bacteria.[371] Synthesis of the corrin ring is described briefly in Chapter 24, and the chemistry of the ligands to cobalt at the "top" of the vitamin $B_{12}$ molecules is considered in Chapter 16, Section B.

***Thiamin.*** Investigation of the biosynthesis of thiamin has been difficult because only minute amounts are formed by microorganisms such as *E. coli* or yeast. Furthermore, significant differences in the routes of synthesis in different organisms have caused confusion.[372,372a] The pathways outlined in Fig. 25-21 are incomplete.

The pyrimidine portion of thiamin (Fig. 25-21) is distinct in structure from the pyrimidines of nucleic acids. In bacteria it originates from the purine precursor 5-aminoimidazole ribotide, which is converted into a hydroxymethylpyrimidine (Fig. 25-21)[373] which is

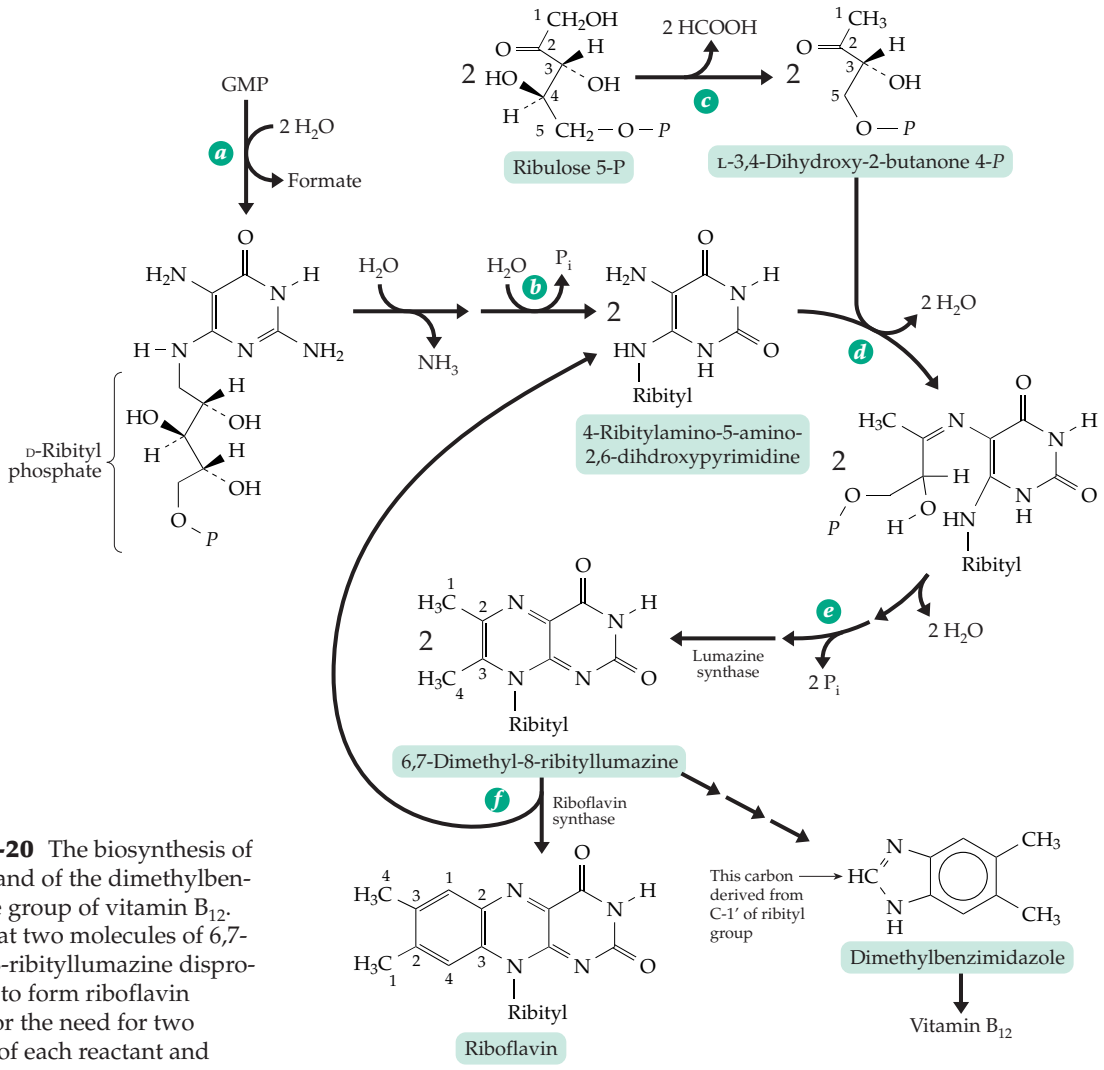
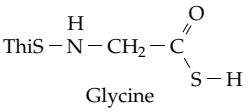



**Figure 25-20** The biosynthesis of riboflavin and of the dimethylbenzimidazole group of vitamin $B_{12}$. The fact that two molecules of 6,7-dimethyl-8-ribityllumazine disproportionate to form riboflavin accounts for the need for two molecules of each reactant and product in many steps.

coupled with the thiazolium ring to form the vitamin. All of the carbon atoms of the substituted pyrimidine can be derived from the 5-aminoimidazole ribotide, but the pathway is uncertain. Both glycine and formate enter the pyrimidine, but labeling patterns are different in *E. coli* and in yeast.[372]

The thiazole ring is assembled on the 5-carbon backbone of **1-deoxyxylulose 5-phosphate**, which is also an intermediate in the alternative biosynthetic pathway for terpenes (Fig. 22-2) and in synthesis of vitamin $B_6$ (Fig. 25-21). In *E. coli* the sulfur atom of the thiazole comes from cysteine and the nitrogen from tyrosine.[374] The same is true for chloroplasts,[375] whereas in yeast glycine appears to donate the nitrogen.[372] The thiamin biosynthetic operon of *E. coli* contains six genes,[372a,376] one of which (***ThiS***) encodes a protein that serves as a sulfur carrier from cysteine into the thiazole.[374] The C-terminal glycine is converted into a thiocarboxylate:




The *ThiI* gene, which encodes aother sulfurtransferase protein, is also needed.[374a] The enzymology of the insertion of this sulfur into the thiazole is uncertain but may resemble that involved in synthesis of biotin, lipoic acid, and molybdopterin.[374] Linkage of the two parts of the thiamin molecule (step *d*, Fig. 25-21) is catalyzed by thiamin phosphate synthase, evidently via an $S_N2$ type reaction.[377–377b]

***Pyridoxol (vitamin $B_6$).*** Again 1-deoxyxylulose 5-*P* serves as a precursor.[378] In *E. coli* only two genes have been implicated in the condensation of this compound with 4-(phosphohydroxy)-L-threonine (Fig. 25-21, step *f*).[378a] One is an $NAD^+$-dependent dehydrogenase that acts on the second substrate prior to the condensation. Significant differences from the path-

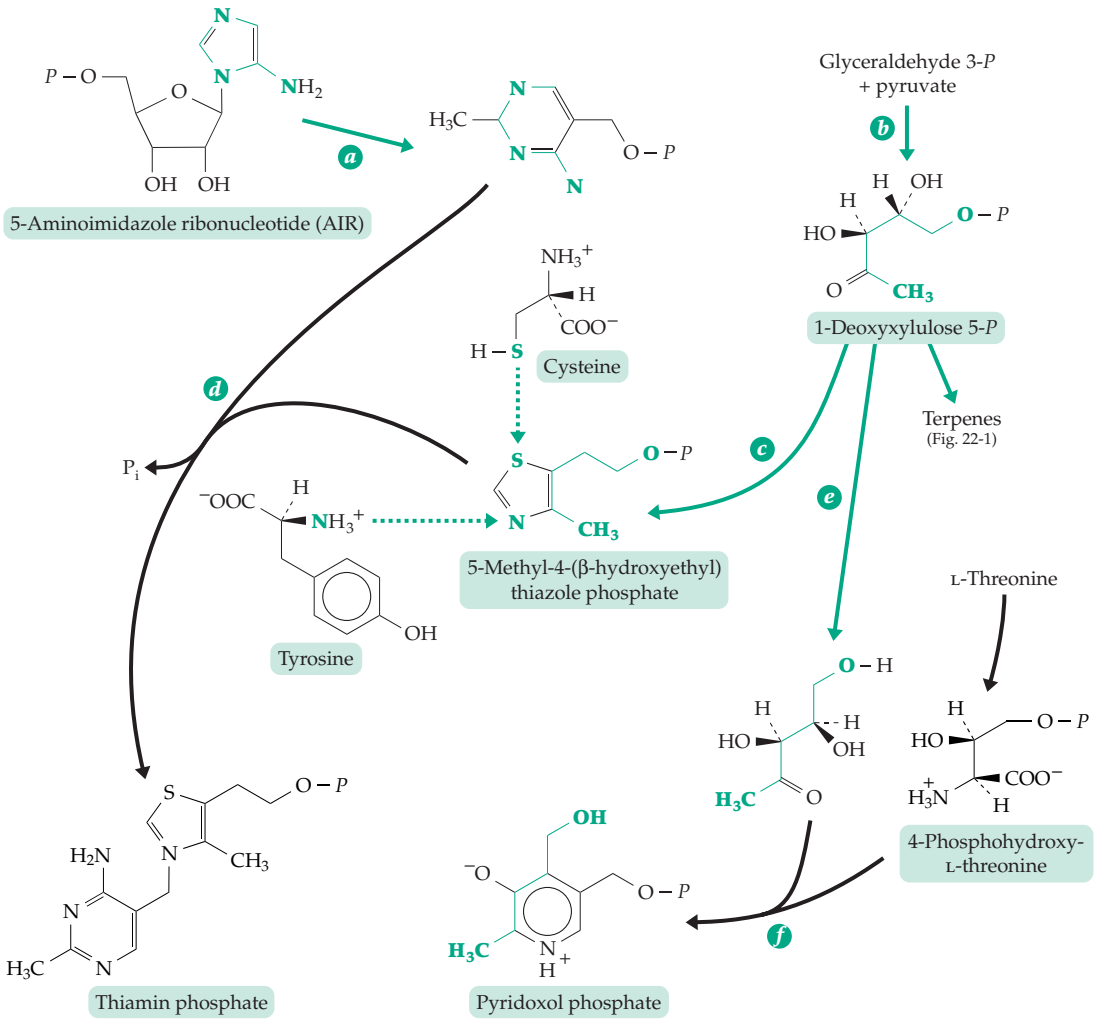
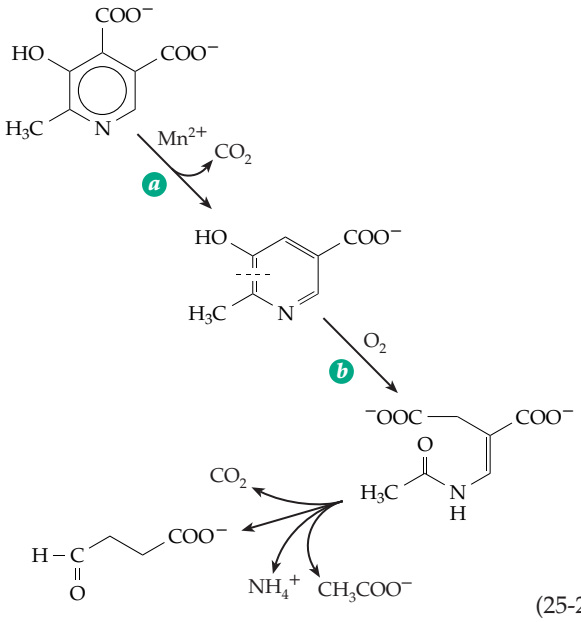



**Figure 25-21** Proposed pathways for biosynthesis of thiamin phosphate and pyridoxol phosphate.

way shown in Fig. 25-21 exist in yeast and other fungi.[378b,c] Interconversion of pyridoxal phosphate and other forms of vitamin $B_6$ is discussed in Chapter 14.

The degradation of pyridoxol by bacteria has been investigated in detail.[379–381] In one pathway the hydroxymethyl group in the 5 position and the substituent in the 4 position are both oxidized in the early steps to carboxylate groups. Then, as indicated in Eq. 25-24, a decarboxylation is followed by the action of an unusual dioxygenase.

Isolated from a strain of *Pseudomonas*, this enzyme contains bound FAD, which must be reduced by external NADH. Like a typical dioxygenase the enzyme introduces two atoms of oxygen into the product. However, it also uses the reduced FAD to reduce the double bond system (either before or after the attack by oxygen).[381] Another enzyme of the same bacterium is remarkable in hydrolyzing the product of the oxygenation reaction to four different products without the accumulation of intermediates.




(25-24)

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## Study Questions

1. The vitamin niacin (nicotinic acid) is converted to NAD through the intermediate, desamido NAD.

   a) Propose an enzymatic synthesis of desamido NAD beginning from niacin and ribose 5-phosphate
   b) Propose an enzymatic synthesis of NAD beginning from desamido NAD

2. AMP is formed from IMP in an ATP requiring reaction. The introduced nitrogen atom is derived directly from aspartate.

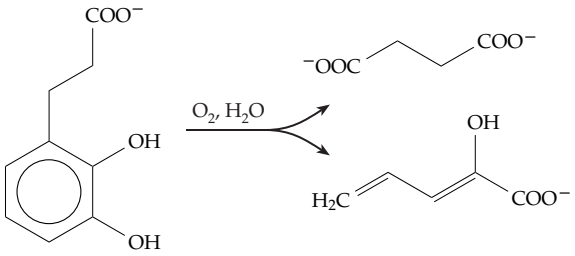
   a) Propose a reaction mechanism based on an analogous reaction sequence occurring during amino acid metabolism.
   b) Identify the analogous enzymes upon which you have based your answer by names of the enzymes or by the names of the substrates and products.

3. One of the mechanisms proposed for the decarboxylation of orotidine 5'-phosphate to UMP involves initial addition of an enzyme nucleophile to the pyrimidine ring. Describe and criticize this mechanism. Hint: The proposal has some similarity to the thymidylate synthase mechanism.

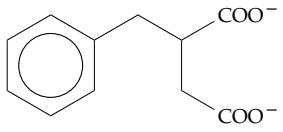
4. Name the enzymes and describe the chemical reaction that occurs for each of the four steps in the following pathway:

$$\text{UDP} \xrightarrow{1} \text{dUDP} \xrightarrow{2} \text{dUTP} \xrightarrow{3} \text{dUMP} \xrightarrow{4} \text{dTMP}$$

5. The following reaction is catalyzed by a dioxygenase from *E. coli*. The dioxygenase reaction opens the ring and the intermediate is cleaved hydrolytically. Propose a structure for the intermediate and a mechanism for the hydrolytic cleavage. See Fleming *et al*.[382]




6. Anaerobic breakdown of toluene by denitrifying bacteria begins by the addition of toluene to fumarate to form benzylsuccinate. Benzylsuccinate synthase has an amino acid sequence homologous to that of pyruvate formate lyase (p. 799–801), contains a glycyl radical, and is activated in a manner similar to activation of pyruvate formate lyase. Propose a mechanism for formation of benzylsuccinate. See Krieger *et al.*[383]




Benzylsuccinate

7. Write out, using structural formulas, a step-by-step reaction sequence for the conversion of *O*-succinylbenzoate into isochorismate as indicated in Fig. 25-4.

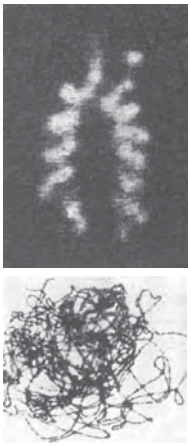
Right: The human **karyotype**, a full set of chromosomes numbered according to size and content and showing characteristic banding patterns. The 22 autosomes plus either an X or Y chromosome make up the haploid set which, for the female, contains ~ 3,500 Mbp of DNA. Each diploid cell contains 46 chromosomes. From Alberts *et al.* "Molecular Biology of the Cell", Third edition, Garland, New York, 1994, p 356.

Upper Left: A pair of mitotic sister chromatids in a section stained with an antibody to topoisomerase II. Notice that the two chromatids are coiled with opposite helical handedness. Lower Left: Meiotic chromosomes of a lily at the pachytene stage in which sister chromatids are connected along their length in a **synaptonemal complex.** From Kleckner, N. (1996) *Proc. Natl. Acad. Sci. U.S.A.* **93**, 8167–8174

# Contents

# Biochemical Genetics

# 26



The most exciting biological discoveries of the 20th century include the unraveling of the genetic code (Chapter 5) and the understanding of the ways in which nucleic acids and proteins are synthesized. The biosynthesis of both nucleotides and amino acids has been considered in Chapters 24 and 25 and the basic chemistry of the polymerization processes in Chapters 12 and 17. Chapters 27–29 deal with the ways in which these polymerization reactions are controlled and by which the correct sequences of nucleotides or amino acids are obtained. The understanding of these matters is a development of genetics[1–2b] as well as of biochemistry; hence this introductory and historical chapter.

## A. Historical Sketch

The discovery of deoxyribonucleic acid dates to 1869, when Miescher isolated a new chemical substance from white blood cells that he obtained from pus and later from sperm cells.[3] The material, which became known as nucleic acid, occurred in both plants and animals, thymus glands and yeast cells being among the best sources. Chemical studies indicated that the nucleic acids isolated from thymus glands and from yeast cells were different. As we now know, thymus nucleic acid was primarily DNA and yeast nucleic acid primarily RNA. For a while it was suspected that animals contained only DNA and plants only RNA, and it was not until the early 1940s that it was established that both substances were present in all organisms.[3–6]

### 1. DNA as the Genetic Material

In 1928, Griffith, using cells of *Diplococcus pneumoniae*, showed that genetic information that controls properties of the capsular polysaccharides could be transferred from one strain of bacteria to another. A material present in killed cells and in cell-free extracts permanently altered the capsular properties of cells exposed to the material.[7] This **transformation** of bacteria remained a mystery for many years. At the time of the experiments there was no hint of the genetic role of nucleic acids, which were generally regarded as strange materials. Furthermore, the covalent structure of nucleic acids was uncertain. A popular idea was that a tetranucleotide served as a repeating unit for some kind of regular polymer. Genes were most often thought to be protein in nature.

However, in 1944, Avery and associates showed that purified DNA extracted from pneumococci could carry out transformation.[8–12] The transforming principal appeared to contain little protein. It was not inactivated by proteolytic enzymes but was inactivated by deoxyribonuclease. Avery was 67 at the time of this discovery, refuting the popular contention that all important scientific discoveries are made by young people.

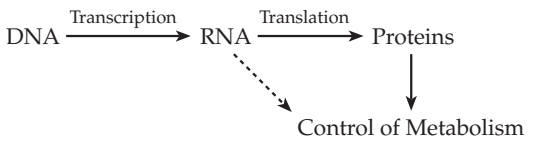
Other experiments also pointed to the conclusion that DNA was the genetic material. DNA was found localized in the nuclei of eukaryotic cells. The absolute amount per cell was constant for a given species. Studies of bacteriophage replication pointed strongly to DNA as the genetic material.[11] In 1952 Hershey and Chase showed that when a phage particle infects a cell the viral DNA enters the bacterium, but the protein "coat" remains outside.[13] This was demonstrated by

preparing two types of labeled bacteriophage T2. One contained $^{32}P$, which had been incorporated into the DNA, and the other $^{35}S$ incorporated into the proteins. Cells of *E. coli* were infected with the labeled phage preparations and were then agitated violently in a blender to shear the phage particles off of the bacteria. Over 80% of the $^{35}S$ was removed from the bacteria by this treatment, but most of the $^{32}P$ entered the bacteria and could be recovered in the next generation of progeny bacteriophage.[14]

## 2. The Double Helix

Development of newer methods of investigation of the chemical composition of nucleic acids led Chargaff to an important discovery. Although the base composition of DNA was extremely variable from organism to organism, the molar ratio of adenine to thymine was nearly always 1:1, as was that of cytosine to guanine![15] This observation provided the basis for the concept of base pairing in the structure of DNA. The final important information was supplied by X-ray diffraction studies of stretched fibers of DNA, which showed that molecules of DNA were almost certainly helical structures containing more than one chain. The crucial experiments were done by Rosalind Franklin[16–17b] and M. H. F. Wilkins, whose data were used by Watson and Crick in 1952 in constructing their model of the double helical structure[12,18–20] (Fig. 5-6). Once established, the structure of DNA itself suggested both the nature of the genetic code and a replication mechanism. *The genetic code had to lie in the sequence of nucleotides. Base pairing provided a mechanism by which the two mutually complementary strands could be separated. Biosynthesis of a new complementary strand alongside each one would result in a precise replication of each gene and of the entire genome.* In a similar fashion, RNA could be synthesized alongside a DNA "template" and could then be used to direct protein synthesis.

The presence of RNA in the cytoplasm had been linked to protein synthesis by experiments done in the early 1940s. After the discovery of the double helix, the concept followed quickly that DNA was the master "blueprint" from which secondary blueprints or **transcripts** of RNA could be copied. The RNA copies, later identified as **messenger RNA** (mRNA), provided the genetic information for specifying protein sequence. The flow of information from DNA to RNA to proteins could be symbolized as in Eq. 26-1.




(26-1)

Proteins, in one way or another, control nearly all of metabolism. This includes the reactions that form the nucleotide precursors of the nucleic acids and that lead to polymerization of the amino acids and nucleotides, reactions catalyzed by **protein enzymes** and **ribozymes**. Thus, the flow of information from DNA to proteins is only part of a larger loop of metabolic processes (p. 973). Genetic information flows from DNA out into the cell, and copies of the master blueprint are passed nearly unchanged from generation to generation. The simple concepts implied by Eq. 26-1 quickly caught the imagination of the entire community of scientists and led to a rapid blossoming of the field of biochemical genetics.[20a,b]

## 3. Ribonucleic Acids and Proteins

By 1942 it was clear from ultraviolet cytophotometry developed by Caspersson[21] and from cytochemical work of Brachet[22,23] that RNA had something to do with protein synthesis. Use of radioautography with $^{3}H$-containing uridine showed that RNA was synthesized in the nucleus of eukaryotic cells and was transported out into the cytoplasm.[24,25]

Ribosomes were discovered by electron microscopists examining the structure of the endoplasmic reticulum using ultrathin sectioning techniques. Their presence in cells was established by 1956, and the name ribosome was proposed in 1957. At first it was difficult to study protein synthesis *in vitro* using isolated ribosomes. No *net* synthesis could be detected until Hoagland and associates measured the rate of incorporation of $^{14}C$-labeled amino acids into protein.[26] This sensitive method permitted measurement of very small amounts of protein synthesis in cell-free preparations from rat liver and paved the way toward studies with ribosomes themselves.

Immediately after the Watson–Crick proposals were made in 1953, it was generally thought that ribosomal RNA (rRNA), which constitutes up to 90% of the total RNA of some cells, carried the genetic message from the nucleus to the cytoplasm. By 1960 it seemed unlikely. For one thing the size and composition of rRNA was similar for different bacteria, despite differences in base composition of the DNA (Chapter 5).[27] It had been concluded that a relatively unstable, short-lived form of RNA must carry the message. Ribosomal RNA, however, was quite stable.[28]

***Messenger RNA.*** In 1956, Volkin and Astrachan[29,30] detected a rapidly labeled and labile RNA in phage-infected bacterial cells. Studies of enzyme induction also suggested the existence of mRNA. Many bacteria, including *E. coli*, when grown on glucose as the sole source of energy and then suddenly switched to lactose, are unable to utilize the new sugar

immediately. However, transfer to lactose induces, within a period of two minutes, the synthesis of new proteins needed for the metabolism of this sugar. Among the new proteins is a **permease** for lactose and a **β-galactosidase** (Chapter 12)[31] that cleaves the disaccharide to glucose and galactose. When the lactose is exhausted, the level of the induced enzymes drops almost as quickly. These results suggested that the RNA that carries the genetic message for synthesis of the new enzymes must be unstable. It must be produced rapidly in response to the presence of the inducing sugar and must disappear rapidly in its absence.

In 1961, Jacob and Monod postulated messenger RNA (mRNA) as a short-lived polynucleotide.[30,32,33] An abundance of additional evidence supported the proposal. For example, RNA molecules produced after infection of *E. coli* by bacteriophage T4 underwent hybridization (Chapter 5) with denatured DNA of the bacteriophage. Furthermore, this virus-specific mRNA became associated with preexisting bacterial ribosomes and provided the template for synthesis of phage proteins.[34] The experiment provided direct evidence for transcription of mRNA from genes of the viral DNA.

***Transfer RNA.*** Crick[35] suggested, in 1957, that special "adapter" molecules might be needed to align amino acids with their codons in the RNA transcript. He thought that the adapters might be polynucleotides. At the same time chemical studies of the RNA of cells revealed that a low-molecular-weight RNA made up 15% of the total RNA of *E. coli*. This RNA was recognized in the same year as constituting the needed adapters, when Hoagland demonstrated enzymatic "activation" of amino acids and their subsequent incorporation into protein. The name transfer RNA (tRNA; Figs. 5-30, 5-31) was proposed.[36]

## 4. Deciphering the Genetic Code

The genetic code consists of triplets of DNA base pairs (codons), each corresponding to a single amino acid. The triplets are consecutive, do not overlap, and are not separated by any "punctuation." Although this represented one of the simplest possibilities for a code, it required much effort more than a period of more than 10 years to prove it. Even after the triplet nature of the genetic code became evident, many questions remained. Were all of the 64 possible codons used by the living cell? If so, were they all used to code for amino acids or were some set aside for other purposes? How many codons were used for a single amino acid? Was the code universal or did different organisms use different codes? How could one decipher the code? Despite the complexity of these questions, they seem to have been almost completely answered.

In an important experiment[37] Nirenberg and Matthaei, in 1961, isolated ribosomes from *E. coli* and mixed them with crude extracts of soluble materials, also from *E. coli* cells. The extracts included tRNA molecules and aminocyl-tRNA synthases. The 20 amino acids, ATP, and an ATP-generating system (PEP + pyruvate kinase) were added. Nirenberg showed that under such conditions protein was synthesized by ribosomes in response to the presence of added RNA. For example, RNA from tobacco mosaic virus (Chapter 7) was very effective in stimulating protein synthesis. The crucial experiment, which was done originally simply as a "control," was one in which a synthetic polynucleotide consisting solely of uridylic acid units was substituted for mRNA. In effect, this was a synthetic mRNA containing only the codon UUU repeated over and over. The ribosomes read this code and synthesized a peptide containing only phenylalanine. Thus, poly(U) gave polyphenylalanine, and *UUU was identified as a codon specifying phenylalanine*. The first nucleotide triplet had been identified! In the same manner CCC was identified as a proline codon and AAA as a lysine codon. Study of mixed copolymers containing two different nucleotides in a random sequence suggested other codon assignments. A few years later, after Khorana had supplied the methods for synthesis of oligonucleotides and of regular alternating polymers of known sequence, the remaining codons were identified.

Another important technique was based on the observation that synthetic trinucleotides induced the binding to ribosomes of tRNA molecules that were "charged" with their specific amino acids.[38,39] For example, the trinucleotides UpUpU and ApApA stimulated the binding to ribosomes of $^{14}$C-labeled phenylalanyl-tRNA and lysyl-tRNA, respectively. The corresponding dinucleotides had no effect, an observation that not only verified the two codons but also provided direct evidence for the triplet nature of the genetic code. Another powerful approach was the use of artificial RNA polymers, synthesized by combined chemical and enzymatic approaches.[40] For example, the polynucleotide CUCUCUCUCU · · · led to the synthesis by ribosomes of a regular alternating polypeptide of leucine and serine.

Table 5-5 shows the codon assignments, as we now know them, for each of the 20 amino acids. Table 5-6 shows the same 64 codons in a rectangular array. In addition to those codons assigned to specific amino acids, three are designated as **chain termination codons**: UAA, UAG, and UGA. These are frequently referred to as "nonsense" codons. The termination codons UAA and UAG are also known as *ochre* and *amber*, respectively, although these names have no scientific significance.[41] The codons AUG (methionine)

and much less often GUG (valine) serve as the **initiation codons** in protein synthesis. Consequently, the N-terminal amino acid in most newly synthesized eukaryotic proteins is methionine, and in bacterial proteins it is *N*-formylmethionine. As explained in Chapter 29, *N*-formylmethionyl-tRNA is specifically bound to initiation sites containing the AUG codon in bacterial mRNA-ribosome complexes.

A number of studies suggested that the genetic code as worked out for *E. coli* might be universal. For example, in the laboratories of Wittman and of Fraenkel-Conrat, RNA extracted from tobacco mosaic virus was treated with nitrous acid, a procedure known to deaminate many cytosine residues to uracil (Eq. 5-12). Such treatment could change the codon UCU (serine) to UUU (phenylalanine) and the codon CCC (proline) to CUC (leucine). When the nitrous acid-treated RNA was used to infect tobacco plants and virus particles were prepared in quantity from the resultant mutant strains, it was found that the amino acid sequence of the virus coat protein had been altered,[42] and that many of the alterations were exactly those that would be predicted from Table 5-6. Likewise, the amino acid substitutions in known defects of hemoglobin (Fig. 7-23) could be accounted for, in most cases, by single base alterations. Thus, hemoglobin S arose as a result of the following change in the sixth codon of the globin β chain gene: GAG (Glu) → GTG (Val).[43] Another argument favoring a universal code was based on the observation that mRNA coding for a globin chain could be translated by ribosomes and tRNA molecules from *E. coli.* The resulting protein was authentic mammalian globin.[44]

As often happens, a well-established conclusion may have to be modified. There are exceptions to the universal genetic code in mitochondrial DNA and in some protozoa (Chapter 5).[45]

## B. Genetic Methods

Our present knowledge of molecular biology has depended greatly on the methods of genetics. The following introduction begins with a consideration of mutations.

### 1. Mutations

Changes in the structure of DNA occur only rarely. The average gene may be duplicated $10^6$ times before some mistake results in a single detectable mutation.[46] Nevertheless, by using bacteria or bacterial viruses, it is possible to screen enormous numbers of individuals for the occurrence of mutations. If one million virus particles are spread on an agar plate under conditions where mutation in a certain gene can be recognized, on the average one mutant is found. The most common mutations are **base-pair switches** or **point mutations** that result from incorporation of the wrong base during replication or repair. In these mutations one base of a triplet codon is replaced by another to form a different codon, causing the substitution of one amino acid by another in the corresponding protein as was seen for hemoglobin S. Changes involving replacement of one pyrimidine by another (C → T or T → C) or of one purine by another are sometimes called **transition mutations**, whereas if a pyrimidine is replaced by a purine, or vice versa, the mutation is known as a **transversion**. An example is the previously mentioned A → T in hemoglobin S. Transition mutations are by far the most common, one possible cause being pairing with a minor tautomer of one of the bases (Chapter 5). For example, A could pair with a minor tautomer of C, causing a mutation from T to C. Note that substitution of an incorrect base in one strand will lead, in the next round of replication, to correct pairing again but with an AT pair replaced by GC, or vice versa, in one of the daughter DNA duplex strands. A base substitution does not always cause an amino acid replacement because of the "degeneracy" of the code, i.e., the fact that more than one codon specifies a given amino acid.

From the observed rate of appearance of point mutations (one mutation per $10^6$ gene duplications), we can estimate that one mutation occurs per $10^9$ replications at a single nucleotide site. Point mutants tend to "back mutate," often at almost the same rate as is observed for the forward mutation. That is, one in $10^9$ times a mutation of the same nucleotide will take place to return the code to its original form. The phenomenon is easy to understand. For example, if T should be replaced by C because the latter formed a minor tautomer and paired with A, the mutation would appear in progeny duplexes as a GC pair. When this pair was replicated, there would be a finite probability that the C of the parental DNA strand would again assume the minor tautomeric structure and pair with A instead of G, leading to a back mutation.

Although the rates of spontaneous mutation are low, they can be greatly increased by mutagenic chemicals (Chapter 27) or by irradiation. It is perfectly practical to measure the rates of both forward and back mutation. When this was done, it was found that certain chemicals, e.g., acridine dyes, induce mutations that undergo reverse mutation at a very much lower frequency than normal. It was eventually shown that these mutations resulted either from **deletions** of one or more nucleotides from the chain or from **insertions** of extra nucleotides. Deletion and insertion mutations often result from errors during genetic recombination and repair at times when the DNA chain is broken.

Mutations involving deletion or insertion of one or a few nucleotides are called **frame-shift mutations**.

Messenger RNA is read by the protein synthesizing machinery from some starting point. As is illustrated in Chapter 5, Section E,1 the codons are read three bases at a time, and the proper amino acid corresponding to each codon is inserted. *When a deletion or insertion in the mRNA is met, all subsequent codons may be misread because the reading frame has shifted forward or backward by one or two nucleotides.* The protein synthesized bears little resemblance to that formed by the nonmutant organism and is usually completely nonfunctional. Mutations are considered further in Chapter 27.

## 2. Mapping the Chromosome of a Bacteriophage

Intensive work on the "T-even" phage T2, T4, and T6 (Box 7-C) was begun in 1938 by Delbrück and associates. The genetic information for these viruses is carried in a single linear DNA molecule, which in the case of T4 contains ~1.7 x $10^5$ base pairs (170 kb), enough for about 200 genes. Before the sequences of the viral DNA were known, the positions of more than 60 of these genes were mapped in the following way. When a bacteriophage infects a cell of *E. coli*, it injects its DNA through the cell wall and into the cytoplasm. About 20 minutes later the cell bursts, and ~100 fully formed replicas of the original virus particle are released. This rapid rate of production of progeny is so fast that it is possible to carry out in a test tube in 20 min a genetic experiment that would require the entire population of the earth if done with humans. The approach is explained nicely by Seymour Benzer, the man who first mapped the fine structure of a gene.[47] Bacteriophage particles, like bacteria, can be "plated out" on agar plates, which must contain a uniform suspension of bacteria susceptible to the virus. Wherever a virus particle lies, a bacterium is infected. Soon the infection spreads to neighboring bacteria with production of a transparent "plaque" (Fig. 26-1). The number of active virus particles present in a suspension can be determined easily by plating and counting of the plaques.

Mutant bacteriophages can be identified in various ways. Some biochemical traits affect the appearance of the plaque. Other easily detected traits include alteration in the specificity toward strains of the host bacterium. A key discovery that made genetic mapping possible for bacteriophage was that *genetic recombination between two phage particles can take place within a host bacterium*. When large numbers of bacteriophages of two different mutant strains were grown and were mixed together in excess with many bacteria, a few of the progeny phage were found to contain both mutant traits in the same virus and an equal number were "wild type." Although recombinations between mutations that are located close together in the DNA are rare, their frequency still greatly exceeds that of new mutations. While this type of experiment gave no hint about the nature of the events involved, it showed that recombination had occurred.

Study of recombination frequencies between different strains of phage soon revealed that some sites of mutation are **closely linked**. Recombination between these sites occurs only rarely. Other sites are weakly linked, and recombination occurs often. This behavior was reminiscent of that established many years earlier for genes of the fruit fly *Drosophila*, maize, and other higher organisms. Recombination by "crossing over" in the chromosomes of *Drosophila* was established by Morgan and associates in 1911.[1,2] The basic idea behind chromosome mapping in any organism is the assumption that *recombination frequencies between two mutations are directly proportional to the distance between them on the genetic map*. For the T4 phage a recombination frequency of 1% is taken as one unit. The total T4 map is 700 units long. The fact that this is greater than 100% means that if genes are located at opposite ends of the chromosome multiple recombination events can occur between them. However, a maximum of 50% crossing over is observed for distant gene pairs, and the approximate linearity of map distance and recombination frequency holds only for distances of 10 units or less.[48]

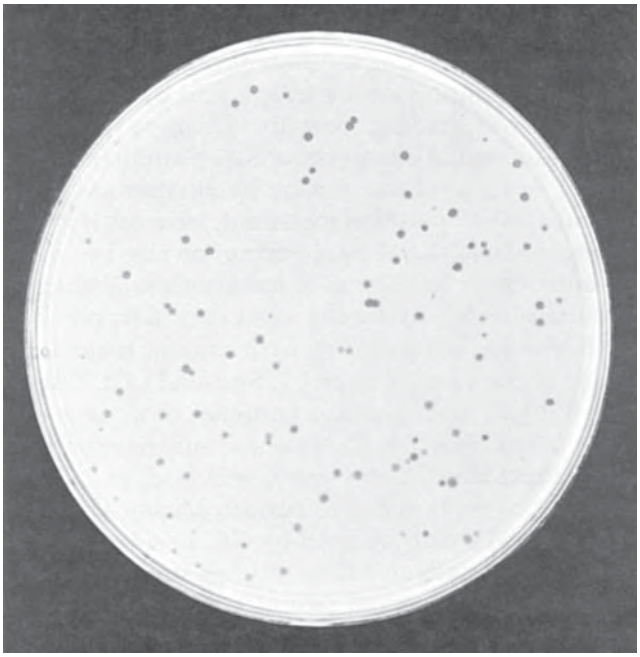


**Figure 26-1** Plaques formed by bacteriophage ϕ11 growing on *Staphylococcus aureus*. Each transparent (dark) plaque is the result of lysis of bacteria by the progeny of a single bacteriophage particle. Courtesy of Peter Pattee.

How can recombinant bacteriophage be identified rapidly? Benzer used two strains of *E. coli*, the B strain and the K strain, as hosts. Mutants in gene *rII* form characteristic plaques on strain B but do not grow on strain K. To determine the recombination frequency between two different *rII* mutants, the viruses were added to liquid cultures of B cells (in which they replicate), and recombination was allowed to occur. Recombination permitted the emergence not only of a phage containing *both* mutations but also of a wild-type phage in which both mutations had been eliminated by the recombination process. Since only recombinants of the latter type grow in strain K, it was possible to detect a single recombinant among one billion progeny. Since the total DNA length in phage T4 is 166 kb, there are 237 base pairs for each of the 700 units of map length. Thus, a recombination frequency at 0.01% between two mutations meant that the two mutations were no more than three base pairs apart in the DNA. Benzer concluded that he had observed the expected recombination frequencies for mutations even of immediately adjacent bases in the DNA.

To make fine genetic mapping practical, a series of bacteriophage containing deletion mutations involving large segments of the *rII* gene were isolated. Using these, it was possible to establish in which segments of the gene a particular mutation lay. Then, recombination experiments with previously identified mutations in that same general region allowed the mutations to be pinpointed. In this way Benzer identified over 300 sites of mutations within the *rII* region. He concluded that the minimum distance between two mutable sites was compatible with the Watson–Crick structure of the gene.[47]

## 3. The Cistron

How can one tell whether two mutations are in the same gene or in nearby or adjacent genes? The answer can be supplied by a test of **complementation**. If two mutant bacteriophage are altered in different genes, they can often reproduce within a host if the bacterium is infected with both of the mutant phage. Since each one has a good gene for one of the two proteins involved, recombinant phage, in which all of the gene functions are fulfilled, will be formed. On the other hand, if both mutant phages are defective in the same gene (although at different locations), they usually cannot complement each other at high frequencies in a coinfection. The experiment is referred to as a ***cis–trans* comparison**. The coinfection with the two different mutants is the trans test. A control, the *cis* test, uses a recombinant containing both of the mutations in the same DNA and coinfection with a standard phage. Normal replication is expected in this instance.

When the complementation (or *cis–trans*) test was applied to various mutants in the *rII* region, it became clear that there are two genes, *rIIA* and *rIIB*. The name **cistron** was proposed by Benzer to represent that length of DNA identifiable in this fashion as a genetic unit. *For most purposes the terms gene and cistron are nearly synonymous.* When mapping of the *rII* region was done, there was no information about the functions of the proteins specified by these two cistrons. However, both the *rIIA* and *rIIB* proteins have since been shown to become incorporated in the membranes of phage-infected bacterial cells.[49,50] There they affect the ease of lysis of the infected cells and, in that manner, cause *rII* plaques to be larger and to have sharper edges than standard plaques.

## 4. Nutritional Auxotrophs

The beginning of biochemical genetics is often attributed to Beadle and Tatum, who, in 1940, discovered mutants of *Neurospora* with specific growth requirements. They X-rayed one parent strain to form mutants, then tested individual spores for their possible need of a specific nutrient for growth. The 299th spore tested required pyridoxine! Many other mutants requiring vitamins, amino acids, and nucleic acid bases were then discovered.[1,51] A few years later similar **nutritional auxotrophs**, as the mutants are called, were discovered for *E. coli*. Ordinary wild-type cells of *E. coli* can grow on a minimal medium containing a carbon compound as a source for energy together with inorganic nutrients. Irradiation with ultraviolet light or treatment with mutagenic chemicals produces many mutant cells that fail to grow on such a minimal medium. However, addition of one or more specific compounds, such as an amino acid or vitamin, usually permits growth. Selection of such nutritional auxotrophs can be accomplished by plating out large numbers of the irradiated or chemically treated cells on a solid, rich nutrient medium. Colonies (**clones**) are allowed to develop by multiplication of the individual bacteria. The auxotrophs are selected by **replica plating**.[2] In this procedure a sterile velveteen pad is pressed against a nutrient agar plate containing small colonies of bacteria and is used to "print" replica plates containing a minimal medium. The colonies on the initial and replica plates are compared and the colonies of auxotrophs (which do not grow on the minimal medium) are selected. In a second stage, the auxotrophs may be replica plated to minimal medium supplemented with various nutrients (amino acids, purines, pyrimidines, vitamins, etc.). Selection is made easier by pretreatment of the irradiated cells suspended in minimal medium with penicillin. Penicillin kills the growing cells, but the auxotrophs, which do not grow on the minimal medi-

um, survive. The penicillin is then destroyed by adding penicillinase (a β-lactamase; Box 20-G) leaving a suspension much enriched in the percentage of auxotrophic mutants.[2]

A nutritional auxotroph of a bacterium often has a defect in a gene specifying a protein needed for the biosynthesis of the required nutrient. Individual genes recognized in this way are named with a genetic symbol. For example, gene *trpA* specifies one of the two protein subunits of tryptophan synthase. Other kinds of mutations, e.g., those affecting motility or other properties of the cells, can also be detected and are given appropriate symbols. A few of these genetic symbols are indicated on the *E. coli* chromosome map in Fig. 26-4, and many others are used throughout this book. On the basis of such nutritional experiments Beadle, by 1945, had proposed his famous one-gene-one-enzyme hypothesis.[1]

## 5. Establishing the Correspondence of a Genetic Map and an Amino Acid Sequence

Although the studies of the *rII* region of the T4 chromosome established that genetic mapping could be carried to the level of individual nucleotides in the DNA, it was still necessary to prove a linear correspondence between the nucleotide sequence in the DNA and the amino acid sequence in proteins. This was accomplished by Yanofsky[52,53] and associates through study of the enzyme tryptophan synthase of *E. coli.* Tryptophan synthase (Fig. 25-3) consists of two subunits, α and β, the former containing only 268 amino acids and encoded by the *trpA* gene. To obtain a fine structure map of the A gene, a large series of tryptophan auxotrophs unable to grow in the absence of added tryptophan were isolated. Genetic crosses were carried out with the aid of a **transducing bacteriophage** Plkc. Transducing bacteriophage (Section E,3), while multiplying in susceptible bacteria, sometimes incorporate a portion of the bacterial chromosome into their own DNA. Then, when the virus infects other bacteria, some of the genetic information can be transferred through recombination into the chromosome of bacteria that survive infection. Use of a series of deletion mutants, as in the *rII* mapping, permitted division of the A gene into a series of segments, and observation of recombination frequencies permitted fine structure mapping.

The second part of the proof of colinearity of DNA and protein sequences was the determination of the complete amino acid sequence of tryptophan synthase and peptide mapping (Chapter 3) of fragments of the mutant enzymes. From the peptide maps it was possible to identify altered peptides and to establish the exact nature of the amino acid substitutions present in a variety of different tryptophan auxotrophs. When this was done, it was found that those mutations that mapped very close together had amino acid substitutions at adjacent or nearly adjacent sites in the peptide chain.

The same problem was approached by Sarabhai and associates[54] through the nonsense mutations (Section 6), which lead to premature chain termination during protein synthesis. During late stages of the infection of *E. coli* by phage T4, most of the protein synthesis is that of a single protein of the virus head. Synthesis of protein by infected cells was allowed to proceed in the presence of specific $^{14}C$-labeled amino acids. Then cell extracts were digested with trypsin or chymotrypsin, the head-protein peptides were separated by electrophoresis, and autoradiograms were prepared. A series of T4 nonsense mutants that mapped within the head-protein gene were shown to give rise to incomplete head-protein chains. The peptide fragments were of varying lengths. By examining the radioautograms prepared from the enzymatically fragmented peptides, it was possible to arrange the mutants in a sequence based on the length of peptide formed and to show that this was the same as that deduced by genetic mapping. More recently the colinearity of codon and amino acid sequences has been verified repeatedly by comparison of experimentally determined nucleotide sequences in RNA and DNA molecules with the corresponding amino acid sequences for thousands of proteins.

Before the triplet nature of codons had been established, Crick and associates used frame-shift mutations in a clever way to demonstrate that the genetic code did consist of triplets of nucleotides.[7,55,55a] Consider what will happen if two strains of bacteria, each containing a frame-shift mutation (e.g., a −1 deletion), are mated. Genetic recombination can occur to yield mutants containing *both* of the frame-shift mutations. It would be difficult to recognize such recombinants because, according to almost any theory of coding, they would still produce completely defective proteins. However, Crick *et al*. introduced a third frame-shift mutation of the same type into the same gene and observed that the recombinants containing all three deletions (or insertions) were able to synthesize at least partially active proteins. Thus, while introduction of one or two single nucleotide deletions completely inactivates a gene, deletion of three nucleotides close together within a gene shortens the total message by just three nucleotides. The gene will contain only a short region in which the codons are scrambled. The reading frame for the remainder of the protein will not be changed. The protein specified will often be functional because it has a normal sequence except for a small region where some amino acid substitutions will be found and where one amino acid will be completely missing.

## 6. Conditionally Lethal Mutations

Studies of plaque morphology and of nutritional auxotrophs are directed narrowly at one gene or group of genes. It is desirable to have a general means of detecting mutations in the many other genes present within cells. However, most mutations are **lethal,** and this effect cannot be overcome by adding any nutrient. Lethal mutations are very common in higher organisms, but since eukaryotic cells have pairs of homologous chromosomes, they can be carried in one chromosome and the individual survives. With bacteria and viruses there is only one chromosome, and lethal mutants cannot survive.

Nutritional auxotrophs can be described as **conditionally lethal mutants**; they survive only if the medium is supplemented with the nutrient, whose synthesis depends upon the missing enzyme. Other kinds of conditional lethal mutations permit study of almost every gene in an organism. For example, **temperature-sensitive** (*ts*) mutants grow perfectly well at a low temperature, e.g., 25°C, but do not grow at a higher temperature, e.g., 42°C.[41,56] Many temperature-sensitive mutations involve an amino acid replacement that causes the affected protein to be less stable to heat than is the wild type protein. Others involve a loss in protein-synthesizing ability for reasons that may be obscure. Temperature-sensitive mutations occur spontaneously in nature, an example being the gene that controls hair pigment in Siamese cats.[41] The gene (or gene product) is inactivated at body temperatures but is active in the cooler parts of the body, such as the paws, tail, and nose, with the result that the cat's hair is highly pigmented only in those regions.

Screening for conditionally lethal temperature-sensitive mutants of bacteriophage T4 permitted isolation of hundreds of mutants involving sites at random over the entire viral chromosome. Complementation studies permitted assignment of these to individual genes, which at first were identified only by number (Fig. 26-2). Later specific functions were associated with the genes.[57,58] For example, the product of gene 42 was identified as an enzyme required in the synthesis of hydroxymethyl-dCMP (Chapter 5). Genes 20–24, among others, must code for head proteins because mutants produce normal tails but no heads. Gene 23 codes for the major head subunits, while gene 20 has something to do with "capping" the end of the head. These mutants produce cylindrical "polyheads" in place of the normal heads. Mutants of genes 25–29 have defective base plates and do not form tails, while mutants 34–38 lack tail fibers. The specific ways in which some of these gene products are assembled to form the base plates and tails of the phage are indicated in Fig. 7-29. The positions of the *rIIA* and *rIIB* genes are also shown in Fig. 26-2.

A second type of conditionally lethal mutation leads to alteration of an amino acid codon to one of the three **chain termination codons** UAG, UAA, and UGA (Table 5-3).[60,61] These are often called **nonsense mutations** in contrast to **missense mutations** in which one amino acid is replaced by another. A chain termination mutant synthesizes only part of the product of the defective gene because of the presence of the termination codon. A remarkable aspect of chain termination mutations is that they can be **suppressed** by other mutations in distant parts of the virus or bacterial chromosome. Many otherwise lethal mutations of bacteriophage T4 were discovered by their ability to grow in mutant strains of *E. coli*, which contained **suppressor genes**,[62] and their inability to grow in the normal B strain. Three different suppressor genes *supD*, *supE*, and *supF* were found to suppress mutations that formed UAG. These are commonly known as *amber* suppressor genes. A second group of *ochre* suppressor genes, including *supB* and *supC*, suppressed mutations that formed codon UAA. Suppressors for mutations that form codon UGA have also been found.[63] Like the temperature-sensitive mutants, *amber* and *ochre* mutants can be obtained in almost any genes of a bacterial virus. Chain termination mutants of unessential genes in bacteria can be recognized by transferring the genes by conjugation or by viral transduction into a strain ($sup^+$) that contains a desired suppressor gene.

Conditionally lethal mutants have been of great value in developing our understanding of the genetics of bacterial viruses. They have also provided a powerful technique for approaching complex problems of bacterial physiology. For example, we may ask how many genes are required for a bacterium to sense the presence of a food and to swim toward it (Chapter 19). Even though few clues as to the basic chemistry underlying these phenomena can be obtained in this way, the use of temperature-sensitive mutants and complementation tests permits us to establish the total number of genes involved in these complex processes and to map their positions on a bacterial or viral chromosome. This is often an important step toward a more complete understanding of a biological phenomenon.

## 7. The Nature of Suppressor Genes

How can one mutation be suppressed by a second mutation at a different point in the chromosome? Rarely, a mutation is suppressed by a second mutation within the *same* gene. Such **intragenic complementation** sometimes occurs when a mutation leads to an amino acid replacement that disrupts the structural stability or function of a protein. Sometimes a mutation at another site involving a residue, which interacts with the first amino acid replaced, will alter the inter-

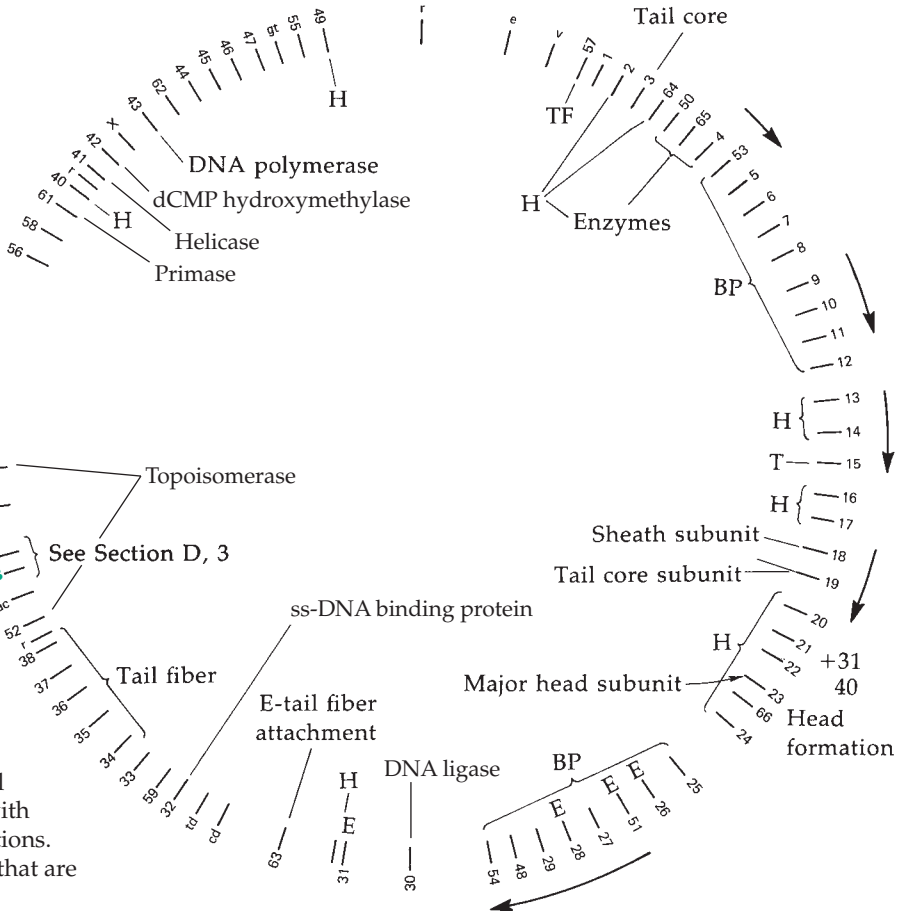



**Figure 26-2** Linkage map for bacteriophage T4. See H. A. Sober and Eiserling and Dickson.[57,59] Abbreviations: H, head; T, tail; TF, tail fiber; BP, base plate; and E, proteins with enzymatic rather than structural functions. The bold arrows indicate some genes that are transcribed together (operons).

action between the two residues in a way that restores function to the protein. For example, if the first amino acid side chain is small and is replaced by mutation with a larger side chain, a second mutation leading to a decrease in the size of another side chain may permit the protein to fold and function properly. An example was found among mutants of tryptophan synthase.[64] Mutants in which Gly 211 of the α chain was replaced by Glu or Tyr 175 of the same chain by Cys both produced inactive enzymes. However, the double mutant with both replacements synthesized active tryptophan synthase. It is known now that these residues are adjacent to one another and form part of the binding site for the substrate indole-3-glycerol phosphate (Figs. 25-2; 25-3). Only the double mutant permits the substituted side chains to pack properly. In other cases, intragenic suppression involves changes in the subunit interactions in oligomeric proteins. These changes may affect the formation of correct quaternary structures of the proteins.

As discussed in the preceding section, the best known suppressor genes are those that suppress chain termination mutations (Section 6). These genes often encode mutant forms of tRNA molecules, which allow incorporation of an amino acid rather than chain termination to occur. They are discussed further in Chapter 29. Suppressor genes are not limited to bacteria. For example, the vermilion eye color mutation of *Drosophila* leads to a loss of brown eye pigments because of the inactivity of tryptophan 2,3-dioxygenase (Eq. 18-38). However, synthesis of the tryptophan dioxygenase from the *vermilion* mutant is inhibited by $tRNA_2^{Trp}$, one of the two tryptophanyl tRNAs. The suppressor mutation alters the tRNA in such a way that the inhibition is relieved.[61]

## C. Plasmids, Episomes, and Viruses

The small pieces of DNA known as plasmids, which replicate independently of the chromosomes, have been discussed briefly in Chapter 5. Plasmids share a number of properties with viruses, and both are important to the techniques of contemporary molecular biology and genetic engineering. Bacterial plasmids may be present as one or several copies for each chromosome. Episomes are plasmids that are able to become integrated into the bacterial chromosome. Some extrachromosomal elements are episomes in one host and plasmids in another. Bacterial

plasmids may be infectious (transferable) or noninfectious. In the former case, they are able to transfer their DNA into another cell and are known as **sex factors** (F agents or fertility factors). A sex factor is able to integrate into a chromosome and later to come out and transfer other genes with it. In this property it resembles a transducing phage.

Plasmids and episomes vary in size. The F sex factor is a 100-kb circular molecule of supercoiled DNA. Colicinogenic factors,[65] which may also be present in *E. coli* in as many as 10–15 copies per bacterial chromosome, are often much smaller (6–7.5 kb). Some larger colicinogenic plasmids are also sex factors. They carry genes for toxic protein antibiotics known as **colicins** (Box 8-D) which attack other strains of *E. coli*, providing a selective advantage for the strain producing the colicin. They also carry a gene or genes conferring on the host bacterium resistance to antibiotics such as penicillin and chloramphenicol. Penicillin is inactivated because the plasmids carry a gene encoding a penicillinase that hydrolytically cleaves the β-lactam ring (Box 20-G). Chloramphenicol (Fig. 25-10) is inactivated by the action of chloramphenicol *O*-acyltransferase.

## 1. Bacterial Sex Factors

Bacteria usually reproduce by a simple cell division. The DNA in the chromosome is doubled in quantity and the cell divides, each daughter cell receiving an identical chromosome. However, in 1946 Lederberg and Tatum showed that sexual reproduction is also possible.[66,67] They studied nutritional auxotrophs of *E. coli* strain K-12, which lacked the ability to synthesize amino acids or vitamins. When cells of two different mutants were mixed together and allowed to grow for a few generations, a few individual bacteria regained the ability to grow on a minimal medium. Since each of the two strains had one defective gene, the creation of an individual with neither of the two defects required combining of genetic traits from both strains. The existence of bacterial conjugation was recognized. Later it was established that true **genetic recombination** had occurred, i.e., genes from the two mating cells had been integrated into a single molecule of bacterial DNA.

This transfer of DNA between bacterial cells requires the presence of a plasmid sex factor (F agent), whose presence confers a male character to the individual cell. The F agent is large enough to contain about 90 genes and has a length of ~30 μm, ~2.5% that of the *E. coli* chromosome. Among other things, the *E. coli* F agent contains the genes needed to direct the synthesis of the **F pili** (sex pili). These tiny appendages, 8.5 nm in diameter (see Fig. 7-9), grow out quickly during a period of 4–5 min to a length of about 1.1 μm. The end of an F pilus becomes attached to a female cell (a bacterium lacking the F agent) and may induce the transfer of DNA into the female cell. The mechanism of transfer has been uncertain. It may involve formation of a cytoplasmic bridge between cells in close contact. The pilus may be retracted into the membrane of the male cell, pulling the two cells close together. The DNA probably flows through the pilus into the female.[68,69]

On rare occasions an F agent becomes integrated into the chromosome of a bacterium. Both the F agent and the chromosome have been shown by electron microscopy to be circular. The integration process requires the enzymatic cleavage of the DNA of both the chromosome and the F agent and the rejoining of the ends in such a way that a continuous circle is formed (Fig. 26-3). The enzymes that catalyze these reactions are considered in Chapter 27. Different F agents can be incorporated into the chromosome at different points around the circle. A strain of bacteria containing an integrated F agent is known as an *Hfr* (high frequency of recombination) strain.

When an Hfr strain conjugates with an $F^-$ (female), replication of the entire male chromosome commences at some point near the end of the integrated F agent, and genes of the bacterial chromosome followed by those of the F factor are transferred into the female. Only a single strand of DNA (customarily referred to as the **plus strand**) is transferred from the donor cell and into the recipient cell (Fig. 26-3). There the complementary **minus strand** is synthesized to form a complete double-stranded DNA molecule bearing the genes from the Hfr cell. Only rarely does a copy of the entire chromosome of the donor cell enter the female cell. More often the DNA strand, or perhaps the pilus itself, breaks and only part of the chromosome is transferred.

Partial chromosome transfer from a male cell transforms the $F^-$ cell into a partial diploid (**merozygote**) containing double the usual number of some of the genes. Within this partial diploid genetic recombination between the two chromosomes takes place (Fig. 26-3) by the mechanisms discussed in Chapter 27. The end result of the recombination process is that the daughter cells formed by subsequent division contain only single chromosomes with the usual number of genes. However, some genes come from each of the two parental strains. Thus, an $F^-$ mutant unable to grow on a medium deficient in a certain nutrient may receive a gene from the male and now be able to grow on a minimal medium. Even though the number of such recombinants is small, they are easily selected from the very large number of mutant bacteria that are mixed together initially.

One result of DNA transfer from Hfr into $F^-$ bacteria is sometimes the introduction of a complete copy of the F agent into the female bacterium. Since this con-

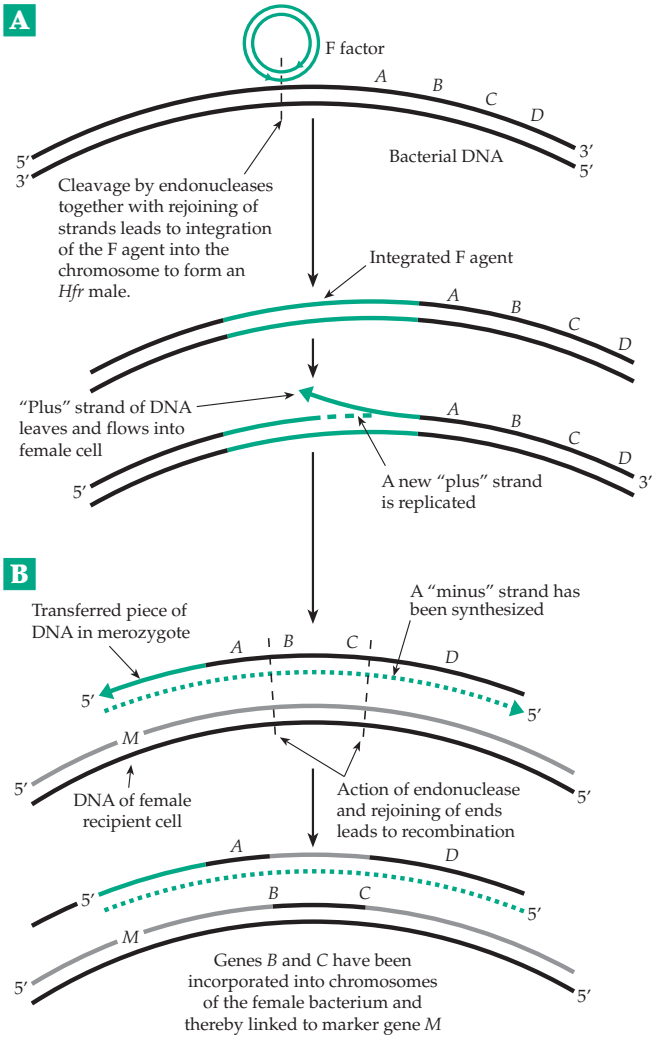



**Figure 26-3** Integration of an F agent into a bacterial chromosome and transfer of some bacterial genes into another cell. (A) Incorporation of the F agent into *E. coli* genome and transfer of the "plus" strand of DNA out to a female recipient cell. (B) Genetic recombination between a piece of transferred DNA and the genome of the recipient cell.

verts the recipient into a male, Brinton referred to "bacterial sex as a virus disease." In fact, infectious plasmids and viruses display many similarities. For example, filamentous bacteriophages (Chapter 5; Fig. 7-7) adsorb to the F pili of male bacteria and the DNA, flowing in a direction opposite to that in bacterial conjugation, enters the cell.[70] The bacteriophage carry genes for the protein subunits of their coats (Figs. 26-2, 7-29), while F factors carry genes for synthesis of pilins. Pilins accumulate within the cell membrane and are extruded to generate F pili, just as viral subunits are extruded to form the virus coats. There is also a close similarity between episomes that can be integrated into bacterial chromosomes and the **temperate bacteriophages** considered in the next section.

## 2. Temperate Bacteriophage; Phage Lambda

When DNA from a typical bacteriophage enters a bacterial cell, it seizes control of the metabolic machinery of the cell almost immediately and directs it entirely toward the production of new virus particles. This leads within a period of about 20 min to the production of one or two hundred progeny viruses and to the lysis and death of the cell. However, the DNA from a temperate phage may become repressed and, like an F factor, be integrated with the bacterial genome (Fig. 26-3). In the resulting **prophage** or **lysogenic** state, the repressed phage DNA is replicated as part of the bacterial genome but does no harm to the host cells unless some factor "activates" the incorporated genetic material by release of the repression. Replication of the phage and lysis of the bacterium then ensues. Temperate phage may also exist as plasmids (e.g., plasmid P1).

The decision for lysis or lysogeny, which is very important for the survival of the bacteriophage, is governed primarily by the nutritional status of the host. For a bacterium growing in a relatively rich environment such as the colon, lysis will increase the chances of daughter phage encountering host bacteria. However, in soils, *E. coli* grows very slowly, and a bacteriophage capable of entering a lysogenic state has an increased chance of survival until the host bacterium finds a richer growth medium.

The best known temperate phage is **phage lambda** of *E. coli*.[70–72] A tailed virus resembling the T-even phages (Box 7-C), phage λ has a smaller (~48.5 kb) DNA genome.[73] Within the bacterial cell the ends of the λ DNA may be joined to form a circular replicative form of the virus. In ~30% of the infected cells the λ DNA becomes integrated into the *E. coli* chromosome at the special site, *att λ*, which is located at 17 min on the *E. coli* chromosome map (Fig. 26-4). The incorporated phage DNA now occupies a linear segment amounting to about 1.2% of the total length of the *E. coli* chromosome. It is replicated along with the rest of the chromosome and for, the most part, goes unnoticed.

The host, *E. coli* K12, contains useful *amber* suppressors that make it easy to detect mutations in the bacteriophage. The integrated prophage can undergo mutations of almost any type, including large deletion mutations, and can still be investigated through complementation studies with other strains of virus. Thus, a family of modified **defective λ phage** was developed. When the λ prophage is excised from the bacterial chromosome, adjacent bacterial genes are occasionally carried with it. This allowed development of **λ transducing phage,** which can carry genes and transfer them into bacteria lacking these genes. More recently an important series of cloning vehicles have been derived from phage λ.

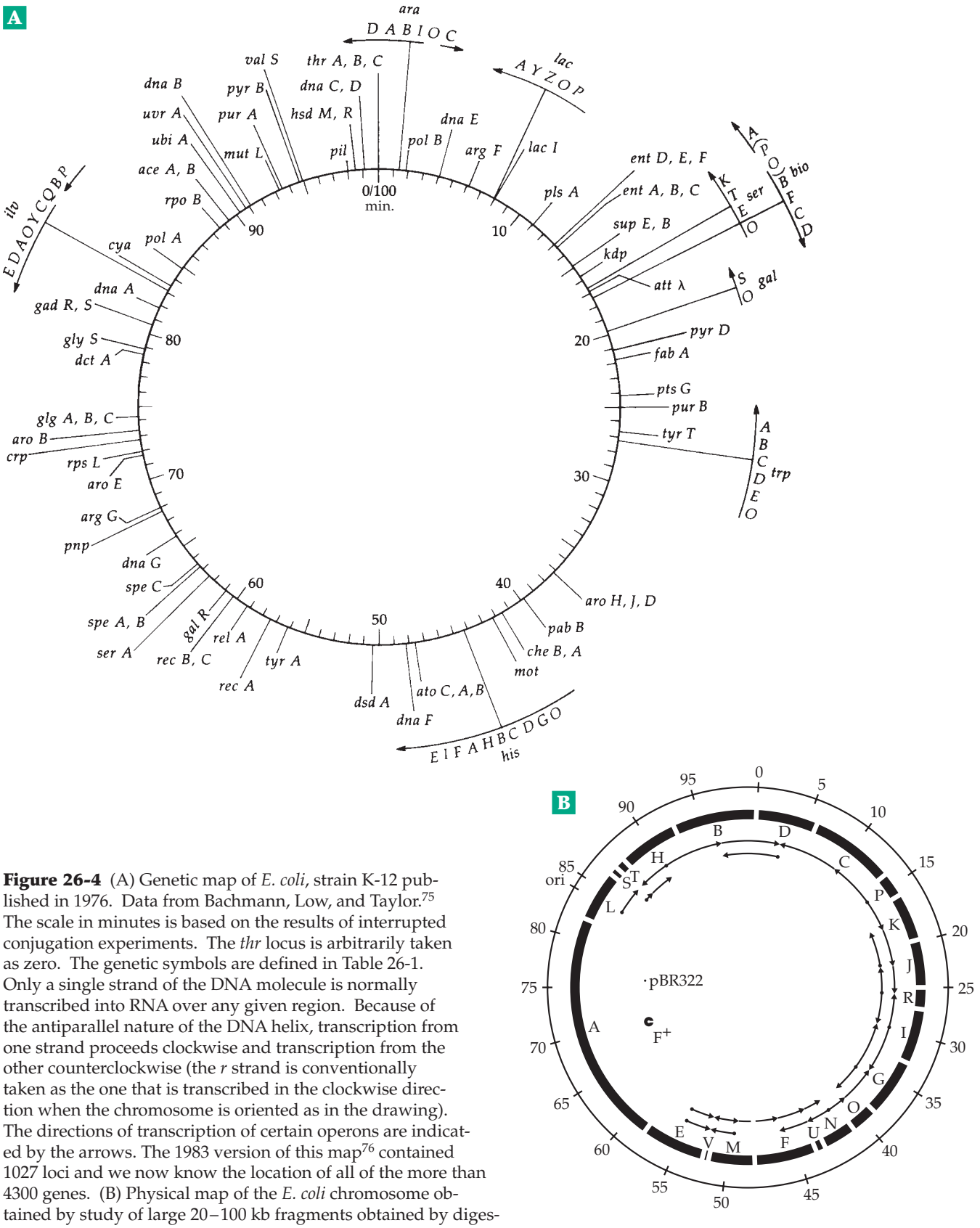



**Figure 26-4** (A) Genetic map of *E. coli*, strain K-12 published in 1976. Data from Bachmann, Low, and Taylor.[75] The scale in minutes is based on the results of interrupted conjugation experiments. The *thr* locus is arbitrarily taken as zero. The genetic symbols are defined in Table 26-1. Only a single strand of the DNA molecule is normally transcribed into RNA over any given region. Because of the antiparallel nature of the DNA helix, transcription from one strand proceeds clockwise and transcription from the other counterclockwise (the *r* strand is conventionally taken as the one that is transcribed in the clockwise direction when the chromosome is oriented as in the drawing). The directions of transcription of certain operons are indicated by the arrows. The 1983 version of this map[76] contained 1027 loci and we now know the location of all of the more than 4300 genes. (B) Physical map of the *E. coli* chromosome obtained by study of large 20–100 kb fragments obtained by digestion with restriction endonuclease *Not*I and separated by pulsed field gel electrophoresis. The fragments A–V were ordered using genetic information and overlapping fragments from partial *Not*I digests (arrows). On the outside the genetic map has been superimposed after distortion of the scale in minutes to make the two maps coincident. The 100-kb $F^+$ plasmid and the 4.5-kb plasmid pBR322 are also drawn to show relative sizes. From Smith *et al.*[77] A finer restriction map, based on 3400 cloned fragments, was prepared by Kohara, Akiyama, and Isono in 1987.[78]

## TABLE 26-1
## Some Genes of *E. coli*[a]

| Gene symbol | Mnemonic | Map position (min)[a] | Phenotypic trait affected |
|---|---|---|---|
| *aceA* | Acetate | 89 | Isocitrate lyase |
| *aceB* | Acetate | 89 | Malate synthetase A |
| *araA* | Arabinose | 1 | L-Arabinose isomerase |
| *araB* | Arabinose | 1 | L-Ribulokinase |
| *araC* | Arabinose | 1 | Regulatory gene |
| *araD* | Arabinose | 1 | L-Ribulose-5-phosphate-4-epimerase |
| *araI* | Arabinose | 1 | Initiator locus |
| *araO* | Arabinose | 1 | Operator locus |
| *argF* | Arginine | 6 | Ornithine carbamoyltransferase |
| *argG* | Arginine | 68 | Argininosuccinic acid synthetase |
| *aroB* | Aromatic | 73 | Dehydroquinate synthetase |
| *aroD* | Aromatic | 37 | Dehydroquinate dehydratase |
| *aroE* | Aromatic | 71 | Dehydroshikimate reductase |
| *aroH* | Aromatic | 37 | DAHP synthetase (tryptophan-repressible isoenzyme) |
| *aroJ* | Aromatic | 37 | Probable operator locus for *aro*H |
| *atoA* | Acetoacetate | 48 | Coenzyme A transferase |
| *atoB* | Acetoacetate | 48 | Thiolase II |
| *atoC* | Acetoacetate | 48 | Regulatory gene |
| *attλ* | Attachment | 17 | Integration site for prophage λ |
| *bioA* | Biotin | 17 | Group II; 7-oxo-8-aminopelargonic acid (7 KAP) → 7,8-diaminopelargonic acid (DAPA) |
| *bioB* | Biotin | 17 | Conversion of dethiobiotin to biotin |
| *bioC* | Biotin | 17 | Block prior to pimeloyl-CoA |
| *bioD* | Biotin | 17 | Dethiobiotin synthetase |
| *bioF* | Biotin | 17 | Pimeloyl-CoA → 7 KAP |
| *bioO* | Biotin | 17 | Operator for genes *bio*B through *bio*D |
| *bioP* | Biotin | 17 | Promoter site for genes *bio*B through *bio*D |
| *cheA* | Chemotaxis | 42 | Chemotactic motility |
| *cheB* | Chemotaxis | 42 | Chemotactic motility |
| *crp* | | 73 | Cyclic adenosine monophosphate receptor protein |
| *cya* | | 83 | Adenylate cyclase |
| *dctA* | | 79 | Uptake of $C_4$-dicarboxylic acids |
| *dnaA* | DNA | 82 | DNA synthesis; initiation defective |
| *dnaB* | DNA | 91 | DNA synthesis |
| *dnaC* | DNA | 99 | *dnaD*; DNA synthesis; initiation defective |
| *dnaE* | DNA | 4 | *polC*, DNA polymerase III and mutator activity |
| *dnaF* | DNA | 48 | *nrdA*; ribonucleoside diphosphate reductase |
| *dnaG* | DNA | 66 | DNA synthesis |
| *dsdA* | D-Serine | 50 | D-Serine deaminase |
| *entA* | Enterochelin | 13 | 2,3-Dihydro-2,3-dihydroxybenzoate dehydrogenase |
| *entB* | Enterochelin | 13 | 2,3-Dihydro-2,3-dihydroxybenzoate synthetase |
| *entC* | Enterochelin | 13 | Isochorismate synthetase |
| *entD,E,F* | Enterochelin | 13 | Unknown steps in conversion of 2,3-dihydroxybenzoate to enterochelin |
| *fabA* | | 22 | β-Hydroxydecanoylthioester dehydratase |
| *gadR* | | 81 | Regulatory gene for *gadS* |
| *gadS* | | 81 | Glutamic acid decarboxylase |
| *galE* | Galactose | 17 | Uridine diphosphogalactose 4-epimerase |
| *galK* | Galactose | 17 | Galactokinase |
| *galO* | Galactose | 17 | Operator locus |
| *galT* | Galactose | 17 | Galactose 1-phosphate uridyltransferase |
| *galR* | Galactose | 61 | Regulatory gene |
| *glgA* | Glycogen | 74 | Glycogen synthetase |
| *glgB* | Glycogen | 74 | α-1,4-Glucan: α-1,4-glucan 6-glucosyltransferase |
| *glgC* | Glycogen | 74 | Adenosine diphosphate glucose pyrophosphorylase |
| *glyS* | Glycine | 79 | Glycyl-transfer RNA synthetase |
| *hisA* | Histidine | 44 | Isomerase |
| *hisB* | Histidine | 44 | Imidazole glycerol phosphate dehydrase: histidinol phosphatase |
| *hisC* | Histidine | 44 | Imidazole acetol phosphate aminotransferase |
| *hisD* | Histidine | 44 | Histidinol dehydrogenase |
| *hisE* | Histidine | 44 | Phosphoribosyl-adenosine triphosphate-pyrophospho-hydrolase |
| *hisF* | Histidine | 44 | Cyclase |
| *hisG* | Histidine | 44 | Phosphoribosyl-adenosine triphosphate-pyro-phosphorylase |
| *hisH* | Histidine | 44 | Amidotransferase |
| *hisI* | Histidine | 44 | Phosphoribosyl-adenosine monophosphate-hydrolase |
| *hisO* | Histidine | 44 | Operator locus |
| *dsdM* | Host specificity | 98 | Host modification activity: DNA methylase M |
| *hsdR* | Host specificity | 98 | Host restriction activity: endonuclease R |
| *ilvA* | Isoleucine—valine | 83 | Threonine deaminase (dehydratase) |
| *ilvB* | Isoleucine—valine | 83 | Acetohydroxy acid synthetase I |
| *ilvC* | Isoleucine—valine | 83 | α-Hydroxy-β-oxo acid reductoisomerase |
| *ilvD* | Isoleucine—valine | 83 | Dehydrase |
| *ilvE* | Isoleucine—valine | 83 | Aminotransferase B |
| *ilvO* | Isoleucine—valine | 83 | Operator locus for genes *ilv*A,D,E |
| *ilvP* | Isoleucine—valine | 83 | Operator locus for gene *ilv*B |
| *ilvQ* | Isoleucine—valine | 83 | Induction recognition site for *ilv*C |
| *ilvY* | Isoleucine—valine | 83 | Positive control element for *ilv*C induction |
| *kdp* | K accumulation | 16 | Defect in potassium ion uptake |
| *lacA* | Lactose | 8 | Thiogalactoside transacetylase |
| *lacI* | Lactose | 8 | Regulator gene |
| *lacO* | Lactose | 8 | Operator locus |
| *lacP* | Lactose | 8 | Promoter locus |
| *lacY* | Lactose | 8 | Galactoside permease (M protein) |
| *lacZ* | Lactose | 8 | β-Galactosidase |
| *mot* | Motility | 42 | Flagellar paralysis |
| *mutL* | Mutator | 93 | Generalized high mutability (AT → GC) |
| *pabB* | *p*-Aminobenzoate | 40 | Requirement |
| *pil* | Pili | 98 | Presence or absence of pili (fimbriae) |
| *plsA* | Phospholipid | 11 | Glycerol-3-phosphate acyltransferase |
| *pnp* | | 68 | Polynucleotide phosphorylase |
| *polA* | Polymerase | 85 | DNA polymerase I |
| *polB* | Polymerase | 2 | DNA polymerase II |
| *ptsG* | Phosphotranserase | 24 | Catabolite repression system |
| *purA* | Purine | 93 | Adenylosuccinic acid synthetase |
| *purB* | Purine | 25 | Adenylosuccinase |
| *pyrB* | Pyrimidine | 95 | Aspartate carbamoyltransferase |
| *pyrD* | Pyrimidine | 21 | Dihydroorotic acid dehydrogenase |
| *recA* | Recombination | 58 | Ultraviolet sensitivity and competence for genetic recombination |
| *recB* | Recombination | 60 | Ultraviolet sensitivity, genetic recombination; exonuclease V subunit |
| *recC* | Recombination | 60 | Ultraviolet sensitivity, genetic recombination; exonuclease V subunit |
| *relA* | Relaxed | 59 | Regulation of RNA synthesis |
| *rpoB* | RNA polymerase | 89 | RNA polymerase: β subunit (*rif* gene) |
| *rpsL* | Ribosomal protein, small | 72 | Ribosomal protein S12 (*strA* gene, streptomycin resistance) |
| *serA* | Serine | 62 | 3-Phosphoglyceric acid dehydrogenase |
| *serO* | Serine | 20 | Operator locus |
| *serS* | Serine | 20 | Seryl transfer RNA synthetase |
| *speA* | Spermidine | 63 | Arginine decarboxylase |
| *speB* | Spermidine | 63 | Agmatine ureohydrolase |
| *speC* | Spermidine | 63 | Ornithine decarboxylase |
| *supB* | Suppressor | 15 | Suppressor of *ochre* mutations |
| *supE* | Suppressor | 15 | Suppressor of *amber* mutations (*su-2*) |
| *thrA* | Threonine | 0 | Aspartokinase I-homoserine dehydrogenase I complex |
| *thrB* | Threonine | 0 | Homoserine kinase |
| *thrC* | Threonine | 0 | Threonine synthetase |
| *trpA* | Tryptophan | 27 | Tryptophan synthetase, A protein |
| *trpB* | Tryptophan | 27 | Tryptophan synthetase, B protein |
| *trpC* | Tryptophan | 27 | *N*-(5-Phosphoribosyl) anthranilate |
| *trpD* | Tryptophan | 27 | Phosphoribosyl anthranilatetransferase |
| *trpE* | Tryptophan | 27 | Anthranilate synthetase |
| *trpO* | Tryptophan | 27 | Operator locus |
| *tyrA* | Tyrosine | 56 | Chorismate mutase T-prephenate dehydrogenase |
| *tyrT* | Tyrosine | 27 | Tyrosine transfer $RNA_1$ (*su-3* gene; amber suppressor) |
| *ubiA* | Ubiquinone | 90 | 4-Hydroxybenzoate → 3-octaprenyl 4-hydroxybenzoate |
| *uvrA* | Ultraviolet | 91 | Repair of ultraviolet radiation damage to DNA, UV endo-nuclease |
| *valS* | Valine | 95 | Valyl-transfer RNA synthetase |

[a] This list contains 126 of more than 1027 genes that had been mapped by 1983. (Bachmann, B. J. (1983) *Bacteriol. Rev.* **47**, 180—230). Their positions are shown diagrammatically in Fig. 26-4.

## D. Mapping of Chromosomes

Let us now consider how knowledge of bacterial sex factors and of phage λ permitted the mapping of bacterial chromosomes. Together with the use of restriction endonucleases these techniques gave us the first precise physical maps of bacterial chromosomes and pointed the way toward the determination of complete genome sequences.

### 1. The Chromosome Map of *E. coli*

There are about 4,639,221 nucleotide pairs in the circular DNA molecule that is the chromosome of *E. coli* strain K-12. We now know the complete sequence, which includes all of the individual genes that are present.[74] However, our first knowledge of the location of these genes in the chromosome depended upon construction of a **linkage map** (Fig. 26-4A). Construction of this map, with 126 genes, began with the study of nutritional auxotrophs whose defective genes are located at many points on the chromosome. By 1983, 1027 genes had been mapped. In 1997, when the complete nucleotide sequence became known, 4288 protein coding genes could be recognized.[74] The map in Fig. 26-4 was established 30 years earlier, largely by use of interrupted bacterial mating.[79,80] In this procedure *Hfr* cells carrying specific mutations are mixed with wild-type F cells, and conjugation is allowed to proceed for a certain length of time. Then the cells are agitated violently, e.g., in a Waring blender. This breaks all of the conjugation bridges and interrupts the mating process. Mating is interrupted at different times, and the recipient bacteria are tested for the presence of genes transferred from the donor strain. Using this technique it was found that complete transfer of the chromosome takes ~100 min at 37°C, and that the approximate location of any gene on the chromosome can be determined by the length of time required for transfer of that gene into the recipient cell. It is a little more complex than this. Because complete chromosome transfer is rare, substrains of *E. coli* K-12 with an F agent integrated at different points were used. With certain F factors those genes lying clockwise around the circle in Fig. 26-4 immediately beyond the point of integration are transferred quickly and with high frequency.

The **time-of-entry map** in Fig. 26-4A is based not only on interrupted matings but also on the use of **transduction** by bacteriophage P1.[76,79] Transduction by phage permits the tranfer of a short fragment of DNA, about 2 min in length, on the *E. coli* map. Joint transduction, i.e., joint incorporation of two genes into the chromosome of the receptor, occurs with a frequency related to the map distance between these two genes. Thus, finer mapping was done within many segments of the *E. coli* chromosome. Meanings of the gene symbols used in the figure are given in Table 26-1. Similar maps were prepared for *Salmonella typhimurium* and *Bacillus subtilis*.

### 2. Restriction Endonucleases

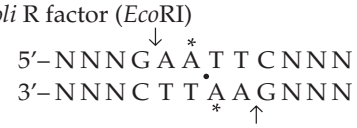
Many of the procedures for cloning genes, synthesizing more copies of a DNA ("amplifying" the DNA), making genetic maps, and generating mutants, are dependent upon **restriction endonucleases**. The name comes from a property of bacteria, which often can digest and destroy DNA of invading viruses or DNA that has been injected during mating with a bacterium of an incompatible strain. Investigation of this phenomenon, known as **restriction**, revealed that the DNA of viruses that are able to replicate within a particular host is *marked* in some fashion at specific sites in the molecule. The marking often consists of the presence of methyl groups. Properly methylated DNA is not degraded, but unmethylated DNA is cleaved by a highly specific endonuclease at the same sites that are normally methylated. Each species of bacteria (and often an individual strain within a species) has its own restriction enzymes. Restriction enzymes are very specific and cut DNA chains at unique base sequences. Three types are recognized.[80a]

Type I restriction enzymes, such as those encoded in the chromosome of *E. coli*, are large 300- to 400-kDa proteins composed of at least three kinds of polypeptide chain. They bind at specific sites of a foreign DNA and apparently cleave the chain randomly nearby. They require ATP, $Mg^{2+}$, and *S*-adenosylmethionine and have the unusual property of promoting the hydrolysis of large amounts of ATP.[81,82] The significance of these properties is still unknown.

The type II restriction endonucleases, which are the ones most widely used in molecular biology, are relatively small 50- to 100-kDa monomeric or dimeric proteins. About 2400 different enzymes with 188 different specificities had been isolated by 1995.[83,84] The sites of attack, in most instances, are nucleotide sequences with a twofold axis of local symmetry.[85] For example, the following sites of cleavage have been identified for two restriction endonucleases encoded by the DNA of R-factor plasmids of *E. coli* and for a restriction enzyme from *Hemophilus influenzae*. In the diagrams ↓ are sites of cleavage, ∗ are sites of methylation, and • are local twofold axes (centers of palindromes); N can be any nucleotide with a proper base pairing partner.

Restriction enzymes often create breaks in each of the two strands in positions symmetrically arranged around the local twofold axis. This is what we might expect of a dimeric enzyme that binds in the major or minor groove of the double helix, each active site

*E. coli* R factor (*Eco*RI)



*E. coli* R factor (*Eco*RII)

*H. influenzae* (*Hin*dIII)

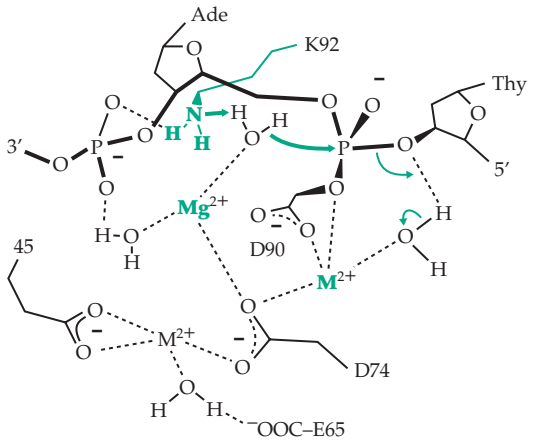
attacking one of the polynucleotide chains. In fact, the two 277-residue subunits of the *Eco*RI enzyme[86] bind primarily in the major grooves of the DNA, one active site on each strand. Each recognition unit makes 12 hydrogen bonds to the DNA. Each base pair forms two of these hydrogen bonds. Four arginines and two glutamates participate.[87] This provides a net charge of +2, which may be important for electrostatic interaction of the protein with backbone phosphate groups of the DNA. The binding of the protein affects the conformation of the DNA, widening the major groove from that in B DNA, and causing a torsional kink with some unwinding of the double helix.[88] It appears that the specificity for the GAATTC hexanucleotide is in part a result of direct complementary interactions between functional groups in the major groove (see Fig. 5-3), bound water molecules, and amino acid side chains from the enzyme (Fig. 26-5). Methylation of the 6-amino groups of the adenines in the center of the recognition sequence prevents cleavage by the *Eco*RI endonuclease, but appears to alter the interaction with the protein only slightly.[89]

Although they often share little sequence similarity and have quite different specificiities, many restriction enzymes have similar three-dimensional structures as well as mechanisms of action. This is true for the *Eco*RI, *Bam*HI (Fig. 26-5),[83,90] *Eco*RV,[91,91a] and *Cfr*10I enzymes,[84] and presumably many others. The specifically shaped and tightly packed active sites in the enzyme–substrate complexes ensure specificity. For example, the *Eco*RV endonuclease cleaves DNA at its recognition site at least a million times faster than at any other DNA sequence.[91] As mentioned in Chapter 12, restriction endonucleases require a metal ion, preferably $Mg^{2+}$, and probably act via a hydroxyl ion generated from $Mg^{2+}$–$OH_2$ at the active site. Three conserved active site residues, Asp 91, Glu 111, and Lys 113, in the *Eco*RI endonuclease interact with the DNA near the cleavage site. Lys 113 is replaced by Glu 113 in the *Bam*HI enzyme.[83,90]

The corresponding conserved residues in the smaller *Eco*RV enzyme are Asp 74, Asp 90, and Lys 92. They are shown in the following diagram that represents one of several possible metal-ion dependent mechanisms.[91–93] The metal-coordinated hydroxyl ion is generated by proton transfer to the $-NH_2$ group of Lys 92 and carries out an in-line attack on the backbone phospho group of thymidine at the cleavage point. At least two metal ions are needed, and three may be present, as shown in this diagram from Sam and Perona.[93]

**TABLE 26-2**
**Some Commonly Used Restriction Endonucleases, Their Sources, and Cleavage Sites**

| Enzyme | Source | Cleavage Site |
|---|---|---|
| *Alu*I | *Arthrobacter luteus* | 5' - - AG↓CT - - 3' |
| *Bam*HI | *Bacillus amyloliquefaciens H* | G↓GATCC |
| *Bcl*I | *Bacillus caldolyticus* | T↓GATCA |
| *Bgl*II | *Bacillus globigii* | A↓GATCT |
| *Cfr*10I | *Citrobacter freundii* | Pu↓CCGGPy |
| *Eco*RI | *Escherichia coli* | G↓AATTC |
| *Eco*RV | *Escherichia coli* | GAT↓ATC |
| *Hae*III | *Haemophilus aegypticus* | GG↓CC |
| *Hin*dIII | *Haemophilus influenzae* | A↓AGCTT |
| *Kpn*I | *Klebsiella pneumoniae* | GGTAC↓C |
| *Mbo*I | *Moraxella bovis* | ↓GATC |
| *Pst*I | *Providencia stuartii* | CTGCA↓G |
| *Sal*I | *Streptomyces albus* | G↓TCGAC |
| *Sau*3AI | *Streptococcus aureus* | ↓GATC |
| *Sfi*I | *Streptomyces fimbriatus* | GGCCNNNN↓NGGC |




Restriction enzymes that cleave DNA at a large number of specific sequences are available commercially. A few are listed in Table 26-2. Another group of restriction enzymes have similar recognition sequences but cut the dsDNA that they recognize at a specific neighboring site rather than within the recognition sequence. An example is *Fok*I, which recognizes the nonpalindromic $\frac{GGATG}{CCTAC}$, but cuts the chains 9 and 13 base pairs to the right. This enzyme has been used by Szybalski and associates to devise a system for cutting ssDNA precisely at a desired point and converting it to ds fragments.[95]

## 3. Restriction Mapping

The calibration of the *E. coli* genetic map in minutes was a temporary expedient. It was followed by **physical maps** expressed directly as micrometers of DNA length (total length ~1.6 mm) or thousands of nucleotide units (kb). A physical map obtained by **restriction enzyme mapping** is shown in Fig. 26-4B. To obtain this map DNA fragments were prepared using specific restriction endonucleases (Section E, 1).

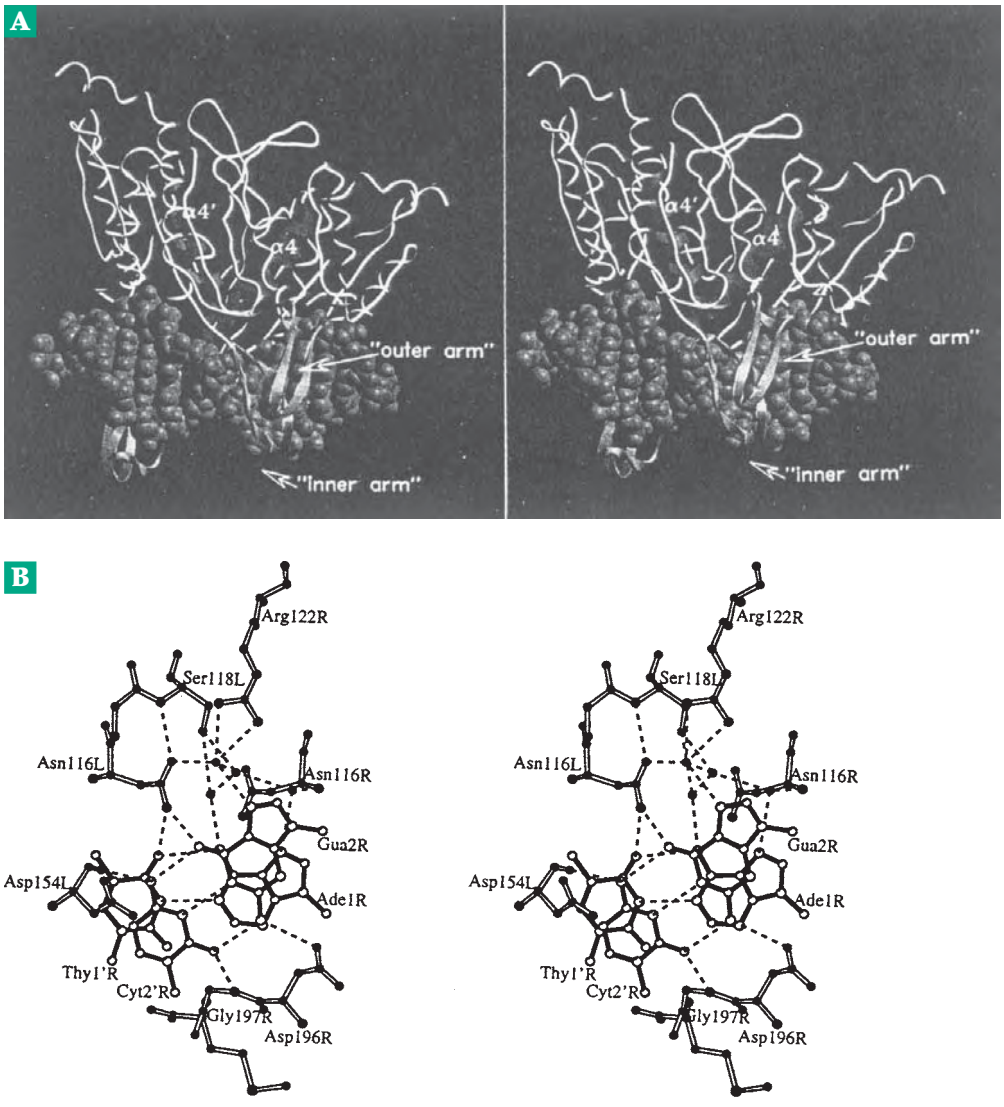



**Figure 26-5** (A) Stereoscopic ribbon drawing of the dimeric *Eco*RI restriction endonuclease in a complex with DNA. The equivalent helices marked α4 and α4' point into the major groove of the DNA double helix while the inner and outer "arms" wrap around the DNA. From Bozic *et al.*[84] based on coordinates of Kim *et al.*[94] (B) Stereoscopic view of the two base pairs T•A and C•G of the right end of the recognition motif 5'-CGATCC-3' (Table 26-2) bound to the *Bam*HI restriction endonuclease. The third base pair C•G lies below the two that are shown. Notice the numerous hydrogen bonds, some of which bind atoms of the DNA directly to atoms of the protein and also hydrogen bonds to water molecules (filled circles). The tight packing of complementary charged and dipolar groups of protein, nucleic acid, water, and $Mg^{2+}$ ions (not seen in this drawing) thoughout the complex accounts for the high specificity of these enzymes. From Newman *et al.*[83] Courtesy of Aneel Aggarwal.

The fragments were aligned using genetic markers, and their lengths were estimated by their electrophoretic mobilities.[77] The time-of-entry map has been added on a distorted scale in Fig. 26-4B. There are, on the average, 46.4 kb of DNA per minute, but this amount varies around the chromosome between 38 and 61 kb / min.

Mapping with restriction endonucleases was for many years an essential step in determination of the complete sequence of a piece of DNA. To make a restriction map the DNA, which may have been cut from a chromosome by a restriction endonuclease, is cloned. This permits isolation of a large amount of the DNA, which is then cut by other restriction endonucleases with differing specificities. Overlapping fragments resulting from the cleavages by single restriction enzymes are ordered to provide a map such as that of yeast mitochondrial DNA shown in Fig. 5-48. Any piece from the mapped DNA can now be cloned, and the exact sequence determined. The development of pulsed-field electrophoresis (Chapter 5), with its ability to separate DNA fragments 2000 kb or greater in length, allows restriction mapping with enzymes that cut at rare intervals to give very large fragments. For example, the *Not*I restriction endonuclease cuts the 4.7 Mb *E. coli* K12 genome into 22 fragments that were used to construct the complete restriction map of Fig. 26-4B.[77] Sequences of many viruses and plasmids, mitochondrial and plastid DNAs, and several bacterial genomes have been determined by use of restriction mapping and sequencing of the restriction fragments. Restriction fragment patterns have also been important to determine eukaryotic genome maps including the first genetic linkage map,[96] the first physical map of the human genome,[97] and the complete human genome sequences (Section G).

## 4. Electron Microscopy

Physical mapping by electron microscopy has been applied to bacteriophage, which can be obtained with large deletions in various parts of the genome.[98,99] The method can also be applied to cloned pieces of DNA. DNA might be isolated from two different phage strains, for example, from wild-type λ and from a mutant phage with a particular gene or genes deleted. The λ DNA can be denatured readily and separated into *r* strands and *l* strands by isopycnic centrifugation. If the isolated *l* strand of one strain is mixed with the *r* strand of another strain and annealed, a double-stranded DNA will be formed and, if there is a deletion in one strain, the homologous region in the normal λ DNA will form a single-stranded loop that can be visualized in the electron microscope. Figure 26-6 shows an example of a micrograph of such a **heteroduplex** molecule with a deletion loop and also a "bubble," where a segment of nonhomologous

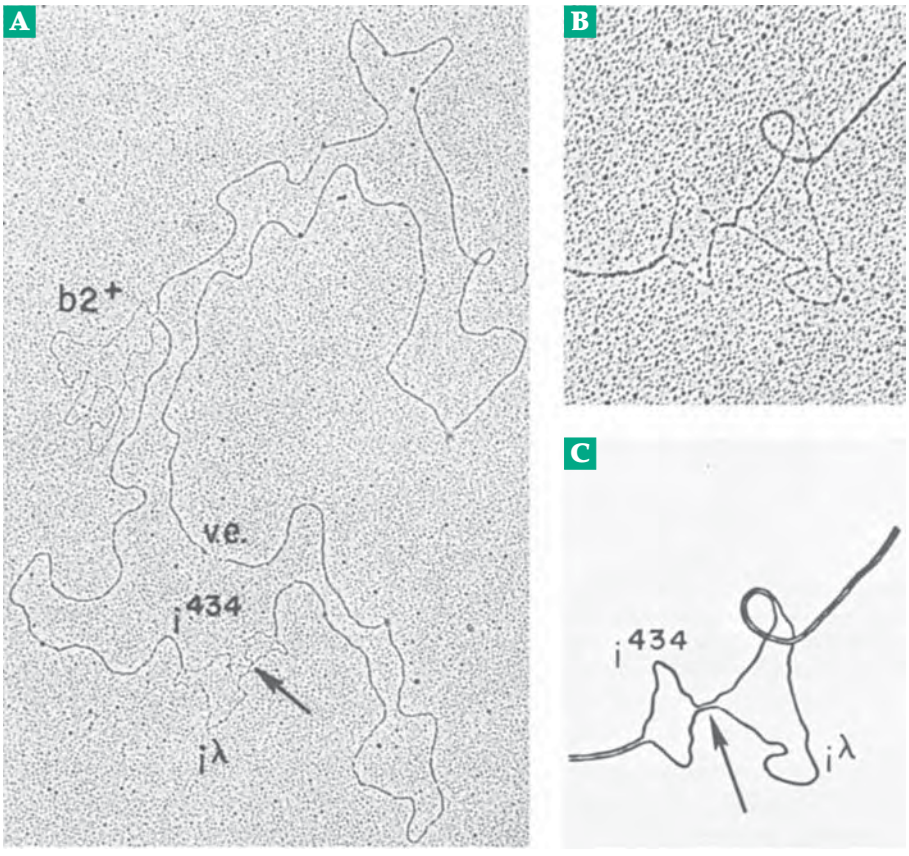



**Figure 26-6** (A) Electron micrograph of a heteroduplex DNA molecule constructed from complementary strands of phages λb2 and λ*imm*434. In λb2 a segment of λ DNA has been deleted producing a deletion loop (labeled b2) and in λ*imm*434 a piece of DNA from phage 434 has been substituted for λ DNA resulting in a "nonhomology bubble" (labeled $i^{434}$ / $i^{\lambda}$). The vegetative (cohesive) ends of the DNA are labeled v.e. (B) Enlargement of the nonhomology bubble. (C) Interpretative drawing of view in (B). Arrow marks a short (20–150 nucleotide) region of apparent homology. From Westmoreland, Szybalski, and Ris.[98]

DNA has been substituted in one strand.[98] Since distances can be measured accurately on the electron micrographs, rather precise (±50–100 bp) physical maps can be obtained. The chromosome map of phage λ was mapped in this way initially; now its complete nucleotide sequence is known. Another electron microscopic method is useful for location of AT-rich regions that denature readily. In a suitable concentration of formamide these regions melt to form visible single-stranded **denaturation loops** similar to the bubbles in Figure 26-6.

An important technique is to hybridize pieces of mRNA with DNA. If this is done with denatured (single-stranded) DNA and processed mRNA, which has been transcribed from genes with intervening sequences, the intervening sequences will form single-stranded loops in the DNA–RNA hybrid. A related technique depends upon the increased stability of DNA–RNA hybrids in high concentrations of formamide. Under these conditions an RNA segment will hybridize with its complementary strand of the DNA duplex displacing the other strand of DNA, which then appears as a visible **R-loop**. Intervening sequences appear as undisturbed DNA duplexes.

### 5. Optical Mapping

The long DNA molecules of bacterial or eukaryotic chromosomes are easily broken by vigorous stirring. However, if handled carefully very large fluorescently stained DNA molecules of 0.4–1.4 Mb lengths can be stretched out on a glass surface, and their lengths measured by optical microscopy. The technique depends upon binding of one of the ends of the DNA to the glass surface. If biotin is covalently attached to one end of the DNA, it will bind to a steptavidin-coated plate. However, unaltered DNA also binds to a vinyl-silane or a trichlorosilane coating under suitable conditions.[100,101] If DNA is incubated with a submerged silanized coverslip, which is then pulled out of the liquid mechanically at a constant speed, the DNA molecules are "dyamically combed" so that they are aligned for easy observation and measurement.[101] DNA can be transferred directly from agarose gels used for electrophoretic separation to the plates.[102] Used in combination with restriction enzymes these procedures allow rapid automated construction of physical maps. An example of whole-genome optical mapping is provided by the restriction map of the genome of the radiation-resistant bacterium *Deinococcus radiodurans*. The genome consists of two circular DNA molecules of 2.6 and 0.415 megabases and a smaller 176-kilobase DNA.[103] These were mapped without the laborious subcloning required by conventional restriction mapping. For example, the *E. coli* restriction map required analysis of 3400 phage clones.

Mapping of eukaryotic chromosomes has involved additional methods which are discussed in Sections E and G,1. These incluse **radiation hybrid mapping**,[104] use of **meiotic recombination**, identification of **restriction fragment length polymorphisms** (**RFLPs**; described in Section E,7), and use of **expressed sequence tags** (**ESTs**), short DNA sequences deduced from mRNA molecules transcribed from the DNA.[105,106]

## E. Cloning, Modifying, and Transferring Genes

A true revolution in biology and in medicine is in progress as a result of our ability to clone, sequence, mutate, and manipulate genes at will. Methods of sequence determination are discussed in Chapter 5 as is the laboratory synthesis of oligonucleotides and of complete genes. Both of these techniques are essential to present-day genetic engineering as are the techniques of cloning,[99,107–113] which are considered in this section.

A diploid cell contains only two copies of many genes, and these two copies are often not identical. In a gram of any tissue, which may contain $10^9$ cells, we will have only ~1 ng of a 1 kb (1 kilobase) gene. To isolate this gene we would have to fish it out from a huge excess of other genes. Present-day cloning techniques offer a way to locate the gene, increase its quantity by many orders of magnitude, learn its sequence, induce any desired mutations at any points, and transfer the gene into other organisms in such a form that it can be expressed, i.e., be transcribed and induce synthesis of proteins. The methods have taken years to develop and continue to be improved. Only some basic procedures and concepts are described here. Numerous manuals[99,108,109,111,114–118] as well as commercial "kits" are available.

### 1. Joining DNA Fragments

The cloning and manipulation of genes usually depends upon the precise cutting of DNA into discrete fragments by restriction endonucleases. Many restriction enzymes generate **cohesive ends** (sticky ends). Thus, *Eco*RI produces DNA fragments with the single-stranded "tails" shown here at the 5'-ends of the cut duplexes:

```
5′ ----- G 3′                    5′ pA A T T C ----- 3′
3′ ----- C T T A Ap 5′                      3′ G ----- 5′
```

These cohesive ends can be used to join together different restriction fragments. It is easy to see that the complementary single-stranded tails can form base pairs to regenerate the original hexanucleotide

sequence cleaved by *Eco*RI. There will still be nicks between G and A at the specific cleavage points, but these nicks can be closed enzymatically using DNA ligase. Thus, the original DNA cleaved by *Eco*RI can be reformed, or another piece of DNA that also has tails generated by *Eco*RI can be grafted onto an end.

Many of the other enzymes in Table 26-2 also form cohesive ends. Five of them (*Bam*HI, *Bcl*I, *Bgl*II, *Mbo*I, and *Sau*3A) have at the center of their recognition sites the same tetranucleotide: GATC. Enzymes *Sau*3A and *Mbo* I are called **isoschizomers** because they have just the same 4-base recognition sequence and also yield the same restriction patterns. Notice that they will both cut all of the *Bam*HI, *Bcl*I, or *Bgl*II sites, but *Bam*HI and *Bgl*II will not cut all *Mbo*I or *Sau*3A sites. However, cohesive ends made by any of these enzymes can be joined. The gaps left during the joining of certain of the fragments can be ligated enzymatically. *Sau*3A will cut at either methylated or unmethylated sites but *Mbo*I will not cut at methylated sites.

Two enzymes (*Kpn*I and *Pst*I in the list in Table 26-2) form 3'-cohesive ends rather than 5'-cohesive ends. In addition, there are three (*Alu*I, *Eco*RV, and *Hae*III) that cut at the local twofold axis; they form no cohesive ends but leave **blunt ends** (flush ends). Blunt end fragments are also much used in genetic engineering. "Linkers" that provide cohesive ends can be added.[119] The *Sfi*I endonuclease cuts between two 4-bp palindromes in a 13-bp recognition sequence (Table 26-2).[120]

***Some useful enzymes.*** Several enzymes of use in cloning[121] are listed in Table 26-3. The detailed chemistry of most of these is discussed in Chapter 27. Among these are the **ligases** that allow DNA fragments to be joined. They act on DNA strands with adjacent 3'-OH and 5'-phosphate termini. The *E. coli* ligase seals single stranded nicks using $NAD^+$ as an energy source (Eq. 27-5). It is therefore able to ligate DNA fragments with cohesive ends. The T4 DNA ligase, which is obtained from *E. coli* infected with phage T4, not only can seal nicks but can ligate pieces of DNA with blunt ends. Its activity is linked to cleavage of ATP. If two DNA strands are joined, but with gaps in one or both strands, the gaps can be filled efficiently by the 109-kDa **DNA polymerase I** from *E. coli*. Most often the 76-kDa **Klenow fragment**, which is lacking the 5' → 3' exonuclease activity, is used. **T4 DNA polymerase** has similar properties.

A problem with DNA fragments with cohesive ends is that they spontaneously form closed circles, a process that may compete with a desired joining to another piece of DNA. One solution to this problem is to hydrolyze off the 5'-phosphate groups with an **alkaline phosphatase** (Chapter 12). This prevents formation of covalently closed circles. However, pieces of DNA that retain their 5'-phosphate groups can be ligated to these dephosphorylated pieces. **T4 polynucleotide kinase**[122] can be used to put a phospho group back onto the 5' end of a chain. A $^{32}P$ end label can be added to such a polynucleotide using $^{32}P$-labeled ATP.

***Forming homopolymeric tails.*** Chromosomal DNA may be cleaved with restriction enzymes that leave blunt ends or it may be cleaved randomly by shearing. In either case the blunt ends can be treated first with **λ-exonuclease**, then with **terminal deoxynucleotidyl transferase**[123] isolated from calf thymus. The exonuclease treatment cuts off a few nucleotides from the 5' termini leaving short single-stranded 3'−OH termini. The terminal transferase, a polynucleotide polymerase that acts on ssDNA, is nonspecific and requires no template. Using an appropriate nucleotide triphosphate, it will add a single-stranded tail of either deoxyribonucleotides or ribonucleotides to the exposed 3' termini of a polynucleotide of three or more residues. If deoxyATP is used, a 3' poly(dA) tail will be added to each 5' terminus. Such a poly(dA)-tailed DNA fragment can be annealed and ligated to DNA carrying poly(dT) tails. This approach has been used widely to insert a piece of DNA into a cloning vehicle. For example, a circular plasmid (Fig. 26-7) can be opened by a single cleavage with *Eco*RI or other suitable restriction enzyme. The opened plasmid is treated with exonuclease, and poly(dA) tails are added. The piece of DNA to be cloned is tailed with poly(dT). After annealing and ligation recombinant plasmids carrying the **passenger DNA** will be formed.

If DNA is cleaved with *Pst*I or *Kpn*I (Table 26-2), the resulting 3' cohesive ends can be extended with a poly(dC) tail. If the cloning vehicle also has a site for *Pst*I or *Kpn*I, it can be opened and poly(dG) tails can be applied. A useful feature is that after annealing, filling in the gaps, and ligation the original *Pst*I or *Kpn*I sites are restored. This provides for easy recovery of the cloned fragments (Fig. 26-8).

***Preparing material for cloning.*** DNA may be prepared for cloning by (1) random cleavage by shearing or by enzymatic attack, (2) cleavage by one or more restriction endonucleases, (3) preparation of cDNA from mRNA, or (4) nonenzymatic chemical synthesis of DNA segments. The use of random cleavage has largely been replaced by cleavage with restriction enzymes. A major problem is the separation of the very large number of different restriction fragments formed from a large piece of DNA or from an entire genome. The creation of "libraries" of such fragments is described in Section 5. Considerable simplification comes from separation of individual eukaryotic chromosomes before the library is prepared. Careful purification of DNA to be used in cloning is helpful. This may be done by electrophoresis in agarose or

polyacrylamide gels or using HPLC. One technique is to embed cells directly in a gel, to diffuse in proteases and restriction enzymes that lyse the cells and release the DNA, and cleave it, and then to conduct the electrophoresis (see Chapter 5, Section H,1). DNA fragments of very large size can be separated. In addition to isolation of a fragment to be cloned the cloning vehicle must be prepared. This often involves release of a plasmid by lysis of bacteria that carry it and isolation using a suitable column. Likewise, after the DNA has been cloned and the content of DNA has been increased by growing a large bacterial culture, the plasmids must be released and purified, and the cloned DNA excised with a restriction enzyme. Alternatively, the cloning vehicle may be a virus, which must be isolated and disrupted to release the DNA. Use of the polymerase chain reaction (PCR; Fig. 5-47) allows cloning and amplification of DNA fragments with a minimum of purification.

## 2. Cloning Vehicles (Vectors)

Many cloning vehicles, more commonly referred to as **vectors**, originated with naturally occurring, independently replicating plasmids or viruses (replicons). More recently artificial chromosomes have been developed as cloning vehicles. Plasmids and viruses have been extensively engineered to provide convenience and safety. A large number of specialized

**TABLE 26-3**
**Some Enzymes Used in Molecular Cloning**

| Name | Source | Reaction |
|---|---|---|
| T4 DNA polymerase | *E. coli* infected with bacteriophage T4 | 5'→ 3' chain growth<br>3'→ 5' exonuclease |
| *E. coli* DNA polymerase I and Klenow fragment | *E. coli* | 5'→ 3' chain growth<br>3'→ 5' exonuclease<br>5'→ 3' exonuclease (lacking in Klenow fragment) |
| Reverse transcriptase | RNA tumor viruses, e.g., avian myoblastosis virus | 5'→ 3' DNA chain growth |
| Ribonuclease H | *E. coli* | Cuts RNA in DNA-RNA hybrid |
| Lambda and T7 exonucleases | Bacteriophages | |
| Bal 31 nuclease | *Alteromonas espejiano*, a marine bacterium | Degrades both 3' and 5' termini of dsDNA |
| T7 RNA polymerase | Bacteriophage T7 | DNA-dependent RNA polymerase |
| Terminal deoxyribonucleotide transferase | Thymus gland, plants | Limited 5'→ 3' chain growth; template independent addition of tails to DNA fragments |
| T4 DNA ligase | *E. coli* carrying an engineered λ phage | Ligation of DNA, either blunt or cohesive ends; uses ATP |
| *E. coli* DNA ligase | *E. coli* | Ligation of DNA with cohesive ends; uses $NAD^+$ as energy source |
| RNA ligase | Bacteriophage T4 | Ligation of RNA and DNA |
| T4 polynucleotide kinase | Bacteriophage T4 | Phosphorylation of 5′-OH terminus of a polynucleotide (DNA or RNA) |
| *Eco*RI methylase | *E. coli* | Transfer $CH_3$ from *S*-adenosylmethionine to adenines in *Eco*RI sites |

See Chapter 12 for general discussion of nucleases.

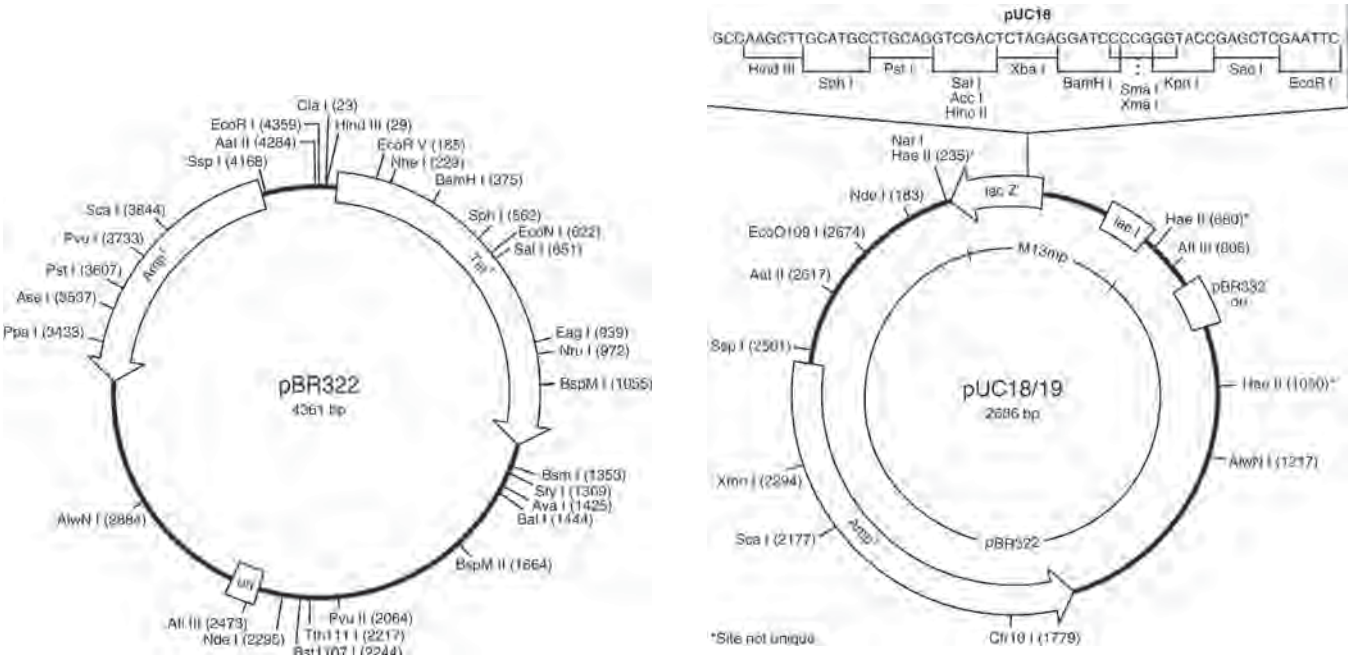



**Figure 26-7** Genetic map of cloning plasmids pBR322 and pUC18. Abbreviations: ori, origin of replication; Amp$^r$, ampicillin resistance gene; Tet$^r$, tetracycline resistance gene. Other abbreviations are for sites cleaved by specific restriction endonucleases, a few of which are defined in Table 26-2. The nucleotide sequence numbers and directions of transcription are also indicated. Reproduced by permission of Amersham Pharmacia Biotech Inc.

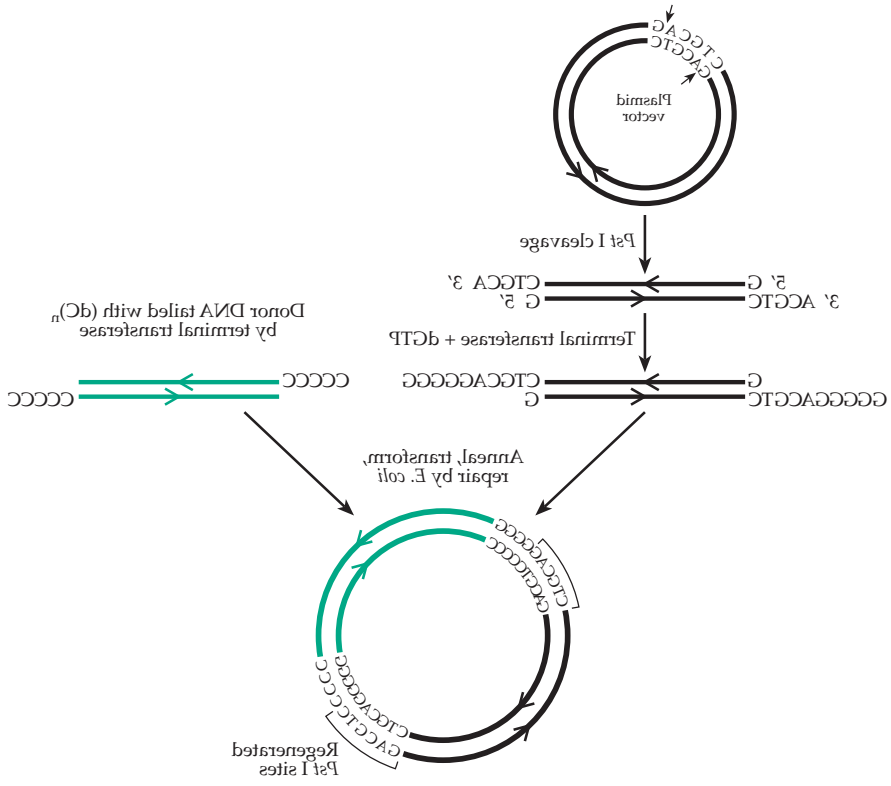



**Figure 26-8** Regeneration of a *Pst* I site by dG:dC tailing with terminal transferase. See Glover.[99] Arrowheads indicate 5′→3′ directions.

vehicles have been devised. Only a few will be described briefly here. Suitable books and manuals must be consulted for details.[5,99,108–110,114,115,121,123a]

### *Plasmids related to ColE1.*

The small colicinogenic plasmid, ColE1 of *E. coli*, is attractive for cloning because there are 20 copies per bacterial cell. If chloramphenicol is added during the logarithmic phase of growth, the *E. coli* cells will make 1000–3000 copies. Thus, a relatively small culture of the bacteria containing the cloned DNA in a ColE1 plasmid will yield a large amount of the desired DNA. In the past one of the most widely used cloning vehicles[124] has been plasmid pBR322, which was derived from a close relative of ColEl. The genome size of the original plasmid was reduced by deletion of genes unnecessary for its successful replication. Transposons

that might permit accidental transfer of DNA to other organisms were inactivated. Unneeded or undesirable restriction enzyme sites were eliminated, and useful restriction sites were introduced by point mutations. The resulting pBR322 has only one site of cleavage each for *Bam*HI, *Sal*I, *Pst*I, *Pvu*I, and *Eco*RI. These are at known positions in the 4363-nucleotide plasmid.[125]

Plasmid pBR322 contains two different antibiotic-resistance genes that were brought in from bacterial R-factors. These are used in selecting bacterial colonies that carry the desired recombinant plasmids. One of these is the β-lactamase gene, which confers resistance to ampicillin (*Amp*$^r$); the other provides resistance to tetracycline (*Tet*$^r$). Their positions are indicated in the pBR322 gene map (Fig. 26-7) as is the essential origin of replication (*ori*). The drug resistance genes are used as follows. If a unique restriction site such as that for *Bam*HI or *Sal*I that lies within the *Tet*$^r$ gene is used to introduce the passenger DNA, the resistance to tetracycline is lost but that to ampicillin is retained. Thus, after incubation with the recombinant plasmids under conditions that favor their uptake by the host bacteria, the bacteria are plated onto an ampicillin-containing medium. Only those harboring the pBR322 plasmid with its *Amp*$^r$ gene can grow. After these have produced small colonies, a replica plate is made on a tetracycline-containing medium. On this medium the desired recombinants do *not* grow because the *Tet*$^r$ gene has been inactivated. This allows selection of colonies containing passenger DNA (Fig. 26-9). A further selection procedure is required to establish that the piece of DNA inserted into the recombinant plasmid is one that is desired.

***Typical cloning procedure with pBR322.*** In much simplifed form the procedure might go as follows: (1) Purchase or isolate plasmid. (2) Cleave plasmid with *Bam*HI; heat at 70°C to inactivate the enzyme. (3) Treat with alkaline phosphatase to remove the 5'-phospho groups. (4) Mix with passenger DNA with cohesive ends generated by *Bam*HI, anneal, and join with DNA ligase. Although the resulting circular recombinant DNA contains a nick as a result of the missing 5'-phospho group, it will be taken up by bacteria and repaired. (5) Incubate joined DNA with cells of host *E. coli* that have been made permeable to DNA by treatment with $Ca^{2+}$ ions. This type of transformation is called **transfection** and is widely used in cloning. (6) Plate transfected cells onto agar containing the first antibiotic, in this case ampicillin. (7) Make replica plate on medium containing second antibiotic, in this case tetracycline. (8) Screen selected colonies for desired DNA fragment. In one procedure a small sample from each of the selected colonies is placed onto spots on a nitrocellulose filter. Several colonies can be placed on one filter and the bacteria lysed, hybridized with a radioactive probe, and then viewed by autoradiography.

***Selecting clones using β-galactosidase in pUC cloning vehicles.*** The newer **pUC** vehicles[126] contain the origin of replication and the ampicillin-resistance gene from pBR322. In addition, a segment of DNA from the *E. coli lac* operon (Fig. 28-2) has been grafted into an intergenic region (Fig. 26-7). It contains the lac control region as well as the coding sequence for the first 145 residues of β-galactosidase. Within bacteria containing the pUC DNA an N-terminal portion of β-galactosidase is synthesized. The specially designed host cell contains (in an episome) the gene for another defective β-galactosidase, one lacking the N-terminal portion. This defective enzyme, together with the N-terminal portions encoded in the pUC DNA, forms an active galactosidase. When the chromogenic substrate 5-chloro-4-bromo-3-indolyl-β-D-galactoside ("X-gal") and a suitable inducer, such as isopropylthio-β-galactoside (IPTG), are present, the unoccupied plasmid vehicles generate blue colonies. However, if passenger DNA is inserted within the galactosidase gene segment, formation of the enzyme will be disrupted and white colonies will appear. The restriction sites for cloning are placed in a **polylinker** near the 5' terminus of the galactosidase gene. The polylinker has been carefully engineered to maintain the correct reading frame and to avoid disruption of the galactosidase activity. It contains several different restriction sites for insertion of passenger DNA (Fig. 26-7). Such insertion does destroy the galactosidase activity allowing the user to detect the recombinant DNA from the white plaques.

***Filamentous bacteriophages.*** An important series of cloning vehicles have been derived from the circular replicating forms of the filamentous bacteriophage M13 (Chapter 5; Fig. 7-7; Chapter 27).[127] Although the genome contains only a short intergenic region that can be deleted, up to 50 kb of passenger DNA can be inserted into these vehicles. Since long inserted sequences may be deleted spontaneously, M13 is most useful for cloning about 300- to 400-nucleotide chains. Many of the M13 vehicles also use the β-galactosidase blue-white screening technique. These modified viruses are highly infective, but the infected *E. coli* cells are not killed. Rather they produce large numbers of virus particles with single-stranded DNA representing one of the two DNA chains of the parental phage. These are widely used for sequencing by the chain-termination procedure of Sanger *et al.* (Chapter 5). The procedure requires a primer sequence. If M13 recombinants are sequenced, the primer consists of a synthetic oligonucleotide that can be annealed to the galactosidase gene fragment at its 3' end just in front of the DNA segment that is to be sequenced.

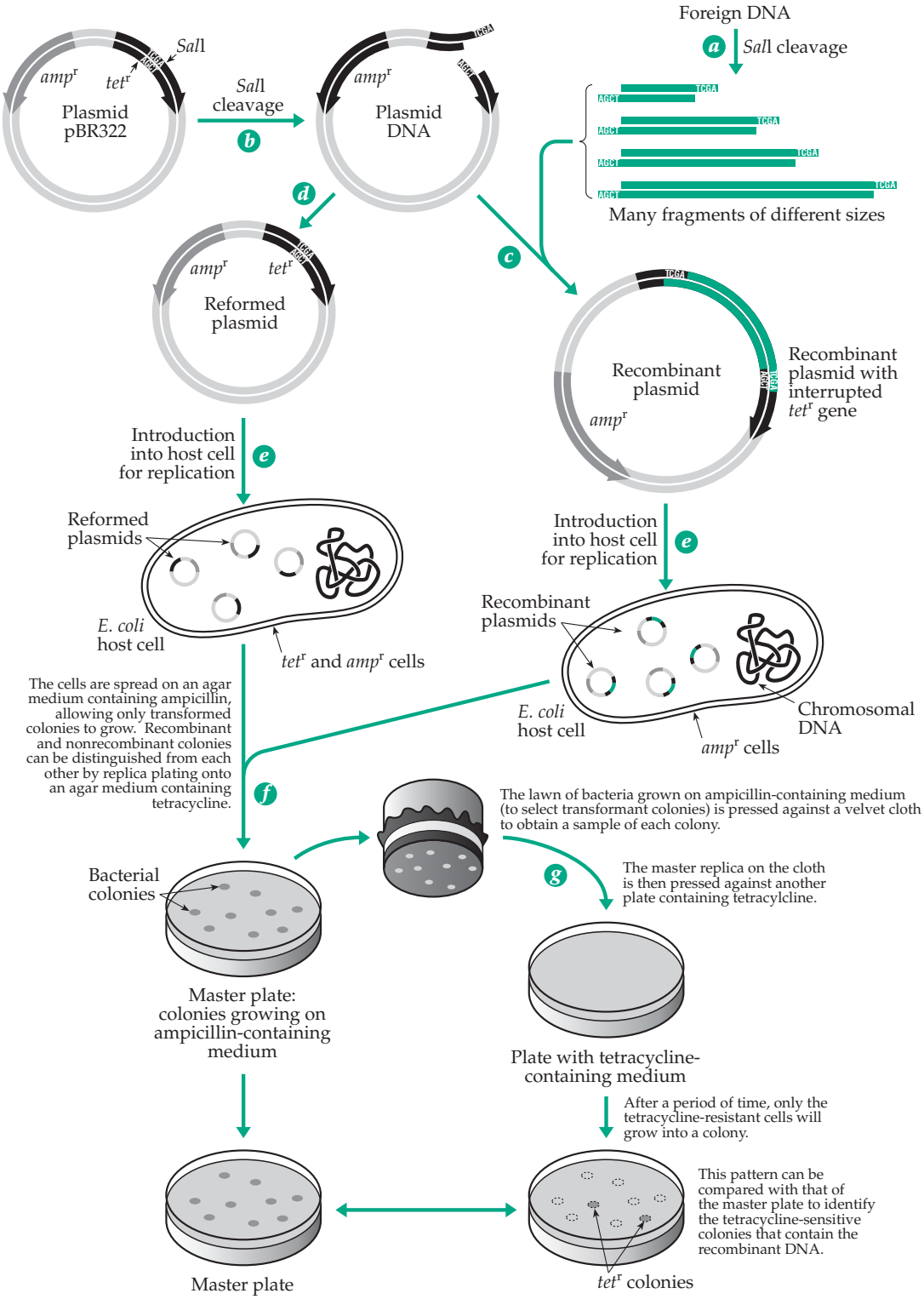



**Figure 26-9** A classical scheme for cloning DNA in a pBR322 plasmid vehicle. A DNA sample is digested (step *a*) with one of the restriction endonucleases (e.g., *Sal*I), that cuts the cloning vehicle within the *Tet*$^r$ gene (see Fig. 26-7). The plasmid is also cut with the *Sal*I restriction enzyme (step *b*). After mixing with the digested DNA sample and annealing, recombinant molecules are formed (step *c*). Some plasmids are reformed (step *d*). Both recombinant and reformed plasmids transform *E. coli* cells (step *e*). The transformed cells are plated on an agar medium containing ampicillin (step *f*). Only cells containing the ampicillin-resistance gene grow (step *g*). A replica plate is made and is pressed onto another plate containing tetracycline. The recombinant colonies do not grow on the medium because the tetracycline-resistance gene has been interrupted. By comparing the two plates recombinant colonies can be selected. These can be tested, using a suitable probe, to determine whether they carry a desired gene. After Atherly, Girton, and McDonald.[2]

***Lambda cloning vehicles.*** Many cloning vehicles have been derived from the 48,502-bp[128] DNA from the temperate *E. coli* bacteriophage λ. The DNA from phage particles are taken up efficiently by *E. coli* cells, much more so than by transfection. The virus has a complex life cycle, which is discussed in Chapter 28. Within the phage head the λ DNA exists in a folded linear form with 12-base 5' cohesive ends (Fig. 28-11). After entrance into the bacterial cell the DNA cyclizes through its cohesive ends and is ligated by the *E. coli* ligase. Replication of the circular forms ensues. Later in the cycle, rolling circle replication (Eq. 27-7 and associated discussion) produces long concatamers with several phage genomes joined as a single chain. This DNA is "packaged" into new phage heads. As this is done, a nuclease cuts the concatamers at *cos* sites forming monomeric genomes with cohesive ends. This is the **lytic cycle** of the phage. In the alternative **lysogenic cycle** the DNA becomes integrated into the *E. coli* genome. Maintenance of the lysogenic state depends in part on gene *cI*, which encodes a repressor (see Chapter 28) that prevents expression of the genes required in the lytic pathway. Since only the lytic cycle is needed for cloning, it is convenient to place a cloning site within the *cI* gene. The screening of recombinant phage particles is done by examination of plaques. A phage without inserted DNA will be able to undergo both the lytic and lysogenic cycles and will form turbid plaques. However, if passenger DNA is inserted in the *cI* gene, the lysogenic cycle is prevented, and clear plaques are formed.

Of the ~50 genes present in native λ, only about half are necessary for replication in the lytic cycle. Thus, it is possible to delete about 1/3 of the genome to make room for more passenger DNA. However, to form mature phage particles the length of the DNA must be at least 75% of the native length. No more than 110% of the native amount may be present. The total DNA must fall between 38 and 53 kb in length. To accommodate these packaging requirements **replacement vectors** containing unnecessary "stuffer DNA" between two lambda "arms" are used. The unneeded stuffer piece has the same kind of restriction site or sites at each end so that it can easily be cut out and replaced by the passenger DNA. This permits cloning of DNA segments up to about 22 kb in length. Lambda vehicles have all been engineered to eliminate undesired restriction sites and to reduce the number of sites for *Eco*RI and other restriction enzymes commonly used for cloning. The widely used **Charon series**[129] have been further engineered so that they will grow only in strains of bacteria that cannot survive in the human intestinal tract. For example, amber mutations (Section B,6) are incorporated into genes needed for phage assembly, and the bacterial hosts must contain an amber suppressor gene. The bacteria are also nutritional auxotrophs with absolute requirements for thymidine and diaminopimelic acid in the medium. The latter compound is not found in the intestinal tract. The purpose of these alterations is to prevent the spread of recombinant DNA into the environment.

To sequence DNA carried in a lambda vehicle or to study it in other ways, it is often necessary to cut it with restriction enzymes, to prepare a restriction fragment map, and to subclone the fragments into a plasmid vehicle. Lambda vehicles, which will automatically transfer the passenger DNA into an M13 vehicle when propagated in a host carrying a special helper virus, have been devised.[130] The helper virus encodes proteins that recognize and cleave sequences that mark the initiation and termination of M13 DNA synthesis. These are used to mark the ends of the passenger DNA. As DNA synthesis occurs the displaced passenger DNA and M13 genes are excised, circularized, and converted into a replicating form of an M13 cloning vehicle.

***Cosmid vehicles and in vitro packaging.*** Cosmids[131] are hybrids of a plasmid vehicle and phage λ. They contain the *cos* sites that are cleaved during packaging of λ DNA. A cosmid cleaved at a restriction site will form upon ligation a range of different sized DNA molecules that contain *cos* sites on both sides of a piece of passenger DNA, which may be up to 45 kb in length (Fig. 26-10). This can be cut at the *cos* sites and packed into heads using an *in vitro* packaging system. In this system the unassembled subunits of the phage particle are produced in special strains of bacteria and

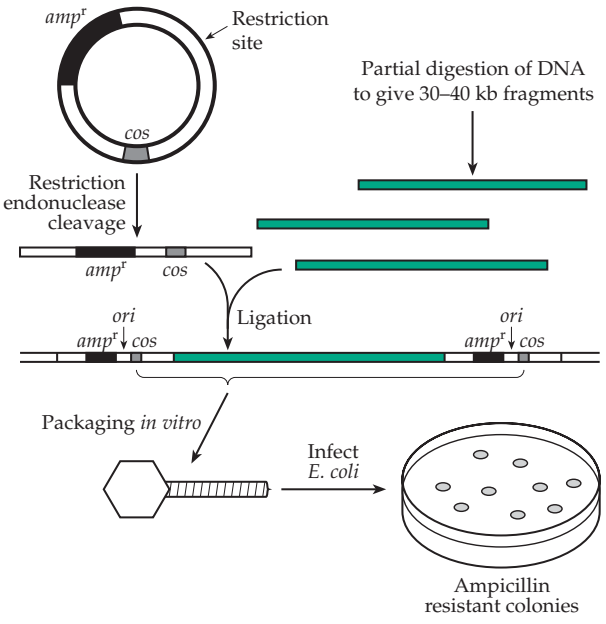



**Figure 26-10** Cloning DNA in cosmids. See Glover.[99]

are allowed to assemble and package the cosmid DNA. Since cosmids contain relatively large pieces of DNA, they are useful for preparing sequentially overlapping clones that allow the investigator to "walk" along the DNA hunting for a target gene. Cosmid vehicles have been designed to allow both efficient genomic walking and restriction mapping.[132]

## 3. Expression of Cloned Genes in Bacteria

A major goal in recombinant DNA technology is the production of useful foreign proteins by bacteria, yeast, or other cultured cells. Protein synthesis depends upon both transcription and translation of the cloned genes and may also involve secretion of proteins from the host cells. The first step, transcription, is controlled to a major extent by the structures of promoters and other control elements in the DNA (Chapter 28). Since eukaryotic promoters often function poorly in bacteria, it is customary to put the cloned gene under the control of a strong bacterial or viral λ promoter. The latter include the λ promoter $P_L$ (Fig. 28-8) and the *lac* (Fig. 28-2) and *trp* promoters of *E. coli*. These are all available in cloning vehicles.

It is often useful to create hybrid proteins fused to the *E. coli* β-galactosidase gene. If another gene is spliced in at either the N terminus or the C terminus of the galactosidase (*lac*Z) gene but is kept under control of the *lac* promoter, the resulting hybrid protein will have galactosidase activity, which can be used for screening. In addition, the hybrid protein will often react with antibodies directed against the protein whose gene is being cloned. Another kind of hybrid fuses the cloned gene to that of β-lactamase, for example at the *Pst I* site of plasmid pBR322 (Fig. 26-6). The β-lactamase activity will be gone, but the hybrid protein will be secreted because β-lactamase normally is secreted into the periplasmic space and its N-terminal signal sequence is now fused to the cloned protein. Engineering of a suitable site for cleavage by a protease can release the foreign protein in an active form. A variety of other **expression systems**, often using **reporter gene** products, have been developed.[133]

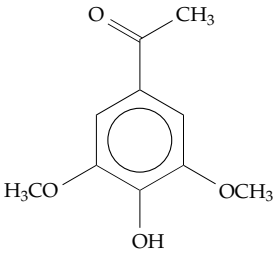
Bacteria often degrade foreign proteins by hydrolytic attack. One way in which such damage has been minimized is to clone multiple fused copies of the gene for the desired product. The resulting polyprotein may be resistant to degradation, and if the gene has been correctly engineered, may be cut apart by cyanogen bromide (Eq. 3-17) or by a specific protease.

For many years most cloning was done in *E. coli*, but cloning systems have now been developed for many other bacteria including *Bacillus* and other gram-positive bacteria, and also for yeast, insect cells, animals, and plants.[133–135]

## 4. Cloning and Transferring Eukaryotic Genes

Eukaryotic genes cloned into bacterial plasmids are often poorly expressed. It is advantageous to clone such genes in eukaryotic cells, where the cutting and splicing of hnRNA to remove intervening sequences during formation of mRNA (Chapter 28) does occur. This permits expression of the cloned gene, something that is possible in bacteria only if cDNA that lacks the introns is cloned. The need for posttranslational modification of many proteins also interferes with expression in bacteria. Many methods of gene transfer and cloning have been developed.[136] The yeast *Saccharomyces cerevisiae* is often an ideal host for cloning. It grows rapidly in either its haploid or diploid stage (Chapter 1). Some strains carry a 2-μm 6.3-kb circular plasmid with 50–100 copies per cell.[109] This has been developed as a cloning vehicle. Recombinant plasmids can be used as **shuttle vehicles** for transferring genes cloned in *E. coli* into the yeast plasmid. Genes may also be cloned as minichromosomes, such as **yeast artificial chromosomes** (**YACs**). Artificial chromosomes contain origins of replication from yeast, human, or bacterial chromosomes as well as telomeres and centromeres[137] (see also Chapter 27). YACs have been widely used and became popular because they can accomodate 600 kbp or more of DNA.[137–139] However, their use in the human genome project resulted in serious problems of instability.[140] Bacterial artificial chromosomes (BACs), which accommodate only 200–300 kbp, **P1 artificial chromosomes** (**PACs**),[141,142] and human minochromosomes are more stable.[143] Another problem, which affects the use of yeast for production of eukaryotic proteins, is the tendency for poor removal of introns.

***Plant genes.*** Much of the cloning in higher plant cells has made use of the **Ti plasmid** of *Agrobacterium tumefaciens*, a soil bacterium that enters wounds in dicotyledenous plants causing tumors known as **crown galls**.[144–146c] A related species *A. rhizogenes* harbors a similar plasmid that causes "hairy root" disease.[146c,147] The infecting bacteria respond to the synthesis of certain phenolic compounds such as **acetosyringone**, which are produced in plant wounds, by entering the plant cells.[148,149] Only bacteria carrying the Ti (tumor-inducing) plasmid cause tumors. The plasmid carries a 13-kb region




Acetosyringone, a compound that induces infection by *Agrobacterium*