1
Nitrogen Metabolism

Some microorganisms are capable of reducing nitrogen gas to ammonium, which can then be incorporated into amino acids, and thence into other organic nitrogenous compounds, including purines, pyrimidines, amino sugars, phospholipid bases and a variety of cofactors and coenzymes that are vitamins for animals. Plants and other microorganisms can incorporate ammonium and inorganic nitrates and nitrites into amino acids and other nitrogenous compounds. Animals cannot utilize inorganic nitrogen compounds to any significant extent, but rather are reliant on plant foods (and also, to some extent, microorganisms) for amino acids for the synthesis of tissue proteins and other nitrogenous compounds, including purines and pyrimidines. Other organic nitrogenous compounds in plant foods can be utilized to a greater or lesser extent.

Ruminants are able to make use of inorganic nitrogen compounds indirectly, because of their large intestinal population of commensal bacteria that can synthesize amino acids from ammonium. This is economically important, since chemically synthesized urea fed to ruminants releases more expensive protein-rich oil-seed cake and protein from bacteria, yeasts and fungi for human consumption, or as feedstuff for monogastric livestock.

The major end products of amino acid catabolism by animals are relatively simple organic compounds such as urea, purines and uric acid, as well as ammonium salts (and in some cases ammonia gas) and nitrate and nitrite salts. Various microorganisms can oxidize ammonia to nitrogen gas, reduce nitrites and nitrates to nitrogen gas or catalyze a reaction between ammonia and nitrite to produce nitrogen gas.

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There is, thus, a cycle of nitrogen metabolism:

- nitrogen gas is fixed as ammonium;
- ammonium is incorporated into amino acids;
- other nitrogenous compounds are synthesized from amino acids;
- this is followed by catabolism, ultimately yielding ammonium and nitrates, then denitrification reactions releasing nitrogen gas.

This nitrogen cycle is shown in Figure 1.1.

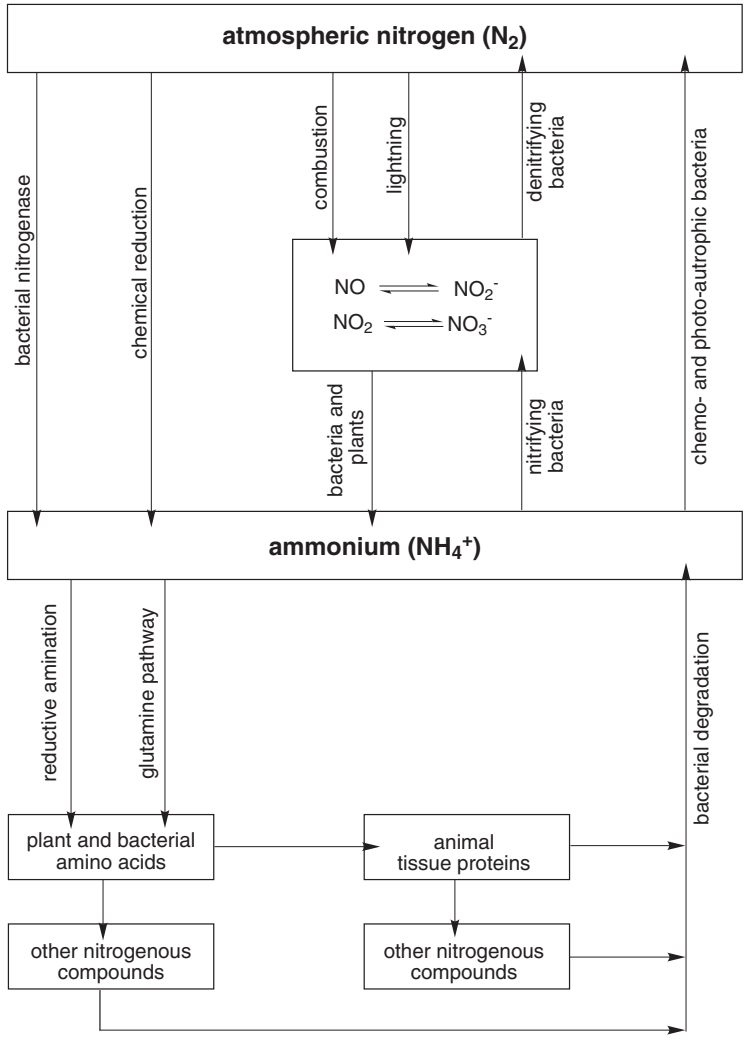

Figure 1.1 The nitrogen cycle.
Nitrogenase EC 1.18.6.1 (ferredoxin-linked), 1.19.6.1 (flavodoxin-linked).

As a result of human activity, the nitrogen cycle is no longer in balance. There is an excess of nitrogen fixation overdenitrification, resulting in the accumulation of fixed nitrogen in rivers, lakes and oceans and of nitrogen oxides in the atmosphere. Global production of nitrogen fertilizers was 80×10^6 million tonnes in 1997, and is projected to rise to 134×10^6 million tonnes by 2020; half of all the chemically synthesized nitrogen fertilizer used up until 1990 was used between 1980 and 1990.

The burning of fossil fuels and biomass accounts for release into the atmosphere of some 20×10^6 tonnes of nitrogen oxides each year, and lightning probably produces about half as much. It is estimated that terrestrial ecosystems produced $90–140 \times 10^6$ tonnes of fixed nitrogen a year prior to human activity and that widespread cultivation of legume crops has added $32–55 \times 10^6$ tonnes of fixed nitrogen per year. Marine ecosystems are estimated to fix $30–300 \times 10^6$ tonnes of nitrogen a year. Overall, human activities are estimated to fix 210×10^6 tonnes of nitrogen a year, compared with 140×10^6 tonnes from biological nitrogen fixation and the action of lightning (Galloway *et al.*, 1995; Vitousek *et al.*, 1997).

There are two consequences of this excess of nitrogen fixation overdenitrification. Nitrous oxide (N_2O) is a greenhouse gas, and hence it contributes to global warming and climate change. It also catalyzes the destruction of ozone in the stratosphere. Nitrates in drinking water present a health hazard; gastric microorganisms reduce nitrate (NO_3^-) to nitrite (NO_2^-), which can react with haemoglobin to yield methaemoglobin, which does not transport oxygen. Although mammals have methaemoglobin reductase and can regenerate active haemoglobin, young infants are especially at risk from excessive nitrate intake, because foetal haemoglobin is considerably more sensitive to nitrite than is adult haemoglobin.

A nitrate concentration greater than 10 mg N/l of water is considered to pose a threat to public health. Nitrites are also able to react with amines under the acidic conditions of the stomach to form carcinogenic nitrosamines, although it is not clear whether the small amounts of nitrosamines formed from dietary amines and nitrites pose a significant health hazard. There is therefore great interest in bacteria that can be used to denitrify drinking water (section 1.2; Martinez-Espinosa *et al.*, 2011).

1.1 Nitrogen fixation

The N ≡ N triple bond in nitrogen gas is extremely stable, with a bond energy of 0.94 MJ (225 kcal) per mol; this is the bond that has to be broken to fix nitrogen. The Haber-Bosch process for synthesis of ammonia (the basis of the chemical fertilizer industry) uses temperatures of 300–550°C and pressures of 15–25 MPa (150–250 atm), with an iron catalyst, to reduce nitrogen with hydrogen gas to form ammonia:

$$N_2 + 3H_3 \rightarrow 2NH_3$$

Nitrogen-fixing microorganisms (diazotrophes) catalyze the same reaction at temperatures as low as 10°C and 100 kPa (1 atm) pressure. This bacterial nitrogen fixation accounts for some 100×10^6 tonnes of nitrogen per year. As shown in Table 1.1, the bacteria and cyanobacteria (formerly known as blue-green algae) that catalyze nitrogen fixation occupy a wide variety of ecological niches. Among heterotrophic bacteria, diazotrophes may be obligate or facultative anaerobes or obligate aerobes, and autotrophic diazotrophes may be aerobic or anaerobic, photosynthetic or non-photosynthetic. Non-photosynthetic autotrophic diazotrophes include those that can reduce sulphate to sulphide (e.g. *Desulphovibrio* spp.) and the methanogenic archaea.

Although the ability to fix nitrogen is found in bacteria and archaea occupying a wide variety of ecological niches, only a few hundred prokaryotic species (and no eukaryotes) are diazotrophic. Free-living heterotrophic bacteria have proven to be the easiest organisms in which to study nitrogen fixation, but they make a relatively minor contribution to global nitrogen fixation compared with photoautotrophic and symbiotic organisms.

A number of plant-bacteroid symbiont pairs are also diazotrophic. The best known is the symbiotic association of *Rhizobium* spp. in root nodules of legumes (section 1.1.1.7), but a number of other diazotrophic organisms (e.g.

Table 1.1 Some organisms capable of fixing nitrogen.

Free-living heterotrophes	obligatory aerobic	*Azotobacter* spp., *Mycobacterium* spp.
	facultatively anaerobic	*Klebsiella pneumoniae, Bacillus polymyxa*
	obligatory anaerobic	*Clostridium pasteurianum, Clostridium butyricum*
Free-living autotrophes	obligatory aerobic	cyanobacteria: *Anabaena* spp., *Nostoc* spp., *Plectonema* spp.
	facultatively anaerobic	*Rhodospirillum* spp., *Rhodopseudomonas* spp.
	obligatory anaerobic	*Chromatium* spp., *Chlorobium* spp.
Symbiotic associations	fungi (lichens), liverworts, tropical grasses, *Azolla* spp.	cyanobacteria
	plant leaf nodules	*Klebsiella* spp.
	roots and leaves of plants	*Azotobacter* spp.
	legume root nodules	*Rhizobium* spp.
	non-legume root nodules	*Frankia* spp.

Frankia spp.) form symbiotic associations with non-leguminous plants. *Rhizobium* and *Frankia* are obligate symbionts, and are not capable of independent existence. A number of organisms that are both capable of independent existence and capable of fixing nitrogen when free-living, such as *Azotobacter* spp. and cyanobacteria, frequently form symbiotic associations in leaf nodules of higher plants or around the roots of aquatic plants. Many lichens, which are symbionts of fungi with bacteria or cyanobacteria, are diazotrophic.

Some nitrogen-fixing endophytic bacteria form nodule-independent associations with cereal crops, but it is unclear whether the effect on plant growth is due to nitrogen fixation or to the synthesis of bacterial metabolites that act as plant growth hormones by the bacteria.

A major challenge for plant science is the possibility of engineering nitrogen fixation into non-leguminous crops. There are two possible approaches to this (Beatty & Good, 2011). It may be possible to transfer nitrogen-fixing genes directly into cereal crops and ensure their expression in the roots (section 1.1.1.1), or it may be possible to bio-engineer cereal crops to produce the same chemo-attractants for nitrogen-fixing bacteria as are produced by legumes (section 1.1.1.7).

Some wood-eating insects (e.g. termites) and molluscs (e.g. the shipworm, *Teredo* spp.) have symbiotic diazotrophic bacteria that may make a significant contribution to the host's nitrogen nutrition. Commensal bacteria in ruminants fix nitrogen, but there is no evidence that non-ruminant mammals (including human beings) harbour any significant number of intestinal nitrogen-fixing bacteria.

There are three requirements for nitrogen fixation: the enzyme nitrogenase, which catalyzes the reduction of N_2 to NH_4^+; a source of reductant; and an electron carrier to couple the reductant with the enzyme. In addition, there is a requirement for 16 × ATP per mol of nitrogen reduced to ammonium. In *Clostridium* spp. as much as 30 per cent of the metabolic energy derived from anaerobic fermentation may be utilized in nitrogen fixation.

1.1.1 Nitrogenase

There are three related families of proteins that catalyze the reduction of nitrogen gas to ammonia. The most studied contains both molybdenum and iron, but there are also nitrogenases that contain vanadium instead of molybdenum, and some that contain only iron. These different nitrogenases are encoded by different genes and, in some microorganisms, all three enzymes are expressed. There is considerable sequence homology between the different nitrogenases and also between the same types of nitrogenase (Mo-Fe, V-Fe and Fe) from different organisms. Nitrogenases may utilize either ferredoxin or flavodoxin as the reductant (Eady, 1996; Howard & Rees, 1996).

The reaction catalyzed by nitrogenase is:

$$N_2 + 8H^+ + 8e^- + 16ATP \rightarrow 2NH_3 + H_2 + 16ADP + 16P_i$$

Two separate proteins make up nitrogenase: an iron-containing protein that is a homodimer with two ATP binding sites and a single iron-sulphur cluster (4Fe4S) shared between the two subunits; and the iron-molybdenum protein, which is a hetero-tetramer ($2\alpha2\beta$) with two iron-sulphur clusters (8Fe7S) and two mol of the molybdenum coenzyme (7Fe-Mo-9S-homocitrate). The two $\alpha\beta$ subunits of this protein seem to be independent; both catalyze the reduction of nitrogen, so that the tetramer has two catalytic sites.

The main function of the iron protein is to transfer reducing equivalents to the molybdenum-iron protein. It is sometimes called nitrogenase reductase, but it is also required for the synthesis of the iron-molybdenum cofactor and its insertion into the iron-molybdenum protein. Each of the eight electron transfer reactions required for the reduction of 1 mol of nitrogen involves association between the iron protein and the iron-molybdenum protein, then dissociation of the complex (Burgess & Lowe, 1996; Howard & Rees, 1996; Rubio & Ludden, 2008).

In the reduced iron protein, the (4Fe4S) cluster is in the +1 oxidation state, and the protein binds two mol of MgATP. Hydrolysis of both mol of ATP causes transfer of one electron to the iron-molybdenum protein. The oxidized iron protein, with the iron-sulphur cluster in the +2 oxidation state and $2 \times$ ADP bound, then dissociates from the iron-molybdenum protein. It is reduced back to the +1 oxidation state by ferredoxin or flavodoxin (and *in vitro* by a variety of other reducing agents as well), and the 2 mol of ADP are replaced by ATP.

The iron-sulphur cluster of the iron-molybdenum protein is reduced by reaction with the iron protein, and then transfers electrons to the iron-molybdenum cofactor, which is the site of nitrogen binding and reduction. Nitrogen only binds to the cofactor when it has undergone three electron transfer reactions (i.e. three single electron reductions). One mol of ammonia is released when the cofactor has undergone five electron transfer reactions, and the second is released after seven electron transfer reactions (Seefeldt *et al.*, 2009).

Nitrogenase also catalyzes the reduction of acetylene (ethyne) to ethylene (ethene), a reaction that is commonly used to study the enzyme *in vitro*, and of ethylene to ethane. Acetylene binds to the enzyme when it has undergone only two electron transfer reactions. In the absence of nitrogen or any other substrate, all of the electrons passing through nitrogenase reduce protons to hydrogen. Even when nitrogen is present, 25 per cent of the electron flux goes to proton reduction, with no more than 75 per cent to nitrogen reduction.

Carbon monoxide is normally a potent inhibitor of nitrogenase, but a point mutation in the iron-molybdenum protein leads to an enzyme that will

catalyze the reduction of carbon monoxide to methane, and onwards to form higher hydrocarbons such as ethane, ethylene, propylene (propene) and propane (Yang *et al.*, 2011).

A separate type of nitrogenase has been isolated from *Streptomyces thermoautotrophicus*. The reduction of nitrogen to ammonia is catalyzed by an oxygen-insensitive molybdenum-containing enzyme (as discussed in section 1.1.1.3, nitrogenase from most organisms is extremely sensitive to oxygen), and the ATP requirement for nitrogen reduction is considerably lower than for the enzymes discussed above. Nitrogen reduction is coupled to the oxidation of carbon monoxide, reducing oxygen to superoxide. The superoxide is then re-oxidized to oxygen, with transfer of electrons to nitrogenase for reduction of nitrogen to ammonia (Ribbe *et al.*, 1997).

1.1.1.1 The nitrogen fixation gene cluster As shown in Table 1.2, the nitrogen-fixing (*nif*) gene cluster in *Klebsiella pneumoniae* consists of a total of 20 separate, but coordinately expressed, genes, arranged in seven operons. In addition to the genes for the nitrogenase proteins discussed above, these genes code for enzymes involved in the synthesis of the molybdenum-iron cofactor, its insertion into the molybdenum-iron protein and the enzymes involved in the synthesis of other cofactors required for nitrogen fixation, including ferredoxin and flavodoxin, and proteins that regulate nitrogenase activity.

1.1.1.2 Regulation of nitrogenase by the availability of fixed nitrogen and ATP Nitrogen fixation is highly ATP expensive, as is transcription and translation of the multiple genes involved, so in most nitrogen-fixing microorganisms there is repression of the expression of nitrogen-fixing genes by the availability of fixed nitrogen. No more nitrogen will be fixed into ammonium than can be incorporated into amino acids. However, in *Rhizobium* in legume root nodules, there is no repression of nitrogen-fixing genes by ammonium and the symbiotic microorganisms fix more nitrogen than they can incorporate into amino acids for their own use. This diffuses across the symbiosome membrane (section 1.1.1.7) into the host cell cytosol. A downward concentration gradient is achieved partly by the pH difference between the interior of the symbiosome and the host cell cytosol, and partly by the removal of ammonium as it is incorporated into amino acids (Udvardi & Day, 1997).

In addition to transcriptional control of nitrogenase in response to the intracellular concentration of fixed nitrogen, there is short-term regulation of existing nitrogenase protein in some organisms. Low fixed nitrogen is detected by an accumulation of 2-oxoglutarate, which is the key substrate for incorporation of ammonia into amino acids (section 1.3.2). When the concentration of 2-oxoglutarate is low, nitrogenase is inhibited. As the concentration of 2-oxoglutarate rises, so regulatory proteins are displaced from

Table 1.2 The proteins encoded by the *nif* genes of *Klebsiella pneumoniae*, in the order in which they occur in the genome. The 20 genes are arranged in seven operons.

Gene	Protein function
nifJ	Pyruvate oxido-reductase, required for generation of reducing equivalents from pyruvate oxidation.
nifH	The peptide chain of the iron-protein of nitrogenase.
nifD	The α-subunit of the iron-molybdenum protein of nitrogenase.
nifK	The β-subunit of the iron-molybdenum protein of nitrogenase.
nifT	Unknown; may be involved in formation of the iron-molybdenum-homocitrate cofactor.
nifY	A protein associated with the apo-protein of the iron-molybdenum protein of nitrogenase that dissociates when the iron-molybdenum-homocitrate cofactor is inserted.
nifE	Forms heterotetramer with *nifN* product that acts as a template for synthesis of the iron-molybdenum-homocitrate cofactor.
nifN	See *nifE*.
nifX	Negative regulator (repressor) of *nif* operon in response to oxygen and NH_4^+.
nifU	Required for full activity of nitrogenase, probably concerned with the iron-sulphur centre of the iron-protein.
nifS	A pyridoxal phosphate-dependent enzyme that catalyzes desulphuration of cysteine to alanine, concerned with forming the iron-sulphur centre of the iron-protein of nitrogenase.
nifV	Catalyzes synthesis of homocitrate from 2-oxoglutarate for the iron-molybdenum-homocitrate cofactor.
nifW	Associates with the nitrogenase molybdenum-iron protein under conditions of oxygen stress.
nifZ	Involved in insertion of the iron-molybdenum-homocitrate cofactor into nitrogenase.
nifM	Involved in activation of the iron protein of nitrogenase.
nifF	The flavodoxin that accepts electrons from pyruvate oxido-reductase.
nifL	Regulatory flavoprotein that represses expression of the whole *nif* complex, especially in response to oxygen. In the oxidized form, it prevents binding of the *nifL* gene product to the promoter regions of the *nif* operons.
nifA	Transcriptional activator that binds to promoter regions of the *nif* operons, and so induces expression of the whole *nif* complex.
nifB	Involved in synthesis of the iron-molybdenum-homocitrate cofactor.
nifQ	Uptake of molybdenum for synthesis of the iron-molybdenum-homocitrate cofactor.

nitrogenase, permitting increased reduction of nitrogen to ammonia. ATP acts synergistically with 2-oxoglutarate, reflecting the high ATP cost of nitrogen fixation (Leigh & Dodsworth, 2007).

In some microorganisms, the iron protein of nitrogenase is regulated by ADP-ribosylation. A specific nitrogenase reductase, ADP-ribosyltransferase, is activated in response to an increase in the concentration of ammonium, asparagine or glutamine. The ADP-ribosylated iron protein is inactive, so

halting nitrogen fixation. A fall in the ATP : ADP ratio also activates the ADP-ribosyltransferase. The inhibition of the iron protein is reversed by a glycohydrolase that is activated in response to a decrease in the concentration of ammonium or an increase in 2-oxoglutarate. The ADP-ribosyltransferase and glycohydrolase are encoded on the same operon, and must be reciprocally regulated in response to fixed nitrogen and 2-oxoglutarate (Ludden, 1994; Wang & Noren, 2006).

1.1.1.3 Protection of nitrogenase against oxygen Both the iron protein and the molybdenum-iron protein of nitrogenase are irreversibly damaged by oxygen, as a result of generation of superoxide and other reactive oxygen species when oxygen binds to the metal-sulphur centre and undergoes reduction. For anaerobic microorganisms, this does not present a problem. Anaerobic photosynthetic organisms, including sulphur bacteria that oxidize sulphides and inorganic sulphur to sulphates, and also non-sulphur anaerobic photosynthetic organisms, do not produce oxygen, so these can fix nitrogen in the light.

Facultative anaerobes only express the nitrogenase genes in the absence of oxygen, so that they only fix nitrogen under anaerobic conditions, or when they are essentially anaerobic because they are respiring at such a rate that they have reduced the oxygen concentration to near zero.

Aerobic heterotrophic and photosynthetic microorganisms have evolved a variety of ways to combine oxygen sensitive nitrogen fixation with the presence or production of oxygen. In some photosynthetic organisms, nitrogenase is protected by ADP-ribosylation in response to light; the ADP-ribosylated enzyme undergoes a conformational change that protects the iron-sulphur cluster against oxygen. In other organisms, there are conformational changes in response to light similar to those seen in response to oxygen stress in heterotrophic organisms (section 1.1.1.5).

1.1.1.4 Respiratory protection in aerobic microorganisms *Azotobacter* spp. are obligatory aerobes that fix nitrogen. They have two terminal electron transport chain cytochromes that react with oxygen; one is associated with phosphorylation of ADP and inorganic phosphate to ATP, while the other is not. The cytochrome that is not associated with ADP phosphorylation has a higher K_m for oxygen than that the one that is associated with phosphorylation; thus, as the concentration of oxygen increases, the less efficient branch of the electron transport chain becomes more important. This means that as the concentration of oxygen increases, so the rate of oxidation of substrates, and consumption of oxygen, increases to maintain the same level of ATP formation. When the availability of oxygen rises to such an extent that it cannot be removed by this respiratory protection, the resultant oxygen stress leads to conformational protection of nitrogenase (Robson & Postgate, 1980).

1.1.1.5 Conformational changes in nitrogenase In many diazotrophic organisms, there is a conformational switch to protect nitrogenase from oxygen. Oxygen stress leads to an interaction between a protective iron-sulphur protein and the two components of nitrogenase (the iron protein and the molybdenum-iron protein), to form a complex that is catalytically inactive, but in which the reactive centres of the nitrogenase proteins are protected against oxygen binding and damage. As the oxygen concentration falls, so this complex dissociates, releasing active nitrogenase (Robson & Postgate, 1980).

1.1.1.6 Heterocyst formation in filamentous cyanobacteria Cyanobacteria are photosynthetic organisms that generate oxygen. When filamentous cyanobacteria are grown in the presence of fixed nitrogen, all cells along the filament appear the same, and all are photosynthetic vegetative cells. However, when they are grown in the absence of fixed nitrogen, individual cells at more or less regular intervals along the filament differentiate into larger cells known as heterocysts, which fix nitrogen. Approximately 10 per cent of the cells typically become heterocysts, although, in the symbiotic association between *Anabaena* and the water fern *Azolla*, up to 30 per cent of the cells of the cyanobacterium become heterocysts. This symbiotic association between *Anabaena* and *Azolla* has been used to enhance rice production in paddy fields for centuries (Burris & Roberts, 1993; Golden & Yoon, 2003).

The heterocysts have photosystem I, which produces ATP, but they lack photosystem II, which produces oxygen and reduces carbon dioxide to glucose. The heterocysts are surrounded by a glycolipid layer that prevents the entry of oxygen. However, they have to import carbon substrates from, and export fixed nitrogen to, vegetative cells through pores between adjacent cells in the filament. To minimize oxygen damage to nitrogenase, there is a 'honeycomb' of membranes in the heterocyst that contains various oxygenases (Wolk, 1996).

1.1.1.7 Symbiotic *Rhizobium* spp. in root nodules Legume roots secrete flavonoids (section 9.2.2) that act as chemo-attractants for free-living *Rhizobium* in the soil. In response to this stimulus, *Rhizobium* synthesizes signalling compounds that act on the legume root hairs, causing them to curve inwards. This permits *Rhizobium* to invade the root and cause an inflammatory response that leads to dedifferentiation of quiescent root cortical cells into actively dividing meristem and nodule formation.

There is considerable specificity as to which *Rhizobium* species will invade, and become symbiotic with, which legume species. This is partly determined by the flavonoid chemo-attractants secreted by the legume, and partly by the nodulation factors secreted by *Rhizobium*. Within the nodules, the bacteria are enclosed in a membrane synthesized by the plant, and they divide and

differentiate into nitrogen-fixing bacteroids. This organelle, consisting of the plant-derived membrane and the bacteroids, is called the symbiosome (Gibson *et al.*, 2008).

Some non-leguminous plants also form symbiotic associations with nitrogen-fixing organisms, commonly *Frankia* spp., in a similar way to legume root nodule formation. These are commonly trees or woody shrubs, including the alder (*Alnus* spp.), *Elaeagnus* spp. and *Ceanothus* spp.

Leghaemoglobin in legume root nodules is an oxygen-binding haem protein with considerable sequence homology with mammalian haemoglobins. It is at the surface of the *Rhizobium* bacteroids, and it serves to deliver oxygen as required for oxidative phosphorylation to produce the ATP required for nitrogen fixation, while also preventing irreversible damage to nitrogenase by maintaining a very low concentration of free oxygen. There are similar haemoglobin-like proteins in nitrogen-fixing non-legume root nodules. The protein is synthesized by the host plant, in response to *Rhizobium* infection, but the haem prosthetic group is synthesized by the bacteroids. Nodules that contain highly effective *Rhizobium* have a pink or red colour as a result of their content of leghaemoglobin (Appleby, 1984; Wittenberg *et al.*, 1974).

1.2 Nitrification and denitrification

Nitrification is the process of oxidizing ammonia to nitrite and nitrate; denitrification is the process of reducing nitrate to nitrogen gas. Three main groups of microorganisms catalyze nitrification reactions, oxidizing ammonia to nitrite (NO_2^-) via hydroxylamine (NH_2OH). Chemolithotrophic bacteria consume only inorganic substrates for energy metabolism. Ammonia-oxidizing chemolithotrophic organisms fix inorganic carbon by linking ATP production to the oxidation of ammonia using molecular oxygen. Methanotrophic bacteria oxidize methane as their principal energy-yielding pathway, but also oxidize ammonia to nitrite by a co-metabolic process (i.e. they do not gain energy directly from the oxidation of ammonia). Heterotrophic ammonia-oxidizing microorganisms metabolize organic carbon compounds and also oxidize ammonia to nitrite.

A variety of nitrite-oxidizing microorganisms oxidize nitrite to nitrate (NO_3^-), which is then a substrate for denitrification. Many microorganisms and fungi use nitrate and nitrite as terminal electron acceptors, forming nitric oxide, nitrous oxide and then nitrogen:

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$$

These organisms flourish in anaerobic environments, especially where the concentrations of nitrate and organic carbon are relatively high (Stein & Yung, 2003).

1.2.1 The anammox (ANaerobic AMMonium OXidation) reaction

A novel denitrification reaction was discovered in a waste water treatment plant in the Netherlands in 1986 – an anaerobic reaction between nitrite and ammonium to form nitrogen gas:

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

The microorganism concerned was identified as *Brocadia anammoxidans*, and the reaction has now been identified in a number of other microorganisms. Indeed, it is estimated that 50–70 per cent of the denitrification activity of oceans and lakes may be due to the reduction of nitrite to nitric oxide, followed by reaction with ammonium to yield hydrazine (N_2H_2), which is then oxidized to nitrogen. The oxidation of hydrazine is linked to the reduction of ferredoxin, and it produces a proton-motive force that can be used to form ATP from ADP and inorganic phosphate. Microorganisms that catalyze this anammox (anaerobic ammonium oxidation) reaction are now exploited as a way of denitrifying drinking water (Jetten *et al.*, 2009; Kuenen, 2008; Op den Camp *et al.*, 2006).

1.3 The incorporation of fixed nitrogen into organic compounds

1.3.1 Utilization of nitrite and nitrate in plants

Nitrates applied to the soil as fertilizer, and washed into the soil together with nitrites formed by the atmospheric oxidation of nitrogen or bacterial oxidation of ammonium, are taken up by the roots by active transport using a pH gradient generated by an ATPase. Nitrate is reduced to ammonium before being used by plants and microorganisms for amino acid synthesis. The two enzymes involved – nitrate reductase and nitrite reductase – are widely distributed in plants and microorganisms. Nitrate induces synthesis of nitrate and nitrite reductase and the nitrate transport proteins. There are two nitrate transport proteins in most plants, with low and high affinities, and the soil concentration of nitrate can vary between 10 µmol/l to 100 mmol/l.

Nitrate reductase, which catalyzes the NADH-dependent reduction of nitrate (NO_3^-) to nitrite (NO_2^-), is a cytosolic enzyme in both leaves and roots. It has three redox centres – FAD, haem and a molybdenum-pterin cofactor – and it uses NADPH as the reductant. Nitrate reductase activity falls in the dark and during carbon dioxide depletion as a result of phosphorylation of the enzyme. However, the purified phosphorylated enzyme is active *in vitro*;

inhibition requires binding of an inhibitory protein to the phosphorylated enzyme. In light, or when carbon dioxide is available, the enzyme is rapidly dephosphorylated and reactivated, since the inhibitory protein does not bind to the dephosphorylated enzyme.

Nitrite reductase catalyzes the reduction of nitrite to ammonium, and again occurs in both roots and leaves. It contains haem and an iron-sulphur redox centre. The reductant is ferredoxin, which only occurs in green parts of the plant, and is reduced by photosystem I in the chloroplasts. However, there is a ferredoxin-like electron carrier in roots, as well as an NADPH-dependent ferredoxin reductase (Oaks & Hirel, 1985).

Nitrate reductase also catalyzes the reduction of chlorate (widely used as a herbicide) to chlorite, which is toxic to plants. Chlorate-resistant plants lack either nitrate reductase or its molybdenum cofactor.

1.3.2 Incorporation of ammonium into organic compounds

There are two main ways in which ammonium can be incorporated into organic compounds: reductive amination of 2-oxoglutarate catalyzed by glutamate dehydrogenase (the glutamate pathway – see section 1.3.2.1); and synthesis of glutamine from glutamate and ammonium, followed by synthesis of glutamate by reductive transfer of the amide group of glutamine onto 2-oxoglutarate (the glutamine pathway – see section 1.3.2.4). While many bacteria use the glutamate pathway, most plants, algae, fungi and some insects use the glutamine pathway.

In organisms that have both pathways, the reductive pathway is favoured when ammonium concentrations are high, and the glutamine pathway is used when ammonium concentrations are low. Glutamine synthetase has a considerably lower K_m for ammonium than does glutamate dehydrogenase. However, the glutamine pathway (Figure 1.4) has an additional cost of $1 \times$ ATP for each mol of ammonium incorporated, compared to the glutamate dehydrogenase pathway (Figure 1.2).

Some microorganisms have other amino acid dehydrogenases that can catalyze the incorporation of ammonium, and the reaction of aspartase (Figure 1.3) is reversible and can function in the direction of ammonium incorporation.

Legumes fall into two groups: amine exporters, which export glutamine, asparagine or 4-methylene-glutamine from the root nodules to the rest of the plant, and ureide formers, which synthesize allantoin, allantoic acid or citrulline for export to the rest of the plant. The synthesis of citrulline from glutamate is discussed in section 5.9. As we will see in section 1.4.2, allantoin and allantoic acid are the products of purine catabolism (Schubert, 1986).

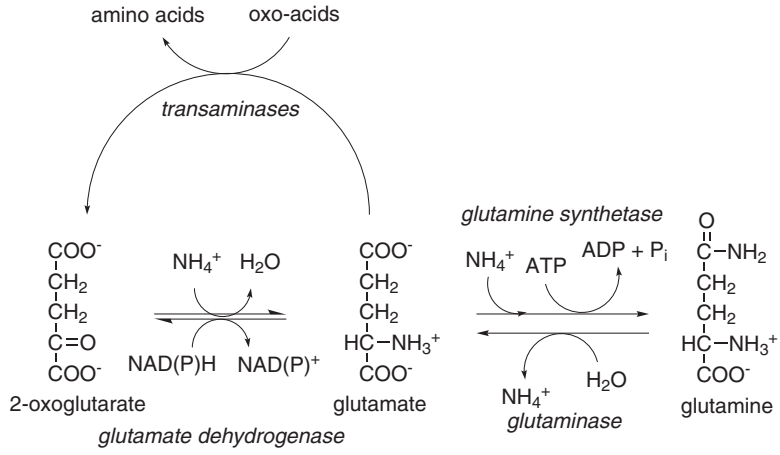

Figure 1.2 Incorporation of ammonia into glutamate and glutamine.
Glutamate dehydrogenase EC 1.4.1.2 (NAD-linked), EC 1.4.1.4 (NADP-linked), EC 1.4.1.3 (linked to either NAD or NADP), glutamine synthetase EC 6.3.1.2, glutaminase EC 3.5.1.2.

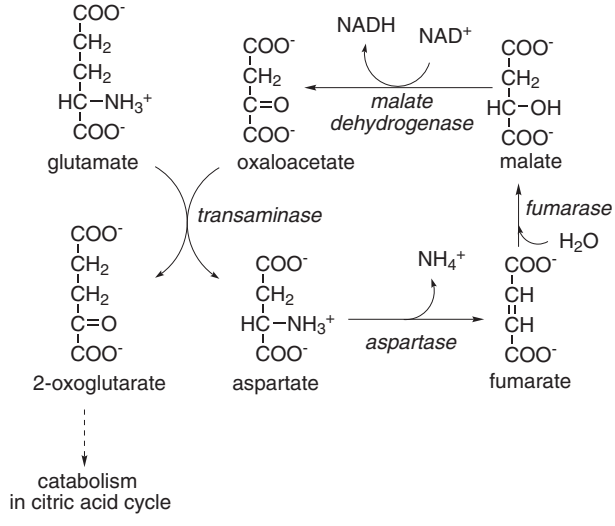

Figure 1.3 The catabolism of glutamate.
Glutamate-oxaloacetate transaminase EC 2.6.1.1, aspartase (aspartate ammonia lyase) EC 4.3.1.1, fumarase EC 4.2.1.2, malate dehydrogenase EC 1.1.1.37.

1.3.2.1 Reductive amination – the glutamate pathway of ammonium incorporation In some bacteria, and also in mammals, the main pathway for incorporation of ammonium into amino acids is reductive amination of 2-oxoglutarate to glutamate, catalyzed by glutamate dehydrogenase, followed (in many cases) by amidation of glutamate to glutamine, as shown in Figure

1.2. The reaction of glutamine synthetase is one of those in which it is easy to explain the role of ATP in an endothermic reaction. Although, overall, the amidation of glutamate is linked to hydrolysis of ATP to ADP and inorganic phosphate, the reaction proceeds by way of intermediate phosphorylation of glutamate to γ-glutamyl-phosphate. As discussed in Chapter 5, glutamate is the precursor for synthesis of proline, ornithine and arginine, as well as providing the amino groups of most amino acids by transamination of the corresponding oxo-acid (see section 3.3).

Mammals cannot utilize ammonium for net synthesis of amino acids, but ammonium arising from deamination of amino acids in peripheral tissues (see section 1.5) is used to synthesize glutamate and glutamine for transport to the liver. Liver cells adjacent to the central vein, which drains the liver into the main venous circulation, have active glutamate dehydrogenase and glutamine synthetase, so as to ensure that little or no ammonium enters the bloodstream. Glutamine is the major source of nitrogen to most tissues, and it is also a major metabolic fuel for rapidly dividing cells of the immune system and gastro-intestinal tract (Chwals, 2004).

The hydrolysis of glutamine to ammonium and glutamate, catalyzed by glutaminase, occurs in both the liver and the kidneys. There are different isoenzymes of glutaminase in these two tissues. The liver enzyme is induced in response to starvation (when amino acids arising from tissue proteins are being catabolized as metabolic fuel) or a high protein diet (when there are surplus amino acids to be deaminated and used for synthesis of fatty acids and glucose), while the kidney enzyme responds to metabolic acidosis (Curthoys & Watford, 1995).

In the liver, glutaminase occurs in periportal cells (those adjacent to the hepatic portal vein, which receives blood from the gastro-intestinal tract) and acts to release ammonium for synthesis of urea for excretion (section 1.6.2.1). In the kidney, part of the response to metabolic acidosis is increased expression of glutaminase and glutamate dehydrogenase (to act in the direction of oxidative deamination, producing ammonium), and of ammonium transporters, so as to increase ammonium excretion in the urine. Onward metabolism of the 2-oxoglutarate arising from glutamine catabolism produces bicarbonate to increase blood buffering capacity. 2-Oxoglutarate dehydrogenase is activated by hydrogen ions and, in response to a fall in pH, the concentration of 2-oxoglutarate in renal cortical tubules falls rapidly, so enhancing deamidation of glutamine and deamination of glutamate, yielding ammonium (Curthoys & Gstraunthaler, 2001; Ibrahim *et al.*, 2008; Karim *et al.*, 2005; Lowry & Ross, 1980; Nissim, 1999).

Both glutamate and glutamine can be used as nitrogen donors for synthesis of a variety of amino acids. The utilization of the amino group of glutamate in transamination reactions is discussed in section 3.3. Plants and most bacteria can synthesize all the amino acids they require for protein synthesis. As

discussed in section 2.2, mammals can synthesize only those amino acids for which they can synthesize the oxo-acid carbon skeletons; others (the essential or indispensable amino acids) have to be provided in the diet. In many, if not all, of the reactions in which glutamine acts as a nitrogen donor, the reaction proceeds in two stages, with one catalytic site catalyzing the hydrolysis of glutamine to glutamate and ammonium, and another catalyzing the (commonly ATP-dependent) incorporation of ammonium. As discussed in section 5.3.2.1, there is an ammonium tunnel through the enzyme connecting the two catalytic sites.

1.3.2.2 Glutamate dehydrogenase Glutamate dehydrogenase from plants and animals can generally use either NAD or NADP as the electron carrier; the relative activity with the two coenzymes depends on the species and tissue of origin of the enzyme. In bacteria and yeasts, the enzyme generally uses only one of the nicotinamide nucleotides, depending on species.

In *Neurospora crassa*, there are two separate isoenzymes of glutamate dehydrogenase. One uses NADPH and functions primarily in the direction of glutamate synthesis (reductive amination, the incorporation of ammonium). The other is a catabolic enzyme, acting primarily in the direction of oxidative deamination; it uses NAD^+. The two enzymes are induced and repressed reciprocally in response to the presence of ammonium or glutamate in the culture medium.

In *E. coli*, glutamate dehydrogenase acts only in the direction of glutamate synthesis, incorporating ammonium. In a strain capable of growth on glutamate as the sole carbon source, glutamate dehydrogenase synthesis is repressed and the glutamate is catabolized by transamination with oxaloacetate, yielding 2-oxoglutarate and aspartate. Ammonium is then liberated by aspartase, yielding fumarate (Figure 1.3), which is used to regenerate oxaloacetate as shown in Figure 1.10. Growth on a glutamate-rich medium induces synthesis of aspartase.

This pathway also occurs in other microorganisms, but not in mammals, which lack aspartase. The aspartase reaction is readily reversible and it may represent a significant route for incorporation of ammonium into amino acids in some microorganisms (Vender *et al.*, 1965).

Aspartases from different microorganisms show significant sequence homology with each other, and also with other class II fumarases such as argininosuccinase in the urea cycle and arginine synthesis (see sections 1.6.2.1 and 5.9.1) and adenylosuccinate lyase (see section 1.4.1). As well as being a substrate, aspartate also activates the enzyme by binding to a separate regulator site together with a divalent metal ion. The reverse reaction provides an industrially important source of aspartate for synthesis of the sweetener aspartame (β-methylaspartyl phenylalanine). With substrates such as hydrox-

ylamine and hydrazine, aspartase can be used for synthesis of *N*-substituted aspartate derivatives that are of potential pharmaceutical interest (Viola, 2000; Weiner *et al.*, 2008).

There are two isoenzymes of glutamate dehydrogenase in plants, an NADH-dependent enzyme in mitochondria and an NADPH-dependent enzyme in chloroplasts. Both enzymes have a relatively high K_m for ammonium, and thus function mainly to release ammonium from glutamate for incorporation into glutamine. They may also have a protective role in roots, to detoxify excessive amounts of ammonium absorbed from the soil or formed by reduction of nitrate (section 1.3.1; Lam *et al.*, 1996).

1.3.2.3 Mammalian glutamate dehydrogenase Mammalian liver glutamate dehydrogenase is a polymer containing six active sites, which do not show cooperativity. It is activated by ADP and 5′AMP, and inhibited by GTP, which change the affinity of the enzyme for the nicotinamide nucleotide coenzyme. This pattern of regulation in response to the energy charge of the cell suggests that the principal function of the enzyme is catabolic, catalyzing the deamination of glutamate to the citric acid cycle intermediate 2-oxoglutarate. Catabolism of glutamate (and other amino acids) will be enhanced when the energy charge is low, as indicated by increasing concentrations of ADP and 5′AMP, and it is inhibited when there is adequate GTP, which is formed in the liver by substrate-level phosphorylation in the citric acid cycle. Glutamate dehydrogenase is also activated by elevated intracellular concentrations of leucine, synergistically with ADP; as we will see in section 2.1.6.6, leucine has a role in regulating overall amino acid and protein metabolism (Plaitakis *et al.*, 2000; Plaitakis & Zaganas, 2001).

Although the regulation of mammalian glutamate dehydrogenase suggests it acts mainly in the oxidative direction, reductive amination of 2-oxoglutarate to glutamate is important in liver and the central nervous system, and the kinetics of glutamate dehydrogenase are such that the direction of the reaction depends very much on the relative concentrations of glutamate and 2-oxoglutarate, and especially on the concentration of ammonium. Even a relatively modest increase in the plasma ammonium concentration, from a normal range of below 50 μmol/l up to 80–100 μmol/l, which is far too small to have any effect on plasma pH, results in disturbance of consciousness. In patients whose plasma concentration rises above about 200 μmol/l, ammonia intoxication can lead to coma and convulsions, which may be fatal. This is mainly due to depletion of 2-oxoglutarate by formation of glutamate (and hence impaired activity of the citric acid cycle) and inadequate ATP formation to maintain nervous system activity. Increased formation of glutamate in the central nervous system may also be important, since glutamate is an excitatory neurotransmitter.

Glutamate dehydrogenase has some activity towards other amino acids, including alanine. Inhibition of glutamate dehydrogenase by GTP increases its activity towards alanine (Hudson & Daniel, 1993).

There is a nerve-tissue specific isoenzyme of glutamate dehydrogenase which has a lower K_m for glutamate than the liver enzyme and is more sensitive to activation by ADP and leucine, but is insensitive to inhibition by GTP. It is only in tissues that catalyze gluconeogenesis that succinyl CoA synthetase in the citric acid cycle catalyzes substrate-level phosphorylation to yield GTP; in other tissues, the enzyme catalyzes phosphorylation of ADP to ATP. This brain-specific glutamate dehydrogenase is involved in the catabolism of glutamate acting as a neurotransmitter (Plaitakis & Zaganas, 2001; Zaganas *et al.*, 2009).

1.3.2.4 Glutamate synthase – the glutamine pathway of ammonium incorporation Directly or indirectly, glutamate and glutamine provide fixed nitrogen for almost all of the nitrogenous compounds in bacteria and plants. Glutamate provides the amino groups for all of the amino acids and for about half of the nitrogen in purines (see section 1.4.1) and pyrimidines (section 1.4.3). Glutamine provides the remaining nitrogen for purines and pyrimidines, the amide nitrogen of asparagine (section 1.3.2.5) and the heterocyclic nitrogen of histidine (section 8.1) and tryptophan (section 9.1.3).

In plants, glutamate dehydrogenase (see section 1.3.2.1) is a relatively minor route for ammonium incorporation compared with the glutamate synthase pathway (Figure 1.4). Bacteria such as *E. coli* can use either pathway, depending on the conditions. The glutamate synthase pathway is more ATP expensive and may account for 15 per cent of total ATP utilization. It is used in growth on an energy-rich substrate; glutamate dehydrogenase is used in growth on a substrate providing adequate ammonium but limited energy. In yeast, the glutamate dehydrogenase pathway is similarly preferred when there is ample glucose available, and mutations in glutamate synthase have no effect on the rate of ammonium incorporation or growth. When glucose is limiting, the availability of 2-oxoglutarate falls. This favours the glutamate synthase pathway, since glutamate synthase has a three-fold higher affinity for 2-oxoglutarate than does glutamate dehydrogenase (Magasanik, 2003).

Glutamine synthetase activity is regulated by cumulative feedback inhibition by end products including alanine, serine, glycine, AMP, carbamoyl phosphate, CTP, glucosamine 6-phosphate, histidine and tryptophan, all of which act as competitive inhibitors at either the glutamate or the ATP substrate site. Apart from alanine and serine, all of these inhibitors are products of glutamine metabolism; nucleotide synthesis accounts for 75 per cent of glutamine requirements (ignoring its role in glutamate synthase), as well as 40 per cent of glycine requirements and most of the consumption of one-carbon units

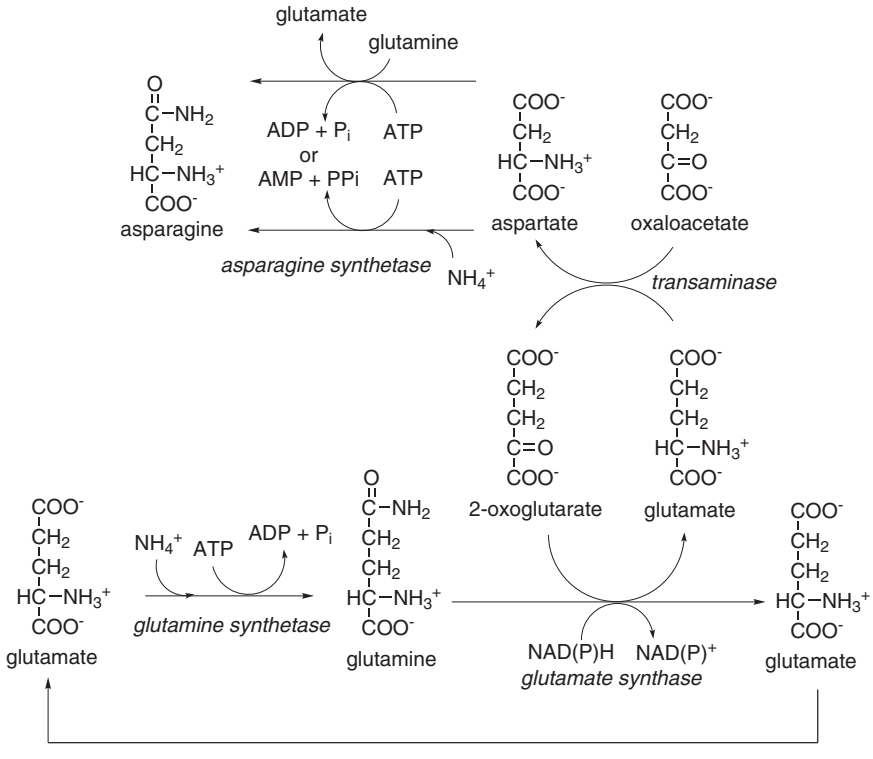

Figure 1.4 The synthesis of glutamine and asparagine.
Glutamine synthetase EC 6.3.1.2, glutamate synthase EC 1.4.1.13, glutamate-oxaloacetate transaminase EC 2.6.1.1, asparagine synthase EC 6.3.1.1 (ammonia utilising), EC 6.3.5.5 (glutamine utilizing).

derived from glycine and serine (see section 4.4). The enzyme is also regulated by adenylation; each of the 12 subunits of the enzyme can be adenylated on a tyrosine residue, which inhibits that subunit and renders the others more susceptible to feedback inhibition (Reitzer, 2003).

Glutamate synthase in root nodules of legumes and root plastids of non-leguminous plants is involved in the assimilation of ammonium from the symbiotic bacteria, the soil, or the reduction of nitrite and nitrate. In chloroplasts, its main function is re-assimilation of ammonium produced by photorespiration. The enzyme catalyzes the reaction in two stages: hydrolysis of glutamine to glutamate and ammonium, followed by reductive amination of 2-oxoglutarate to glutamate. There are two separate active sites for the two activities, connected by a 32 Å long ammonium transporting tunnel through the enzyme (Raushel *et al.*, 2003; van den Heuvel *et al.*, 2004; Vanoni & Curti, 1999).

There are three types of glutamate synthase (Suzuki & Knaff, 2005):

1 An NADPH-dependent enzyme in bacteria that contains both FAD and riboflavin phosphate, as well as three different iron-sulphur clusters. This enzyme consists of separate α- and β-chains that form a heterodimer with three active sites. NADPH binds at site 1 in the smaller β-chain and reduces riboflavin phosphate, which in turn reduces the iron-sulphur clusters and the FAD at the reductive amination site. Site 2 is the amination site, which catalyzes the formation of 2-iminoglutarate from 2-oxoglutarate and ammonium, followed by reduction to glutamate. Site 3 is the amidotransferase site, catalyzing the hydrolysis of glutamine to glutamate and ammonia. Glutamine only binds at this site when 2-oxoglutarate is bound to site 2 of the reduced enzyme.

2 A ferredoxin-dependent enzyme in cyanobacteria, algae and higher plants, which consists of a single polypeptide chain, similar to the bacterial α-chain. However, it has fewer iron-sulphur clusters and contains only riboflavin phosphate, not FAD. In green parts of the plant, this enzyme is mainly involved in salvage of the ammonium released by photorespiration, but it also functions in roots for primary incorporation of ammonium.

3 An NADH-dependent enzyme in yeasts, fungi, non-green parts of plants and some insects which consists of a single polypeptide chain that seems to have arisen as a result of fusion of the genes for the α- and β-chains of the NADPH-dependent enzyme.

Photorespiration is an apparently wasteful reaction in which two molecules of glycine react to yield one molecule of serine and one molecule each of carbon dioxide and ammonium. There is thus loss of both ammonium that has been incorporated into amino acids (at the expense of ATP and reduced coenzymes) and also carbon dioxide that has been fixed by photosynthesis (Keys, 2006; Keys *et al.*, 1978).

Photorespiration may have a valuable role in providing carbon dioxide and lowering reducing equivalents in plants that are under stress from drought or salinity, when the stomata close, so that carbon dioxide becomes limiting, and photosynthesis slows down, but photoexcitation of chlorophyll continues, and if unchecked would lead to bleaching and damage to chloroplast enzymes (Allan *et al.*, 2009).

RuBisCO catalyzes oxygenation of ribulose *bis*-phosphate to yield glycerate 3-phosphate and glycolate 2-phosphate in the chloroplast. After dephosphorylation, glycolate is exported to the peroxisome, where it is oxidized to

glyoxylate and transaminated to yield glycine. The glycine is taken up by mitochondria and cleaved by the glycine cleavage system and serine hydroxymethyltransferase (see sections 4.2.1 and 4.2.2) to yield carbon dioxide and ammonium.

The photorespiratory ammonium is re-assimilated by way of formation of glutamine, and then glutamate, aspartate and asparagine, as shown in Figure 1.4. Plant mutants that lack glutamine synthetase or glutamate synthase cannot re-incorporate this ammonium formed by photorespiration, and the mutations are lethal when the plants are grown under conditions that increase photorespiration (Lam *et al.*, 1996). The tobacco wildfire toxin produced by *Pseudomonas tabaci* causes chlorosis and leaf death in plants exposed to the bacterium (and convulsions in experimental animals). It is an inhibitor of glutamine synthetase, so that ammonium produced by photorespiration in the infected plants cannot be re-assimilated and consequently accumulates to toxic concentrations (Sinden & Durbin, 1968).

1.3.2.5 Synthesis of aspartate and asparagine As shown in Figure 1.4, aspartate is synthesized by transamination of oxaloacetate, at the expense of glutamate, and, as discussed in Chapter 6, it is the precursor for the synthesis of lysine, methionine, threonine and isoleucine in plants and microorganisms. It can also act as a nitrogen donor in a variety of reactions (e.g. Figures 1.7, 1.9, 1.17, 5.17).

Asparagine is formed from aspartate either by amidotransfer from glutamine or by incorporation of ammonium directly, the reaction of asparagine synthetase. There are two families of asparagine synthetases: those that utilize ammonium as the nitrogen donor, linked to utilization of ATP (forming AMP and pyrophosphate); and those that utilize glutamine as the nitrogen donor. In prokaryotes, these enzymes form AMP and pyrophosphate, while in eukaryotes they form ADP and phosphate.

In microorganisms that express both types of asparagine synthetase, growth on a nitrogen-limited medium leads to low expression of the ammonium-dependent enzyme and high expression of the glutamine-dependent enzyme. When high concentrations of fixed nitrogen are available, it is mainly the ammonium-dependent enzyme that is expressed. This suggests that the ammonium-dependent enzyme functions at least in part as a means of removing excess ammonium (Reitzer & Magasanik, 1982).

Like glutamate synthase (see section 1.3.2.4), the glutamine-dependent asparagine synthetases catalyze a two-step reaction, with hydrolysis of glutamine to glutamate and ammonium at one catalytic site, and ATP-dependent amination of aspartate at a separate site, connected to the first by an ammonium tunnel (Raushel *et al.*, 2003).

In most plants, asparagine is the main form in which fixed nitrogen is transported around the plant, the main form of nitrogen released in seed

germination and the main nitrogen storage compound. The activity of asparagine synthetase increases considerably in nitrogen-fixing root nodules and germinating seeds. Exposure to light leads to increased expression of glutamine synthetase and glutamate synthetase, but decreased expression of asparagine synthetase and glutamate dehydrogenase. As a result, concentrations of glutamine are higher in light-grown plants, while asparagine concentrations are higher in dark-grown plants (Lam *et al.*, 1996).

Although most prokaryotes are capable of synthesizing glutamine and asparagine, these amino acid amides are not incorporated directly into tRNA. Rather, the parent acidic amino acid (glutamate or aspartate) is attached to the appropriate tRNA, then amidated in an ATP-dependent reaction utilizing ammonium liberated from aspartate by the action of asparaginase as the nitrogen donor. In eukaryotes, the amino acid amides are incorporated into tRNA directly (Sheppard *et al.*, 2008).

In many tumours, the capacity to synthesize asparagine is limited, and recombinant microbial asparaginase has been used as part of chemotherapy of leukaemia and some other cancers since the 1960s, to lower plasma concentrations of asparagine and so inhibit tumour growth. Measurement of asparagine synthetase activity in cultured cells permits determination of those cancers that are likely to be susceptible to asparaginase therapy and those that will not respond (Lorenzi & Weinstein, 2009). An alternative approach to cancer chemotherapy, with fewer side effects, is the use of inhibitors of asparagine synthetase (Richards & Kilberg, 2006). Asparaginase also catalyzes deamidation of glutamine to glutamate, and part of its effectiveness in cancer chemotherapy may be due to depletion of glutamine, which is essential for purine and pyrimidine synthesis (see sections 1.4.1 and 1.4.3; Cory & Cory, 2006).

An alternative route of asparagine synthesis in plants is via the incorporation of cyanide into β-cyano-alanine, by displacement of the sulphydryl group of cysteine as hydrogen sulphide, followed by the action of either cyanoalanine hydratase to form asparagine, or nitrilase to yield aspartate and ammonium. Cyanoalanine synthase is a pyridoxal phosphate-dependent enzyme, catalyzing a β-elimination reaction (see section 3.4). Cysteine synthase (section 6.3.1.1) also catalyzes the synthesis of cyanoalanine, and cyanoalanine synthase catalyzes the synthesis of cysteine from acetylserine and sulphide (Figure 6.14; Warrilow & Hawkesford, 2000).

Cyanide is a metabolic by-product in biosynthesis of the plant hormone ethylene (section 6.3.4), and it is also released from cyanogenic glycosides, which are present in a large number of plants. The pathway shown in Figure 1.5 is thus primarily one for cyanide detoxication rather than assimilation of inorganic nitrogen. In insects, the activity of mitochondrial cyanoalanine synthase is correlated with tolerance to cyanide (Meyers & Ahmad, 1991; Piotrowski, 2008).

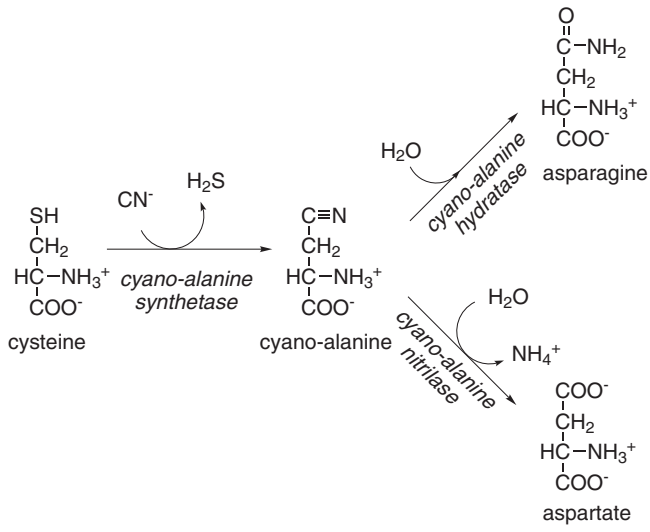

Figure 1.5 The role of cyanide in nitrogen incorporation.
Cyano-alanine synthetase EC 4.4.1.9, cyano-alanine hydratase EC 4.2.1.65, cyano-alanine nitrilase EC 3.5.5.4.

1.4 The synthesis and catabolism of purine and pyrimidine nucleotides

Apart from their role in protein synthesis, quantitatively the most important use of amino acids is in the synthesis of purine and pyrimidine nucleotides. These are required for the synthesis of DNA and RNA, as well as the purine nucleotide coenzymes (ATP, GTP and UTP) and the purine moieties of other coenzymes, including the nicotinamide nucleotide coenzymes NAD and NADP (see section 9.4.4.4), flavin coenzymes and coenzyme A. As will be discussed in section 1.6.1, synthesis of purines is the main route of excretion of surplus nitrogenous compounds in some animals. Inhibitors of purine and pyrimidine synthesis are widely used in cancer chemotherapy and as antiviral agents. The pathways of purine (Figures 1.6 and 1.7) and pyrimidine (Figure 1.11) synthesis illustrate the various ways in which the early products of incorporation of inorganic nitrogen – glutamine and aspartate – act as nitrogen donors.

The pathways of purine and pyrimidine metabolism are highly conserved, with the same steps in prokaryotes, plants and animals, although there are some differences in regulation. Also, some sequential reactions that, in prokaryotes and plants are catalyzed by separate enzymes are, in mammals, catalyzed by multifunctional proteins (Zrenner *et al.*, 2006).

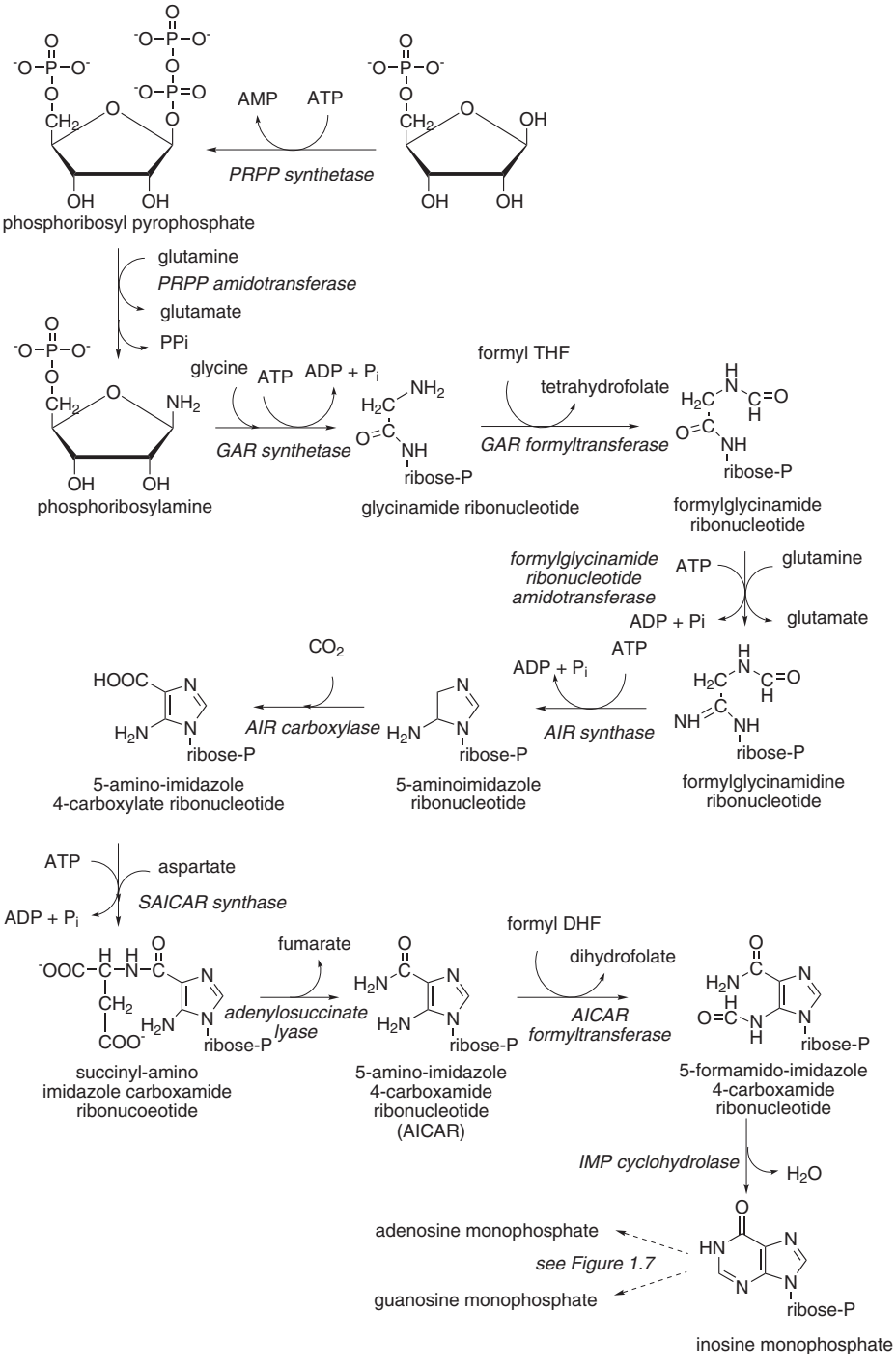
AMP
ATP
PRPP synthetase
phosphoribosyl pyrophosphate
glutamine
PRPP amidotransferase
glutamate
PPi
phosphoribosylamine
glycine
ATP
ADP + Pi
GAR synthetase
glycinamide ribonucleotide
formyl THF
tetrahydrofolate
GAR formyltransferase
formylglycinamide ribonucleotide
formylglycinamide ribonucleotide amidotransferase
ATP
glutamine
ADP + Pi
glutamate
CO2
AIR carboxylase
5-amino-imidazole 4-carboxylate ribonucleotide
5-aminoimidazole ribonucleotide
ATP
ADP + Pi
AIR synthase
formylglycinamidine ribonucleotide
ATP
aspartate
ADP + Pi
SAICAR synthase
succinyl-amino imidazole carboxamide ribonucoeotide
fumarate
adenylosuccinate lyase
5-amino-imidazole 4-carboxamide ribonucleotide (AICAR)
formyl DHF
dihydrofolate
AICAR formyltransferase
5-formamido-imidazole 4-carboxamide ribonucleotide
IMP cyclohydrolase
H2O
adenosine monophosphate
see Figure 1.7
guanosine monophosphate
inosine monophosphate

Figure 1.6 Purine synthesis.
AICAR = aminoimidazole carboxamide ribonucleotide, AIR = aminoimidazole ribonucleotide, GAR = glycinamide ribonucleotide, THF = tetrahydrofolate; phosphoribosylpyrophosphate (PRPP) synthetase EC 2.7.6.1, PRPP amidotransferase EC 2.4.2.14, glycinamide ribonucleotide (GAR) synthetase EC 6.3.4.13, GAR formyltransferase EC 2.1.2.2, formylglycinamide amidotransferase EC 6.3.5.3, aminoimidazole ribonucleotide (AIR) synthase EC 6.3.3.1, AIR carboxylase EC 4.1.1.21, N^5-carboxyaminoimidazole ribonucleotide synthetase EC 6.3.4.18, N^5-carboxyaminoimidazole ribonucleotide mutase EC 5.4.99.18, succinyl-aminoimidazole carboxamide ribonucleotide (SAICAR) synthase EC 6.3.2.6, adenylosuccinate lyase EC 4.3.2.2, aminoimidazole carboxamide ribonucleotide (AICAR) formyltransferase EC 2.1.2.3, IMP cyclohydrolase EC 3.5.4.10.

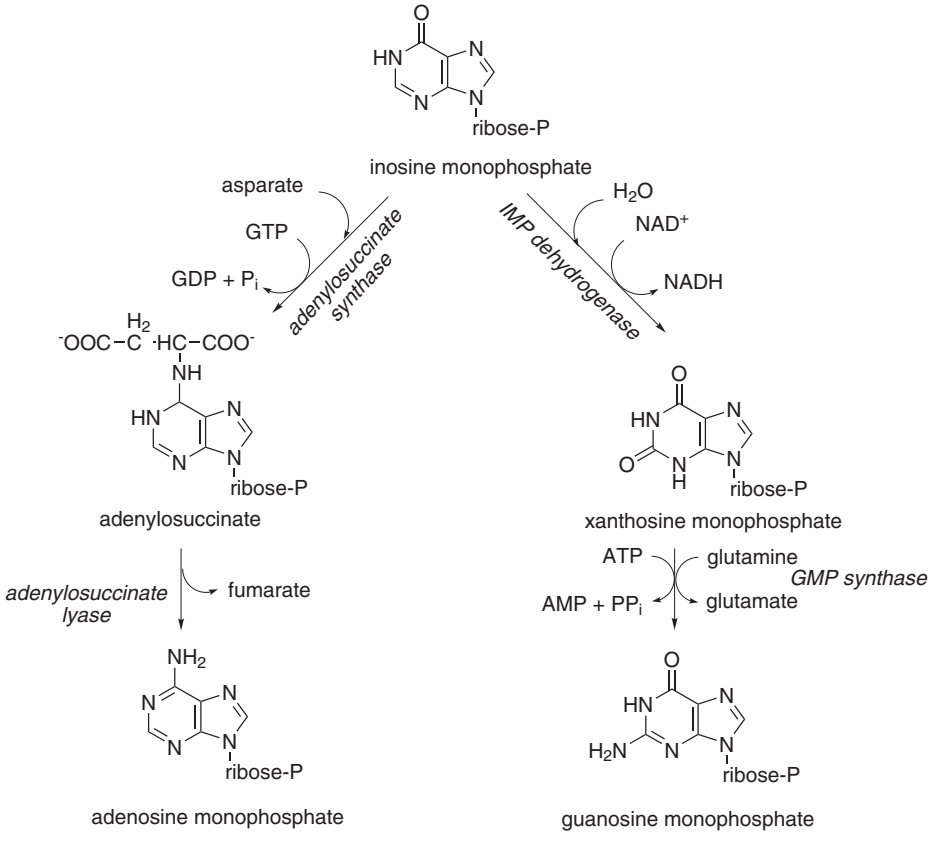

Figure 1.7 Synthesis of AMP and GMP from IMP.
Adenylosuccinate synthetase EC 6.3.4.4, adenylosuccinate lyase EC 4.3.2.2, IMP dehydrogenase EC 1.1.1.205, GMP synthase EC 6.3.4.1 (ammonia-utilizing), EC 6.3.5.2 (glutamine-utilizing).

Table 1.3 Inhibitors of nucleotide metabolism in cancer chemotherapy.

methotrexate	Folic acid antagonist; inhibits methylation of dUMP→TMP and two methylation reactions in purine synthesis.
azaserine, diaza-*nor*-leucine	Glutamine analogues, mechanism-dependent (suicide) inhibitors of glutamine-utilizing reactions.
mercaptopurine	Substrate for hypoxanthine: guanine phosphoribosyltransferase, forming a nucleotide analogue that is an pseudo-end product inhibitor of PRPP amidotransferase, adenylosuccinate synthase and IMP dehydrogenase.
fluoro-uracil	Metabolized to fluoro-dUMP and inhibits methylation of dUMP → TMP.
adenine and cytosine arabinosides	Nucleotide analogues with arabinose in place of ribose; metabolized to triphosphates and inhibit DNA polymerase.

In purine synthesis, the purine ring system is assembled stepwise attached to ribose phosphate. By contrast, a complete pyrimidine ring is synthesized before reaction with phosphoribosyl pyrophosphate to form a pyrimidine nucleotide.

Apart from purinotelic animals, which excrete purines as the main end product of nitrogen metabolism (section 1.6.1), excretion of uric acid provides an index of purine turnover. There is no similar index of pyrimidine turnover in mammals, since the end product of pyrimidine catabolism is urea – the same as the end product of amino acid catabolism (see section 1.6.2).

Knowledge of the pathways of urine and pyrimidine biosynthesis has permitted the development of a number of compounds for cancer chemotherapy (shown in Table 1.3) that will inhibit specific steps of nucleotide synthesis and so prevent the growth of rapidly dividing cells (including tumour cells).

1.4.1 Purine synthesis

Most mammalian tissues are capable of *de novo* purine synthesis, although phagocytes are reliant on salvage of purines from engulfed microorganisms. Many parasitic organisms are unable to synthesize purines, although they can interconvert them, so they are reliant on the host for a supply of purines. In tropical legumes, purines are synthesized and partially catabolized to allantoin and allantoic acid (Figure 1.8), which act as transport and storage compounds for fixed nitrogen. In the tissues away from the root nodules, allantoin and allantoic acid are catabolized to carbon dioxide and ammonium, which is re-incorporated into amino acids by way of glutamine formation (section 1.3.2.4). In some plants, methylxanthine derivatives such as caffeine and

theobromine are important in protecting leaves from attack by insects, and these are released into the soil to inhibit the germination of competing plants.

As shown in Figure 1.6, there are three steps in which glutamine acts as a nitrogen donor and one that utilizes aspartate. There are two steps in which a single carbon unit is introduced from formyl tetrahydrofolic acid (see section 4.4).

De novo synthesis of purines is regulated by the activity of the first step in the pathway, the synthesis of phosphoribosylamine from phosphoribosyl pyrophosphate and glutamine. Onward metabolism of IMP to either AMP or GMP is regulated by feedback inhibition of each of the enzymes that leads to a branch of the pathway by its end product. Adenylosuccinate synthetase is inhibited by its end product, AMP, while IMP dehydrogenase, which leads to the formation of GMP, is inhibited by GMP. A further level of integration of the synthesis of AMP and GMP is provided by the use of GTP as the phosphate donor in the reaction of adenylosuccinate synthetase (Figure 1.7). The final two steps of IMP synthesis, formyltransferase and IMP cyclohydrolase, are catalyzed by a bifunctional protein.

The reaction of adenylosuccinate synthetase involves phosphorylation of IMP to 6-phospho-IMP, followed by displacement of the phosphate group by the amino group of aspartate. There is only one adenylosuccinate synthetase in prokaryotes, but in vertebrates there are two isoenzymes with different isoelectric points, tissue distribution, kinetics and *in vivo* regulation. The acidic isoenzyme is mainly active in the *de novo* synthesis of purines; its expression is coordinated with increased purine synthesis. The basic isoenzyme is mainly concerned with ammonia generation through the purine nucleotide cycle (Figure 1.9), and its activity is increased in response to a high protein intake (Baugher *et al.*, 1980).

Adenylosuccinate lyase catalyzes two steps in purine synthesis: the conversion of succinylaminoimidazole carboxamide ribonucleotide to aminoimidazole carboxamide ribonucleotide (Figure 1.7) and of adenylosuccinate to AMP (Figure 1.8). Deficiency of the enzyme leads to the accumulation of succinylaminoimidazole carboxamide and succinyladenosine in body fluids and variable degrees of psychomotor delay, convulsions and mental retardation. The relative concentrations of these two metabolites determines the severity of the disease. Succinylaminoimidazole carboxamide appears to be harmful, while succinyladenosine may provide some degree of protection (van den Berghe *et al.*, 1997).

In prokaryotes, the enzymes of purine synthesis are monofunctional, apart from a bifunctional enzyme that catalyzes the reactions of AICAR formyltransferase and IMP cyclohydrolase. In higher eukaryotes, there are is one trifunctional enzyme and two bifunctional enzymes which form a cluster in the cytosol – a large multi-enzyme complex that has been called the purinosome (An *et al.*, 2008), consisting of:

- a trifunctional enzyme that catalyzes the reactions of glycinamide ribonucleotide synthetase, GAR formyltransferase and AIR synthase;

- a bifunctional enzyme that catalyzes the reactions of AIR carboxylase and SAICAR synthase;

- a bifunctional enzyme that catalyzes the reactions of AICAR formyltransferase and IMP cyclohydrolase, as in prokaryotes.

The reaction catalyzed by the AIR carboxylase domain of the bifunctional enzyme in higher eukaryotes requires two separate enzymes in bacteria, yeasts and fungi: N^5-carboxyaminoimidazole ribonucleotide synthetase, which introduces a carboxyl group onto N-5 of the imidazole ring, and an isomerase that transfers the carboxyl group onto C-4. It is not clear whether the AIR carboxylase domain of the bifunctional enzyme introduces the carboxyl group directly onto C-4 or acts via the intermediate formation of N^5-CAIR.

The reaction of AICAR formyltransferase is unusual in that the one-carbon unit is transferred from formyl dihydrofolate, rather than the tetrahydrofolate, as is the case with most formyltransferases (see section 4.4), including GAR formyltransferase. Formyl tetrahydrofolate is oxidized to formyl dihydrofolate by oxidized cytochrome c and, after the formyltransferase reaction, the dihydrofolate is reduced back to tetrahydrofolate by dihydrofolate reductase (Baggott & Tamura, 2010).

1.4.1.1 Phosphoribosyl pyrophosphate (PRPP) synthetase Phosphoribosyl pyrophosphate (PRPP) is the initial substrate for purine synthesis and, as discussed below, the availability of PRPP is a major regulatory factor in the rate of purine nucleotide synthesis. There are two highly homologous isoenzymes of PRPP synthetase, which form a multi-enzyme complex with two PRPP synthetase-associated proteins. They are coded for by genes on the X chromosome, and recessive genetic defects resulting in low activity impair purine (and pyrimidine) synthesis in affected males. This results in peripheral neuropathy, sensorineural hearing loss and loss of vision – the Charcot-Marie-Tooth disease first described in the late 19th century (de Brouwer *et al.*, 2007; Kim *et al.*, 2007).

By contrast, there are a number of dominant genetic conditions in which the activity of PRPP synthetase is elevated. In most cases, the problem is over-expression of the gene for PRPP synthetase I, with no effect on the kinetics of the reaction. In other cases, this is the result of either an increase in the V_{max} of the enzyme, with no difference in the values of K_m for the substrates, or sensitivity to inhibitors, or reduced sensitivity of the enzyme to feedback inhibition by ADP and GDP, which can be considered to be end products of PRPP metabolism. The excessive or uncontrolled activity of

PRPP synthetase leads to excessive synthesis (and catabolism) of purines, with elevated blood concentrations of uric acid, the early development of gout (see section 1.4.2.2) and urate renal stones. In some cases, there are also neurodevelopmental problems (Ahmed *et al.*, 1999; Becker *et al.*, 1986; 1996; Zoref *et al.*, 1975).

PRPP synthetase has an absolute requirement for inorganic phosphate for activity, presumably reflecting the sequestration of phosphate in its product, which, if phosphate were limiting in the cell, would lead to impaired phosphorylation of ADP to ATP. It is also inhibited by PRPP, although significant inhibition is only observed at concentrations of PRPP that are unlikely to be achieved under physiological conditions. The end products of pathways that utilize PRPP act as feedback inhibitors of PRPP synthetase, and 2,3-bisphosphoglycerate may also be important in controlling its activity (Becker, 2001; Wyngaarden, 1976).

PRPP synthetase I is more sensitive to feedback inhibition by ADP and GDP than is PRPP synthetase II, but the liver enzyme is less sensitive to inhibition than would appear from its subunit composition. This is because relatively high concentrations of magnesium ions overcome the inhibition by GDP almost completely and partially overcome inhibition by ADP, so that the enzyme is activated by magnesium ions. In response to growth promoters and mitogens, there is a considerable increase in the expression and activity of PRPP synthetase because of the need for increased purine synthesis (Sonoda *et al.*, 1998).

Only a small number of mammalian enzymes utilize PRPP (see Table 1.4), and impaired activity of any of them will lead to an increase in the intracellular concentration of PRPP and, hence, increased *de novo* purine synthesis.

Table 1.4 Mammalian enzymes that utilize phosphoribosyl pyrophosphate.

Enzyme	EC number	Pathway
adenine phosphoribosyltransferase	2.4.2.7	purine salvage, Figure 1.10
hypoxanthine guanine phosphoribosyltransferase	2.4.2.8	purine salvage, Figure 1.10
nicotinamide phosphoribosyltransferase	2.4.2.12	NAD(P) synthesis, Figure 9.17
nicotinic acid phosphoribosyltransferase	2.4.2.11	NAD(P) synthesis, Figure 9.17
orotic acid phosphoribosyltransferase	2.4.2.2	pyrimidine synthesis, Figure 1.11
PRPP amidotransferase	2.4.2.14	*de novo* purine synthesis, Figure 1.6
quinolinic acid phosphoribosyltransferase	2.4.2.19	NAD(P) synthesis from tryptophan, Figure 9.17

1.4.1.2 PRPP amidotransferase The synthesis of phosphoribosylamine from PRPP is the first committed step of purine synthesis, and the main regulatory step. It is also the first step for synthesis of the pyrimidine ring of thiamin (vitamin B_1). Bacteria lacking PRPP amidotransferase are still capable of synthesizing thiamin, but they are reliant on an exogenous source of purines, suggesting that the alternative pathway for phosphoribosylamine synthesis from ribose 5-phosphate can meet the need for synthesis of thiamin but not purines, which are required in considerably larger amounts (Koenigsknecht *et al.*, 2007). The activity of human PRPP amidotransferase is exquisitely sensitive to the intracellular concentration of PRPP, having an apparent K_m of 140 µmol/l, compared with a normal intracellular concentration of 2–30 µmol/l.

PRPP amidotransferase catalyzes a two-stage reaction, with two separate catalytic sites. Glutamine is hydrolyzed at one site and the ammonia is channelled to the second site, where it reacts with PRPP. Unlike carbamoyl phosphate synthetase (section 1.4.3) and tryptophan synthase (section 9.1.3), the channel is not a permanent feature of the enzyme, but is only formed in response to glutamine and PRPP binding at their active sites. The channel is hydrophobic, so it is likely that what is transferred between the active sites is ammonia rather than ammonium. Again, this is unlike carbamoyl phosphate synthetase, where ammonium is transferred through a hydrophilic channel (Smith, 1998).

When PRPP is bound to the enzyme, the K_m for glutamine is 1.6 mmol/l – considerably below the usual intracellular concentration of glutamine (4–7 mmol/l). However, in the absence of PRPP at the active site, the K_m for glutamine is some 200-fold higher, so that glutamine is only hydrolyzed when PRPP is available to accept ammonia. In the ligand-free enzyme, the glutamine site is closed so that glutamine cannot bind. Binding of PRPP leads to a conformational change that opens the glutamine binding site and both lowers the K_m for glutamine 100-fold and increases the catalytic efficiency three-fold. It also opens the hydrophobic ammonia tunnel between the two active sites (Bera *et al.*, 2000; Smith, 1998).

PRPP amidotransferase is inhibited by the end products of purine synthesis, AMP and GMP. When either is present, the substrate/velocity curve becomes significantly sigmoid (a Hill coefficient of 2.7, compared with 1.1 in the absence of purine nucleotides), and the apparent K_m for PRPP increases to 480 µmol/l. There are two nucleotide binding sites: one overlaps the region that binds the ribose phosphate moiety of PRPP and the other the pyrophosphate binding site. In bacteria, the enzyme is inhibited synergistically by ADP and GMP when both are bound. Binding of GMP increases the affinity for ADP some 20-fold (Chen *et al.*, 1997).

Increasing concentrations of PRPP can overcome the inhibition of the mammalian enzyme caused by AMP and GMP. There are two forms of the

enzyme, with molecular masses of 133 and 270 kDa. AMP and GMP act to convert the enzyme to the larger, less active form, while PRPP acts to convert it back to the smaller, more active, form. The normal intracellular concentrations of AMP and GMP are close to those that give half-maximal inhibition of the enzyme (Holmes *et al.*, 1973a, 1973b). Interestingly, in birds, the PRPP amidotransferase dimer is stabilized by PRPP and has maximum activity; in the presence of AMP and GMP, this dissociates to a smaller, less active monomer (Wyngaarden, 1976). This presumably reflects the difference between the regulation of purine biosynthesis in mammals and birds; birds are uricotelic, and their major excretory product of nitrogen metabolism is uric acid (see section 1.6.1).

1.4.2 Purine catabolism and salvage

IMP accumulating in excess of needs for the synthesis of AMP or GMP (i.e. when both adenylosuccinate synthetase and IMP dehydrogenase are inhibited by their end products) is rapidly dephosphorylated to inosine by phosphomonoesterase, followed by the action of purine nucleotide phosphorylase to yield hypoxanthine. GMP in excess of requirements is also a substrate for phosphomonoesterase and purine nucleotide phosphorylase, yielding guanine, which is deaminated to xanthine. AMP in excess of requirements is a substrate for AMP deaminase, yielding IMP, or may be a substrate for phosphomonoesterase, liberating adenosine, which is deaminated by adenosine deaminase.

The muscle isoenzyme of AMP deaminase is important in exercise to prevent accumulation of ADP and disturbance of the ATP : ADP ratio. As ADP begins to accumulate, it is the substrate for adenylate kinase, catalyzing the reversible reaction of $2 \times \text{ADP} \rightleftharpoons \text{ATP} + 5'\text{AMP}$. Although 5′AMP acts as an important metabolic signal of the energy state of the cell, it is removed by AMP deaminase to prevent the back reaction to ADP. IMP accumulates in fast-twitch muscle during exercise, then falls as it is converted back to AMP, then ATP. Deficiency of muscle AMP deaminase is a relatively common cause of exercise-induced myopathy; 1–2 per cent of all muscle biopsy samples sent for pathological investigation show deficiency of the enzyme (Hancock *et al.*, 2006).

As shown in Figure 1.8, hypoxanthine is oxidized to xanthine, then uric acid, by xanthine oxidase, which also has general aldehyde oxidase activity. It is a molybdenum-containing flavoprotein with two iron-sulphur centres. The reaction involves reduction of Mo^{VI} to Mo^{IV} by transfer of electrons from the substrate, followed by electron transfer to the flavin via the iron-sulphur centres and reduction of oxygen to hydrogen peroxide. Xanthine oxidase can be converted to xanthine dehydrogenase, which reduces NAD^+ rather than

oxygen, by formation of disulphide bridges catalyzed by glutathione-dependent thiol disulphide oxidoreductase. Xanthine dehydrogenase can be converted back to the oxidase by reduction of the disulphide bridges. The dehydrogenase is also irreversibly converted to the oxidase by partial proteolysis.

When lysosomes are disrupted during tissue homogenization, almost all of the enzyme is recovered as the oxidase, with little or no dehydrogenase activity detectable. The dehydrogenase form of the enzyme reacts rapidly with NAD^+ and only slowly with oxygen, while the oxidase form reacts rapidly with oxygen but only slowly with NAD^+. The flavin radical is stabilized in the dehydrogenase form of the enzyme, and when the flavin radical form of the enzyme reacts with oxygen rather than NAD^+, the result is formation of superoxide. Hydrogen peroxide is formed when the fully reduced flavin form of the enzyme reacts with oxygen (Nishino *et al.*, 2008; Rajagopalan, 1988a, 1988b).

In most mammals, uric acid is further oxidized to allantoin, then hydrolyzed to allantoic acid. However, human beings and other primates lack uric acid oxidase, and they excrete uric acid as the end product of purine catabolism. See section 1.4.2.2 for a discussion of uric acid and gout.

Plants store nitrogen rather than eliminating it, and allantoin and allantoic acid are important nitrogen storage compounds in tropical legumes. Allantoic acid undergoes onward metabolism to carbon dioxide and ammonium, which can be reincorporated into amino acids as discussed in section 1.3.2. Xanthine is important in many plants as the precursor for the methylxanthines caffeine and theobromine, which are secondary metabolites involved in protecting leaves against insect predators and which, when diffusing from seeds, prevent the germination of seeds of competing species (Zrenner *et al.*, 2006).

There is an obvious need to maintain an appropriate balance between the intracellular concentrations of adenine and guanine nucleotides – and also, because of their role in metabolic regulation, to maintain appropriate concentrations of both AMP and GMP as well as adenosine, which has a major role in cell signalling. In order to achieve this, there is continual catabolism of AMP and GMP. Hypoxanthine is salvaged by the action of hypoxanthine: guanine phosphoribosyltransferase (HGPRT), and the resultant IMP can be converted back to AMP, as shown in Figures 1.7 and 1.9. Guanine is also a substrate for HGPRT, forming GMP. In both cases, the reaction uses phosphoribosyl pyrophosphate as the donor of ribose phosphate. The purine nucleotide cycle shown in Figure 1.9 is a significant source of ammonium for urea synthesis (see section 1.6.2.1).

The *de novo* synthesis of purines is suppressed by purine salvage because the resultant nucleotides inhibit PRPP amidotransferase. Conversely, purine salvage is suppressed by *de novo* synthesis as a result of depletion of intracellular pools of PRPP (Yamaoka *et al.*, 2001).

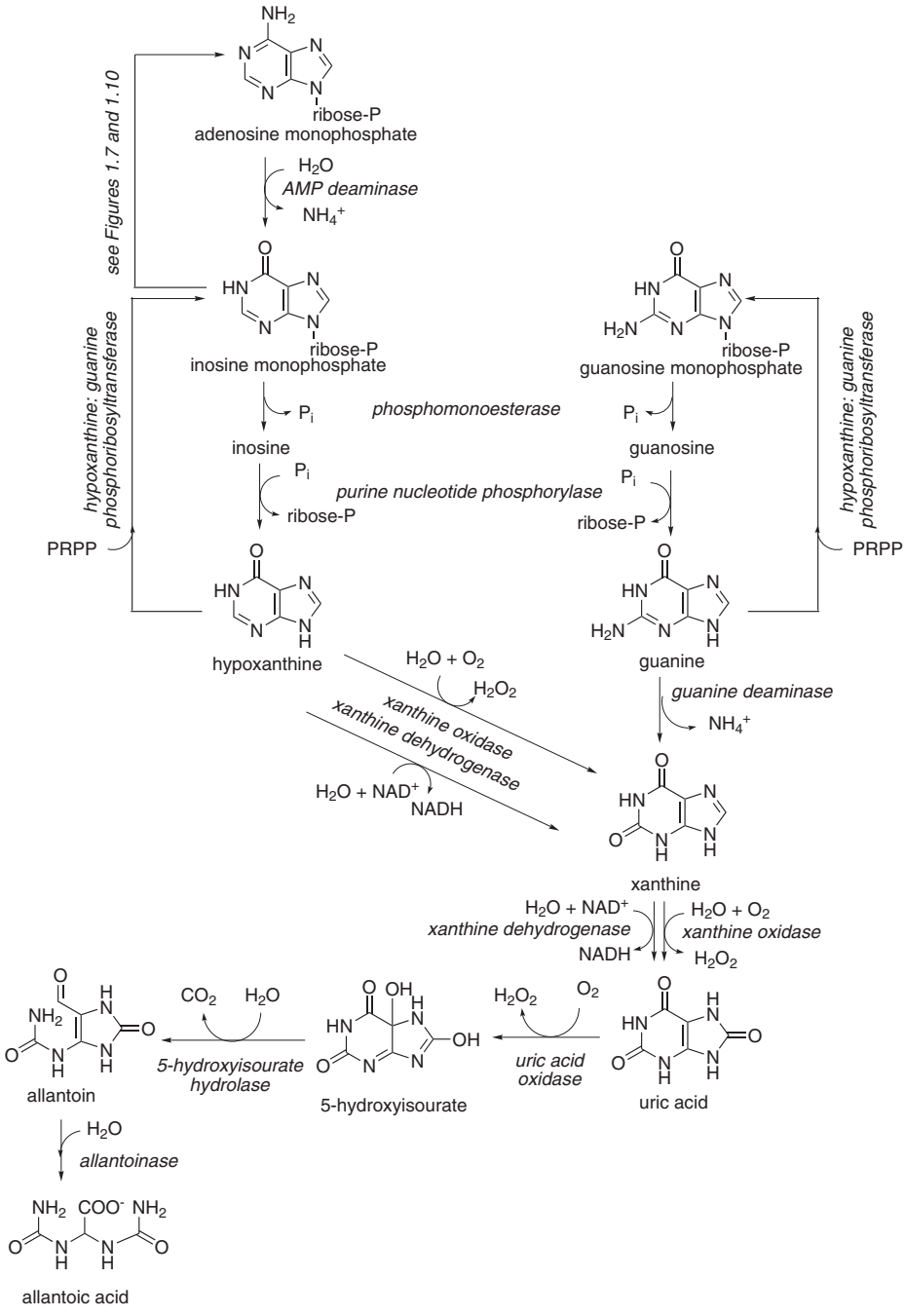

Figure 1.8 Purine catabolism.
AMP deaminase EC 3.5.4.6, xanthine dehydrogenase EC 1.17.1.4, xanthine oxidase EC 1.17.3.2, uric acid oxidase EC 1.7.3.3, 5-hydroxyisourate hydrolase EC 3.5.2.17, allantoinase EC 3.5.2.5.

The reaction of AMP deaminase provides an alternative route for deamination of a wide variety of amino acids and the generation of ammonium for synthesis of urea (see section 1.6.2.1). As shown in Figure 1.9, the fumarate liberated from adenylosuccinate in the reaction catalyzed by adenylosuccinase can be metabolized to oxaloacetate, which acts as amino acceptor for a wide variety of transaminases, forming aspartate that can then be used for re-synthesis of AMP from IMP. This is an energy-efficient pathway for deamination; there is a cost of 1 mol of GTP for the synthesis of adenylosuccinate, but a yield of ≈2.5 mol of ATP from re-oxidation of the NADH formed in the reaction of malate dehydrogenase. By contrast, the formation of ammonium from glutamine involves a cost of 1 mol of ATP for glutamine synthesis, with no gain in ATP in the reaction of glutaminase (see Figure 1.2).

1.4.2.1 Adenosine deaminase deficiency – severe combined immune deficiency Lack of adenosine deaminase is rare, but it results in more or less complete loss of cell-mediated immunity – severe combined immune deficiency. A variety of different mutations have been identified in the small number of patients who lack the enzyme, including point mutations, premature stop codons, RNA splicing errors and deletion mutations. In some cases, there is a higher than normal concentration of adenosine deaminase mRNA in cells, but the enzyme is unstable, so that there is little or no active enzyme. In all cases where the activity of the enzyme is less than 5 per cent of normal, the result is more or less complete loss of B- and T-lymphocyte activity, and hence severe combined immune deficiency. In most cases, the condition develops in early infancy, leading to early death if treatment is not initiated. However, in 10–15 per cent of cases, the condition develops 6–24 months after birth, and in a small number of cases the condition does not develop until four years of age or older. Partial lack of adenosine deaminase usually results in normal immune cell function, but in some cases it may be associated with late-onset immunodeficiency.

Lymphocytes are mainly reliant on purine salvage from engulfed microorganisms rather than *de novo* purine synthesis, so that lack of adenosine deaminase results in reduced availability of IMP, and hence GMP. More importantly, in the absence of adenosine deaminase, dAMP from catabolism of the DNA of engulfed microorganisms accumulates and is cytotoxic. dAMP is a substrate for phosphorylation to dATP, which inhibits both ribonucleotide reductase and also synthesis of *S*-adenosylmethionine (section 6.3.2). As a result of the inhibition of ribonucleotide reductase, there is failure to synthesize the other deoxynucleotides needed for DNA synthesis. As a result of the inhibition of *S*-adenosylmethionine synthesis, there is a failure of many methyl transfer reactions (Cohen *et al.*, 1978; Mitchell *et al.*, 1978).

Adenosine deaminase deficiency was one of the first genetic diseases to be treated by gene therapy, by inserting the gene into stem cells from the patient's

own bone marrow *in vitro*, and then transplanting them back into the patient. In a small number of cases, leukaemia developed as a result of the activation of oncogenes in the process of inserting the adenosine deaminase gene, but more than 30 patients worldwide have been treated, with a successful outcome in most cases (Ferrua *et al.*, 2010; Silver & Flotte, 2008).

1.4.2.2 Gout and hyperuricaemia Gout is a painful inflammatory condition caused by crystallization of uric acid salts in joints, as nodules under the skin, and sometimes in the kidney, leading to kidney failure, as a result of a blood concentration that is above the low solubility product of uric acid and its salts. Human beings and other primates which lack uricase (Figure 1.9) normally maintain a plasma concentration close to its solubility limit. A relatively modest increase in uric acid synthesis, or reduction in its excretion, can

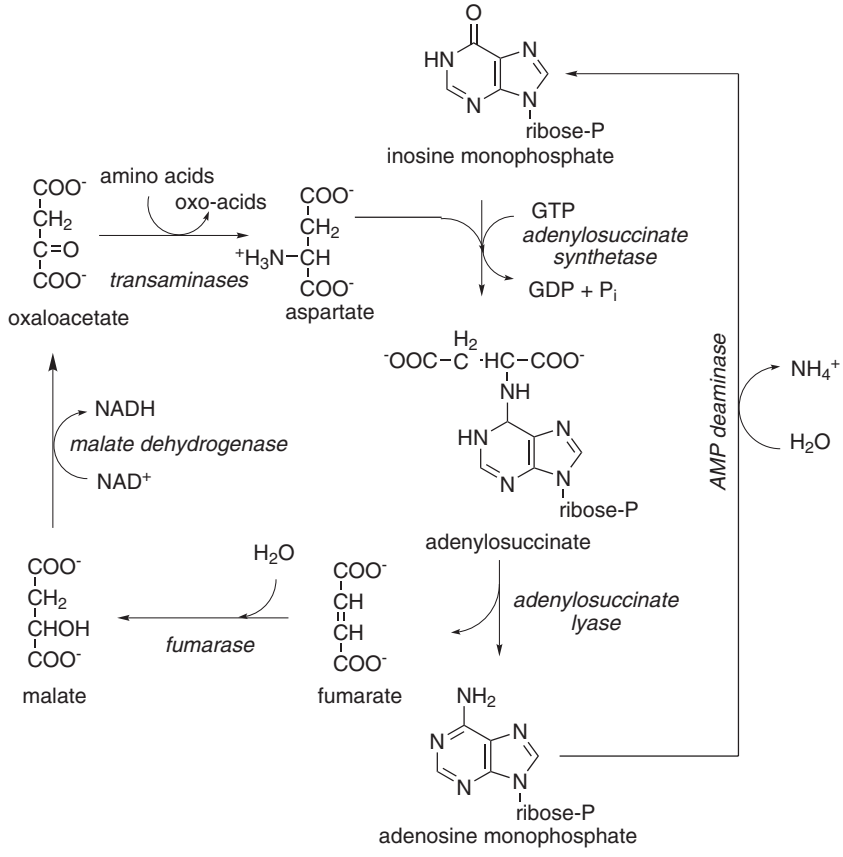

Figure 1.9 AMP deaminase as a source of ammonia.
Adenylosuccinate synthetase EC 6.3.4.4, adenylosuccinate lyase EC 4.3.2.2, fumarase EC 4.2.1.2, malate dehydrogenase EC 1.1.1.37, AMP deaminase EC 3.5.4.6.

result in crystallization of uric acid salts in joints and elsewhere. The underlying cause of gout may be either impaired urinary excretion or increased synthesis and catabolism of purines.

Uric acid is handled in the kidney in an unusual way for what appears to be a metabolically useless end product. It is completely filtered in the glomerulus, but is then more or less completely reabsorbed in the proximal renal tubule and is actively secreted in the distal renal tubule. This, as well as the loss of uricase in primate evolution, suggests that there may be selective advantage in maintaining a high plasma concentration of uric acid. It acts as an antioxidant, forming allantoin non-enzymically by reaction with reactive oxygen species, and it also stimulates the innate immune system. It has been suggested that loss of uricase in primate evolution led to stimulation by uric acid of the foraging response to starvation – a distinct selective advantage (Alvarez-Lario & Macarron-Vicente, 2010; Johnson *et al.*, 2009).

The active secretion of uric acid in the distal renal tubule is inhibited by lactic and other acids. Conditions that are associated with persistent lactic acidosis (such as some types of glycogen storage disease) lead to impaired excretion of uric acid and the early development of gout. Premenopausally, women are less at risk of gout than are men, although this gender difference is lost after the menopause. There is evidence that oestrogens lower serum uric acid and, while some studies show increased uric acid excretion in response to oestrogen administration, others do not.

Increased purine synthesis may result from a genetic defect of PRPP amidotransferase that results in reduced sensitivity to inhibition by AMP and GMP (see section 1.4.1.2) or a partial defect in HGPRT (Figure 1.10), which results in less utilization of PRPP and, hence, an increased concentration which overcomes the inhibition of PRPP amidotransferase by its end products. As noted in section 1.4.1.2, genetic defects that result in increased activity of PRPP synthetase also lead to increased purine synthesis and catabolism, and hence may be a cause of gout.

Large amounts of fructose can increase uric acid production because the phosphorylation of fructose to fructose 1-phosphate is unregulated, leading to depletion of intracellular inorganic phosphate, accumulation of ADP and AMP and increased catabolism via adenosine deaminase. It is not clear whether normal, more modest, intakes of fructose may also be a factor in the development of hyperuricaemia and gout (Henry *et al.*, 1991).

Patients with gout have elevated levels of xanthine oxidase, but it is not clear whether this is a cause of the condition or a result of increased concentrations of xanthine and hypoxanthine. However, the usual treatment for gout, whatever the underlying cause, is administration of allopurinol and more modern inhibitors of xanthine oxidase. This is effective because xanthine is more soluble than uric acid, so allowing accumulation of a higher concentration of an end product of purine catabolism without the risk of

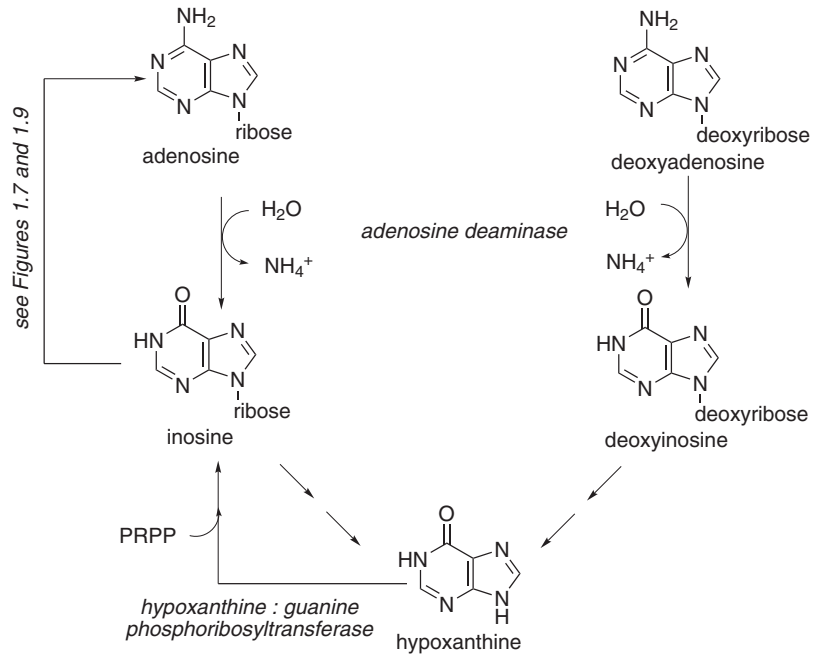

Figure 1.10 Purine salvage.
Adenosine deaminase EC 3.5.4.4, hypoxanthine : guanine phosphoribosyltransferase EC 2.4.2.8.

crystallization. Alterative or additional treatments include sulfinpyrazone to increase the urinary excretion of uric acid.

1.4.2.3 HGPRT deficiency – the Lesch-Nyhan syndrome More or less complete deficiency of HGPRT (less than 1.5 per cent of control activity) leads to the Lesch-Nyhan syndrome, an X-linked recessive genetic disease. Affected (male) children develop hyperuricaemia and gout from an early age, because of increased *de novo* purine synthesis as a result of increased availability of PRPP. In addition, they suffer from delayed motor development, severe spasticity and choreic, athetoid or dystonic movement disorders and compulsive self-mutilation.

Less severe deficiency of the enzyme (at least 8 per cent of control activity) leads to the Kelley-Seegmiller syndrome. Affected children develop hyperuricaemia and gout from an early age, but do not show the neurological signs associated with Lesch-Nyhan syndrome. Patients with residual HGPRT activity between 1.5–8 per cent of control show varying neurological signs, with the severity inversely related to the activity of the enzyme. The development of gout and hyperuricaemia, but not the neurological signs of the disease, can be controlled by administration of allopurinol to inhibit xanthine oxidase (Torres & Puig, 2007).

Part of the basis of the neurological problems in Lesch-Nyhan syndrome is lack of GTP for the synthesis of tetrahydrobiopterin, the cofactor for hydroxylation of the aromatic amino acids to form the catecholamines and serotonin (see section 9.2.3). Patients with the malignant (unresponsive) variant of phenylketonuria (section 9.2.3), who cannot synthesize tetrahydrobiopterin, show many of the same neurological defects as those with Lesch-Nyhan syndrome, but not the compulsive self-mutilation.

Provision of 5-hydroxytryptophan as a precursor for serotonin synthesis (section 9.4.3) has a temporary beneficial effect in Lesch-Nyhan syndrome, although there is a relapse after about three weeks. By contrast, the administration of dihydroxyphenylalanine as a precursor for catecholamine synthesis (section 9.2.4) leads to a dramatic worsening of the condition. A number of studies have shown that patients with Lesch-Nyhan syndrome have very few dopaminergic nerve terminals and cell bodies, in all regions of the central nervous system.

Treatment of neonatal rats with the dopaminergic neurotoxin 6-hydroxydopamine leads to self-mutilating behaviour similar to that seen in Lesch-Nyhan syndrome, when they are challenged with dihydroxyphenylalanine as adults, suggesting hyper-sensitivity of remaining dopamine receptors (Breese *et al.*, 1990; Ernst *et al.*, 1996).

1.4.3 Pyrimidine synthesis

As shown in Figure 1.11, the first reaction of pyrimidine synthesis is formation of carbamoyl phosphate from carbon dioxide and ammonium. In mammals, there are two isoenzymes of carbamoyl phosphate synthetase: a cytosolic enzyme that is involved in pyrimidine synthesis, and a mitochondrial enzyme (in liver and kidney) that is involved in the synthesis of urea in the liver and arginine in the kidney (sections 1.6.2.1 and 5.9.1). As might be expected, the cytoplasmic carbamoyl phosphate synthetase is inhibited by pyrimidine nucleotides, which increase the K_m for ATP. It is also activated by PRPP, which reduces the K_m for ATP. This means that, as well as being inhibited by the end product of the pathway, carbamoyl phosphate synthetase is only significantly active when there is an adequate amount of PRPP available for utilization of the orotic acid that is the immediate precursor of UMP. The activity of cytosolic carbamoyl phosphate synthetase is upregulated by phosphorylation at two distinct sites in response to signals for cell proliferation. Phosphorylation by MAP kinase leads to increased efficacy of PRPP as an activator and decreased inhibition by UTP. Phosphorylation by protein kinase A abolishes inhibition by UTP, but also reduces activation by PRPP (Huang & Graves, 2003; Jones, 1980).

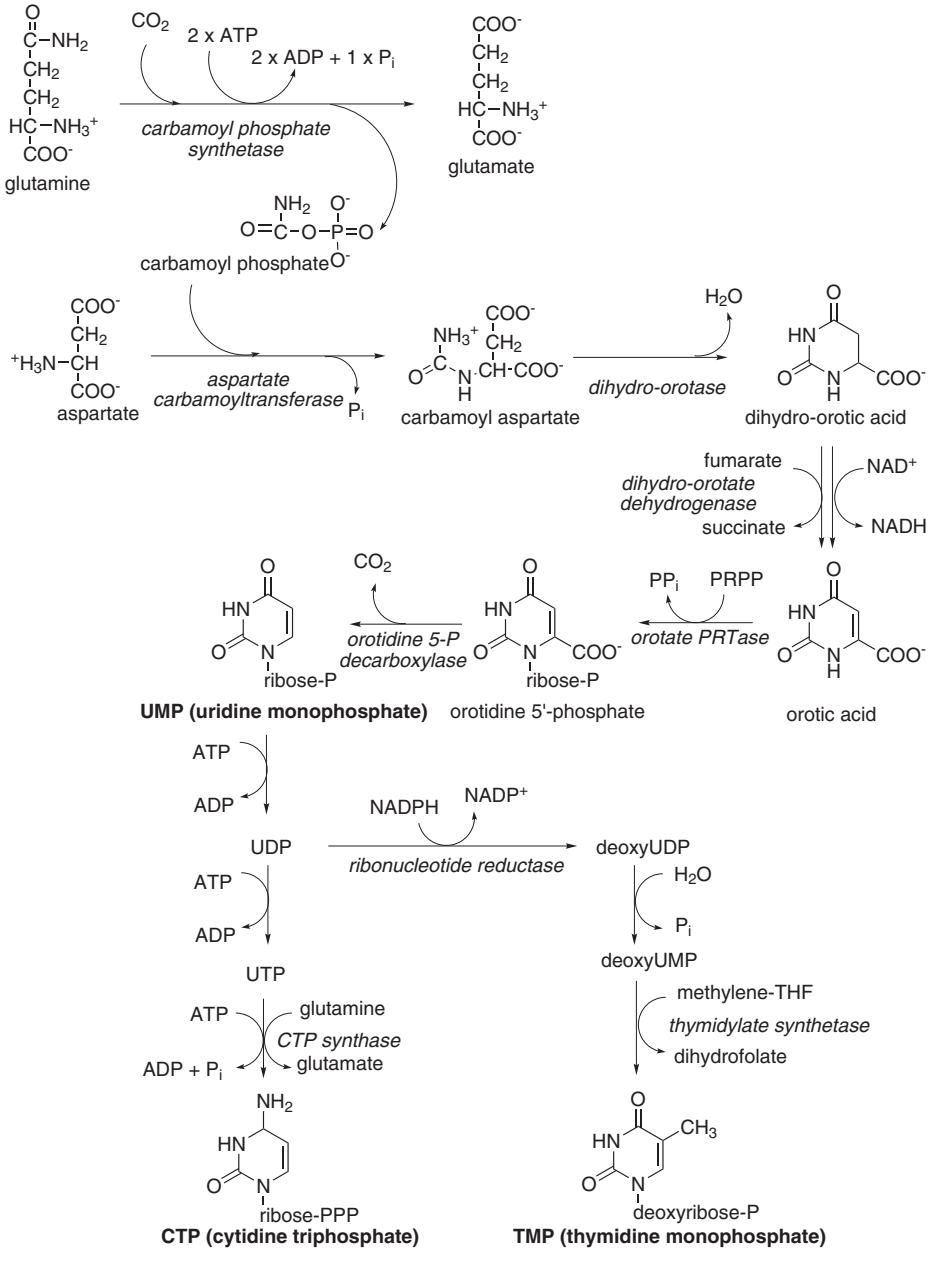

Figure 1.11 Pyrimidine synthesis.
Carbamoyl phosphate synthetase EC 6.3.5.5, aspartate carbamoyltransferase EC 2.1.3.2, dihydro-orotase EC 3.5.2.3, dihydro-orotate dehydrogenase EC 1.3.98.1 (fumarate-linked), EC 1.3.1.14 (DAD-linked), EC 1.3.1.15 (NADP-linked), orotidine 5′-phosphate decarboxylase EC 4.1.1.23, CTP synthase EC 6.3.4.2, ribonucleotide reductase EC 1.17.4.1, thymidylate synthetase EC 2.1.1.45.

The reaction of cytosolic carbamoyl phosphate synthetase involves three catalytic sites and two intramolecular tunnels. Site 1 catalyzes the hydrolysis of glutamine to yield ammonium, which is channelled through a hydrophilic tunnel to site 2. Site 2 catalyzes the phosphorylation of carbon dioxide to carboxyphosphate, which reacts with ammonium to form carbamate. The carbamate is then channelled through the second tunnel to site 3, where it is phosphorylated to yield carbamoyl phosphate. The enzyme is a hetero-dimer, with site 1 (glutaminase) in the smaller subunit, and the two ATP utilizing sites (2 and 3) in the larger subunit. The formation of carboxyphosphate at site 2 triggers a conformational change in the protein that is transmitted to the smaller subunit, activating the glutaminase site. This means that glutamine is not hydrolyzed until there is carboxyphosphate available to undergo the next step in the reaction sequence (Huang *et al.*, 2001; Rubio, 1993).

In plants and prokaryotes, there is only a single isoenzyme of carbamoyl phosphate synthetase for the synthesis of both pyrimidines and arginine. As in animals, the enzyme utilizes glutamine and has two separate active sites, one of which hydrolyses glutamine to yield ammonium, while the other utilizes the ammonium for synthesis of carbamoyl phosphate. Like the enzyme in animals, carbamoyl phosphate synthetase in plants and prokaryotes is inhibited by UMP, but this inhibition is overcome by ornithine, so allowing arginine synthesis independently of the requirement for pyrimidine synthesis. Plants synthesize relatively large amounts of uridine nucleotides because of the role of UDP-glucose in the synthesis of sucrose and starches. For example, when potato tubers are detached from the plant and are no longer synthesizing starch, the synthesis of uridine nucleotides falls (Zrenner *et al.*, 2006).

In prokaryotes, each step of pyrimidine synthesis is catalyzed by a separate enzyme. In yeasts and fungi, a bifunctional enzyme catalyzes the reactions of carbamoyl phosphate synthetase and aspartate carbamoyltransferase. The yeast enzyme is inhibited by UTP, which binds to a regulatory site near the carbamoyl phosphate synthase site (the third step of the reaction) and a conformational change reduces the affinity for ATP at that site and for aspartate at the carbamyltransferase site (Serre *et al.*, 2004). In plants, the first three steps of the pathway are catalyzed by separate enzymes, as in prokaryotes.

In many bacteria, regulation of the pyrimidine synthesis operon does not involve DNA-binding repressor or activator proteins, as is the case for the control of most bacterial operons. The operon is regulated by the availability of pyrimidine nucleotides, sensed directly by RNA polymerase. During transcription of leader regions upstream of each gene in the operon, alternative structures in the RNA determine whether the full gene will be transcribed or, in the presence of adequate amounts of pyrimidines, whether there will be premature termination of transcription. Depending on the availability of ATP and CTP, the leader region may either undergo a conformational change, leading to the termination of transcription, or form an anti-termination loop

that allows transcription to continue. Alternative pyrimidine-dependent structures of the full-length transcript determine the efficiency of translation of the mRNA (Turnbough & Switzer, 2008).

In animals, there are only three genes associated with the synthesis of UMP from glutamine, which encode:

- the CAD multi-enzyme protein, which catalyzes the reactions of carbamoyl phosphate synthetase, aspartate carbamoyltransferase and dihydro-orotase;
- dihydro-orotate dehydrogenase;
- a bifunctional enzyme, UMP synthetase, which catalyzes the synthesis of orotidine 5-phosphate from orotidine and PRPP, followed by decarboxylation to UMP.

The carbamoyl phosphate synthetase domain of the CAD protein is inhibited by UTP and allosterically activated by PRPP, which is the substrate for a later reaction not catalyzed by the CAD protein. It is also phosphorylated by MAP kinase and protein kinase A. Phosphorylation has no effect on the catalytic activity of the protein, but affects its sensitivity to allosteric regulation. This permits synchronization of pyrimidine synthesis with the cell cycle in response to different receptor signalling pathways. Phosphorylation by either kinase abolishes feedback inhibition by UTP. Phosphorylation by protein kinase A on serine^{1046} decreases sensitivity to activation by PRPP, while the result of MAP kinase phosphorylation on threonine^{456} increases the sensitivity of the enzyme to PRPP.

The two kinases are mutually antagonistic, so that the enzyme is phosphorylated by one or the other but not both, either because phosphorylation by one leads to a conformational change that prevents phosphorylation by the other, or because both kinases can form stable complexes with the CAD protein and binding of one prevents the binding of the other. The CAD protein is also subject to autophosphorylation of sites other than those phosphorylated by MAP kinase and protein kinase A. This leads to increased sensitivity to feedback inhibition by UTP and decreased activation by PRPP (Sigoillot *et al.*, 2002a, 2002b, 2003).

The CAD protein and UMP synthetase are cytosolic enzymes (CAD also occurs in the nucleus), while dihydro-orotate dehydrogenase is a mitochondrial enzyme, associated with the outer face of the inner mitochondrial membrane. Dihydro-orotic acid crosses the outer mitochondrial membrane and is reduced at the expense of an enzyme-bound flavin, which is re-oxidized by reduction of ubiquinone. Hence, like the reaction of succinate dehydrogenase

in the citric acid cycle, it directly feeds into the mitochondrial electron transport chain. Orotic acid crosses out of the mitochondrion as the substrate for UMP synthetase. Apart from dihydro-orotic acid and orotic acid, none of the other intermediates of the pathway occurs in free solution – all are channelled from one active site of the appropriate multi-functional enzyme to the next (Jones, 1980).

UMP is phosphorylated to UDP and then to UTP. CTP is formed from UTP in a glutamine-dependent amidotransferase reaction. UDP is also a substrate for ribonucleotide reductase to yield deoxy-UDP, which is dephosphorylated to deoxy-UMP, then methylated to TMP in a methylene-tetrahydrofolate-dependent reaction. This means that in folic acid deficiency, there is impaired synthesis of thymidine nucleotides and, hence, impaired synthesis of DNA. Together with failure of purine synthesis because of the two folic acid-dependent steps in the purine biosynthetic pathway (see section 1.4.1), this explains the development of megaloblastic anaemia in folic acid deficiency and the efficacy of folic acid antimetabolites in cancer chemotherapy (Table 1.3).

The methylene tetrahydrofolate-dependent reaction of thymidylate synthetase is interesting, in that it is an example of a folic acid-dependent reaction in which the methylene group is reduced to a methyl group at the expense of tetrahydrofolate being oxidized to dihydrofolate, which is reduced back to tetrahydrofolate by dihydrofolate reductase. The anti-cancer drug methotrexate acts mainly as an inhibitor of dihydrofolate reductase, although it also inhibits the conjugation of folic acid with glutamate. The antibacterial agent trimethoprim also inhibits dihydrofolate reductase. It has a considerably higher affinity for the bacterial enzyme than for the mammalian enzyme, so that it inhibits TMP synthesis in bacteria at doses that have little effect on the ability of human cells to synthesize TMP (Bertino, 2009; Gangjee & Jain, 2004; McGuire, 2003).

1.4.3.1 Orotic aciduria Genetic deficiency of UMP synthetase leads to orotic aciduria, which is characterized by urinary excretion of orotic acid and megaloblastic anaemia that is unresponsive to folate or vitamin B_{12}, as well as circulating microcytic, hypochromic red blood cells. The anaemia is presumably due to failure of erythroblast maturation as a result of a lack of pyrimidine nucleotides, and it responds to administration of uridine. This also reduces the urinary excretion of orotic acid, because of inhibition of carbamoyl phosphate synthetase by pyrimidine nucleotides.

Orotic aciduria also occurs as a result of genetic lack of mitochondrial ornithine carbamoyltransferase, a key enzyme in urea synthesis (see section 1.6.2.1). Although the intermediates of the reactions catalyzed by the CAD protein do not enter into free solution, carbamoyl phosphate synthesized in

the mitochondria that cannot be used for ornithine synthesis is exported to the cytosol, where it binds to the aspartate carbamoyltransferase active site, leading to increased synthesis of orotic acid and the excretion of orotic acid as an end product of nitrogen metabolism. Patients lacking ornithine carbamoyltransferase suffer potentially fatal hyperammonaemia, especially after consuming moderate amounts of protein. However, their synthesis of pyrimidine nucleotides is unaffected and they do not develop the anaemia associated with UMP synthetase deficiency.

Orotic acid is a normal constituent of bovine milk. Heterozygosity for deficiency of UMP synthetase is common in Holstein-Friesian cattle, leading to orotic aciduria and orotic acidaemia during lactation, as well as abnormally high concentrations of orotic acid in the milk. The heterozygotes are apparently unaffected by the condition, but homozygous calves are either still-born or die shortly after birth (Harden & Robinson, 1987a, 1987b).

1.4.4 Pyrimidine catabolism and salvage

Uracil and thymidine are released from their nucleosides by nucleosidases and are then catabolized by parallel pathways, leading to the release of nitrogen as ammonium and the formation of malonyl CoA from uracil and methylmalonyl CoA from thymine (Figure 1.12). Malonyl CoA is decarboxylated to acetyl CoA, and methylmalonyl CoA is isomerized by a vitamin B_{12}-dependent enzyme to yield the citric acid cycle intermediate succinyl CoA. Unlike purines, there are thus no unique metabolites of pyrimidines.

In prokaryotes, cytosine liberated by nucleosidase action is deaminated to uracil. Plants and animals lack cytosine deamidase, but do have cytidine deamidase. The resultant uridine is then a substrate for nucleosidases to yield uracil.

In plants and microorganisms, the β-alanine formed from uracil may be utilized in the synthesis of the vitamin pantothenic acid, which is the precursor for synthesis of coenzyme A and the functional moiety of the acyl carrier protein for fatty acid synthesis. However, a possibly more important source of β-alanine in bacteria is β-decarboxylation of aspartic acid. In mammals, which do not synthesize pantothenic acid, β-alanine is mainly incorporated into the dipeptide carnosine (β-alanyl-histidine – see section 8.5).

Uridine, cytidine, deoxycytidine and thymidine, arising from the diet or intracellular catabolism of nucleic acids, can be phosphorylated by kinases that utilize ATP as the phosphate donor. Unlike purines, there is little salvage of pyrimidines in PRPP-dependent reactions in mammals. There is a uracil phosphoribosyltransferase in prokaryotes, yeast and plants, and a human homologue of the yeast enzyme has been identified in foetal brain cDNA libraries (Li, J. *et al.*, 2007).

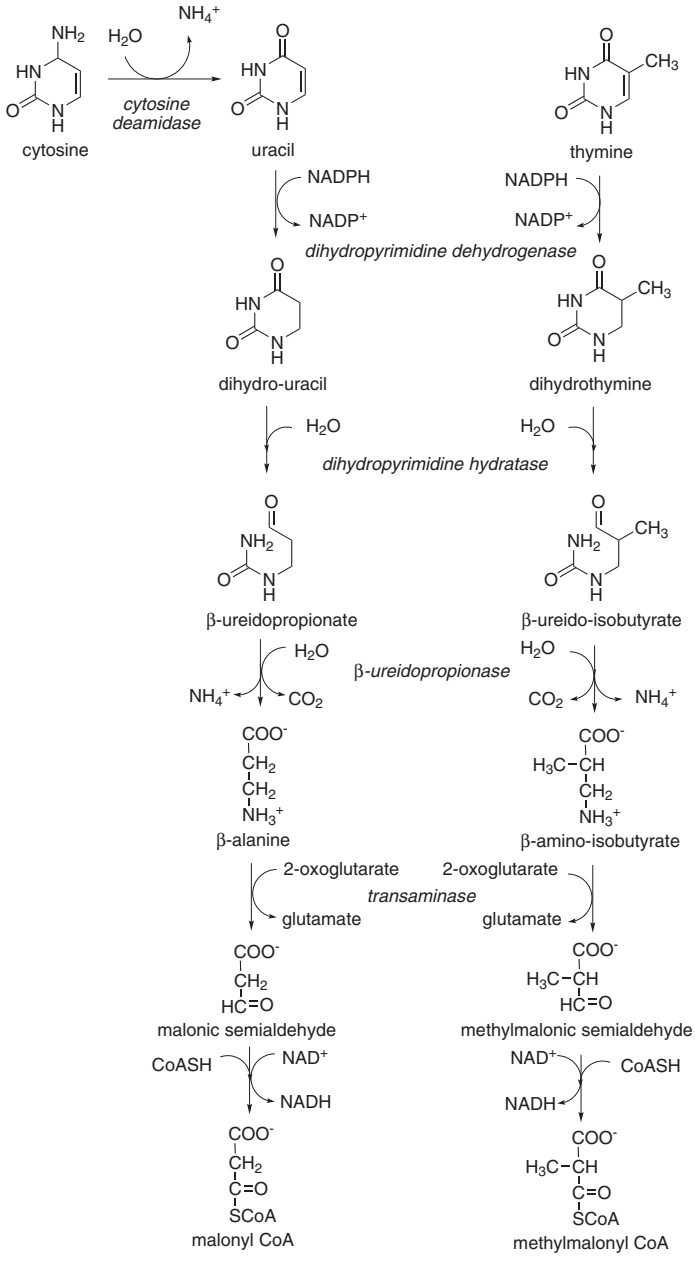

Figure 1.12 Pyrimidine catabolism.
Cytosine deamidase EC 3.5.4.1, dihydropyrimidine dehydrogenase EC 1.3.1.1 (NAD-linked), EC 1.3.1.2 (NADP-linked), dihydropyrimidine hydratase EC 3.5.2.2, β-ureidopropionase EC 3.5.1.6.

1.5 Deamination of amino acids

There is little storage of amino acids in animals, so amino acids in excess of immediate requirements for synthesis of proteins and other nitrogenous compounds (including purines and pyrimidines) will be deaminated, directly or indirectly leading to the formation of ammonium, and their carbon skeletons will be available for energy-yielding metabolism. In addition to the amino acid oxidases discussed below, serine and threonine undergo non-oxidative deamination (see sections 4.6.2 and 6.1.5). The adenosine deaminase cycle shown in Figure 1.10 also allows deamination of amino acids linked to transamination of oxaloacetate to aspartate.

Formation of ammonium by the reaction of glutamate dehydrogenase (see section 1.3.2.2) is associated with a yield of ≈2.5 × ATP from re-oxidation of the NADH formed in the reaction. There is no ATP yield from the formation of ammonium by the amino acid oxidases (section 1.5.1) or amine oxidases (section 1.5.2), since the redox state of the flavin coenzyme is unchanged at the end of the reaction cycle. The adenosine deaminase cycle (Figure 1.10) has a net yield of ≈1.5 × ATP; there is a gain of NADH (equivalent to ≈2.5 × ATP in the mitochondrial electron transport chain), but a need for GTP for the synthesis of adenylosuccinate. When glutamine is formed in peripheral tissues for transport to the liver, there is an additional cost of 1 × ATP for glutamine synthesis (Figure 1.2).

1.5.1 Amino acid oxidases

There are five mammalian flavoprotein amino acid oxidases: L-amino acid oxidase; D-amino acid oxidase; D-aspartate oxidase; glycine oxidase; and lysine oxidase. These catalyze oxidation of the amino group of an amino acid to yield ammonium and the corresponding oxo-acid, as well as the oxidation of water to hydrogen peroxide (Figure 1.13). All five enzymes are peroxisomal, so the potentially cytotoxic hydrogen peroxide is removed by catalase and peroxidases. The substrate amino acid is oxidized to the imino acid at the expense of FAD being reduced. The reduced FAD is then re-oxidized by reaction with oxygen, yielding hydrogen peroxide, and the imino acid undergoes non-enzymic hydrolysis to yield the oxo-acid and ammonium.

L-amino acid oxidase has a broad specificity, but generally a low activity, and is relatively unimportant in amino acid metabolism. D-amino acid oxidase catalyzes oxidative deamination of basic and neutral D-amino acids, and D-aspartate oxidase the oxidative deamination of D-aspartate, D-asparagine, D-glutamate and *N*-methyl-D-aspartate (Homma, 2007).

Modest amounts of D-amino acids occur in bacterial proteins and the bacterial cell wall, as well as in peptide antibiotics and a number of marine invertebrates (D-aspartate in the nervous system of cephalopods and

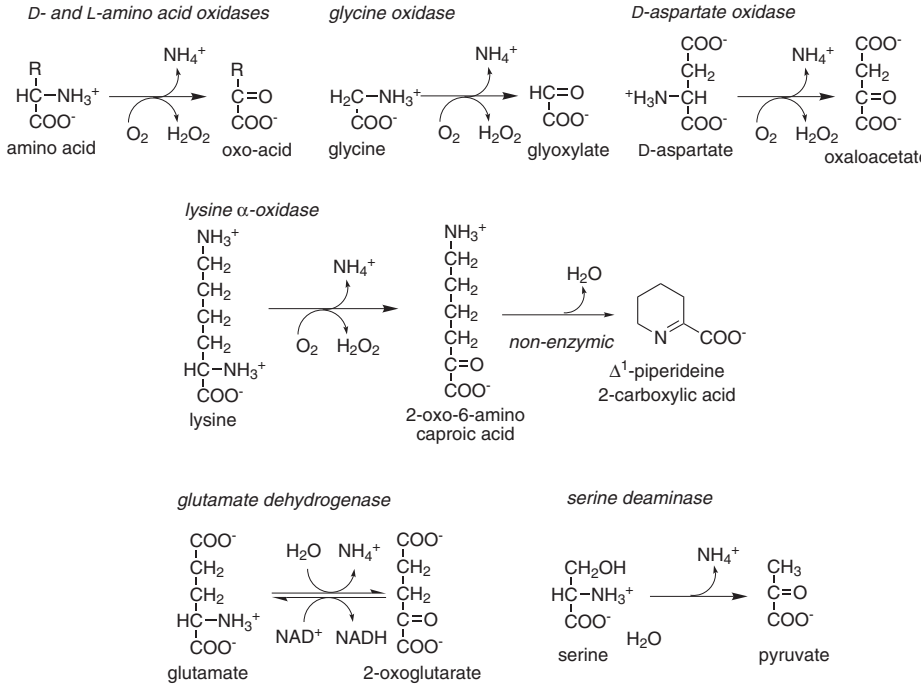

Figure 1.13 Deamination of amino acids.
L-amino acid oxidase EC 1.4.3.2, D-amino acid oxidase EC 1.4.3.3, glycine oxidase EC 1.4.3.19, D-aspartate oxidase EC 1.4.3.1, L-lysine oxidase EC 1.4.3.14, glutamate dehydrogenase EC 1.4.1.2 (NAD-linked), EC 1.4.1.4 (NADP-linked), EC 1.4.1.3 (linked to either NAD or NADP), serine deaminase EC 4.3.1.17.

D-alanine in muscle and hepatopancreas of crustaceans). They are absorbed from the gastro-intestinal tract, and many act as inhibitors of enzymes that catalyze reactions of the corresponding L-amino acids. A number of studies have shown that the activity of D-amino acid oxidase is low in germ-free animals, and that feeding D-amino acids leads to its induction, suggesting that the main role of this enzyme is detoxication of the (small) amounts of D-amino acids that are absorbed.

There is some spontaneous isomerization of amino acids in proteins. D-aspartate and D-hydroxyproline accumulate in tissues such as dentine, tooth enamel and the lens of the eye with increasing age (D'Aniello *et al.*, 1993; Pilone, 2000; Wolosker *et al.*, 2000).

D-serine and D-aspartate are neurotransmitters in the central nervous system, and D-amino acid oxidase and D-aspartate oxidase have roles in regulating the concentrations of these neurotransmitters. D-amino acid oxidase knockout mice have higher than normal concentrations of D-serine in the central nervous system, and D-aspartate oxidase knockout mice have abnor-

mally high concentrations of D-aspartate in the central nervous system (Katane *et al.*, 2008).

A further physiological role of D-amino acid oxidase and D-aspartate oxidase is to permit the isomerization of D-amino acids to the corresponding L-amino acids, and hence their use in protein synthesis. The oxo-acids formed by oxidative deamination of D-amino acids are symmetrical compounds and are substrates for transaminases, yielding the L-isomers (see section 3.3). The importance of this for human nutrition is unclear, but experimental animals can meet at least a part of their requirement for methionine and other essential amino acids from the D-isomers. It is unclear to what extent this is the result of D-amino acid oxidase in the liver and kidney, as opposed to bacterial amino acid racemases in the large intestine (section 3.2).

In some bacteria (including *Helicobacter pylori* and *E. coli*) there is a D-amino acid dehydrogenase, an iron-sulphur flavoprotein that catalyzes oxidation of D-amino acids to yield the oxo-acid, ammonium and, initially, H_2. The hydrogen is then ionized and its electrons are transferred onto ubiquinone and thence through the electron transport chain, so providing a source of ATP (Tanigawa *et al.*, 2010).

Lysine oxidase provides the main pathway for lysine catabolism in the brain (see section 6.3.2.1), and it has been investigated as a possible anticancer agent, acting to deplete lysine and minimize tumour growth. The protein marinocine is a lysine oxidase which, in some organisms, has antibacterial activity as a result of both the hydrogen peroxide generated and depletion of lysine (Lucas-Elio *et al.*, 2006).

Glycine oxidase is important in the deamination of a wide variety of amino acids. As shown in Figure 1.14, the glyoxylate formed by oxidative deamination of glycine can be a substrate for transamination back to glycine at the expense of a variety of amino acids (see section 3.3 for a discussion of transamination). The importance of glycine oxidase in amino acid catabolism is shown by the genetic disease primary hyperoxaluria (type I). The defect in this condition is a lack of alanine-glyoxylate transaminase. As a result, glyoxylate accumulates, and is a substrate for lactate dehydrogenase, forming oxalate (section 4.3.1). Calcium oxalate crystallizes in the kidneys, leading to renal failure. Apart from those patients who are vitamin B_6 responsive, the only treatment is combined liver and kidney transplantation.

1.5.2 Amine oxidases

As discussed in section 3.4, decarboxylation of amino acids leads to the synthesis of amines. Some of these, such as histamine (see section 8.4), 5-hydroxytryptamine (serotonin, section 9.4.3) and the catecholamines (section 9.2.4) are neurotransmitters. Others, including phenylethylamine, tyramine and tryptamine, are synthesized by bacteria and may have potent

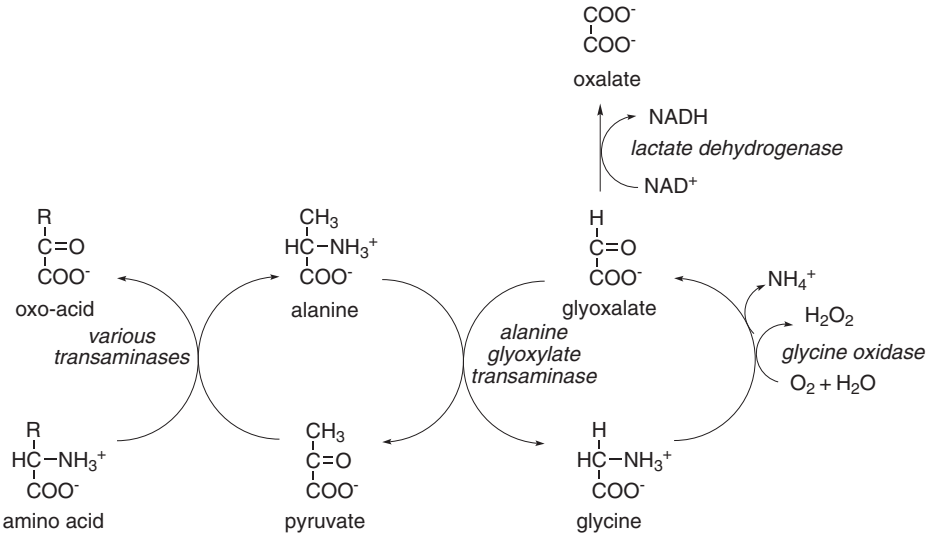

Figure 1.14 Transdeamination – transamination linked to glycine oxidase. Glycine oxidase EC 1.4.3.19, lactate dehydrogenase EC 1.1.1.27.

pharmacological actions if they enter the bloodstream. The action of the neurotransmitter amines is terminated by oxidation to aldehydes catalyzed by monoamine oxidase, a mitochondrial flavoprotein. This catalyzes the oxidation of a primary amine to the corresponding aldehyde, liberating ammonium and forming hydrogen peroxide by re-oxidation of the reduced flavin with oxygen (section 9.2.4.2). Potentially hazardous amines absorbed from the gastro-intestinal tract are oxidized by monoamine oxidase in the liver.

The copper-containing amine oxidases and diamine oxidase catalyze the same reaction as mono-amine oxidase, but linked to the reduction of a quinone coenzyme. The quinone cofactor is formed by post-synthetic modification of tyrosine or tryptophan residues in the precursor protein (see section 9.5). Some of these enzymes occur in plasma and may have a role both in detoxication of amines absorbed from the gastro-intestinal tract and in histamine (section 8.4) and other amines released by mast cells; others are cell surface enzymes. Lysyl oxidase catalyzes oxidation of the ε-amino group of lysine residues to an aldehyde, an essential process in cross-linkage of collagen and elastin (section 6.2.4.1).

1.5.3 Glutamate and alanine dehydrogenases

The reaction of mammalian glutamate dehydrogenase (see Figure 1.2 and section 1.3.2.3) is readily reversible. The enzyme can act either reductively, to

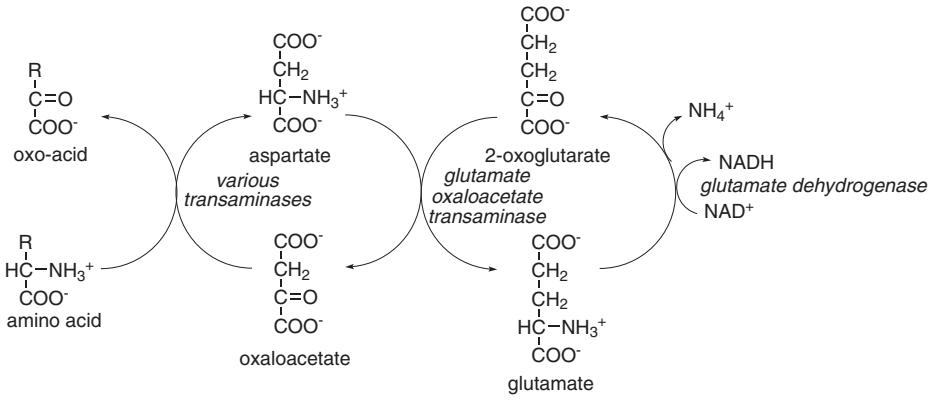

Figure 1.15 Transdeamination – transamination linked to glutamate dehydrogenase. Glutamate dehydrogenase EC 1.4.1.2, glutamate-oxaloacetate transaminase EC 2.6.1.1.

incorporate ammonium into glutamate, or oxidatively, liberating ammonium. The direction of the reaction depends mainly on the concentration of ammonium in the cell, although the relative concentrations of glutamate and glutamine, and the NAD^+:NADH ratio, are also important determinants of the direction of reaction.

Like glycine oxidase, glutamate dehydrogenase can lead to the oxidative deamination of a wide variety of amino acids, since the 2-oxoglutarate formed is a substrate for transamination. A number of transaminases with specificity for different amino acids are linked to 2-oxoglutarate as the amino acceptor (section 3.3). Other amino acids are substrates for transaminases linked to oxaloacetate as the amino acceptor, with glutamate-oxaloacetate transaminase providing the link to glutamate dehydrogenase, as shown in Figure 1.15.

In bacteria, alanine dehydrogenase similarly provides a route for deamination of a variety of amino acids, with transaminases linked to pyruvate as the amino acceptor. Mammals lack alanine dehydrogenase.

1.5.4 Non-oxidative deamination of amino acids

Serine deaminase catalyzes non-oxidative deamination of serine (and, in mammals, also threonine), liberating ammonium and water, and yielding pyruvate from serine and 2-oxobutyrate from threonine. As will be discussed in section 4.6.2, deamination of serine is mainly a pathway for provision of pyruvate for gluconeogenesis rather than for disposal of surplus serine. See section 6.1.5 for a discussion of the catabolic and biosynthetic roles of threonine deaminase.

1.5.5 Glutaminase and asparaginase

Both glutamine and asparagine are substrates for deamidases, liberating ammonium and forming glutamate and aspartate respectively. Prokaryote asparaginase has significant glutaminase activity, but the mammalian enzyme does not. There are two mammalian glutaminases: a liver-type enzyme and a kidney-type enzyme. Both are mitochondrial enzymes, and both are activated by phosphate ions.

The liver-type glutaminase is found only in the liver, in the periportal hepatocytes, and is induced by feeding a high-protein diet and in response to starvation. It has a relatively high K_m for glutamine and is not inhibited by glutamate (Curthoys & Watford, 1995). Its function is to liberate ammonium for urea synthesis (see section 1.6.2.1).

The kidney-type glutaminase is found in many tissues, including skeletal muscle, central nervous system and platelets. It has a low K_m for glutamine and is inhibited by glutamate. Incubation of isolated cerebellar mitochondria with [^{14}C]glutamine leads to rapid accumulation of labelled glutamate in the incubation medium with the same specific activity as that of the glutamine substrate, and no mixing of the glutamate produced with intra-mitochondrial glutamate pools. This suggests that the main function of glutaminase in the nervous system is to provide glutamate as a neurotransmitter and as a precursor for GABA synthesis (see section 5.5 and Holten & Gundersen, 2008).

In the kidney, glutamine catabolism increases in response to metabolic acidosis as a result of increased translation of glutaminase mRNA. The 3′-untranslated region of glutaminase mRNA contains a direct repeat of an eight-base AU sequence that acts as a pH response element, binding to a protein that stabilizes the mRNA, so increasing translation. Glutamine catabolism in the kidney results in the formation of two molecules of ammonium (which are secreted into the urine to enhance acid excretion) and two of bicarbonate (which are secreted into the venous blood to provide compensation for the acidosis) (Curthoys & Gstraunthaler, 2001).

There is also phosphate-independent glutaminase activity in some tissues, but this is a partial activity of γ-glutamyl transpeptidase (section 5.4.5) rather than a true glutaminase. It is an extracellular enzyme, and in the distal renal tubule its glutaminase activity increases as the pH of the tubule content decreases. Thus, its function is to hydrolyze glutamine to produce ammonium to buffer the urine pH.

A number of amines can react with a glutamine or asparagine residue in a protein, replacing the amide group and liberating ammonium. This reaction may be important in the incorporation of polyamines into proteins (section 5.8) and possibly in the long-term effects of the hallucinogen mescaline, which can be incorporated into central nervous system proteins. Similarly, in blood clotting, factor XIII stabilizes the fibrin clot by catalyzing the formation of

cross-links in which the ε-amino group of a lysine residue replaces the amide group of glutamine, liberating ammonium (section 5.3.3; Pisano *et al.*, 1969). Obviously, neither of these transglutaminase reactions is a significant source of ammonium.

1.6 Excretion of nitrogenous waste

Fishes and other small aquatic animals that inhabit a relatively large volume of water excrete most of their nitrogenous waste as ammonium. Earthworms, with a relatively high surface : volume ratio, excrete mainly ammonia rather than ammonium. Other organisms have to produce a less toxic end product of nitrogen metabolism.

The tadpole excretes most of its nitrogenous waste as ammonium, but on metamorphosis its terminal nitrogen metabolism changes; adult frogs, which spend much of their time on dry land, excrete mainly urea (section 1.6.2). The *Xenopus* toad, an amphibian that has made a secondary return to a more or less completely aquatic habitat, excretes 70–80 per cent of its nitrogenous waste as ammonium; however, if it is removed from water it can synthesize urea and store it until it returns to water.

1.6.1 Uricotelic and purinotelic species

Birds, reptiles and many insects utilize the purine synthesis pathway shown in Figure 1.8, and onward oxidation to uric acid (Figure 1.9) as their main pathway of nitrogen metabolism. Such animals are termed uricotelic. Arachnids and bats also utilize the purine synthesis pathway for elimination of nitrogen, but they synthesize guanine as their main excretory product; they are purinotelic.

For both uricotelic and purinotelic species, the imperative is to produce an end product of nitrogen metabolism that is relatively insoluble. Water is obviously limited in the eggs of birds and reptiles, so the developing embryo produces uric acid that can crystallize in the egg and thus does not affect osmolarity to the extent that a more soluble end product would. For adult birds, insects, arachnids and flying mammals, the problem is one of water shortage or the osmotic problems caused by high concentrations of urea, so again it is desirable to be able to excrete nitrogenous waste as a slurry of crystals rather than as a relatively large volume of solution. Indeed, the 'urine' of some insects consists of more or less completely dry crystals of uric acid.

Some insects put their nitrogenous waste to specific use. Waste accumulating during pupation is the precursor for synthesis of the pterin pigments of butterfly wings.

1.6.2 Ureotelic species

When water is not a problem, the main end product of nitrogen metabolism is urea, which is readily soluble. Urea synthesis is also important in the regulation of blood osmolarity. If *Xenopus* is subjected to an osmotic shock, such as being placed in salty water, its synthesis of urea increases (and excretion decreases), so raising blood osmolarity and avoiding dehydration. The lungfish does not normally produce urea but, during aestivation, it accumulates urea as a means of maintaining an osmotic gradient for water uptake. By the end of aestivation, as much as one per cent of the fish's body weight is urea.

The crab-eating frog, *Rana cancrivora*, is rare among amphibians in that it can tolerate moderate salinity. When it is moved from fresh to salt water, there is a considerable increase in the expression of carbamoyl phosphate synthetase I and accumulation of urea as an osmolyte. The Lake Magadi tilapia (*Oreochromis alcalicus grahami*) from Kenya has adapted to living in alkaline water (pH 10.5) by excreting its nitrogenous waste as urea rather than ammonia. The main activity of the urea cycle is in muscle, rather than the liver as in other ureotelic species.

In tadpoles, the thyroid hormones that are essential for metamorphosis induce the enzymes of the urea cycle ready for the switch from ammonotelic to ureotelic life. In mammals, although urea is an end product of ammonia metabolism, it is actively reabsorbed in the distal renal tubule as an osmolyte for the reabsorption of water (Lindley *et al.*, 1999; Takiguchi & Mori, 1995; Wright *et al.*, 2004).

The salt content of fish blood is intermediate between that of fresh and salt water, so that, whatever their environment, fishes have problems of osmoregulation. Marine teleosts (bony fish) have a blood osmotic pressure below that of sea water, and they continually drink copious amounts of water, excrete small amounts of hypertonic urine and excrete salts by active transport through the gills. Freshwater teleosts have the opposite problem; water continually enters the body and they drink very little, but they excrete copious amounts of dilute urine and actively absorb salts through their gills.

The elasmobranchs (cartilaginous fish) have tackled the problem in a different way. Marine elasmobranchs synthesize urea as the end product of nitrogen metabolism, and maintain a high blood concentration (up to 300 mmol/l) by actively reabsorbing it from the glomerular filtrate. Freshwater elasmobranchs retain some of the hyperuraemia of their marine ancestors, so that their blood is hypertonic with respect to their environment. Like freshwater teleosts, they excrete copious amounts of dilute urine to remove the water that enters by osmosis.

1.6.2.1 Urea synthesis The pathway of urea synthesis shown in Figure 1.16 was first elucidated by Krebs and Henseleit in 1932; it was the first cyclic metabolic pathway to be described. The complete cycle occurs in the periportal

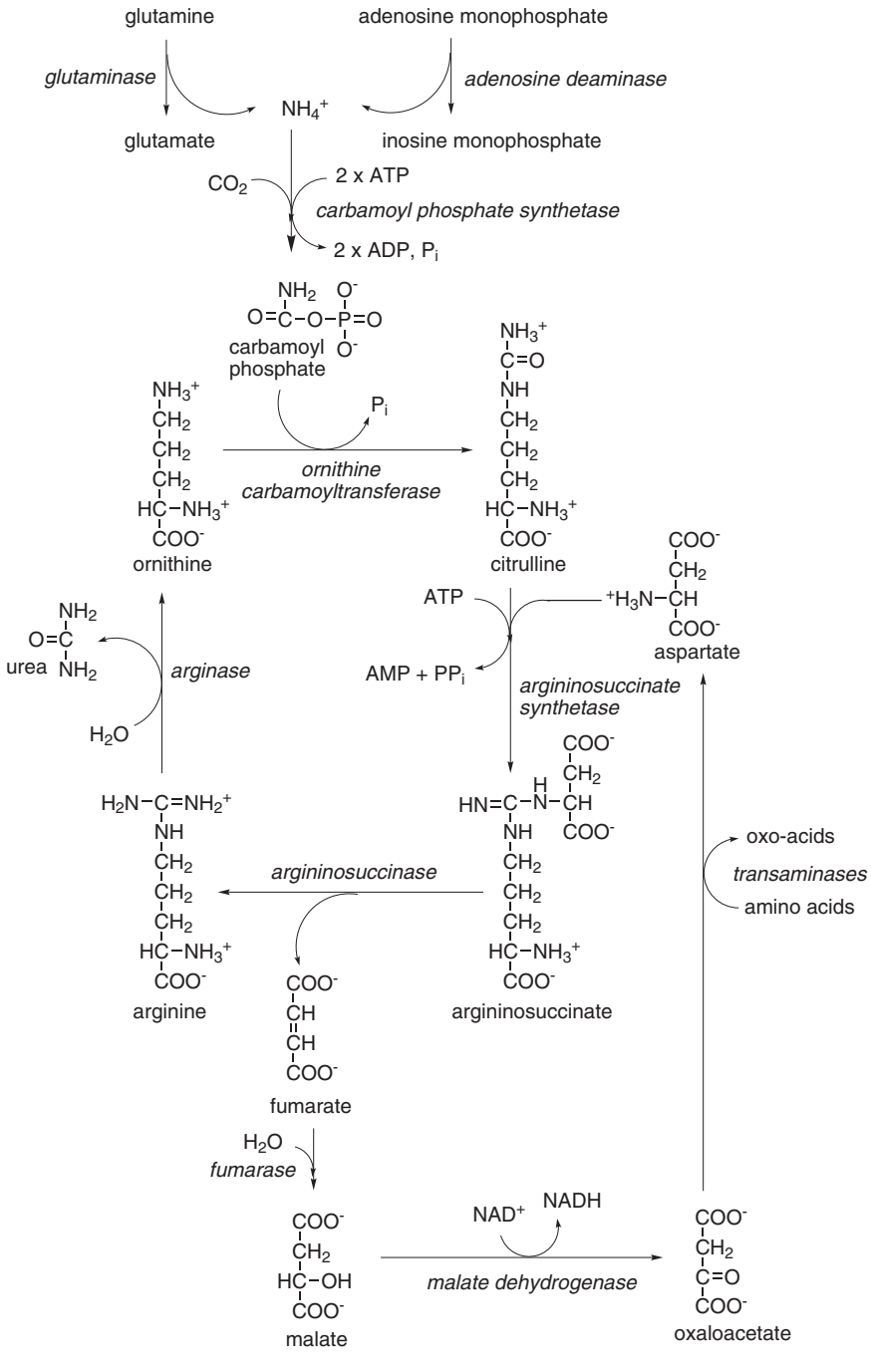

Figure 1.16 The urea synthesis cycle.
Glutaminase EC 3.5.1.2, adenosine deaminase EC 3.5.4.6, carbamoyl phosphate synthetase EC 6.3.4.16, ornithine carbamoyltransferase EC 2.1.3.3, argininosuccinate synthetase EC 6.3.4.5, argininosuccinase EC 4.3.2.1, arginase EC 3.5.2.1, fumarase EC 4.2.1.2, malate dehydrogenase EC 1.1.1.37.

cells of the liver, and also in enterocytes, where its function is synthesis of urea. There is tight channelling of intermediates between one enzyme and the next. There is especially tight channelling of arginine between argininosuccinase and arginase; the addition of a 200-fold excess of unlabelled arginine does not reduce the label from $^{14}CO_2$ in urea, so there is no mixing of the catalytic pool of arginine with the free arginine pool in the liver (Cheung *et al.*, 1989).

As will be discussed in section 5.9, citrulline is synthesized in the small intestinal mucosa and exported to the kidneys, where it is used for the synthesis of arginine. The brain can synthesize urea from citrulline produced by nitric oxide synthase (section 5.9.3), but it lacks ornithine carbamoyltransferase, so it cannot synthesize urea from ammonium.

In the liver, the activities of the enzymes of urea synthesis are increased by a high protein intake, when there is a need for increased urea synthesis to detoxify ammonium arising from amino acids in excess of immediate needs for protein synthesis (see section 2.1.6.4). They are also increased in response to glucagon and glucocorticoid hormones, which are secreted in the fasting state, when there is a need for increased urea synthesis to detoxify the ammonium released from amino acids that are being used for gluconeogenesis. In contrast, in extrahepatic tissues the enzymes are regulated in response to pro- and anti-inflammatory cytokines (Morris, 2002; Takiguchi & Mori, 1995).

There are two sources of ammonia for carbamoyl phosphate synthesis: the action of glutaminase (section 1.5.5) and the reaction of AMP deaminase (shown in Figure 1.9). Carbamoyl phosphate is synthesized from ammonium and carbon dioxide by a mitochondrial carbamoyl phosphate synthetase that is distinct from that involved in pyrimidine synthesis (section 1.4.3) and which uses ammonium rather than glutamine. The activity of mitochondrial carbamoyl phosphate synthetase is increased in response to glucagon and glucocorticoid hormones, acting synergistically. Neither hormone has any significant effect alone but, when both are present, the mRNA is stabilized, so that there is increased synthesis of enzyme protein (Ulbright & Snodgrass, 1993).

Mitochondrial carbamoyl phosphate synthetase I is the main regulator of urea synthesis; it is not sensitive to product inhibition, and the other enzymes of the cycle all operate at substrate concentrations below their K_m, so that they have spare capacity. It is only when there is a genetic defect of one of the enzymes of the cycle, or of the mitochondrial ornithine-citrulline transporter, that carbamoyl phosphate accumulates in the mitochondrion. It can then cross into the cytosol, leading to increased pyrimidine synthesis (section 1.4.3.1; Meijer *et al.*, 1985, 1990).

The sirtuins are NAD-dependent protein deacetylases; SIRT-5 is a mitochondrial enzyme that deacetylates mitochondrial carbamoyl phosphate synthetase, increasing its activity. During fasting, the liver content of NAD

increases, leading to deacetylation of carbamoyl phosphate synthetase and increased urea synthesis to meet the increased catabolism of amino acids for gluconeogenesis. SIRT-5 knock-out mice cannot upregulate carbamoyl phosphate synthetase, and they become hyperammonaemic in fasting (Nakagawa *et al.*, 2009).

Carbamoyl phosphate synthetase I is also induced in fasting in response to glucocorticoids and glucagon (acting via cAMP). There are two glucocorticoid response elements in the gene, and in the absence of cAMP they act additively to increase transcription. cAMP also binds to, and activates, one of the glucocorticoid response elements. This means that, in addition to the induction of key gluconeogenic enzymes and key enzymes of amino acid catabolism such as tyrosine transaminase (section 9.3) and tryptophan dioxygenase (section 9.4.4.1) in response to glucocorticoids, there is also an increase in the capacity for urea synthesis (Schoneveld *et al.*, 2007).

Mammalian mitochondrial carbamoyl phosphate synthetase has an absolute requirement for *N*-acetylglutamate as an allosteric activator; it causes dissociation of the inactive enzyme dimer into the active, but unstable, monomer. As discussed in section 5.9.1, in prokaryotes, plants and animals that are not ureotelic, *N*-acetylglutamate is a precursor for the synthesis of ornithine, and hence arginine, and *N*-acetylglutamate synthetase is inhibited allosterically by arginine.

In ureotelic animals, the ornithine for arginine synthesis is synthesized from glutamate by an alternative pathway (see Figure 5.11), and *N*-acetylglutamate synthetase is activated, rather then inhibited, by arginine. In teleost fishes, where urea is synthesized mainly as a temporary store of nitrogen, there is a glutamine-dependent mitochondrial carbamoyl phosphate synthetase that is activated by *N*-acetylglutamate, but which does not have an absolute requirement for the activator. *N*-Acetylglutamate also activates glutaminase, so increasing the provision of ammonia for urea synthesis (Caldovic & Tuchman, 2003; Caldovic *et al.*, 2010; Meijer *et al.*, 1990).

N-Acetylglutamate synthetase catalyzes the transfer of an acetyl group from acetyl CoA onto the amino group of glutamate. Propionyl CoA and some other acyl CoA derivatives are poor substrates for *N*-acetylglutamate synthetase, but at high concentrations they act as competitive inhibitors. This is a cause of ammonia intoxication in conditions such as propionic aciduria, when tissue concentrations of propionyl CoA are significantly elevated, in some genetic defects affecting fatty acid oxidation, and possibly also in response to valproic acid and other drugs that form CoA derivatives.

N-acetylglutamate synthetase is activated in response to a high protein intake, so increasing the activation of carbamoyl phosphate synthetase and permitting increased urea synthesis from ammonium. Arginine also increases the activity of the enzyme, and it is likely that it is a high mitochondrial concentration of arginine that signals a high protein intake and, hence, the need

for additional synthesis of urea. *N*-acetylglutamate synthetase is also inhibited by its product, *N*-acetylglutamate. The catabolism of *N*-acetylglutamate occurs in the cytosol, so a major factor in controlling the intra-mitochondrial concentration will be the activity of the transport system for its efflux from the mitochondrion (Caldovic & Tuchman, 2003; Morizono *et al.*, 2004).

The second reaction of urea synthesis, catalyzed by ornithine carbamoyltransferase, is also mitochondrial. The remaining reactions are cytosolic, and there is tight channelling of intermediates from one enzyme to the next, and to the transport proteins for import of ornithine into (and efflux of citrulline from) mitochondria, so that the ornithine involved in the urea synthesis cycle is not likely to be available for decarboxylation (section 5.8.1) or transamination.

The activity of ornithine carbamoyltransferase is increased in response to glucagon and glucocorticoid hormones acting synergistically; neither hormone alone has any significant effect. Although the enzyme activity and immunoreactive enzyme protein in the cell increases, there is no change in mRNA, suggesting that the response to the hormones is stabilization of the enzyme protein against catabolism.

Ornithine carbamoyltransferase is also activated by deacetylation catalyzed by SIRT-3 during energy restriction. SIRT-3 knockout mice are unable to upregulate the carbamoyltransferase during energy restriction and develop orotic aciduria as a result of carbamoyl phosphate leaving the mitochondria and being used for increased pyrimidine synthesis (section 1.4.3.1). Acetylation of ornithine carbamoyltransferase on lysine residues decreases its affinity for carbamoyl phosphate and lowers the V_{max}, but it has no effect on the K_m for ornithine (Hallows *et al.*, 2011; Yu *et al.*, 2009).

Argininosuccinate synthetase and argininosuccinase are both induced by glucagon and glucocorticoid hormones, acting synergistically. This is true induction, with increased transcription of the genes to increase the pool of mRNA. The increase in arginase activity in response to glucagon and glucocorticoid hormones (again acting synergistically) is similar to that of carbamoyl phosphate synthetase – increased mRNA and, hence, increased translation, as a result of increased stability of the mRNA rather than increased transcription (Morris, 2002; Ulbright & Snodgrass, 1993).

Argininosuccinate synthetase and argininosuccinase are expressed in many tissues, including vascular endothelium. Here, their function is to recycle the citrulline formed in the nitric oxide synthase reaction (section 5.9.1.1), and argininosuccinate synthetase is induced in response to pro-inflammatory signals rather than amino acids and fasting state hormones (Husson *et al.*, 2003).

As shown in Figure 1.16, the fumarate released by the argininosuccinase reaction is recycled to aspartate by way of malate and oxaloacetate, with a yield of 1 × NADH per mol of aspartate formed; this is equivalent to ≈2.5 mol

of ATP, more than offsetting the ATP cost of argininosuccinate synthesis. Since oxaloacetate is the amino acceptor for a wide variety of transaminases, this provides a route for the disposal of amino groups from most amino acids. The argininosuccinate synthetase reaction involves formation of an enzyme-bound AMP-citrulline intermediate, with the release of pyrophosphate. Tissues contain an active pyrophosphatase, and removal of the pyrophosphate ensures that the reaction (and hence the cycle) proceeds in only one direction.

There are two isoenzymes of arginase; both are manganese metalloenzymes. Arginase I is a cytosolic enzyme which is highly expressed in liver and is involved in urea synthesis. Arginase II is a mitochondrial enzyme with a wide tissue distribution, and it is involved in the provision of ornithine for polyamine synthesis (see section 5.8) and in controlling the amount of arginine available locally for synthesis of nitric oxide (section 5.9.3.2; Cederbaum *et al.*, 2004; Crombez & Cederbaum, 2005).

1.6.2.2 Inborn errors of metabolism affecting the urea synthesis cycle

Genetic defects affecting all of the enzymes of the urea synthesis cycle have been reported. All lead to some degree of ammonia intoxication. Both in relatively prolonged fasting (when amino acids are being deaminated to provide carbon skeletons for gluconeogenesis) and after a moderately protein-rich meal, there is a risk of severe hyperammonaemia, leading to loss of consciousness and convulsions. In most of these conditions, there is also developmental and mental retardation, together with other neurological signs (Endo *et al.*, 2004; Jackson *et al.*, 1986; Meijer *et al.*, 1990).

Because the same enzymes are involved in arginine synthesis (section 5.9.1) and in the urea synthesis cycle (apart from *N*-acetyl glutamate synthetase), arginine is a dietary essential for children affected by any of the defects of the cycle apart from argininaemia. As noted below, arginine supplements are useful in the treatment of inborn errors of the urea cycle.

Lack of mitochondrial carbamoyl phosphate synthetase or *N*-acetyl glutamate synthetase will lead to hyperammonaemia, with no abnormal amounts of intermediates of the urea synthesis cycle appearing in blood or urine. Severe deficiency of the enzyme manifests as infantile-onset disease, while milder deficiency leads to adult-onset disease.

Patients with a genetic defect of *N*-acetylglutamate synthetase suffer severe hyperammonaemia, which may be fatal in the neonatal period because of the failure to activate mitochondrial carbamoyl phosphate synthetase. The *N*-acetylglutamate analogue carbamoylglutamate binds to, and activates, carbamoyl phosphate synthetase; this relieves the hyperammonaemia in both these patients and those with propionic acidaemia, as propionate inhibits *N*-acetylglutamate synthetase. Responsiveness to carbamoylglutamate permits ready differentiation between hyperammonaemia due to lack

of *N*-acetylglutamate synthetase and the clinically identical condition due to lack of carbamoyl phosphate synthetase itself, rather than failure of its activation by *N*-acetylglutamate (Morizono *et al.*, 2004).

Lack of any of the other enzymes of the cycle will lead to accumulation in blood and urine of the substrate of the affected enzyme, and also to excretion of significant amounts of orotic acid. This is because, as carbamoyl phosphate synthesized in the mitochondria that is not used for citrulline synthesis accumulates, it can be exported to the cytosol, where it is a substrate for aspartate carbamoyltransferase and, hence, orotic acid synthesis (Figure 1.12). In some cases, there is a reduced amount of immunologically reactive enzyme protein in tissues; in other cases, there is a normal amount of the enzyme protein present, but it has an abnormally high K_m for its substrate.

The gene for ornithine carbamoyl transferase is on the X chromosome; females who are heterozygous for the condition commonly present between 1–6 years of age with relatively non-specific symptoms: episodic irritability, vomiting, lethargy, delayed growth, protein avoidance and occasional loss of consciousness. Other female carriers are generally unaffected but are at risk of hyperammonaemic coma, especially in childbirth.

There are two variants of argininosuccinic aciduria. The malignant form develops in the first few weeks of life and is characterized by mental and physical retardation, convulsions, episodic loss of consciousness, liver enlargement, and skin and hair abnormalities. There is little or no residual argininosuccinase activity. The milder variant has a later onset and less severe symptoms. There is more enzyme protein present, or, in some cases, a normal amount of enzyme protein but with an abnormally high K_m for argininosuccinate.

Argininaemia is due to lack of cytosolic arginase I in the liver; signs include paraplegia, seizures and mental retardation. There is some degree of compensation in this condition, because arginase II, the mitochondria enzyme that is normally present in the kidney and other extra-hepatic tissues in small amounts, is induced by high tissue concentrations of arginine. This means that, unlike the other inborn errors of the cycle, argininaemia has a late onset, between 2–4 years of age.

The syndrome of hyperammonaemia with hyperornithinaemia and homocitrullinuria is associated with mental retardation and myoclonic seizures. It is due to a defect of the mitochondrial transport protein for uptake of ornithine.

The burden of ammonium to be metabolized will be increased both after a moderately high protein meal and also in the fasting state, and hyperammonaemic coma is especially a problem when affected infants are fasting and have a fever, which increases the need for gluconeogenesis from amino acids. The first approach to treatment of all of the inborn errors of urea synthesis is a relatively low protein intake (adequate for growth but avoiding a signifi-

cant excess of amino acids to be deaminated after a meal) and avoidance of prolonged fasting.

Citrullinaemia, due to a lack of argininosuccinate synthetase, and argininosuccinic aciduria, due to lack of argininosuccinase, can be treated by provision of (relatively large) supplements of arginine. This provides a source of ornithine, so permitting the excretion of 1 mol of nitrogen from ammonium as citrulline, or 1 mol of ammonium and the nitrogen from 1 mol of aspartate as argininosuccinate. As long as arginine is present to provide a source of ornithine, there is now a linear pathway for excretion of 1–2 mol of nitrogen. Theoretically, ornithine should be as effective as arginine, but in practice it seems not to be, presumably because there is little or no hepatic uptake of ornithine. Argininosuccinic aciduria also responds to administration of citrulline.

In all genetic defects of urea synthesis, it is possible to force elimination of excess nitrogen other than through the formation of ammonium. Benzoic acid is conjugated with glycine or alanine and the conjugates are excreted in the urine, so lowering the total body burden of nitrogen. Indeed, as we will see in section 4.1, excessive intakes of benzoic acid can outstrip the body's capacity for glycine synthesis, so that it becomes an essential amino acid. Similarly, phenylacetate is conjugated with glutamine and the resultant phenylacetylglutamine is excreted in the urine, again lowering the total body nitrogen burden. Benzoic acid allows excretion of one nitrogen atom per mol of conjugate, while phenylacetate permits the excretion of two atoms of nitrogen in phenylacetylglutamine (Batshaw, 1994; Batshaw *et al.*, 1982; 2001; Brusilow *et al.*, 1984; Endo *et al.*, 2004).

1.6.2.3 Entero-hepatic circulation of urea Although urea is commonly regarded as the end product of nitrogen metabolism in ureotelic animals, a number of studies have shown that considerably more urea is synthesized each day than is excreted. The biological half-life of [^{14}C]urea injected into rabbits is considerably shorter than that of [^{15}N]urea, as a result of catabolism of urea to yield ammonium and carbon dioxide. There is little reutilization of the labelled carbon dioxide, but a significant proportion of the [^{15}N] is reutilized (Regoeczi *et al.*, 1965).

Studies in which human beings were fed [^{15}N]urea showed enrichment of [^{15}N] in serum albumin, which was reduced after the administration of antibiotics to eliminate intestinal flora. There is no mammalian urease, and these results suggest that urea crosses into the gastro-intestinal tract and is hydrolyzed by bacterial urease, liberating ammonium and carbon dioxide; indeed, some 10 per cent of faecal nitrogen can be attributed to urea derived from the bloodstream. Although urea can enter the intestinal lumen by diffusion, there is also active secretion in pancreatic juice and bile. Teleost fishes, which are ammonotelic, synthesize urea as a temporary store of fixed nitrogen; this

is hydrolyzed in the gastro-intestinal tract, liberating ammonium, which is reabsorbed (Caldovic & Tuchman, 2003).

Up to 25 per cent of total daily urea production undergoes hydrolysis in the large intestine – a total of some 3,600 mg of nitrogen per day. Of this, 10 per cent is lost in faeces, 26 per cent returns to urea (mainly synthesized in the intestinal mucosa) and the remainder is retained in the body in amino acids in tissue proteins (Jackson, 1995). Some of these amino acids are synthesized by intestinal bacteria, although the extent to which they will be available to the host is unclear.

Most amino acids are absorbed in the small intestine, while most of the bacterial population is in the large intestine. However, there is evidence of at least limited amino acid absorption from the large intestine. Much of the ammonium released from urea in the large intestine is absorbed and is trapped as glutamate in the intestinal mucosa and liver. It then enters other amino acids by transamination (Bergen & Wu, 2009). Label from [^{15}N]urea is found in both essential and non-essential amino acids, and it is likely that the formation of essential amino acids represents salvage of the oxo-acids formed by transamination (Fuller & Reeds, 1998).

It is well known that ruminants can utilize urea as a major, if not sole, source of nitrogen, because of their large population of commensal bacteria, and rodents and lagomorphs can similarly make use of intestinal bacterial amino acid synthesis as a result of coprophagy. The nutritional significance of entero-hepatic cycling of urea in human beings is unclear but, if normal adults are maintained on a low-protein diet, there is an increase in nitrogen balance (see section 2.1) when they are fed modest amounts of urea (Meakins & Jackson, 1996). To a limited extent, bacterially synthesized essential amino acids are also available as a result of hydrolysis of bacterial proteins in the large intestine and absorption of the resultant amino acids (Bergen & Wu, 2009).

Most of the intestinal bacteria that have urease use it to liberate ammonium from urea for incorporation into amino acids and bacterial proteins. However, *Helicobacter pylori* uses urease to produce ammonium to neutralize gastric acid, and so permit it to survive in the acid conditions of the stomach (Belzer *et al.*, 2005).

1.6.2.4 Canavanine Canavanine is a toxic insecticidal non-protein amino acid that is an analogue of arginine. It accumulates in the seeds of some legumes and is synthesized by the same enzymes as are involved in the synthesis of arginine and urea (Figure 1.17). No enzyme has been identified for the synthesis of the intermediate canaline by amination of homoserine. Canavanine's toxic action is because it can be incorporated into proteins in place of arginine. Canavanine-insensitive insects have a strongly discriminatory arginyl-tRNA synthetase that does not incorporate canavanine. Canavanine

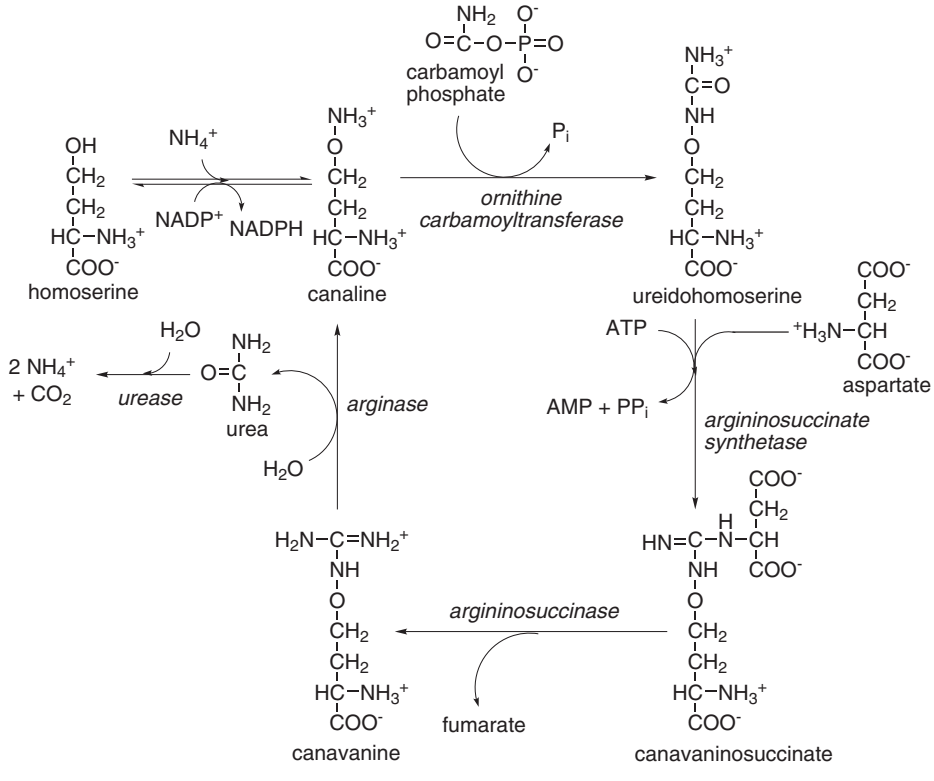

Figure 1.17 The metabolism of canavanine.
Glutaminase EC 3.5.1.2, adenosine deaminase EC 3.5.4.6, carbamoyl phosphate synthetase EC 6.3.4.16, ornithine carbamoyltransferase EC 2.1.3.3, argininosuccinate synthetase EC 6.3.4.5, argininosuccinase EC 4.3.2.1, arginase EC 3.5.2.1.

is also a major store of nitrogen in the seed, and it can be hydrolyzed by arginase and urease to provide ammonium (Rosenthal, 1977, 1990, 1997, 2001).

1.7 Other nitrogenous compounds in human urine

Table 1.5 shows the normal range of excretion of a number of nitrogenous compounds in human urine. Urinary excretion of urea reflects mainly dietary protein intake, or at least catabolism of amino acids in excess of requirements for net protein synthesis, while urinary ammonium excretion reflects acid-base balance and the pH of the glomerular filtrate (see section 1.5.5).

Small amounts of proteins that are small enough to be filtered in the glomerulus, such as amylase and ribonuclease, are excreted in the urine. The

Table 1.5 Average daily excretion of nitrogenous compounds by human beings.

urea (1)	10–35 g	150–600 mmol
ammonium (2)	340–1200 mg	20–70 mmol
amino acids	1.2–3.2 g	1/3 as free amino acids 1/3 as small peptides 1/3 as conjugates
protein	< 60 mg	
uric acid (3)	250–750 mg	1.5–4.5 mmol
amino sugars	10–40 mg	50–250 mg
creatinine	males 1.8 g, females 1.2 g	males 16 mmol, females 10 mmol
creatine	< 50 mg	< 400 µmol

1 Urinary excretion of urea depends largely on protein intake.
2 Urinary excretion of ammonium depends largely on acid-base balance and the pH of the glomerular filtrate.
3 Traces of purines are also excreted: xanthine, hypoxanthine, guanine and adenine.

presence of larger amounts of protein, and especially of proteins as large as serum albumin (M_r 58,000, just above the upper limit for normal glomerular filtration) indicates renal damage or dysfunction.

Creatinine is formed non-enzymically from creatine, the muscle phosphagen (see section 5.9.7), and the amount excreted reflects the total body content of creatine. This, in turn, reflects the body muscle mass. The gender difference in creatinine excretion is due to the lower proportion of body weight that is muscle in women than in men. Creatinine excretion is reasonably constant from day to day, and it is common practice to express urinary excretion of other metabolites per mol of creatinine. Creatine itself is only excreted when muscle tissue breaks down, and excretion of more than about 400 µmol/day indicates muscle atrophy of one kind or another.

1.7.1 Aminoacidurias

Some 70 g of amino acids are filtered by the kidneys each day, almost all of which is actively reabsorbed in the proximal renal tubules. Normally, up to three grams of amino acids are excreted, approximately one-third each as free amino acids, small peptides and conjugates of compounds such as benzoic acid. The excretion of significantly larger amounts of free amino acids (aminoaciduria) usually indicates an inborn error of metabolism. Two different types of aminoaciduria can be distinguished:

1 Excretion of a single amino acid in abnormally large amounts, with a high blood concentration of the amino acid. In such cases, there is a defect in a key enzyme involved in the catabolism of the amino acid, and the amount filtered in the kidney greatly exceeds the amount that can be reabsorbed. This is metabolic aminoaciduria.

2 Excretion of a number of amino acids that have chemically related side-chains, although the plasma concentrations of these amino acids are normal, or, more commonly, lower than normal. This is renal aminoaciduria, due to a defect in one of the amino acid transport systems that reabsorb filtered amino acids from the renal tubule.

From studies of patients with renal aminoacidurias, it is apparent that there are at least five groups of amino acid transporters in the kidney:

- transporting neutral amino acids (there are probably separate transporters for neutral amino acids with small and large side-chains);
- transporting basic amino acids and cystine;
- transporting acidic amino acids;
- the iminoglycine transporter, which transports proline, hydroxyproline and glycine;
- and a transporter for β-amino acids such as taurine (section 6.3.7) and β-alanine.

As we shall see in section 2.5.1, these amino acid transporters have overlapping specificity, and any one amino acid may be transported by more than one carrier (Broer, 2008; Fleck *et al.*, 2003).

One of the first amino acids to be isolated and characterized was cystine, which was isolated from urinary stones by Wollaston in 1810. Patients with cystinuria also excrete abnormally large amounts of arginine, lysine and ornithine, suggesting that there is a common carrier for the basic amino acids and cysteine. Cysteine can be oxidized to cystine, which crystallizes when the concentrated solution reaches the more acidic environment of the distal renal tubule, leading to the formation of kidney stones.

There are two forms of cystinuria. One has a dominant pattern of inheritance and is due to a defect in the transport protein itself; half the normal activity of this protein is not adequate for reabsorption of cysteine from the glomerular filtrate. The other has a recessive pattern of inheritance and is due to lack of one subunit of a protein that activates the transporter; half normal activity of this protein is adequate to maintain cysteine transport (Goodyer, 2004).

The renal clearance of cysteine is greater than that of the basic amino acids, suggesting that in the kidney there is a separate transporter for basic amino acids that does not transport cysteine. This separate basic amino acid transporter seems to be absent from intestinal mucosal cells, and the absorption

of basic amino acids in patients with cystinuria is as low as that of cysteine. In addition, there are high concentrations of bacterial metabolites of the basic amino acids in the faeces.

Further evidence for the existence of a separate basic amino acid transporter that does not transport cysteine comes from patients with lysinuric protein intolerance, who excrete large amounts of lysine, arginine and ornithine, and who develop hyperammonaemia after a meal containing moderate amounts of protein.

Some patients show isolated cystinuria with no excessive excretion of basic amino acids; in these cases, it is assumed that the defect is in a basolateral transporter that is specific for transferring cystine and/or cysteine from the renal epithelial cells into the bloodstream. Defects in basolateral transporters lead to accumulation of abnormal amounts of the affected amino acids in the cytosol of epithelial cells, because the amino acids can enter the cell from the intestinal lumen or renal tubule lumen, but then cannot be transferred to the bloodstream. Defects in the apical (or luminal) transporters mean that amino acids cannot be taken up into the cells from the lumen, so there is no accumulation intracellularly.

Hartnup disease is a defect of the transporter for large neutral amino acids. It is characterized by poor intestinal absorption and massive renal excretion of the aromatic and branched-chain amino acids. Many patients with Hartnup disease develop pellagra, the tryptophan-niacin deficiency disease, as a result of lack of tryptophan for synthesis of the nicotinamide ring of NAD and NADP (section 9.4.5). It was studies of Hartnup disease that led to the discovery of the intestinal absorption of di- and tripeptides by a transport system that is distinct from that involved in absorption of free amino acids. Patients show no increase in the plasma concentration of tryptophan when free tryptophan is given by mouth, but they do show a response to feeding dipeptides containing tryptophan. A number of indolic compounds that are derived from bacterial metabolism of unabsorbed tryptophan are excreted in the urine.

In dicarboxylic aminoaciduria, large amounts of glutamate and aspartate are excreted in the urine, and the excretion of glutamate may exceed the glomerular filtration rate. This reflects active secretion of glutamate into the urine, suggesting that the basolateral transport of glutamate is unaffected. In addition to the high-affinity protein that transports both glutamate and aspartate, there is evidence for a low-affinity glutamate-specific transporter. This may be the protein that is involved in active secretion of glutamate into the urine, since most of the evidence suggests that the same proteins provide both the apical and basolateral transporters. Little dietary glutamate enters the circulation; most is metabolized in the intestinal mucosal cells.

In iminoglycinuria, excessive amounts of proline, hydroxyproline and glycine are excreted in the urine. However, the renal clearance of these three amino acids is significantly lower than the glomerular filtration rate, suggest-

ing that there are additional, unaffected, transporters for these amino acids. Some affected subjects also show impaired intestinal absorption of glycine and proline, while others do not. In some families, heterozygotes also show impaired intestinal and/or renal absorption, while in others, heterozygotes are unaffected. This suggests that there are different common iminoglycine transporters in gut and kidney, as well as glycine-specific and proline-specific transporters.

Further reading

Note: References cited in the text are listed in the bibliography at the end of this book.

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